ENZYME HYDROLYSIS OF CASSAVA PEELS FOR ETHANOL PRODUCTION

By

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This thesis is the results of the authors' original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed date

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To all colleagues in TG103 and HW429…You made it a worthwhile journey.

ABSTRACT

The enzyme hydrolysis of cassava peels for ethanol production provides an interesting research opportunity to convert starch rich lignocellulose waste into renewable fuel production.

The research involved the pretreatment of cassava peels with steam explosion and hot water pretreatment processes as well as combining both amylolytic and cellulolytic enzymes to produce simple sugars.

This research compared different enzyme treatment strategies; a separate hydrolysis that involved the treatment of the peels with either cellulolytic enzymes or amylolytic enzymes, a consecutive hydrolysis process which is a follow up of the separate hydrolysis in which sugars were washed from the initial enzyme treatment (amylase or cellulase treatment) and the cassava peels resuspended for further enzyme treatment was also investigated. Another treatment strategy employed in this study was the simultaneous hydrolysis by amylases and cellulases of the cassava peels. The hydrolysis rate and yield were compared for each process. Minor changes that incorporated steam explosion pretreatment and hot water pretreatment were also studied.

A separate hydrolysis of milled cassava peels treated by amylolytic and cellulolytic enzymes yielded a maximum reducing sugar of 0.41g (as glucose) per gram of peels and 0.31g per gram of peels respectively. Also steam exploded cassava peels treated by amylolytic and cellulolytic enzymes yielded maximum reducing sugars of 0.24g per gram of peels and 0.37g per gram of peels respectively.

Results also showed that a consecutive treatment that incorporates an initial hydrolysis by cellulolytic enzymes followed by a subsequent treatment by amylolytic treatment yielded reducing sugars of 0.64g per gram of milled cassava peels. A reverse treatment where the cellulolytic enzymes were used to first treat the peels before a second treatment by amylolytic enzymes yielded 0.61g reducing sugar per gram of milled cassava peels.

A simultaneous hydrolysis by both cellulolytic and amylolytic enzymes produced a maximum reducing sugar of 0.58g per gram of milled cassava peels. A modification that incorporates hot water pretreatment, simultaneous and consecutive treatment was carried out. The milled cassava peels treated with hot water at 100^0C and amylase enzymes for 2 hours were further subjected to a simultaneous saccharification by cellulases and glucoamylase enzymes yielded a reducing sugar of 0.62g per gram of peels

Fermentation experiments were also carried out with *Kluyveromyces marxianus* at 40^0C and results showed a maximum ethanol yield of 0.12g ethanol per g of cassava peels for a separate hydrolysis and fermentation process and 0.18g ethanol per g of cassava peels for the simultaneous saccharification and fermentation process.

It was concluded that cassava peels presents a very good source of sugars for bioethanol production.

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ABBREVIATIONS

%v/v MWO- concentration of enzymes represented as percentage volume of enzyme by volume of reaction with milled washed and oven dried cassava peels

%v/v SE- percentage volume of enzyme by volume of reaction with steam exploded cassava peels.

ADP -Adenosine diphosphate

AMG- Spirizyme Fuel HS from Novozymes A/S Denmark

AOAC- Association of Official Agricultural Chemists

ATP- Adenosine triphosphate

Cassava C- Viscozyme Cassava C; a cellulase enzyme by Novozymes with endoglucanase, cellobiose and beta glucosidase enzymes from Novozymes A/S Denmark

Cassava R- Viscozyme Cassava R An enzyme mixture of cellulase, hemicellulase and xylanase from Novozymes A/S Denmark

CIEMAT- Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (Centre for Energy Research and Technology Madrid)

DP-Degree of Polymerisation

EtOH- Ethanol

MWO-Milled, washed and oven dried peels

NADH-Nicotinamide adenine dinucleotide

SE- Steam explosion pretreated peels

SHF- Separate hydrolysis and fermentation

SSF- Simultaneous saccharification and fermentation

SSCF- Simultaneous saccharification and co-fermentation

CHAPTER ONE : INTRODUCTION AND LITERATURE

1.1.0. INTRODUCTION: BIOFUELS- PROSPECTS AND RECENT ADVANCES

The rapid growth in industrialization in the last century has caused a dramatic increase in energy consumption all over the world and so the world is in need of alternative sources of energy to meet industrial and transportation needs. Crude oil is known to account for over 50% of the energy needs in the world today however it is a limited source of energy (Yang and Wyman 2008, Sun and Cheng 2002).

Transportation is the second largest energy user and it is by far the largest oil user. The International Monetary Fund (IMF) world economic outlook estimates that there are currently over 1 billion cars in the world. By 2050, it is believed that the estimated number of cars in the world would be 2.9 billion of which 1.9 billion of these cars would be found in developing countries (World Energy Outlook 2011).

The challenges of meeting this rising global energy need for transportation use still remain enormous. Biofuels is believed to be the only large scale alternative when it comes to transportation. Bioethanol is a promising alternative source of energy for the limited crude oil. It is believed to be the most important renewable fuel in terms of volume and market value (Licht 2007).The production of ethanol from starch and sugar based raw materials such as cereals, tubers, sugarcane and sugar beets is not new however in recent times the production of ethanol from lignocellulosic biomass waste has acquired a significant interest. The United States of America and Brazil are the top producers of ethanol from corn and sugarcane respectively. Both countries account for the production of 89% of the world's production in 2009 (www.ethanolrfa.org/page/objects/pdf/RFAoutlook2010 retrieved 2010-04-17).

The process of ethanol production from starch and sugar based raw materials is summarized in the figure 1.1.

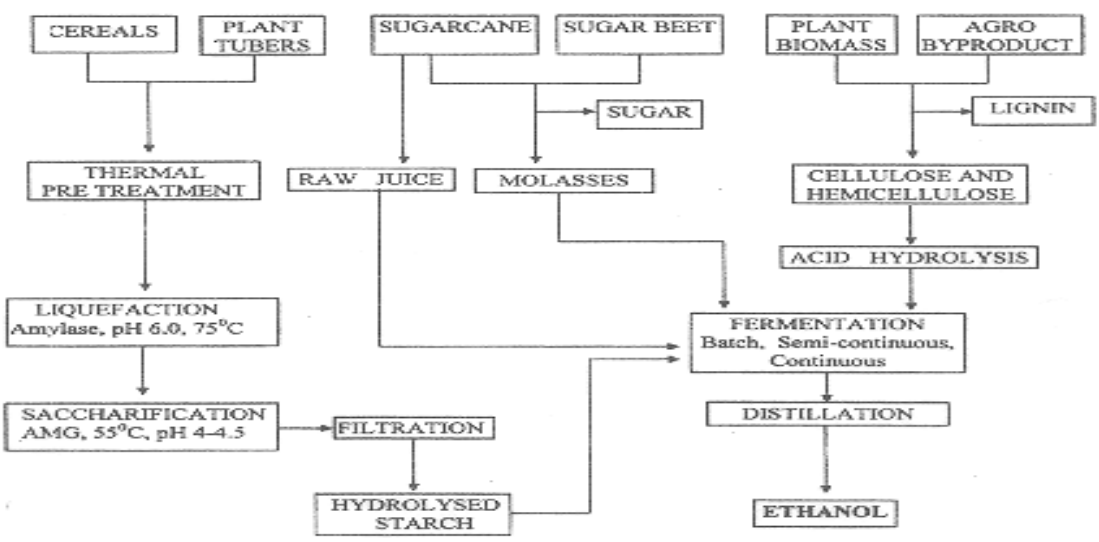


Figure 1.1 Raw materials for ethanol production (Gunasekaran and Raj,1999); Ethanol production from plant biomass and agroby-products may involve other possible routes like the simultaneous saccharification and fermentation where the hydrolysis and fermentation reactions are carried out in the presence of lignin

Considering the rapid rise in world population, the use of starch and sugar based materials brings an added challenge as these materials also serve as sources for food. This has forced the current interest in the use of lignocellulose biomass as raw materials for bioethanol production.

Lignocellulose is believed to be the most abundant plant material resource with an estimated annual production of 1 x 10^{10} metric tonnes and these materials form a large bulk of biomass. There are loosely classified as agricultural residues, forest/wood wastes, municipal wastes (papers) and energy crops dedicated to ethanol and other biofuel production (Sanchez and Cardona 2008).

Considering the recalcitrant and heterogeneous nature of lignocellulosic biomass, research over the past three decades have tried to unravel the chemistry of cellulose to sugar conversion especially with regards to the definition of the roles of substrate properties (degree of polymerisation of cellulose, crystallinity of cellulose, accessible surface area, presence of lignin) in the kinetics and mechanism of lignocellulose conversion to simple sugars (Bansal et al 2009, Zhang and Lynd 2004). However, the past decade has seen increasingly rapid advances in the development of the technology to produce bioethanol from lignocellulose biomass. Research from laboratory and pilot/demonstration scale has advanced into commercial scale in the last year. Novozymes reports that ''The Technology is ready''

(http://www.slideshare.net/FlemingVoetmann/2013-socio-economic-prospects-of-advanced-biofuels-voetmann-wfes retrieved 2013-02-05).

In 2012, The M&G-CHEMTEX launched the Crescentino plant in Italy which is expected to produce an annual 13 million US gallons of ethanol per year from arundo donax and wheat straw. The Shengquan plant in Shanndong China was also launched with a production capacity of 6 million gallons of ethanol per year. Several commercial plants with a combined capacity of 57 million gallons of ethanol are also expected to be launched in the United States and Brazil in 2013 (http://www.slideshare.net/FlemingVoetmann/2013-socio-economic-prospects-of-advanced-biofuels-voetmann-wfes)

Extensive research has been carried out on pretreatment, enzymatic hydrolysis and fermentation of biomass material. Few studies exist on materials that contain starch in addition to cellulose, hemicelluloses and lignin. A few of such materials include sorghum bran, potato peels, yam peels, cassava peels and a host of other agricultural residues.

1.2.0 CASSAVA PEELS- A STARCH RICH LIGNOCELLULOSE WASTE

Cassava (*Manihot esculata*) is a woody shrub extensively cultivated as an annual crop in tropical regions of the world for its edible starchy root. It is known as mandioca (Brazil), Yuca (Columbia), kamoleng kahoy (Philippines) mushu (China) akpu, ege or ugburu (Nigeria) and man sampalang (Thailand). Cassava peel is a by-product from the processing of cassava either for human consumption or starch production. The peel of the cassava is 1-4mm thick and accounts for 10-14% of the total dry matter of the root. (Adegbola et al 1996, Nartey 1979). Food and Agricultural organization (FAO) reports show that Nigeria alone produced 38 million metric tonnes of cassava per annum as at 2004 while current reports from USAID/Market report show that Nigeria currently produces over 45 million metric tonnes of cassava per annum. The Nigerian Government Presidential Cassava Initiative in conjunction with the United Nations Industrial Development Organization developed a Cassava Master Plan in 2006 in which the Projected Cassava production is expected to reach 150 Million Metric tonnes per annum by

2020 (Nigeria Cassava Masterplan 2006). This would in turn generate over 15 million metric tonnes of cassava waste per annum. Currently, cassava waste which includes the peels, leaves and unused leftover stalks from the processing of cassava is used as animal feed and also as manure in small farms in the rural area. Much of the waste is burnt or thrown away. The need to convert this waste into biofuel becomes necessary.

This research will focus on the enzymatic digestion of cassava peels for bioethanol production. This is because it is becoming increasingly difficult to ignore the enormous waste generated from cassava processing in the tropics.

1.3.0 OVERVIEW OF LITERATURE

The following process for conversion of lignocelluloses biomass is required; size reduction, pretreatment and enzymatic hydrolysis by cellulase and other hydrolytic enzymes. The sugars generated from the hydrolysis are then fermented by microorganisms (yeast or other ethanologenic organisms) into ethanol. As stated earlier, few studies exist on biomass that contains starch in addition to cellulose, hemicellulose and lignin. A few of such materials include sorghum bran, potato peels, yam peels and cassava peels. Much of the research up till now on starch rich lignocelluloses biomass (Nuwamanya et al 2012, Kongkiattikajorn and Sornvoraweat 2011, Corredor et al 2007**,** Yoonan and Kongkiattikajorn 2004,) have all been empirical studies showing hydrolysis yield without any reference to the substrate properties. This thesis has focused primarily on enzyme hydrolysis with an aim of having a better understanding of the chemistry that occurs with respect to the substrate.

1.3.1 LIGNOCELLULOSE STRUCTURE AND RECALCITRANCE

A large and growing body of literature abound on various aspects of lignocellulose biomass to ethanol conversion. Several notable reviews on pretreatment (Sun and Cheng 2002, Taherzadeh and Karimi 2008, Yang and Wyman 2008, Zheng and Zhang 2009, Alvira et al 2010) and enzymatic hydrolysis (Bansal et al 2009, Zhang and Lynd

2004, Mosier et al 1999). These reviews show that the key to efficient utilization of lignocelluloses biomass lies in the pretreatment and enzymatic hydrolysis steps.

Lignocellulose biomass is primarily composed of cellulose, hemicellulose and lignin. Cellulose is a polysaccharide consisting of a linear chain of several hundred to over a thousand β-(1-4) linked D-glucose units. Hemicellulose can be any of several heteropolymers such as arabinoxylans. It may contain many different sugar monomers like xylose, mannose, galactose, rhamnose and arabinose. Hemicellulose contains most of the D pentose sugars. Xylose is always the sugar monomer present in the largest amount in hemicelluloses when compared to the other sugars like mannose and galactose usually present in varying amounts. Hemicellulose consist of shorter chains of 500-3000 sugar units when compared to cellulose which has about 7000-15000 glucose residues per polymer. It is also a branched polymer in contrast to cellulose which is unbranched. Lignin, the third component of most lignocellulosic biomass is a complex chemical compound and the most difficult to degrade. ''It is an integral part of the secondary walls of plants'' (Lebo et al 2000). It is heterogenous and lacks a defined primary structure. It fills the spaces in the cell walls of plants between cellulose, hemicellulose and pectin components. Unlike cellulose and hemicellulose it is hydrophobic and a non-carbohydrate polymer with about 40 aromatic subunits which are covalently linked (Wardrop 1970). The barrier to hydrolysis of lignocelluloses is that the sugars are trapped inside the lignocelluloses due to the crosslinking between the polysaccharides (cellulose and hemicelluloses) and the lignin via ester and ether linkages. Ester linkages arise between oxidized sugars, the uronic acids and the phenols and propanols of the lignin. To extract the fermentable sugars one must first disconnect the cellulose from the lignin before the hydrolysis of the sugars to simple monosaccharides.

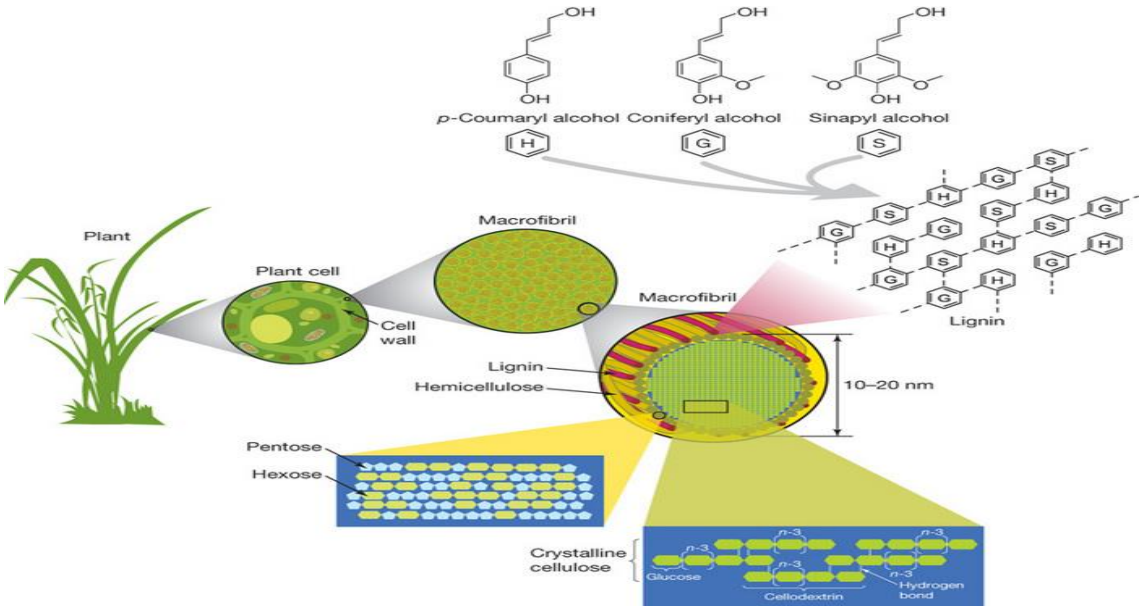


Fig *1.2 Structure of lignocellulose (Rubin E, 2008) Lignin is composed of three major phenolic components, namely p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S).*

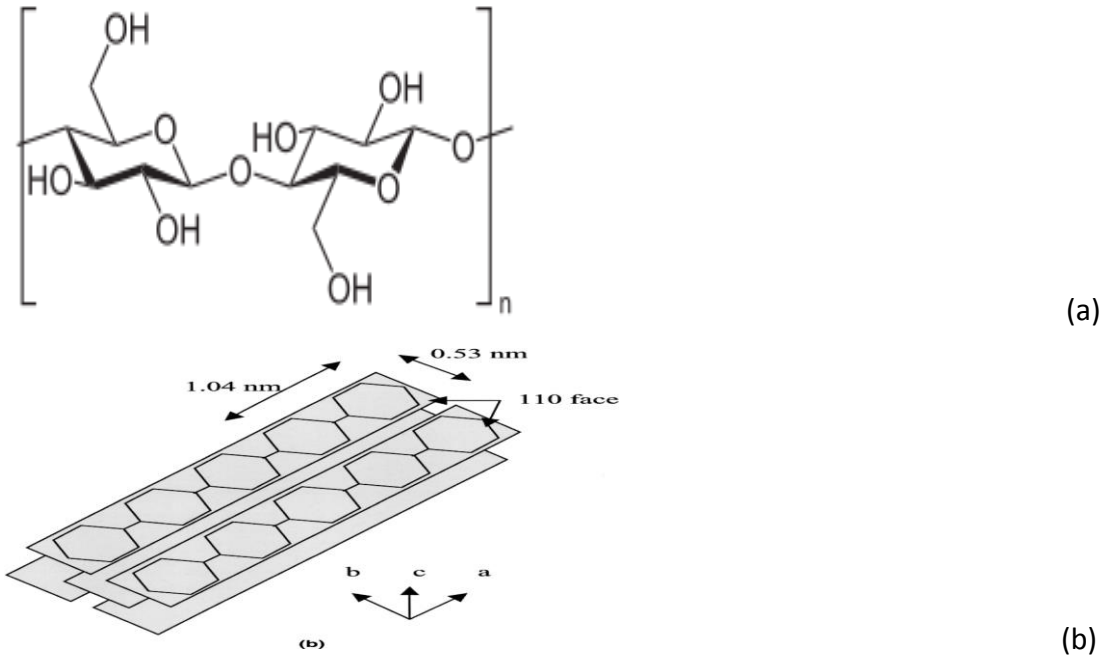


Fig 1.3: a. cellobiose molecule b. glucopyranose sheets (Zhang and Lynd 2004).

Unlike starch, the repeat unit for cellulose is cellobiose and not glucose (Fig 1.3a). This causes neighbouring anhydroglucose units to be rotated at 180^0 with respect to the next anhydroglucose unit. This rotation is responsible for the highly symmetrical form of cellulose as each side of the chain has the same number of hydroxyl groups.

Hydrogen bonding exist both within a chain and also links cellulose chains together producing fibers of great tensile strength (Krassig 1993).

Cellulose in higher plants form structures called microfibrils which are unbranched fibrils with 30-36 glucose chains aggregated laterally by hydrogen bonding and van der Waals forces to form crystalline structures (Somerville et al 2004). These microfibrils are organized into macrofibrils (Rubin 2008*).* X ray studies show that these chains are arranged in layered sheets and the sheets with successive sheets stacked on top of each other by van der Waal forces to form a three dimensional particle (fig 1.3b). This arrangement causes the surface of cellulose to have different ''faces'' that interact with the aqueous environment and cellulose enzymes with the favoured plane being the ab plane. The ac plane which carries the additional faces represents the reducing and non reducing ends of the cellulose chains. (Mosier et al 1999, Zhang and Lynd 2004, Arantes and Sandler 2010).

This structural feature of cellulose and how it affects cellulose hydrolysis has not been fully understood although it is believed that the high degree of aggregation of the fibres causes a more compact structure resulting in smaller internal accessible surface area. Other structural features that are believed to impact hydrolysis rate and yield in cellulose are degree of polymerisation and crystallinity index (Zhang and Lynd 2004).

1.4.0 PRETREATMENT OF LIGNOCELLULOSE BIOMASS

The recalcitrance of lignocellulose biomass necessitates a pretreatment step to overcome this recalcitrance. The goal of pretreatment is to alter the physical features and chemical composition/structure of lignocellulosic materials thus making cellulose more accessible to enzymatic hydrolysis for sugar conversion. Pretreatment is believed to be the most important unit operation in terms of cost followed closely by the cost of enzymatic hydrolysis of pretreated cellulose (Hu and Ragauskas 2012, Yang and Wyman 2008, Wooley et al. 1999). Economic analysis shows that almost 40% of projected cost is associated with releasing sugars from hemicelluloses and cellulose with pretreatment responsible for about half of this total. Studies from several reviews (Galbe and Zacchi 2012, Chiaramonti et al 2012, Alvira et al 2009,

Yang and Wyman 2008, Sun and Cheng 2002) show that pretreatment methods can be broadly classified into three main types; physical, chemical and biological pretreatment methods. A combination of physical and chemical pretreatment gives another pretreatment strategy termed physico-chemical pretreatment.

A combination of two or more pretreatment methods may be appropriate so as to maximise the hydrolysis process without degrading the carbohydrates (Galbe and Zacchi 2007). Recently, a number of researchers have combined microwave irradiation and chemical pretreatment methods to overcome the recalcitrance of lignocellulose biomass. Microwave and alkaline pretreatments were used to treat green tea residue. A further hydrolysis of the residue yielded 89% solubility of the cellulose (Tsubaki and Azuma 2013).

1.4.1 PHYSICAL PRETREATMENT

Physical pretreatment is known to be effective in reducing particle size as well as increasing the accessible surface area of the lignocellulosic materials. Crystallinity and degree of polymerisation of cellulose is also believed to be affected. This includes the use of mechanical comminution to reduce size by a combination of chipping, grinding and milling to reduce cellulose crystallinity (Sun and Cheng 2002). Varieties of milling like ball milling, two-roll milling, colloid milling and vibro energy milling have been used. Other physical pretreatment methods include irradiation by microwaves, ultrasound, electron beam and gamma rays (Taherzadeh and Karimi 2008). However, the major drawback to the use of physical pretreatment methods is that there are believed to be far more expensive than the other pretreatment methods. This is because of the energy requirement which again depends on the final particle size and characteristic of the biomass (Hendricks and Zeeman 2009). Energy consumption is estimated to vary from 130 kWh $tonne^{-1}$ for hardwood reduced from 10-30mm to 1.60 mm and 3.2 kWh $tonne^{-1}$ for corn-stover reduced to 9.5 mm. It is therefore obviously desirable that a chosen or newly developed pretreatment process minimizes or even avoids the need for size reduction or grinding (Chiaramonti et al 2012). An alternative approach to biomass pretreatment currently under investigation is mild torrefaction. Torrefaction is a mild form of pyrolysis at

temperatures typically ranging between 200 and 320 °C. It is believed to improve the grinding of fibrous materials thus reducing the energy demand for grinding. Preliminary experiments demonstrated that torrefaction leads to materials which can be enzymatically hydrolyzed and fermented into ethanol with yields comparable to untreated biomass, even if still significantly lower than steam exploded biomass. Further research is however needed in this field (Chiaramonti et al 2010).

Milling has been combined with other pretreatment methods on starch rich lignocellulose biomass. It was used alone on cassava peels (Yoonan and Kongkiattikajorn 2004). It was also combined with acid and alkaline pretreatment of cassava peels (Srinorakutara et al 2006). Milling and Liquid hot water has also been tested on sorghum bran (Corredor et al 2007). These researchers did not treat pretreatment in detail. Their study was mainly focused on enzymatic hydrolysis and fermentation. Kongkiattijorn and Sornvoraweat 2011 studied the simultaneous saccharification and fermentation of cassava peels. Milling and acid/alkaline pretreatment were combined. The study is rather vague on the specific effect of milling on hydrolysis yield or rate.

1.4.2 BIOLOGICAL PRETREATMENT

This involves the use of microorganisms such as white, brown and soft fungi to degrade lignin and hemicelluloses materials. Investigations have been carried out on several white rot fungi or similar microorganisms that are believed to attack mainly lignin and hemicelluloses. The conversion performance of white-rot fungi on wheat straw shows that 35% of the straw was converted to sugars by *Pleurotus ostreatus*. The time needed for this process was estimated to be five weeks (Hataka and Uusirauva 1982, Taniguchi et al 2005). The hydrolysis rate is very slow in spite of its advantages which include low energy requirement and favourable environmental conditions. Cellulose is more resistant to these microorganisms compared to lignin and hemicellulose which are usually degraded by these microorganisms.

1.4.3 CHEMICAL PRETREATMENT

This remains the most investigated pretreatment method available to date. It includes Acid and alkaline hydrolysis, ozonolysis, organosolv (use of organic solvents; ethanol, acetone, methanol etc), oxidative delignification (peroxidise enzyme in the presence of hydrogen peroxide), use of ionic liquids, wet oxidation (use of oxygen at high temperature) amongst others.

The use of concentrated and dilute acids have been investigated and found to be successful for the pretreatment of several biomass materials (wheat straw, rice husk etc). Dilute acids are however preferred to concentrated acids because it generates lower sugar degradation compounds like furfural, 5-hydroxy methyl furfural (HMF) and aromatic lignin degradation compounds. These are known to affect hydrolysis (Saha et al 2005,Wyman 1996).The main limitations to the use of acids is the requirement for reactors that are resistant to corrosion to be used. The difficulty in recovery of the acids used also make the process economically not feasible and this is also a challenge (Vonsivers and Zacchi 1995). Acid pretreatment is believed to solubilise a high percentage of hemicelluloses and degrade a good percentage of lignin.

Alkaline pretreatment methods using sodium, potassium and calcium hydroxides have been studied with sodium hydroxide being the most investigated. Alkaline pretreatment is believed to cause an increase in the internal surface area, a decrease in crystallinity and degree of polymerisation. A separation of structural linkages between lignin and the carbohydrate is also expected with alkaline pretreatment. Alkaline are however expensive although it has been suggested that calcium hydroxide be used instead as lime is cheap and can be recovered easily. Another advantage with alkaline pretreatment is that the reactions can be carried out at low temperatures and pressure unlike other methods (Carvalheiro et al 2008, Mosier et al 2005a, Kim and Holtzapple 2006, Fan et al 1981).

Other pretreatment methods based on the use of chemicals all possess their unique advantages and shortcomings. Most studies are however inconclusive. Ozonolysis is believed to effectively remove lignin and prevents the formation of toxic residues for

the subsequent hydrolysis step however it is very expensive due to the large amount of ozone required (Vidal and Molinier 1998). Organosolv is also highly favoured when the recovery of lignin is of interest (Zhao et al 2009). Several lignocelluloses materials have been treated with organic solvents (acetone, ethanol, methanol, ethylene glycol) and sometimes a mixture of these chemicals and acid catalyst. These solvents dissolve out the lignin from the lignocellulose matrix. The acid catalyst is believed to accelerate the breaking of hemicellulose bonds. These solvents are however expensive and require appropriate extraction and separation techniques which lead to an added cost. These solvents also need to be removed as they inhibit hydrolysis enzymes (Sun and Cheng 2002).

Another interesting chemical pretreatment that has received considerable attention of recent is ionic liquids pretreatment. Ionic liquids are salts; they contain large organic cations and small inorganic anions which exist as liquids at low temperature. 1butyl-3-methylidazolium chloride is an example. They are sometimes called 'green' liquids because no toxic gases are formed when they are used (Alvira et al 2010). Although lots of research on use of ionic liquids have been mainly on pure crystalline cellulose (Yang and Wyman 2008), reports of their uses on lignocelluloses biomass such as straw (Li et al 2009) and wood (Lee et al 2009) continue to increase recently. The low volatility and high thermal stability of these liquids makes research into their use a worthy option. Perhaps the most serious disadvantage to this pretreatment method is that for now, the process cost is not known and it is still unclear if these liquids will affect hydrolytic enzymes.

Ammonia recycle percolation (use of ammonia through a reactor packed with biomass at elevated temperature of 80-100^{0}C), wet oxidation (oxygen at high temperature and pressure) and other oxidizing agents are continually being researched (Arvaniti et al 2012, Banerjee et al 2011, Qiang and Thomsen 2012a).

1.4.4 PHYSICO-CHEMICAL PRETREATMENT

These methods combine physical and chemical pretreatment methods. They could involve the use of chemicals in a high pressure system and a sudden explosive decompression in the vessel to bring about degradation of the cellulose,

hemicelluloses and lignin transformation. These include steam explosion pretreatment, ammonia fibre explosion and CO_2 explosion. Liquid hot water pretreatment is another hydrothermal treatment which does not make use of rapid decompression and does not employ any catalyst or chemicals. Pressure is applied to maintain water at elevated temperature (100-240^{0}C) and provoke changes in the arrangement of the lignocellulose (Alvira et al 2010).

Recent studies have also involved the combination of microwave with chemical pretreatment. Microwave irradiation has been combined with Ionic liquids, acids, alkalis and salts with good results claimed. A 62.5% (w/w) $CaCl_2$ solution was used in the microwave pretreatment of corn stover. This treatment was believed to increase specific surface area by 168.93%, decrease cellulose crystallinity by 13.91% compared to the untreated corn stover. A follow up hydrolysis yielded 91% from the cellulose. Calcium Chloride was used as the heating medium (Li and Xu 2013). Corn stover was also pretreated with a combination of DMSO and 1-ally-3-methylimidazolium (AmimCl) co-solvents. Again, results showed an improved saccharification yield with decreased crystallinity index of the cellulose. The percentage conversion was 71.4% of cellulose in 14 hours compared to 12.5% of the raw corn in 20 hours. These researchers concluded that microwave assisted treatment with DMSO and AmimCl co-solvents was a feasible method for corn stover pretreatment (Liu et al 2012). Microwave has been combined with steam explosion pretreatment, dilute acids and dilutes ammonia with relative success (Chen et al 2012, Pang et al 2012, Chen, Chen, Ye and Sheen 2012). However, irradiation methods are expensive and the practicality of applying microwave on a large scale is still not feasible.

Steam explosion is however believed to be the most effective pretreatment process for the pretreatment of agricultural residues and hardwood as it has a low energy requirement. In steam explosion, high pressure saturated steam is used to treat the chipped biomass at elevated temperatures of 160-260^{0}C corresponding to pressure of 0.69-4.83MPa for several seconds to a few minutes before the material is exposed to atmospheric pressure. The major disadvantage associated with steam

explosion is that it can generate compounds that may be inhibitory to enzymes and microorganisms in the downstream process. This may include weak acids like acetic acid formed from acetyl groups present in the hemicellulose fraction. Partial degradation of hemicelluloses may also yield 5 hydroxymethylfurfural (HMF). Further degradation of furfural and 5 hydroxymethylfurfural also occur leading to the formation of formic and levulinic acids (Palmqvist and Hahn-Hagerdal 2000a). Although the toxic compounds generated depends on the raw materials, it has been suggested that pretreated biomass needs to be washed with water to remove inhibitory materials however this reduces the overall saccharification yield due to the removal of sugars generated by the hydrolysis of hemicelluloses. An alternative would be to research into obtaining more tolerant microorganisms for fermentation (Liu et al 2012).

Studies have shown that residence time, temperature, chip size and moisture content are the most important factors for steam explosion (Duff and Murray 1996).

CO_2 and Ammonia Fibre Explosion (AFEX) also employ the same physical mechanism of decompression at high pressure as steam explosion. The difference being in the use of CO_2 and liquid ammonia in the CO_2 and Ammonia fibre explosion respectively. Both processes are not as efficient as steam explosion although they are known to produce less inhibitory compounds when compared to steam explosion (Dale et al 1985, Mes-hartree et al 1988). Addition of sulphuric acid, SO_2 or CO_2 is also believed to improve the hydrolysis, decrease the production of inhibitory compounds and lead to more complete removal of hemicelluloses (Morjanoff and Gray 1987). Fig 1.4 below shows a pilot steam explosion scheme operated by an Italian company ENEA.

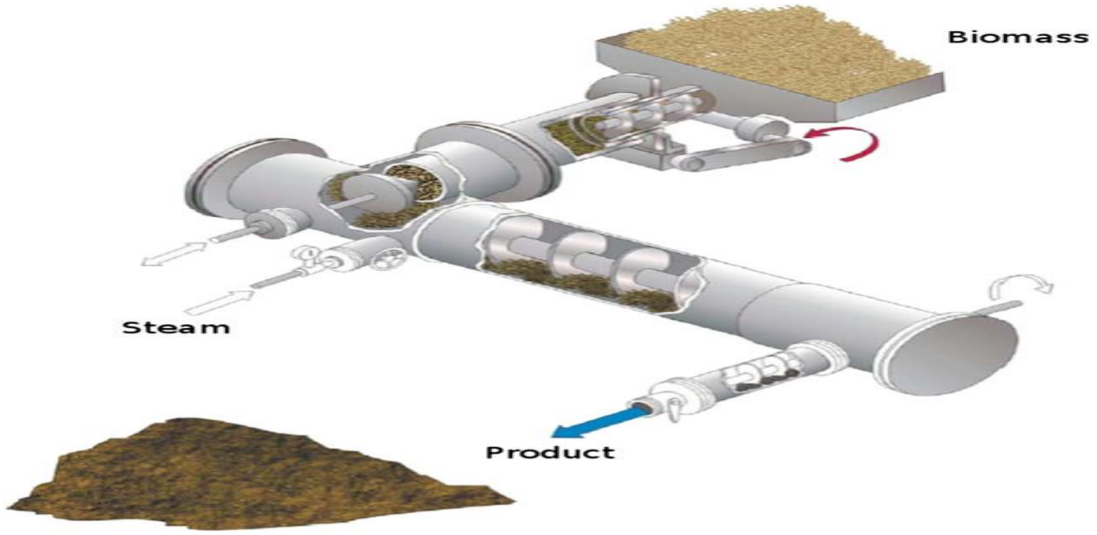


Fig. 1.4 Scheme for the ENEA (an Italian company) pilot facility in Trisaia, Italy for steam explosion pretreatment of different biomass (Chiaramonti et al 2012).

1.4.5 EFFECT OF PRETREATMENT METHOD ON SUBSTRATE PROPERTIES

The constituent physico-chemical properties of lignocellulosic biomaterials will determine to a large extent the type of pretreatment most appropriate to employ. A pretreatment method that works for a particular biomass might be inefficient for another kind of biomass. However the choice of a pre-treatment must have the desired effect on the substrate; producing a more reactive cellulose, minimizing the formation of possible inhibitors to enzymes and fermenting microorganisms. A pretreatment method must also minimize the destruction of cellulose, hemicellulose and starch for cassava peels and other starch rich lignocellulose materials. Of the listed pretreatment methods, milling, acid, alkaline, hot water have been applied to starch rich lignocellulose biomass. Alvira et al 2010 compared the most significant effects the different pretreatment technologies have on the structure of lignocelluloses in the table 1.2 below.

	Milling	Steam explosion	LHW	Acid	Alkaline	AFEX	CO_2 explosion
Increases accessible surface area	H	H	H	H	H	H	H
Cellulose decrystallization	H	N.D	N.D	N.D	N.D	N.D	N.D
Hemicelluloses solubilisation	N.D	H	H	H	L	N.D	H
Generation of toxic compounds	N.D	H	L	H	L	L	N.D

Table 1.2: Effect of Pretreatment on Lignocellulose Structure H: high effect; L: low effect; N.D: not determined; LHW: liquid hotwater AFEX: alkaline fibre explosion; - : Not Measured (Alvira et al 2010)

One question that arises from these studies is which of these factors; accessible surface area, cellulose decrystallization, hemicelluloses solubilisation, lignin structure alteration or generation of toxic materials has the most effect on hydrolysis rate and yield. Although, it is agreed that some of these properties are related. The removal of lignin and hemicelluloses solubilisation would lead to an increase in surface area. A more systematic study that recognises the peculiar properties of a particular biomaterial and perhaps identify how the substrate properties affect hydrolysis would be of much benefit. This might perhaps help in identifying the appropriate pretreatment that will bring about the most desired change identified for the substrate.

The presence of starch in cassava peels might have a major effect on the choice of pretreatment to be used because a pretreatment that could potentially destroy starch should be avoided. For the treatment of sorghum bran, starch was first degraded by alpha amylase and glucosidase enzymes then the substrate was pretreated with liquid hot water and then hydrolysed by a cocktail of enzymes. Results showed that a combination of starch degradation, optimum hot water

pretreatment and enzymatic hydrolysis resulted in maximum total sugar yield of 75% (Corredor et al 2007). The process suggested might be cumbersome with the separation of sugars after the starch degradation then liquid hot water being applied to the substrate before enzymatic hydrolysis is carried out. Perhaps a better experimental design would have been to treat with liquid hot water and then combine enzymatic hydrolysis of cellulose with starch degradation. Kongkiattijorn and Sornvoraweat 2011 studied the simultaneous saccharification and fermentation of cassava peels. Milling and acid/alkaline pretreatment were combined. These researchers did not treat pretreatment in detail. These studies are also rather vague on the effect of pretreatment on hydrolysis.

To date, although there has been little agreement on which substrate properties contributes more to cellulose recalcitrance. Pretreatment studies that are aimed at evaluating the effects on substrate properties would be most useful. The effects of pretreatment methods on starch degradation in cassava peels and how it affects degree of polymerisation, accessible surface area or crystallinity of cellulose will be interesting.

In this study, milling, steam explosion and hot water pretreatment methods were applied to cassava peels.

1.5.0 ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSE

Extensive research has been carried out on cellulose hydrolysis by cellulase enzymes. However, as stated earlier very few studies exist on combining starch and cellulose hydrolysis. Starch hydrolysis carried out by alpha amylase and amyloglucosidase enzymes can be modelled with the Michaelis Menten kinetic model. The Michaelis Menten model which is obeyed by most enzymes assumes a homogenous system where mass transfer (substrate to enzyme and product from enzyme) is not rate limiting therefore only the catalytic step (enzyme substrate complex to enzyme product) is governing the rate of reaction. However cellulose hydrolysis occurs in a heterogeneous system of two phases; the enzymes in an aqueous phase while the cellulose exist in crystalline solid. The physical characteristics of the crystalline cellulose also complicate modelling. The accessible

surface area, degree of polymerisation, degree of crystallinity, substrate and enzyme concentration all contribute significantly to the reaction rate modelling.

Researchers have shown an increased interest in the use of a cocktail of enzymes (cellulases, pectinases, xylanases, lacasses etc) to degrade lignocellulose materials. The existing accounts on starch and cellulose hydrolysis report the combined use of cellulase, alpha amylase, xylanases and pectinase for hydrolysis. However, the studies fail to show how these enzymes interact or specifically how alpha amylase and cellulase enzymes affect the sugar release patterns. The studies also do not take into account the effect of these enzymes on the substrate properties (degree of polymerisation, crystallinity, concentration of chain ends). A more systematic study that identifies how alpha amylase, cellulases and other hydrolytic enzymes interact with these substrate properties would give a mechanistic approach that will help in understanding the kinetics of this reaction.

1.5.1 CELLULOSE HYDROLYSIS: ACTION OF CELLULASE ENZYMES

The enzymatic hydrolysis of cellulose by cellulase involves three types of cellulases; endoglucanases (EC.3.2.14) that randomly cleaves β -1,4-glycosidic bonds on cellulose chains away from the chain ends, cellobiohydrolases(EC.3.2.91) produces cellobiose by attacking cellulose from chain ends. (Cellobiohydrolase I acts from the reducing end while cellobiohydrolase II acts from the non reducing ends of the cellulose chain). Beta glucosidase is the third enzyme in the cellulase system which converts cellobiose to glucose. (Henrisat 1994, Lynd et al 2002, Zhang and Lynd 2004).

The complexity of this system involving three different enzymes acting on cellulose, a heterogenous solid substrate, makes the kinetics of this reaction difficult to comprehend. The mechanism of this reaction is still not fully understood. Studies on the kinetics of this reaction must take into account the molecular architecture of the cellulase enzyme, the role of the substrate properties (crystallinity, degree of polymerisation, accessible area) and the decline in reaction rate that characterise this reaction(Zhang and Lynd, 2004).

Several empirical studies (Sattler et al 1989, Kim and Holtzapple 2006, Berlin et al 2007) that help in quantifying the effects of an individual substrate property and initial rate estimations are available but these do not provide much understanding on the mechanistic details of the process. Researchers have also shown an increased interest in mechanistic or semi-mechanistic kinetic models that take into account the adsorption of enzymes. Zhang and Lynd 2004, points out the imperative need for researchers to seek a functional approach to modelling of enzymatic hydrolysis of cellulose towards identifying rate limiting factors and a deeper understanding at the level of substrate features and multiple enzyme activities.

One of the major challenges of mechanistic models has been the need for improved methodologies for determination of substrate properties like degree of polymerisation, crystallinity and cellulose accessibility. This is a particular problem with materials that have both lignin and cellulose as most measurements of these properties in literature have been on pure cellulose substrate.

Cellulase enzymes are modular proteins with two distinct independent domains; the catalytic module and the cellulose binding module (CBM). The catalytic module is responsible for the hydrolysis of the cellulose chain while the cellulose binding module has the ability to increase the adsorption of cellulolytic enzymes onto the insoluble cellulose (Mosier et al 1999). The two modules are joined by a flexible-glycosylated linker region approximately 3—44 amino acids in length and rich in proline, glycine, serine and threonine. Endoglucanase and cellobiohydrolases are tadpole shaped with the catalytic core forming the head and a wedge-shaped cellulose binding module at the tip of the tail. Endoglucanase enzymes and both Cellobiohydrolase I and Cellobiohydrolase II are very similar however the major difference between two enzymes is believed to be in their catalytic domain. It is hypothesized from experiments conducted using high resolution electron density mapping that ''Cellobiohydrolase I and Cellobiohydrolase II have two large anti parallel Beta sheets that stack face to face and occupy one-third of a 434 residue domain. The curved beta sheets form a flattened cylindrical tunnel 40 A^0 long which accommodates the cellulose chain with glycosyl binding sites of similar residue

structure'' (Zhang and Lynd 2004). Endoglucanase enzymes however are not believed to have this tunnel forming loops instead it is believed to have an open cleft active site. This difference in morphology is believed to be responsible for their differences in mode of operation. The tunnel active site is also believed to be responsible for the production of only cellobiose from cellobiohydrolases as other oligosaccharides like glucose or cellotriose are not formed (Mosier et al. 1999). Other researchers have disagreed with this as it is believed that glucose and cellotriose are formed as well (Medve 1998). Experimental studies of the hydrolysis of oligosaccharides by cellobiohydrolases is believed to yield glucose, cellobiose, and cellotriose from the first cut the enzyme makes after complexing with a chain (Nidetzky et al, 1994). Subsequent processive cuts then form cellobiose.

The exact mechanism of cellulose hydrolysis is not yet known due to insufficient experimental evidence and different interpretations of experimental results. Bansal et al 2009 suggested that from literature the major steps for the mechanism of cellulases are as follows;

1. Adsorption of cellulases onto the substrate through the binding domain (Stahlberg et al 1991).
2. Location of a bond susceptible to cleaving on the substrate surface (Jervis et al 1997). Chain end if cellobiohydrolase, cleavable bond if endoglucanase.
3. Formation of the enzyme-substrate complex (Divne et al 1998, Mulakala and Reilly 2005).
4. Hydrolysis of the beta glucosidic bond and simultaneous forward sliding of the enzyme along the cellulose chain for cellobiohydrolase (Divne et al 1998, Mulakala and Reilly 2005)
5. Desorption of cellulases from the substrate or repetition of step 4 or 2/3 if only the catalytic domain detaches from chain.

This is illustrated in fig 1.5 below.

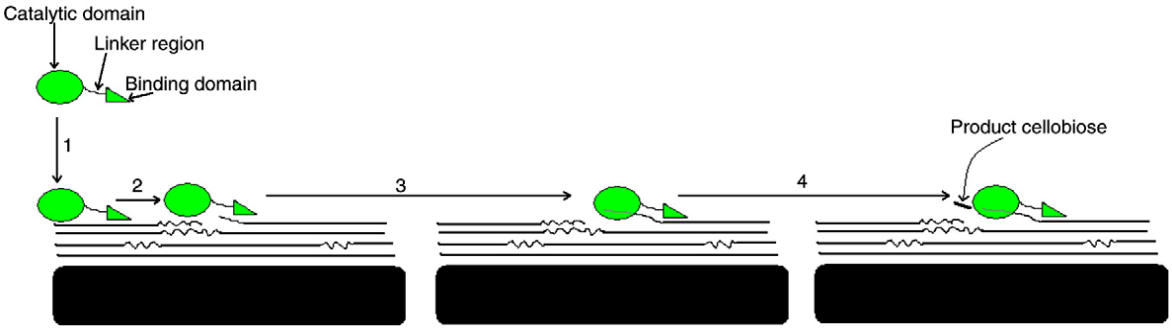


Fig 1.5(Reinikainen et al 1992) Steps 1 to 4 for a cellobiohydrolase acting on a cellulosic substrate (not drawn to scale). For endoglucanase, steps 2 and 3 are different as it does not require chain ends to act on. Step 1 — Adsorption, step 2 — location of chain end, step 3 — formation of enzyme–substrate complex, and step 4 — hydrolysis of the β-glycosidic bond.

Hydrolysis of cellobiose to glucose by β-glucosidase (if present in enzyme mixture) concludes the hydrolysis process.

There have been variations in literature of the mode of action of cellulase enzymes as some researchers have suggested the possibility for the cellulose chain to thread into the catalytic domain by going over the binding domain (Mosier et al. 1999). Others have also suggested that the role of the cellulose binding module in the initial stage of enzymatic hydrolysis goes beyond hydrolysis. Arantes and Saddler, (2010) stated that rather than sequential shaving of cellulose fibrils as postulated by several studies, it was suggested that the recalcitrant cellulose surface is first disrupted or loosened by non-hydrolytic proteins (in this case cellulose binding module). This leads to an increase in cellulose surface area and making it more accessible to the cellulase enzyme complex in a mechanism termed amorphogenesis. The term amorphogenesis is coined by Coughlan (1985) to suggest a mechanism in which the dispersion, swelling or delamination of cellulose substrate occurs resulting in the reduction of the degree of fibrillar aggregation and/or crystallization and the creation of a larger surface by increasing the reactive internal surface.

Fig 1.6A shows the inaccessible bulk of the cellulose being loosened up while they remain molecularly unchanged and then the cellulose network accessible to cellulase enzymes are then cleaved by the synergistic action of endo and exo-glucanases to soluble cello-oligosaccharides (fig 1.6B) which are further hydrolysed

to cellobiose fig 1.6C and finally to glucose by beta glucosidase. The role of the cellulose binding module in the dispersion is proposed by some researchers (Klyosov and Rabinovich 1982, Rabinovich 1980).

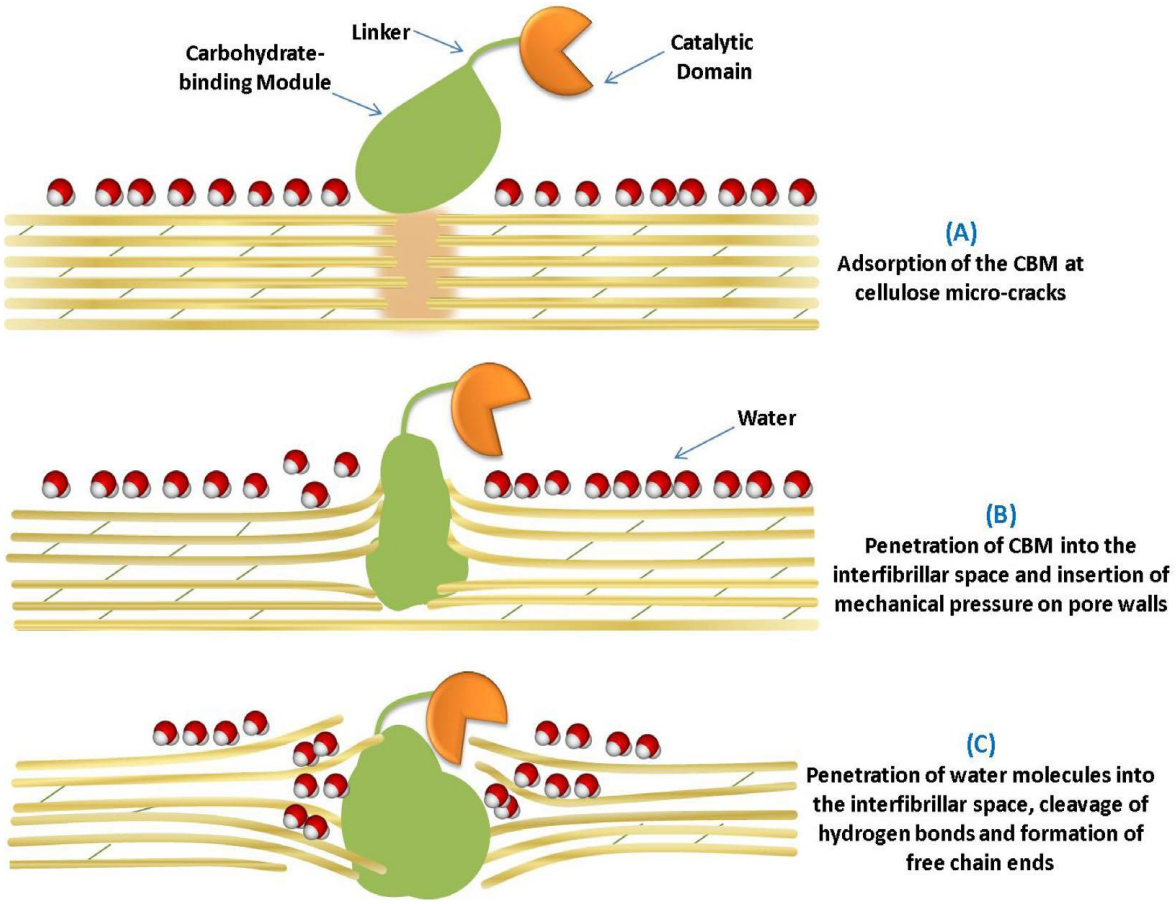


Figure 1.6: Schematic representation of amorphogenesis of cellulose fibers mediated by the carbohydrate-binding module (CBM) of cellobiohydrolase I (CBHI) (adapted from Esteghalian et al 2000). For clarity, the carbohydrate-binding module is oversized compared with the catalytic domain (Arantes and Sandler 2010).

They proposed that cellulases are adsorbed to cellulose defects (disturbance in the crystalline structure of cellulose) and then the cellulose binding module penetrates into the interfibrillar space.

This causes a mechanical action due to the presence of large enzyme within the narrow space leading to swelling in the cellulose structure and allowing more water molecules between the fibrils. The water molecules penetrates further into the capillary space breaking the hydrogen bonds between the cellulose chain therefore

increasing the surface area for the catalytic module to act upon the chain ends for cellobiohydrolase and random cleaving by endoglucanase.

Although a complete mechanism for this reaction has not been accepted, the enzymes; endoglucanase, cellobiohydrolase and beta glucosidase all work to produce soluble sugars through physical and chemical changes in the residual solid phase cellulose.

Chemical changes in cellulose are manifested as changes in degree of polymerisation and chain ends concentration. Endoglucanase increases the concentration of chain ends and significantly decreases the degree of polymerisation by attacking interior portions of cellulose molecules while exoglucanases shorten degree of polymerisation incrementally and only occasionally decrease the concentration of chain ends. It can be concluded that endoglucanases are mostly responsible for chemical changes in cellulose over the course of the reaction but plays a smaller role in solubilisation however exoglucanase is believed to be primarily responsible for solubilisation but has a smaller effect in changing the chemical properties of residual cellulose (Zhang and Lynd 2004)

1.5.2 RATE LIMITATIONS AND SUBSTRATE PROPERTIES

Studies have shown that product inhibition by cellobiose, glucose and ethanol does not account for the significant rate drop associated with cellulose hydrolysis. Enzyme deactivation, decrease in accessible surface area, loss of synergy between cellulases and inaccessibility caused by lignin and hemicelluloses all contribute to the rate slow down. It is also believed that cellulases also adsorb unto lignin (Bansal et al 2009).

Kinetic models that account for adsorption have been proposed. The Langmuir isotherm offers the most common description of cellulase adsorption by single adsorption equilibrium constant and a specific adsorption capacity.

$$E_b = \frac{E_{max} K_{ad} E_f S_c}{1 + K_{ad} E_f}$$

In which E_b is adsorbed cellulase (mg or mol cellulase/L), E_{max} is the maximum cellulase adsorption per g cellulose (mg or mol cellulase / g cellulose), S_c is cellulose concentration (g cellulose/L), E_f is free cellulase (mg or mol cellulase/L), and K_{ad} is the association constant in terms of L/g cellulase)

Langmuir isotherm provides a good fit to data in most cases and can be used to compare the characteristic properties of various cellulose-cellulase systems. It is however used only as a mathematical expression because it does not comply with its underlying assumptions; partially irreversible cellulase adsorption, interactions among adsorbing cellulase components especially at high concentration, multiple type of adsorption sites, entrapments of cellulase by cellulose pores and multiple component cellulase adsorption in which each component has a different constant(Zhang and Lynd 2004). Although several kinetic models like the 2 site adsorption model (Linder 1996, Medve et al 1997, Starlhbeg et al 1991), freudlich isotherm (Medve et al 1994), Langmuir freudlich isotherm (Medve et al 1994) have been proposed, none has accounted for the rate slowdown.

Accessible surface area has been of interest to several researchers in recent times. Recently several mechanistic modelling studies of cellulose hydrolysis (Griggs et al 2012, Levine et al 2011, Levine et al. 2010) have shown considerable interest in how accessible surface area affects hydrolysis yield. Levine et al (2010) provides a unique model that shows that the rate of cellulose hydrolysis is directly dependent on the amount of substrate surface area. The model incorporates the three enzymes; endoglucanase, cellobiohydrolase and beta glucosidase and describes their distinct enzyme adsorption and complexation steps. The model also describes enzymes as having dynamic interactions with the cellulose surface as the reaction progresses. ''The time course of the cellulose surface is captured; as the cellulose particles shrink, new chains are exposed and the total cellulose surface area is reduced."

The model concluded that the total cellulase-accessible cellulose surface area is the governing parameter for enzyme hydrolysis. This was tested experimentally by using 10g/l avicel with 0.16umolg-1 of either Endoglucanase 1 or Cellobiohydrolase I enzymes. A mixture of both enzymes making 0.32umolg-1 was also used for this

hydrolysis experiment at 40^0C in 50mM sodium acetate buffer. Two different experiments using $8m^2g^{-1}$ and $47.6m^2\ g^{-1}$ avicel representing an initial low surface area and an initial high surface area were used to validate the model. Results showed that the activities of the endoglucanase and cellobiohydrolase either acting alone or when in mixed form depended on the amount of accessible surface available for complexation. The competition for glycosidic bonds by both enzymes was also discovered to complicate hydrolysis as the amount of enzyme increases on the cellulose surface. It was noted that at low surface area, adsorptions from both endoglucanase and cellobiohydrolase enzymes resulted in high enzyme surface concentration of both enzymes. This limits the endoglucanase enzymes since it can only adsorb unto chain ends. This in turn leads to the reduction in the concentration of reducing end available for cellobiohydrolases therefore causing a reduction in the synergistic relationship of the endoglucanases and exoglucanase in mixed enzyme preparation. It was therefore suggested that the way cellulolytic enzymes compete for adsorbable sites should be altered. This can be done by adopting a different approach in the design of enzyme mixtures. Enzyme mixtures with endoglucanase and cellobiohydrolases could be mixed in ratios that can reduce the competitive behaviour that leads to enzyme crowding. This is because both endoglucanase and cellobiohydrolase enzymes can adsorb anywhere on the cellulose surface.

The accessible cellulose surface area is therefore different from the total cellulose surface. As hydrolysis time changes, the amount of enzyme that can access the cellulose changes and this would depend on the spatial organization of a given cellulose substrate. The evolution of the enzyme accessible cellulose during the course of reaction would therefore be dependent on the initial organization of the chains i.e degree of polymerisation and how the substrate changes as the reaction progresses (Griggs et al. 2012).

Much of the research carried out on mechanistic modelling of enzymatic hydrolysis of cellulose and centred on rate limitation have been carried out on pure cellulose substrates and as such the effect of lignin and hemicelluloses have not been adequately taken into account. It must also be taken into account that the method

of analysis of substrate properties like crystallinity, accessible surface area and degree of polymerisation in lignocelluloses biomaterials are still unreliable.

The diffusion rates of enzymes are believed to be affected by the presence of lignin and hemicelluloses. The substrate heterogeneity and the partial ''crystallinity'' are all factors that contribute to this hydrolysis reaction occurring in one dimension (Figure 1.7 below). Reactions of this type have been described by a phenomenon termed fractal kinetics by some researchers.

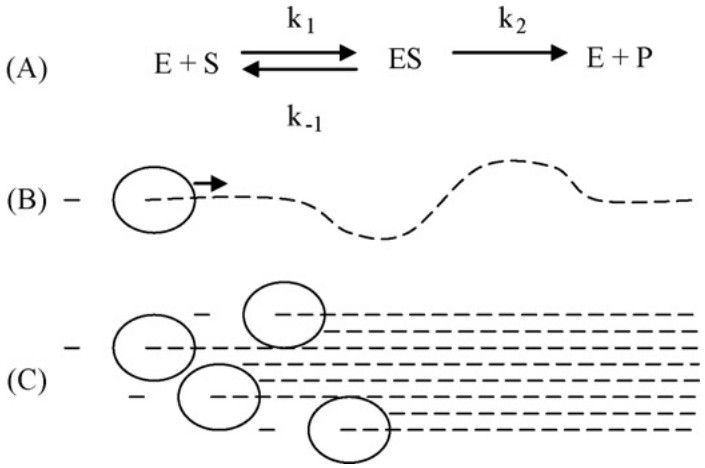


Fig. 1.7. Schematic Michaelis, fractal, and ''jammed'' reactions. (A) Michaelis scheme. E, enzyme; S, substrate; ES, Michaelis intermediate; P, product. (B)Fractal scheme for an enzyme (ellipsoid) acting on chained substrates (dashed curve). The enzyme binds to the chain through its active site tunnel. After cleaving off a substrate unit (bar), the enzyme slides along the chain (in the direction) for the next catalytic cycle. (C) ''Jammed'' scheme for enzymes (ellipsoids) acting on substrate chains (dashed lines) that are packed orderly with defined spacing. Being ''oversized'' in comparison to the interchain space, the enzymes anchored on adjacent chains may jam each other (Xu and Ding 2007).

Fractal kinetics is said to occur when reactions take place in constrained media giving rise to apparent rate orders and time dependent rate constants (Anaker and Kopelman 1987, Kopelman 1986). A recent study to investigate temperature changes that occur for free enzymes and immobilized enzymes using a fractal like kinetic equation showed how the reducing sugar concentration changes with hydrolysis time. The model was successfully fitted with experimental data of both free and immobilized enzymes at temperatures between 37 and 50^0C although the predictions for 53^oC for free enzymes was inaccurate (Zhang et al 2012).

1.5.3 CELLULOSE AND STARCH HYDROLYSIS: A COMPARATIVE REVIEW

Starch molecules are similar to cellulose molecules. There are glucose molecules linked by alpha-1, 4 and alpha-1,6 glucosidic bonds unlike cellulose where the glucose molecules are linked by beta-1,4-glucosidic bonds (Fig 1.8). Starch molecules have different structures due to the two kinds of linkages; the alpha 1, 4 and the alpha 1, 6 glucosidic bonds. A straight chained polymer of glucose with only alpha 1, 4 glucosidic bond is called amylose whereas a branched chained glucose polymer containing alpha -1, 6 glucosidic linkages results in a branched polymer called amylopectin (Fig 1.9). Branching in amylopectin occurs in approximately one per twenty five glucose units in the unbranched segments.

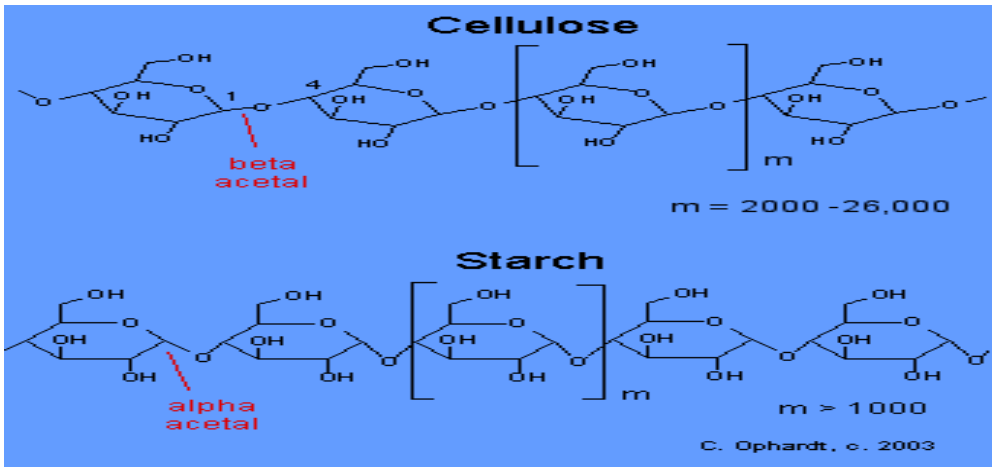


Fig1.8 (http://www.daviddarling.info/images/cellulose_starch.gif retrieved 2010-09-17)

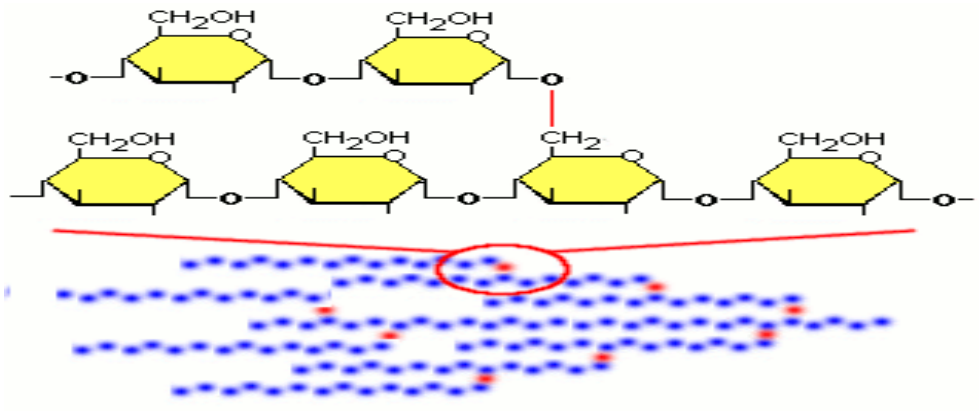


Fig1.9:structureofamylopectin(http://www.vivo.colostate.edu/hbooks/pathphys/digestion/basics/polysac.html retrieved 2010-09-17)

Starch is known to be insoluble in water at room temperature. This is because of the intermolecular and intramolecular hydrogen bonding present in the molecules. Penetration by water and hydrolytic enzymes is not possible however when an aqueous suspension of starch is heated, the hydrogen bonds are weakened and water molecules are absorbed leading to swelling. A process commonly called gelatinization.

Enzymatic hydrolysis of starch by alpha amylase enzymes can yield shorter chains of starch depending on the relative position of the bond under attack as counted from the chain end. The products vary from dextrin, maltotriose, maltose to glucose. Unlike cellulase enzymes, alpha amylase enzymes are produced from a wide variety of organisms including human. They are therefore widely synthesized in nature and represent about 30% of the world's enzyme production (Van der Maarel et al 2002). Several studies have reported that the rates of starch hydrolysis can be about 100 fold faster than the hydrolysis rate of cellulose at same enzyme concentration. The hydrolysis rates of starch and cellulose can be compared on the basis of the following factors; the fraction of bonds accessible to the enzymes, the availability of chain ends and the solubility of the hydrolysis products.

The fraction of accessible glucose-glucose bonds as estimated by Fujii et al 1981 for cellulose is believed to be 8-500 fold lower than for soluble starch. They also reported cellulose to be 5-200 fold less soluble than insoluble starch. This in turn affects the chain end availability (per unit mass). This is because cellulose has a high degree of polymerization with a ratio of glucosyl units per chain ranging from 300-2000 however amylopectin exhibits branching as noted earlier and each branch gives rise to a new chain end and the ratio of glucosyl units to chain ends is approximately 22. Branching for starch occurs every 17-26 glucose units (Bertoldo and Antranikian 2002, Buleon et al 1998). The cellulose hydrolysis is thus limited by the availability of chain ends for cellobiohydrolase (Zhang and Wilson 1997, Valjamae et al 2001, Schulein 2000). Cello-oligosaccharides (cellodextrins) are essentially insoluble at degree of polymerisation greater than 6-10 (Miller et al 1961, Pereira et al 1988, Zhang and Lynd 2003) however maltooligosaccharides are

soluble at degree of polymerisation of 60 (John et al 1982). Again, the planar linear structure of the cellodextrins as compared to the helical branched structure of starch leads to a situation where many bond cleavages need to occur for soluble hydrolysis products to be generated. However for starch molecules, fewer bond cleavages have to take place before soluble products are generated.

The differences observed in hydrolysis rate between starch and cellulose do not lie in the intrinsic difference between the alpha linked glucosidic bonds and the beta linked glucosidic bonds but rather it is the difference in substrate characteristic in starch and cellulose that make the difference (Zhang and Lynd 2004).

1.6.0 REVIEW OF LITERATURE ON CASSAVA PEELS

The composition of cassava peels shows a complex substrate with starch, cellulose, hemicelluloses, lignin and pectins all present in varying proportions each with its own unique chemical bonds of different strengths. Results of compositional analysis are presented in the next chapter (Table 2.1).

Earlier studies on literature on the enzymatic hydrolysis of cassava peels (Srinorakutara et al 2006, Yoonan and Kongkiattikajorn 2004) show that combinations of enzymes (alpha amylases, cellulase, pectinase and xylanase) were employed for saccharification. Acid and alkaline hydrolysis was also used in these studies. More recent studies using microbial hydrolysis (Nuwamanya et al 2012, Olanbiwoninu and Odunfa 2012, Wongskeo et al 2012) have been reported with varying success in hydrolysis yield. Some studies have also included the stems, leaves and the peels as waste and hydrolysed this mixture (Nuwamanya et al 2012). Hermiati el (2012) also studied the hydrolysis of cassava residue using microwave irradiation. None of these studies showed how the hydrolysis yield is related to the substrate properties like the degree of polymerisation.

Srinorakutara et al (2006) analysed the reducing sugars produced by acid, alkaline and enzyme hydrolysis. They compared acid hydrolysis with enzyme hydrolysis using amylolytic and cellulolytic enzymes and concluded that acid hydrolysis produced higher monosaccharides compared to enzyme hydrolysis. They also studied the optimum temperatures and pH of the amylase, cellulase, xylanase and

pectinase enzymes used. Milling was the only pretreatment applied to the peels. The study by Yoonan and Kongkiattikajorn (2004) compared the enzymatic hydrolysis by alpha amylase and glucosidase on one hand and the hydrolysis of cellulase and pectinase on the other hand. The former yielded 30% of reducing sugar and the latter yielded 35% reducing sugars per gram of cassava peels. Both sets of enzymes were combined and yielded 50% reducing sugar per gram of peels. The use of dilute sulphuric acid yielded 60% reducing sugar. Although it is agreed by the researchers that hydrolysis by acid and alkali was not as complex as enzyme hydrolysis especially when a cocktail of enzymes with different optimum operating conditions are required, the maximum reducing sugar production was lower with acid hydrolysis considering that a separate hydrolysis of starch and cellulose yields a combined 65% reducing sugar. Moreover, the severe experimental conditions that require equipment to withstand corrosion makes the process expensive with acid hydrolysis. These authors did not specify the composition of the peels and so it is difficult to know if the total carbohydrate content was approximately 60%.

Some studies (Wongskeo 2012, Nuwamanya et al 2012) have included cassava bagasse or cassava residues or cassava pulp. These materials are also very similar to cassava peels in composition although the amount of starch, cellulose and hemicellulose varies with starch usually above 45%. Cassava bagasse is a by product of starch processing in factories whereby the pulp leftover after the starch has been hydrolysed is composed of fibres and starch residues. This residue can also be combined with the peels that are collected at the start of the industrial process.

Other studies (Wongskeo et al 2012, Olanbiwoninu and Odunfa 2012) used microbial hydrolysis to produce mainly glucose for subsequent ethanol production. Cassava peels were first pretreated with dilute sulphuric acid, sodium hydroxide or methanol with 0.05M sodium carbonate. The peels were then treated with *Pseudomonas florescens B9* or *Aspergillus terreus.* These microbes were actually from rotten cassava peels. Results showed that acid pretreatment combined with either *Pseudomonas florescens or Aspergillus terreus* was between 46-51% reducing sugar per gram of cassava peels (Olanbiwoninu and Odunfa 2012). This study

provides an interesting alternative to the use of industrial enzymes as the microbes could be grown easily on rotten cassava peels. However, the use of chemicals like acids or methanol to pretreat the substrates might increase the cost of the process on an industrial scale considering the effect of acids on corrosion. Also, the effect of these on ethanol producing microbes for the fermentation step is not known. Further studies might be needed to verify the viability of this strategy.

Wongskeo et al 2012 studied the effect of particle size and two bacteria strains A002 and M015 isolated from Thai higher termites, *Microcerotermes* spp on the hydrolysis of cassava residue (a cassava residue with 49.66% starch, 21.47% cellulose, 12.97% hemicellulose, and 21.86% lignin). They reported a glucose yield of 50-66g% of dry cassava residue with both bacterial strains although bacterial strain A002 had slightly higher glucose yield compared to strain M015. They also reported as would be expected a higher glucose yield with cassava peels of a smaller particle size. Here again, the use of microbes to produce sugars was studied. These studies would have been more educative if the authors specified the enzymes being released from the microbes.

As highlighted earlier, the need to relate the effects of enzymatic hydrolysis on substrate properties would also be of immense help to understanding the kinetics of this unique substrate.

In this work hot water and steam explosion pretreatments were employed. The degree of polymerisation of the carbohydrate was also monitored in the solutions and the solid phase

1.7.0 FERMENTATION BY KLUYVEROMYCES MARXIANUS

Over the years, *Saccharomyces cerevisiae* has been the most common industrial microorganism for the production of ethanol from sugars. It is known that the optimum temperature for most *S. cerevisiae* is between 25-35^{0}C however metabolism by these organisms is also known to generate heat and so cooling is often required to cool down the fermentation vessel so that these microorganisms can grow and produce ethanol efficiently (Abdel-Banet et al 2010). The scaling up of the production of fuel ethanol in the last two decades due to the unpredictable

and often times rising cost of fossil fuel has added more pressure on the enormous cost that could be incurred from cooling large industrial systems for efficient ethanol production. It has therefore become imperative that research into identifying thermostable microbes capable of producing ethanol from different substrates be carried out.

S. cerevisiae is still the major microorganism being used in the fermentation of industrial biofuels from grain and molasses. However, developments in converting cellulose to ethanol with glucose inhibition of cellulases have suggested a combination of the simultaneous saccharification and fermentation step in a process termed SSF. Besides the obvious proposed advantage of the simultaneous saccharification and fermentation (SSF) process in preventing the accumulating of glucose, the process is also believed to save cost. The saccharification and fermentation would be carried out in the same vessel instead of two different vessels for saccharification and fermentation. Also, the need for separation of the hydrolysate from the solid residue or waste before fermentation is eliminated. The saccharification and fermentation process would therefore need a thermostable microorganism that is capable of producing ethanol.

A study estimated that for every 5^{o}C increase in fermentation temperature, approximately US$30,000 is saved for a 30,000KL scale ethanol plant that produces ethanol from corn using a separate hydrolysis and fermentation (SHF) method (Babiker et al 2010). These researchers believed that using an SSF process at 40^{0}C instead of at 32-35^{o}C would further save an additional US$500,000 per annum. This was explained by the fact that using glucoamylase has a twofold higher activity at 40°C compared with its activity at 32°C, and if the SSF is performed at 40°C, the cost of using this enzyme could be reduced by up to 50%. The study further proposed that for lignocellulose hydrolyses, since the enzymes are more expensive and require a longer reaction time, an increase in fermentation temperature is most certainly a reasonable option using SSF. Other advantages highlighted by this study also include a lower risk of contamination by other microbes at higher temperature, minimising yeast cell death due to temperature fluctuations and the possibility of

separating ethanol by procedures such as vacuum extraction. They reported that vacuum extraction of ethanol was efficiently achieved at 40°C but not at 35°C suggesting that high-temperature fermentations may help to make the simultaneous fermentation and ethanol extraction process more suitable for fuel ethanol production. A system whereby ethanol can be removed from the vessel will be most desirable for microorganisms with high concentration intolerance for ethanol. It is estimated that 4% of ethanol can be removed by evaporation from a 10% ethanol system in 6 hours at 50^0C (Taylor et al. 1995; Cardona and Sánchez 2007).

Several thermotolerant microorganisms (*S. cerevisiae, K. marxianus* and several other yeast strains) that can be used at high temperatures of 40^0C to about 55^0C have been screened (Ballesteros et al 1991, Oliva et al 2004). Several thermophylic bacteria such as *Bacilus stearothermophilus, Lactobacillus paltarum* and *Geobacillus thermoglucosidasius have shown* promise in producing ethanol at high temperature (Narumi et al 1997, Liu et al 2006, Cripps et al 2009). A genetically modified bacterium *Geobacillus thermoglucosidasius NCIMB11955* has been reported to have the ability to ferment sugars to ethanol at 55-65^0C (Cripps et al 2009).

K. marxianus is believed to have the best performing yeast in terms of growth and fermentation at high temperature. It is reported that several thermotolerant strains of *K. marxianus* grow well at temperatures between 45^0C and 52^0C and ferment ethanol at temperatures between 38^0C and 45^0C (Abdel-fattah et al. 2000; Oliva et al. 2004, Nonklang et al. 2008; Suryawati et al. 2008).

Several researchers have studied the bioethanol potentials of *K. marxianus* as well as other genetically modified strains of this yeast. When compared with *Saccharomyces cerevisiae* at temperatures above 40^0C, *K. marxianus* has been reported to produce and grow better with a higher ethanol yield from different sugars. It however, was believed to have a lower ethanol tolerance (Hacking et al 1984*). K. marxianus* was reported to have maximum growth at 47^0C (Anderson et al. 1986) or 52^0C (Banat et al. 1992). *K.marxianus* is also reported to produce ethanol from galactose and xylose (Schwan and Rose 1994, Margaritis and Bajpai

1982).In terms of quantifying ethanol yields, Studies have reported low to high yield of ethanol using different strains of *K. marxianus*. *The Kluyveromyces marxianus IBM3* is reported to have high yield of ethanol when compared to its parent strain *K. marxianus*. A summary of a few papers is given in the table 1.1 below.

Substrate	**Microbe**	**Ethanol Concentration**	**Maximum Ethanol**	**Fermentation System**	**Reference**
Glucose medium (140 –160 g/L)	*Kluyveromyces marxianus*	_	51.0 (% theoretical yield)	Batch Fermentation at 45^{o}C	Hughes et al. 1984
Diluted Molasses medium (140 – 160 g/L total sugars)	*Kluyveromyces marxianus*	_	51.9 (% theoretical yield)	Batch Fermentation at 45^{o}C	Hughes et al. 1984
Diluted Molasses 200 g/L total sugars	*Kluyveromyces spp.*	44.14-50.54g/l	_	Batch fermentation at 43^{o}C	Saigal,1994
Glucose Medium (10%w/v)	*Kluyveromyces marxianus IBM3*	59g/l	_	Batch fermentation at 45^{o}C	Banat et al. 1992
5%w/v Recycled paper derived material	*Kluyveromyces marxianus CECT 10875*	18g/l	80%theoretical Yield	Simultaneous Saccharification and Fermentation (SSF) at 42^{0}C	Ballesteros et al. 2002
5%w/v cellulose from paper waste and 0.75% cellulase	*Kluyveromyces marxianus IBM3*	10g/l	75% theoretical yield	SSF at 45^{0}C	Baron et al 1995

Table 1.1: Table showing a summary of papers experiments carried out with *Kluyveromyces marxianus* showing ethanol yields

In this study, the simultaneous saccharification and fermentation (SSF) of cassava peels by *K. marxianus* at 40^0C was carried out. Another modified method was also studied whereby the microorganism was introduced after 24 hours of enzyme hydrolysis using both amylases and cellulose enzymes. The study also compared it with a study of a separate hydrolysis and fermentation process (SHF).

1.8.0: OBJECTIVES OF RESEARCH AND OUTLINE OF THESIS

This research is focused at developing a strategy for enzymatic degradation of cassava peels by using a cocktail of amylases, cellulases, hemicellulase and glucoamylase enzymes to completely degrade the carbohydrate component of cassava peels.

The study compared the effect of different concentrations of enzymes, different combination of the enzymes, substrate concentration and monitored the sugars produced as the reaction progressed

This research also focused on the substrate property like the degree of polymerisation with an aim of having a better understanding of the chemistry that occurs with respect to the substrate.

The study also compared steam exploded pre-treated samples, hot water pretreated and milled samples while looking at the sugar release patterns of the hydrolysis reaction.

Finally, we sought to integrate the hydrolysis reaction with the fermentation reaction to streamline the entire process using simultaneous saccharification and fermentation (SSF) with *K. marxianus at* 45^0C

This thesis has been organized into distinct parts although most are intertwined and related. Chapter two describes the methods used throughout the experiments. Chapter three deals with the separate hydrolysis of cassava peels; the enzyme hydrolysis by amylases is done separately and then the hydrolysis of cellulases is carried out as well.

Chapter four describes the simultaneous hydrolysis of both starch and cellulose at the same time by a combination of amylases, cellulases, hemicellulase and glucoamylase enzyme.

Chapter five then presents the consecutive hydrolysis whereby the hydrolysis of starch by amylases is followed by the hydrolysis of cellulose by cellulases. Another alternative investigated was the effect of hydrolysing the cellulose first before hydrolysing the starch. Hot water pretreatment was also treated in depth in chapter five.

Chapter six describes the combination of hydrolysis and fermentation.

CHAPTER TWO: MATERIALS AND METHODS

2.1 MATERIALS USED: EQUIPMENTS AND REAGENTS USED

Cassava peels were obtained from a local farm in Makurdi, Nigeria. These were dried, stored and then transported.

Enzymes: The enzymes used α-amylase, glucoamylase, cellulase and a hemicellulase cocktail were a generous gift from Novozymes A/S Denmark (detailed information on the enzymes are given below).

The reagents used in these experiments were of analytical grade. There were either purchased from Sigma Aldrich. A complete list of equipment used are listed in appendix A

2.2 SAMPLE PREPARATION

Cassava peels cultivated and harvested from a farm in Makurdi Nigeria were sun dried for seven days and transported. The sun dried peels were prepared for enzyme hydrolysis by size reduction using a kitchen blender; A Kenwood BL450 with a grinding mill attachment. The milling condition involved loading 40g of the peels into the mill jar and milling the peels for 3 minutes at room temperature of 25^{o}C into a powdered form with an approximate size of 60-450um.

The milled samples were then washed with water to remove soil contaminants, non-structural sugar, fertilizers and other soluble materials associated with biomass waste. The washing was done by placing 10g of cassava peels in 40ml of water in a 50 ml centrifuge tube. The sample in the centrifuge tubes is then mixed for 10 minutes using a Stuart rotator at 40rpm for 30 minutes. The centrifuge tubes were then transferred to the Beckman Coulter Centrifuge. The samples were centrifuged at 2880 x g for four minutes. The contents were then decanted and the process repeated twice making the sugar concentration in the supernatant drop below 1 g/l. A total of three washes gave a relatively clear liquid as supernatant. The cassava peels were then transferred to Petri-dishes and dried in Gallenkamp 0ven at 50^{0}C

for 24 hours. Moisture content determination is then carried out to make sure the moisture content is not above 10%.

Sampling for analysis or hydrolysis was quite tricky and care was taken to use representative sampling to collect samples for analysis or hydrolysis. Samples were mixed properly and usually collected from different batches of cassava peels that were milled at different times to create subsamples. Samples from different batches of milling were mixed in 50ml centrifuged tubes. The centrifuge tubes are filled halfway with the milled samples and gently rolled at roughly 2 rotations per second to ensure even distribution of particles of different sizes. This method minimised the stratification of the powdered cassava peels. Sampling was found to very critical to results obtained as it was discovered to have an adverse effect on reproducibility of the hydrolysis rate and yield. Random samples from one milling set should not be taken as it affects the reproducibility of results. Samples with large difference in sizes also make reproducibility difficult.

2.3 MOISTURE CONTENT DETERMINATION

Cassava peels can contain varying amount of moisture that change quickly when exposed to air and so the moisture content determination is vital for reproducible results for samples. This procedure is used to determine the amount of total solids remaining after drying at 105^0C.

Petri dishes were pre-dried by being placed in a Gallenkamp Oven at 105^0C for 4 hours and then cooled in a desiccator. They were then weighed to the nearest 0.1 mg and recorded. 10g of cassava peels sample prepared by the sample preparation method as described in 2.2 above were then transferred to the Petri dish, weighed and recorded.

The Petri dish with the sample were then transferred to the oven and dried to a constant weight. This was done in triplicate and the mean value determined. The moisture content determination was also tested on the steam exploded sample.

$$\% \text{ total solids} = \frac{\text{weight of dry Petri dish + sample} - \text{weight of Petri dish}}{\text{Weight of sample}} \times 100$$

% moisture content=100 - weight of dry Petri dish + sample –weight of Petri dish X 100

Weight of sample

The moisture content results are presented in the next chapter.

2.4 DETERMINATION OF THE COMPOSITION OF CASSAVA PEELS

Samples of cassava peel were sent to Ciemat (Centre for Energy Research and Technology) in Madrid Spain for compositional analysis of the peels. The National Renewable Energy Labouratory analytical protocols were used for the determination of starch, lignin, hemicelluloses and other components of the peel. The amount of starch present in the peel was determined by the Megazyme Total Starch assay procedure (amyloglucosidase/α-amylase method) listed as AOAC method 996.11 and uses HPLC for glucose analysis. The cellulose and hemicellulose content were estimated as follows. 0.3g of the cassava peel placed in a pressure tube was hydrolysed by 72% sulfuric acid for 60 minutes. The sample was then diluted to about 4% sulfuric acid by deionized water and then the sample is autoclaved at 121^0C for 60 minutes. The filtrates are collected by vacuum filtration. The hydrolysate was then analysed by HPLC using Biorad Aminex HPX-87P column. Detection was with a refractive index detector with a mobile phase of 0.005N sulfuric acid run at 0.6ml/min. Calibration curves of glucose, xylose, galactose, arabinose and mannose were used to estimate the cellulose and hemicellulose content in the peels. UV Visible microscopy was used to estimate the lignin content. The acetyl content is also determined using acetic acid as standard. The residue is washed by deionized water and placed in a crucible. The ash content is determined by burning in a furnace at 575^0C for 24 hours and the mass of the cassava residue recorded. Further information on this procedure is found on www.nrel/analyticalprocedures.com. This analysis was done after the peel was extracted with water and starch digestion carried out. The composition of cassava peels is presented in table 2.1 below.

Cassava peel waste composition (% on dry weight basis)

	Starch	Cellulose	Hemicellulose[1]	Lignin		Ash	Extractives	Acetyl groups	Others	Total
				Acid-insoluble	Acid-soluble		(other than starch)			
Mean	28.0	23.9	9.4	22.9	1.1	7.4	5.3	0.4	1.6	100.0
Standard deviation	*1.4*	*0.9*	*0.8*	*1.1*	*0.1*	*0.2*	*0.1*	*0.1*		

[1] Xylan	4.1
Galactan	3.0
Arabinan	1.8
Mannan	0.5
Total hemicelluloses	9.4

Table 2.1*:Table showing cassava peel composition.*

The total carbohydrate from the results gave 61.3%.

2.5.0 PROCEDURE FOR THE ENZYME HYDROLYSIS OF CASSAVA PEELS

There are very few papers on enzyme digestion of cassava peels and so finding an appropriate and widely used method for enzyme digestion of cassava peels at this point was a challenge. Most of the papers available do not give detailed information on the procedure used and so vital information like sampling techniques, appropriate substrate concentration or optimal enzyme dosages vary widely in literature. The lignocellulose biomass hydrolysis and fermentation SSF experimental protocol; an analytical protocol developed by the National Renewable Energy Laboratory in the United States was modified for the digestion of cassava peels.

The enzymes used are;

i. Liquizyme SC DS – An alpha amylase with declared activity of 240 KNU-S/g from *Bacillus licheniformis* (KNU-S Alpha amylase Unit)

ii. Spirizyme Fuel HS-A glucoamylase with declared activity of 1425 AGU/g from *Aspergillus niger* (AGU; amyloglucosidase unit)

iii. Viscozyme Cassava R: A product containing a mixture of hemicellulase, cellulase and xylanase with a declared activity of 100 FBG/g(where FBG

is betaglucanase units) from *Aspergillus aculeatus* which would be referred to as cassava R

iv. Viscozyme Cassava C – a cellulase enzyme from *Trichoderma reesei* with a declared activity of 700 EGU/g where EGU is Endoglucanase activity.

The digestion was carried out in a 250ml erlenmeyer flask covered with aluminium foil. 0.05M sodium acetate at different pH ranging from 4 to 6 was used as a buffer. The experiments were carried out at an incubation temperature of 50^0C using a Grant GLS 400 water bath incubator with shaker at 220 strokes per minute. The Grant GLS shaker provides a linear shaking motion. The reaction vessels were at a depth of 50mm and a stroke length of 18mm.

The experimental design involved the investigation of different variables. These include varying different enzyme concentrations, different substrate concentration, different temperatures and pH, varying the combination of the four enzymes and investigating the effect of pretreatment on hydrolysis.

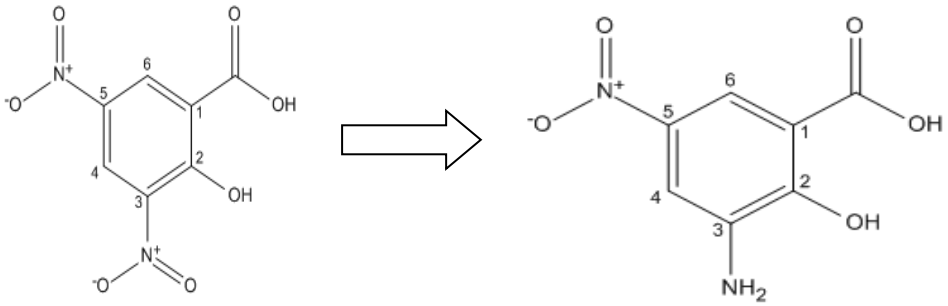
A simple description of the process involves taking a cassava peel sample that had been prepared by the sample preparation method described in section 2.2 above and suspending the cassava peel in 0.05M sodium acetate buffer at a desired pH to get a desired substrate concentration of interest. The cassava peels is then equilibrated to the desired temperature (40-60^0C) using a Stuart Heat Stir CC162. The pH is then adjusted appropriately using the SevenGo pH meter SG2 as the cassava peels is known to be slightly acidic. The calibration of the pH is done at the temperature of the enzyme hydrolysis. The sample in the 250 ml Erlenmeyer flask is then transferred to the water bath at the desired temperature. The enzymes are then loaded to start the reaction. For each batch of experiments, each experiment is started giving a 5 minute interval for sampling to take place.

The enzyme hydrolysis samples were taken at time 0, 5 minutes,10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 24 hours , 48 hours and 72 hours . 2 ml samples are collected in 2 ml eppendorf tubes and centrifuged by an eppendorf 5415 D Bench Top Centrifuge at 13200 x g for 3 minutes. These samples from the supernatant were then analysed either by DNS assay or in some

experiments by both DNS assay and HPLC assay. For the HPLC assay, 0.1M HCl was used to stop the reaction. More information on sampling is presented in the next section.

2.5.1 DINITROSALICYLIC ACID (DNS) ASSAY FOF REDUCING SUGAR MEASUREMENT

The dinitrosalicylic colourimetric method (Miller, 1959) for the determination of glucose was used to test for the presence of free aldehyde group or the reducing sugar as they are called. This involves the oxidation of the aldehyde functional group present in the sugars. Simultaneously the 3,5-dinitrosalicylic acid is reduced to 3-amino- 5-nitrosalicylic acid under alkaline solution.



The presence of the carbohydrate reducing sugar is detected by the spectrophotometric measurement of dinitrosalicylic acid reduction to 3 amino 5 nitrosalicylic acid which displays an absorbance at 575nm. The DNS reagent was prepared by mixing 10 g dinitrosalicylic acid, 10 g sodium hydroxide, 2 g crystalline phenol, 0.5 g sodium sulphite in 1 litre of water. A second solution of 40% w/v sodium potassium tartrate was also made to stabilize the colour developed after heating the DNS reagent. Fresh sodium sulphite solution of 0.5 g/L was always added to the reagent at the time the assay is to be carried out. This is because of the possible atmospheric oxidation of the sulphite present. Its function is to remove dissolved oxygen from aqueous solutions. Glucose solutions of concentration ranging 0.1 g/l to 0.5 g/l were prepared for the dinitrosalicylic assay. 3 ml of the standard solutions were mixed with 3ml of the dinitrosalicylic reagent in a sample bottle and closed. The mixture is heated with a Techno block heater at

100^0C for 15 minutes to develop a red brown colour. 1 ml of 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the colour. The mixture was then cooled to room temperature and the absorbance read with the Beckman Coulter DU 800 UV-Vis Spectrophotometer at 575nm. A calibration curve is then plotted and used for the hydrolysis sample. An example calibration curve is shown in Appendix B.

Hydrolysis samples taken at various times and centrifuged as described above were also analysed with the DNS reagent. To get representative samples, the reaction was mixed vigorously before samples are taken. Samples taken ranging from 20 ul to 100 ul of the supernatant sample is then diluted to 3 ml with water and then 3 ml of the DNS reagent added to stop the reaction. Samples were analysed either immediately or sometimes left after being mixed with DNS reagent. Results showed that analysis done even after four hours didn't show any difference in glucose concentration. However, samples left overnight showed slight reduction in reducing sugar yield. Consequently, most analysis was done within 3 hours of mixing with DNS reagent.

The mixture is heated with the Techno block heater at 100^oC for 15 minutes. 1 ml of the 40%w/v Rochelle salt (potassium sodium tartrate) is then added to the mixture. It is then allowed to cool to room temperature and the absorbance read at 575nm. The absorption is read off the calibration curve and the concentration of the reducing sugar determined.

$$\text{Dextrose Equivalent (DE)} = \frac{\text{g reducing sugar expressed as glucose}}{\text{g dry solid weight of cassava peels}} \times 100\%$$

The dry solid weight was based on the amount of cassava peel used with the particular experiment.

Time points in these experiments are represented by the time the samples are taken. However, in addition to this time, time taken to centrifuge and stop the reaction gives approximately 5 more minutes. For example, if samples are taken at 5 minutes. It would take 3 minutes to centrifuge and roughly 2 minutes to add DNS reagent or 0.1M HCl solution (for Hplc assay) to stop the reaction. Samples at zero

time points were also taken before and after enzymes were added to the reaction. There was however, no difference in hydrolysis yield between these two time points. Subsequently zero time points were taken immediately after enzyme is added.

2.5.2 DETERMINATION OF SUGARS IN LIQUID FRACTION OF HYDROLYSIS BY HPLC

HPLC was used to quantify sugars in the liquid fraction of the hydrolysate. A size exclusion chromatographic column KS 801 was used to monitor the release of oligosaccharides as the reaction progresses. The KS 801 column is also capable of separating saccharides by ligand exchange.

Glucose, maltose and cellobiose purchased from Sigma Aldrich were used as standards. Glucose solutions with concentration range of 2g/l to 20g/l were prepared. Cellobiose and maltose solutions of 1g/l to 20g/l concentrations were also made. Where the solutions were not used immediately after they were made, they were stored in a freezer and removed when needed. They were then thawed and vortexed prior to use. The frozen standards were stored for a maximum of seven days.

Glucose, maltose and cellobiose were analysed by the Waters 2695 HPLC system using a Shodex sugar KS 801 column fitted with a Shodex guard column under the following conditions;

Sample volume -10ul

Mobile Phase HPLC water

Flow rate 1ml/minute

Column Temperature 60^0C

Run time: 15 minutes

Detection was by a Waters 410 refractive index detector.

Calibration curves were obtained from the results of the analysis. Examples of these calibration curves are presented in Appendix B.

The hydrolysis samples taken at time 0, 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 24 hours , 24 hours and 72 hours were used.

2 ml aliquot samples collected in a 2ml eppendorf tubes and 0.1 ml of 1M HCl was added to inhibit the hydrolysis reaction. Experiments conducted in the absence of HCl showed the absence of oligosaccharides of DP 3 and above which were possibly hydrolysed further into glucose. However experiments carried out with 1M HCl showed that the reaction was stopped by 0.1ml of HCl.

The supernatant was then centrifuged by an Eppendorf 5415 D Bench Top Centrifuge at 13200 X g for 3 minutes. The supernatant was then collected and stored in a freezer awaiting HPLC analysis. The samples were thawed and vortexed when needed. They were transferred into autosampler vials and the analysis done by the Waters 2695 Hplc system using a Shodex sugar KS 801 column fitted with a Shodex guard column. The conditions as stated earlier remained the same.

2.6.0 STEAM EXPLOSION PRETREATMENT OF CASSAVA PEELS

The steam explosion of the peels was done by CIEMAT and the machine used for the pretreatment is made up of three units: a steam accumulator, a steam explosion reactor and a discharge cyclone. The steam accumulator supplies steam at a temperature of 210°C to the steam explosion reactor. The steam explosion reactor is the chamber where the lignocellulosic biomass is compressed and suddenly de-pressurised. It consists of a 3" diameter stainless steel 316 vertical pipe, limited by two 3" diameter stainless steel 316 throttle valves. The input valve on the top of the chamber opens and closes by hand and is used to load the ground lignocellulosic biomass in the reactor. The output valve on the bottom of the chamber opens by a triggering and spring device in less than 1 second. The mixture of steam and biomass is thus discharged violently, and passes through a pipe that carries it to the cyclone. The discharge cyclone is built of stainless steel 316. The pretreatment was carried out for 5 minutes.

2.7.0 PROCEDURE FOR THE HOT WATER PRETREATMENT OF CASSAVA PEELS

Cassava peels were also pretreated with liquid hot water. This was done sometimes in the presence of the amylase enzymes. The hot water pretreatment was carried

out in a 250ml Erlenmeyer flask covered with aluminium foil and held with a rubber band. 0.05M sodium acetate at different pH ranging from 4 to 6 was used as a buffer. The experiments were carried out at an incubation temperature of approximately 100^0C.

The experimental design involved the investigation of different variables. These include varying different enzyme concentrations, different pH, varying the combination of the four enzymes and investigating the effect of pretreatment on hydrolysis.

5g cassava peel sample that had been prepared by the sample preparation method described in section 2.2 above was weighed and suspended in 50ml 0.05M sodium acetate buffer. The Erlenmeyer flask, cassava peels and the enzymes are then weighed again. The pH is then adjusted appropriately using the SevenGo pH meter SG2 as the cassava peels is known to be slightly acidic. The Erlenmeyer flask is then transferred to the water bath with an incubation temperature of 99^0C. The Grant GLS 400 Water bath incubator was set to 220 strokes per minute for 2 hours. The Grant GLS shaker provides a linear shaking motion. The reaction vessels were at a depth of 50mm and a stroke length of 18mm.

After 2 hour incubation, the sample is allowed to cool down and weighed. After which deionized water is added to make up for the lost water due to evaporation.

Supernatant samples were again centrifuged and analysed as described in section 2.5.1 above.

Further enzyme hydrolysis of the samples is carried out at different temperatures as described in section 2.5.0 above.

2.8.0 PROCEDURE FOR THE INSOLUBLE SOLID ANALYSIS OF CASSAVA PEELS

The reducing sugars present in the Insoluble solid as hydrolysis progresses was analysed. A modified DNS assay was used to analyse the chain end concentration. These experiments were carried out on a much lower scale.

0.25g of finely grounded cassava peels was suspended in 5ml 0.05M sodium acetate buffer at pH 5 in a 15ml Fisherbrand centrifuge tubes. 40ul of both amylase and

glucoamylase enzymes were used to treat the starch component of the peels for 24 hours. The Fisherbrand centrifuge tubes were incubated in a Stuart SB3 rotator and placed in a Stuart S160 incubator. The mixing speed of the rotator was set at 40 rpm and temperature set at 50^{o}C. The peels were then washed several times with 0.05M sodium acetate to remove all monosaccharides present in the liquid and associated with the residual cellulose. The washing was done by centrifuging with the Beckman Coulter centrifuge and decanted using a pipette. The residual solid is then resuspended in 1.5ml sodium acetate buffer in a 2ml eppendorf tube and treated with either Viscozyme Cassava C or Cassava R enzymes (see section 2.5.0) for different times. Samples were then washed again with the buffer and frozen with liquid nitrogen. The samples were then freeze dried using a VWayirtis benchtop K freeze dryer. The samples were freeze dried for 24 hours after which they were transferred to a weighing boat and weighed.

These experiments were carried out for 3 minutes, 5 minutes, 10 minutes, 30 minutes, 1 hour, 2 hours, 3hours and 24 hours. There were done in duplicates.

The dried samples were then assayed with a DNS Assay for insoluble solids (Wood and Bhat 1988, Ghose 1957)

2.8.1 DINITROSALICYLIC ACID ASSAY FOR INSOLUBLE SOLIDS

The DNS reagent which was prepared as described in section 2.5.1 was used to prepare the standards. However the glucose standards used for this assay were 0.5g/l, 1g/l,1.5g/l and 2g/l. 3ml of these standards were then diluted with 3ml of DNS reagent. The mixture was then heated and mixed with 40% w/v Rochelle salt (potassium sodium tartrate) as described in 2.5.1. It is then allowed to cool to room temperature and diluted 1 in 4 parts and the absorbance read at 575nm.

The calibration was then plotted from the results.

The solid samples from the hydrolysis experiments which have been freeze dried and weighed are suspended in 3 ml of water and allowed to stand for about an hour after which the suspension is mixed with 3ml of DNS reagent. Again, the mixture was heated and diluted in the same way as the standards and absorbance read at 575nm.

$$\text{Dextrose Equivalent (DE)} = \frac{\text{mole of reducing sugar expressed as glucose}}{\text{g dry solid weight of cassava peels}} \times 100\%$$

The DNS assay was modified for solid samples by diluting the samples after heating and mixing with the Rochelle salt solution. It was discovered that 1 in 4 dilutions of the samples could fit into the calibration curve used.

It should also be noted that dextrose equivalent in these experiments were estimated based on expected cellulose and hemicellulose component of the cassava peels. This is because the dextrose equivalent is used in this experiment to estimate the average degree of polymerisation of the cellulose and a more realistic result would be to base the dextrose equivalence on the cellulose and not on the weight of the cassava peels. More information on the assumptions and calculations done are treated fully in chapter 3.

2.9.0 PROCEDURE FOR THE HYDROLYSIS AND FERMENTATION OF CASSAVA PEELS

The procedure for both hydrolysis and fermentation of cassava peels involved two steps. The first being hydrolysis and then a fermentation step with *Kluyveromyces marxianus* CBS712 (NCYC 2791). In this procedure, 3 different approaches were adopted. A separate hydrolysis and fermentation, a simultaneous hydrolysis and fermentation where the enzymes and microorganism were inoculated at the same time and a third approach that involved the inoculation of the microorganism after 24 hours of hydrolysing the cassava peels.

2. 9.1 PREPARATION OF MEDIA, ENZYMES AND YEAST

The composition of the YPD agar and YPD broth were as follows

YPD Agar

-1% w/v Yeast Extract

-2% w/v peptone

-2% w/v Glucose

-2% w/v Agar

YPD Broth

-1% w/v Yeast Extract

-2% w/v peptone

-2% w/v Glucose

The pH of these media was adjusted to 5 with 1M HCl and 1M NaOH. Several 100 mL broth were prepared in 250 mL flask and the media was then autoclaved. The agar was then poured into petri dishes under a lamina flow hood. The enzymes used (amylases, glucoamylase and the cellulase enzymes from section 2.5.0) for hydrolysis were all filter sterilized before use.

Kluyveromyces marxianus CBS712 (NCYC 2791) was bought from the NCYC (National Collection of Yeast Culture). An initial inoculum of the yeast was prepared from the frozen stock culture. Several 250ml flask were used to prepare the initial inoculum from stock culture by transferring the culture into 100 mL of YPD media in a sterile 250 mL flask. The yeast was then incubated in a rotary shaker at 45^0C and for 48 hours and 150rpm. The yeast was then transferred on to the agar plates and grown again at 45^0C in a rotary shaker at 150rpm for 48 hours.

Several 2 mL cryogenic vials were also prepared with 0.45ml of glycerol and autoclaved for 30 minutes at 121^oC. A single colony from the agar plates of *K.marxianus* was then used to inoculate several 250 mL flask with 100mL YPD broth and incubated at 45^0C and 150rpm for 12hours. Under the lamina flow hood, 1.05mL of the *K.marxianus* was then transferred from the 100ml YPD broth into the each cryogenic vial (70% yeast culture and 30% glycerol). The vials are then stored in the -70^0C freezer. Each vial is expected to have a standardized number of cells. One vial was thawed and a cell count performed using a hemacytometer to determine the number of cells contained in the vial. The pH of the broth, glucose concentration and ethanol concentration were noted.

2.9.2 PREPARATION OF SEED CULTURE FOR FERMENTATION EXPERIMENTS

An aerobic fermentation of glucose was used to produce yeast cell mass. This involved a preparation procedure that occurs in two growth stages. The first stage, pre-inoculum, is a flask in which the frozen stock culture, containing a standardized number of cells, is inoculated into YPD (liquid medium). This stage eases the yeast in its transition from stasis to growth phase. The growth phase occurs in a second flask which contains YPD and is inoculated from the first.

In these experiments, one thawed stock vial of *K. marxianus* was inoculated into 50 mL of sterile YPD in a 125 mL Flask (pre-inoculum) and then incubated at 45^0C and 150rpm for 6 hours.

To prepare the second stage flask, 10ml of the pre-inoculum flask is transferred into 90ml sterile YPD broth in a 250ml Flask. The second flask is then incubated at 45^0C and 150rpm for 16 hours. This second flask is the seed culture for fermentation. The inoculations are done under the laminar flow hood. The cultures were always examined under a microscope for microbial growth.

2.9.3 FERMENTORS: BRAUN BIOSTAT Q BIOREACTOR

The Braun Biostat Q Bioreactor (Sartorius Stedim Bioreactor Aubagne Cedex France) is a multi-fermentor system designed for reproducible serial experimentation. The Biostat® Q makes the transition from shaker flasks to stirred tank fermenters simple. It has four 1L bioreactors three of which were used for these experiments. These three bioreactors are autoclavable glass vessels equipped with sensors (temperature, pH and dissolved oxygen- DO), sparger, filters and addition ports. . The supply unit includes peristaltic pumps for acid and base and gas flowmeters. It has a removable vessel tray on top of the drive console which eases handling. The Biostat® Q supports the on-line measurement and control of agitation, temperature, pH and dissolved oxygen. It employs a DCU (*D*igital *C*ontrol *S*ystem) which is a local control system specially tailored to bioprocess (fermentation/cell culture) automation. The DCU 3 is operated using a graphical interface on a flat panel touch-screen. The Standard Biostat Q automation functionality includes

process measurements, calibration routines, and a set of control loops for temperature, pH and dissolved oxygen via stirrer speed.

Three one-Liter Biostat Q Bioreactors were used for the hydrolysis and fermentation of cassava peels using the yeast strain *K.marxianus* at 45^{o}C and at a stirred speed of 300 rpm. The nitrogen gas tank was connected through an autoclaved 0.2 µm filter used to sterilize the nitrogen before reaching the autoclaved cassava media in the vessels. The experiments were done under anaerobic conditions. The nitrogen gas line was set on 1 vvm while the air pump line was off.

The Standard Biostat Q automation was used to record pH, temperature, stir speed, and D.O. (dissolved oxygen). The three D.O. probes were placed in each of the Bioreactor vessels with the cassava peel media. The three pH probes were calibrated before autoclaving the vessels, while the D.O. probes were polarized for about two hours and then calibrated after being autoclaved. After this, the calibration of the probes was completed. The nitrogen gas was turned on at 1 vvm until the vessels' environment was completely anaerobic. Nitrogen gas was then turned down to 0.5 vvm for the experiments. Appendix C shows a picture of the reactor.

2.9.4 PROCEDURE FOR THE SEPERATE HYDROLYSIS AND FERMENTATION OF CASSAVA PEELS

In this method, the cassava peel is hydrolysed first and then the supernatant separated from the residue by centrifuging and filtration after which it is then fermented with the yeast.

Cassava peels of 10%w/v concentration were used for the hydrolysis experiments. 50g of cassava peels in suspended in 500mL of deionized water in the bioreactor. The pH probe of the bioreactor is calibrated. The DO is also calibrated. The pH, temperature and stirring speed of the bioreactor are then set to pH 5, 50^{o}C and 300 rpm.

Amylases, glucoamylase, Cassava C (cellulase enzymes) Cassava R (cellulase and hemicellulase) were diluted in 1:10 ratio. 18 mL of each enzyme was added aseptically to the cassava peel suspension in the bioreactor to start the hydrolysis

part of the experiments. The enzymes were diluted because it was easier to filter sterilize the diluted enzyme compared to the undiluted enzymes. The experiments were run for 48 hours and 4mL samples were taken at 0 hours, 3 hours, 24 hours and 48 hours. After 48 hours, the hydrolysate was centrifuged and filtered. The filtrate was then used for the fermentation experiment. For the fermentation experiment, yeast peptone (YP) media was also prepared to be added to the fermentation media. The yeast peptone media was made up of 10%w/v yeast extract and 20%w/v peptone. The pre-inoculum and seed culture of the *K. marxianus* yeast stain was also prepared as described above.

The yeast extract is added to the filtrate in the bioreactor. The pH of the reactor is then calibrated and then the bioreactor is autoclaved. The pH probes of the bioreactor are then calibrated and the bioreactor is then autoclaved for 30 minutes. After the autoclaving, the reactor is allowed to cool down and then set up. The pH is also allowed to equilibrate. The temperature is set to 45^{o}C and the bioreactor sparged with nitrogen at 1 vvm as described earlier. The DO probes are also calibrated. The reaction is started by aseptically adding the seed culture of the *K.marxianus* strain by the use of a sterilized syringe through the additional ports on the vessel top. 4 mL Samples are taken aseptically by a sterilized syringe at 0 hours, 3 hours, 6 hours, 24 hours, 48 hours and 72 hours. These samples are then centrifuged, filtered and stored and frozen until they are analysed.

The experiment was done in duplicate.

The analysis is done by the HPLC (details shown below)

2.9.5 PROCEDURE FOR THE SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF CASSAVA PEELS

In this procedure the hydrolysis by enzymes and fermentation by *K. marxianus* take place simultaneously. A minor variation of this method is hydrolysing the cassava peels with enzymes and then inoculating the microorganism after 24 hours of saccharification. One advantage of this method is that one vessel is used for both hydrolysis and fermentation. The glucose inhibition of cellulases is also prevented as glucose is being metabolised by the yeast cells. The National Renewable Energy

Laboratory protocol Lap 008 which is titled the simultaneous saccharification and fermentation was modified and used for this experiment (www.nrel.com/lap008) Substrate concentration of 5%w/v and 10%w/v were used for these experiments. Amylases, glucoamylase, Cassava C (cellulase enzymes) Cassava R (cellulase and hemicellulase) were diluted and filter sterilized. A yeast peptone media was also prepared to be added to the fermentation media. The yeast peptone media was made up of 10%w/v yeast extract and 20%w/v peptone. The pre-inoculum and seed culture of the *K.marxianus* yeast stain was also prepared as described above. An example recipe used for these experiments is shown below.

Flask 1

Cassava peels	60g
Yeast Peptone Media	60ml
(Amylase , Glucoamylase	
Cassava C and Cassava R)	72mL
Innoculum	60mL
DI water	348mL
Total	**600g**

Another example where the Cassava Peel concentration is lower is shown below.

Flask 2

Cassava peels	32.38g
Yeast Peptone Media	60ml
(Amylase, Glucoamylase	
Cassava C and Cassava R)	72mL
Innoculum	60mL
DI water	375mL
Total	**600g**

The Recipe in flask 2 is the NREL recommended total cellulose content of 3%w/w. Here the 3% w/w cellulose content was used as 3%w/w carbohydrate content as cassava peels has both starch and cellulose. The cassava peels is first suspended in deionized water. The pH probes of the bioreactor are then calibrated and the bioreactor is then autoclaved for 30 minutes. After the autoclaving, the reactor is allowed to cool down. The pH is also allowed to equilibrate. The temperature is set to 45^{o}C and the bioreactor sparged with nitrogen at 1 vvm as described earlier. The DO probes are also calibrated. The reaction is started by aseptically adding the enzymes and the seed culture of the *K.marxianus* strain by the use of a sterilized

syringe through the additional ports on the vessel top. 4 mL Samples are taken aseptically by a sterilized syringe at 0 hours, 3 hours, 6 hours, 24 hours, 48 hours and 72 hours. These samples are then centrifuged, filtered and stored and frozen until they are analysed.

The analysis is done by the HPLC as shown in the next section.

2.9.6 HPLC ASSAY OF FERMENTATION MEDIUM

HPLC was used to quantify glucose and ethanol in the liquid fraction of the fermentation medium. A REZEK ROA organic acid (150mm) column was used to monitor the fermentation reaction.

Glucose and 99.5% ethanol purchased from Sigma Aldrich were used as standards. Glucose solutions with concentration range of 1.8 g/l to 36g/l were prepared. Ethanol solution of 0.46g/l to 9.4g/l concentrations was also made. Where the solutions were not used immediately after they were made, they were stored in a fridge and removed when needed. They were then vortexed prior to use.

Glucose and ethanol were analysed by A Gilson 234 HPLC system using a REZEK ROA organic acid column fitted with a guard column under the following conditions;

Sample volume -10ul

Mobile Phase-0.005N Sulphuric Acid (H_2S0_4)

Flow rate – 1ml/minute

Column Temperature 60^0C

Run time: 15 minutes

Detection was by a Viscotek VE 3580 refractive index detector.

Calibration curves were obtained from the results of the analysis. Examples of these calibration curves are presented in Appendix B.

The fermentation samples that has been centrifuged, filtered and stored in a fridge are then analysed by the Gilson 234 HPLC system using the conditions as stated above. Detection was by a Viscotek VE 3580 refractive index detector.

Glucose and ethanol yield was calculated as follows:

$$\% \text{ Yield Glucose} = \frac{(\text{Glucose}_f)}{1.111F\ (\text{Mass of peels})} \times 100$$

Where $Glucose_f$ is the mass of residual glucose at the end of reaction

F is the carbohydrate fraction of the cassava peels

1.111 represents the addition of one molecule of water in the conversion of either starch or cellulose to glucose.

$$\% \text{ Yield of Ethanol} = \frac{(EtOH_f) - (EtOH_0)}{0.568F \text{ (Mass of peels)}} \times 100$$

0.568 represents the total ethanol to be possibly gotten from the biomass by yeast.

$EtOH_f$ – total ethanol at the end of reaction in gram

$EtOH_0$ Total ethanol at time zero

F is the carbohydrate fraction of the cassava peels

CHAPTER THREE :RESULTS AND DISCUSSION-SEPARATE HYDROLYSIS

3.0:0 OVERVIEW

A practical strategy for hydrolysing cassava peels that involves a separate hydrolysis of starch and cellulose was investigated and results discussed in this chapter. One advantage of this approach would involve treating amylases and cellulases at their optimum performing conditions (pH and temperature).

An obvious advantage of separate hydrolysis is that after an initial starch hydrolysis, the peels can be further treated with cellulases to hydrolyse the cellulose and hemicellulose components of the peels. Both sets of hydrolysis taking place at the optimum conditions for each set of enzymes. Another variation of this method was treating the cellulose first and then subsequently hydrolysing the starch component. Each treatment approach is expected to have its advantages and drawbacks with regards to how connected starch, cellulose, hemicellulose and lignin are found in cassava peels. This treatment strategy can also be used to assess the effect of the removal of starch on the subsequent hydrolysis of cellulose and vice versa. These reactions were investigated by DNS reducing sugar assay and HPLC analysis of the sugars released. The hydrolysis rates were also estimated and compared. The cassava peels used were pretreated either by size reduction, hotwater pretreatment or steam explosion as described in chapter 2.

3.1.0 MOISTURE CONTENT AND CASSAVA PEEL COMPOSITION

Preliminary experiments carried out on cassava peels to determine moisture content and the composition of cassava peels were carried out and results presented in Tables 3.1 and 3.2

	Air Dried + milled sample	Milled + washed + Oven dried sample	Steam exploded Sample
Mean	8.23	9.30	84.98
Standard deviation	0.2	0.1	0.3

***Table 3.1**: Table showing moisture content of samples (%w/w)*

The moisture content of the milled, washed and oven dried sample and the steam exploded samples were used to determine the mass of substrate to be used for each experiment.

The steam exploded material was frozen after treatment, kept frozen until thawed for the analysis.

Cassava peel waste composition (% on dry weight basis)

	Starch	Cellulose	Hemicellulose[1]	Lignin		Ash	Extractives	Acetyl groups	Others	Total
				Acid-insoluble	Acid-soluble		(other than starch)			
Mean	28.0	23.9	9.4	22.9	1.1	7.4	5.3	0.4	1.6	100.0
Standard deviation	*1.4*	*0.9*	*0.8*	*1.1*	*0.1*	*0.2*	*0.1*	*0.1*		

[1] Xylan	4.1
Galactan	3.0
Arabinan	1.8
Mannan	0.5
Total hemicelluloses	9.4

***Table 3.2**:Table showing cassava peel composition.*

The total carbohydrate from the results gave 61.3%.

3.2.0 REPRODUCIBILITY

One striking observation to emerge from the results was the reproducibility of experiments which showed a general trend that was difficult to explain. The experiments were done at different enzyme and substrate concentrations. It was generally observed that samples at 5% w/v and 14% w/v substrate concentration

showed less reproducible results compared to samples at 10w/v cassava peels. Figures 3.1, 3.2 and 3.3 illustrate this.

Percentage reducing sugar is expressed in the results as gram of reducing sugar per gram of cassava peels with glucose used as the standard. However the addition of one molecule of water to starch/cellulose gives 111% reducing sugar for the complete hydrolysis of pure starch or cellulose. The carbohydrate content of 61.3% in cassava peels will therefore give a maximum yield of 68.0% reducing sugar.

MWO represents milled, washed and oven dried cassava peel and **SE** represents steam exploded cassava peel. Enzyme concentration is in percentage volume by volume (% v/v) as shown in the chart below. Cassava peel concentration is in percentage weight by volume (% w/v). For simplicity alpha amylase will be referred to as amylase.

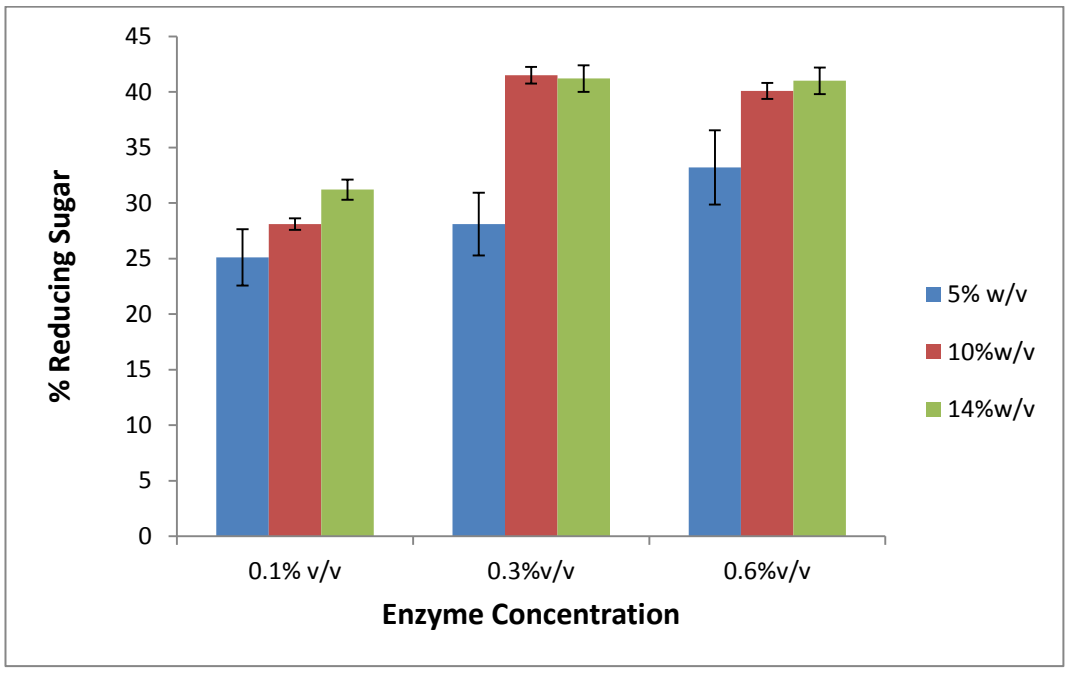


Fig 3.1: *Enzyme hydrolysis of **MWO**-milled, washed and oven dried cassava peels at 5%w/v, 10%w/v and 14%w/v in 0.05M sodium acetate pH 5 at 50^{0}C for 48 hrs by amylase and glucoamylase.*

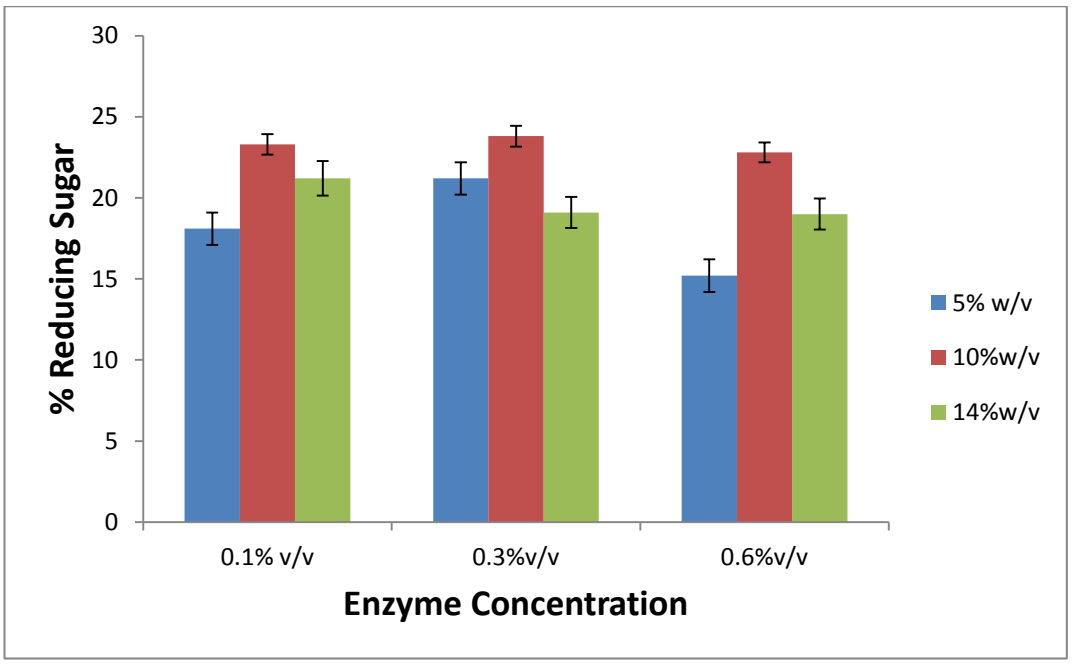


Fig 3.2: *Enzyme hydrolysis of* **SE***-steam exploded sample cassava peels at 5%w/v, 10%w/v and 14%w/v in 0.05M sodium acetate pH 5 at 50^{0}C for 48 hrs by amylase and glucoamylase.*

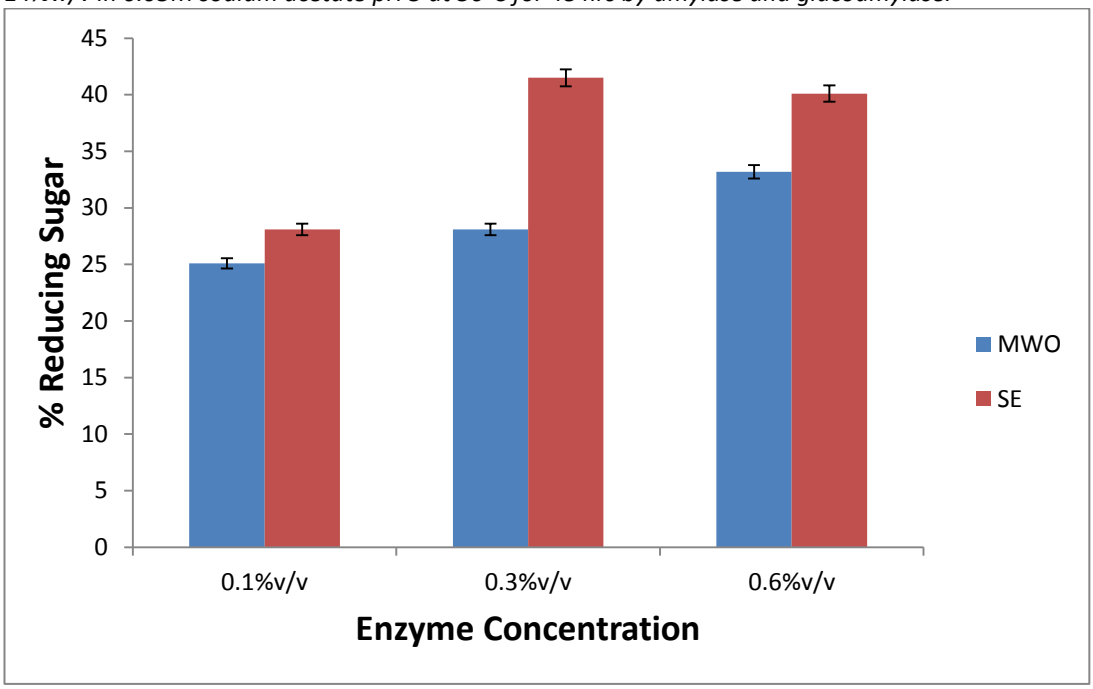


Fig 3.3: *Comparison* of the Enzyme hydrolysis of 10%w/v **SE** cassava peels and 10%w/v **MWO** cassava peels by *amylase and glucoamylase in 0.05M sodium acetate pH 5 at 50^{0}C for 48 hrs.*

Errors were expressed as percentage deviation from a mean of experiments done in triplicate. As can be seen from Figures 3.1 and 3.2, 10%w/v and 14%w/v sample had lower percentage errors compared to experiments with 5% w/v cassava peels. Figure 3.3 also shows that steam exploded samples had marginally higher errors

when compared to MWO samples at the same cassava peel concentrations and enzyme concentrations.

These inconsistencies in results might have been caused by the behaviour of cassava peel suspension in water and the associated mixing conditions. Although at 10%w/v and 14%w/v, reducing sugar yields are close, the viscosity of the hydrolysis at 14% was remarkably higher at the beginning of the experiments for 14%w/v cassava peels content. This made sampling quite tricky as well as mixing becoming uneven. However, about 6 hours of reaction, the suspension becomes a lot less viscous and sampling is easier. This might account for the slight difference in reproducibility for samples at 14%w/v when compared to experiments carried out at 10%w/v substrate concentration. Hydrolysis yield for 5%w/v were much lower compared to 10%w/v and 14%w/v cassava peels concentration. However, when these experiments were conducted at a much smaller scale (2ml-5ml volume), reactions with 5% w/v concentration had similar yields with reactions at 10w/v and 14%w/v cassava peels and were all completed in less than 24 hours. An incubator with a rotator was used for the mixing (for experiments at 2ml-5ml volume) rather than the water bath (with a linear shaking motion). This leads credence to believe that the mixing conditions might have contributed to this anomaly. Fan and Lee (1983) had observed a similar pattern in their experiments and suggested that the hydrodynamics of the system was a more likely cause of these results. They argued that the effect of mixing of heterogeneous suspensions of different sizes in enzyme hydrolysis is difficult to estimate on hydrolysis rate and yield and therefore a culpable factor.

It is generally agreed that for an efficient utilization of lignocellulose for production of ethanol, the fermentable hydrolysate has to have a high glucose concentration to provide a feasible ethanol concentration for subsequent distillation (Rosgaard et al 2007). A low substrate concentration of 5%w/v would generate lower glucose concentrations compared to 10%w/v and 14%w/v substrate concentration.

Results from 10%w/v and 14%w/v cassava peels concentration are subsequently presented and discussed. Most of the experiments were carried out in 50ml

reaction volume. Figures 3.4 and 3.5 shows duplicate experiments at 10%w/v and 14%w/v cassava peels. The enzyme concentration in the experiments is still in %v/v. For example, a representation of 0.15v/v MW0 would represent 0.15%v/v enzyme concentration of an experiment carried out on milled cassava peels and 0.30%v/v SE would represent an enzyme concentration of 0.3%v/v on a steam exploded cassava peel substrate. The cassava peel concentration was always given in % w/v and the % reducing sugar expressed as gram of reducing sugar per gram of cassava peels. Glucose was used as the standard.

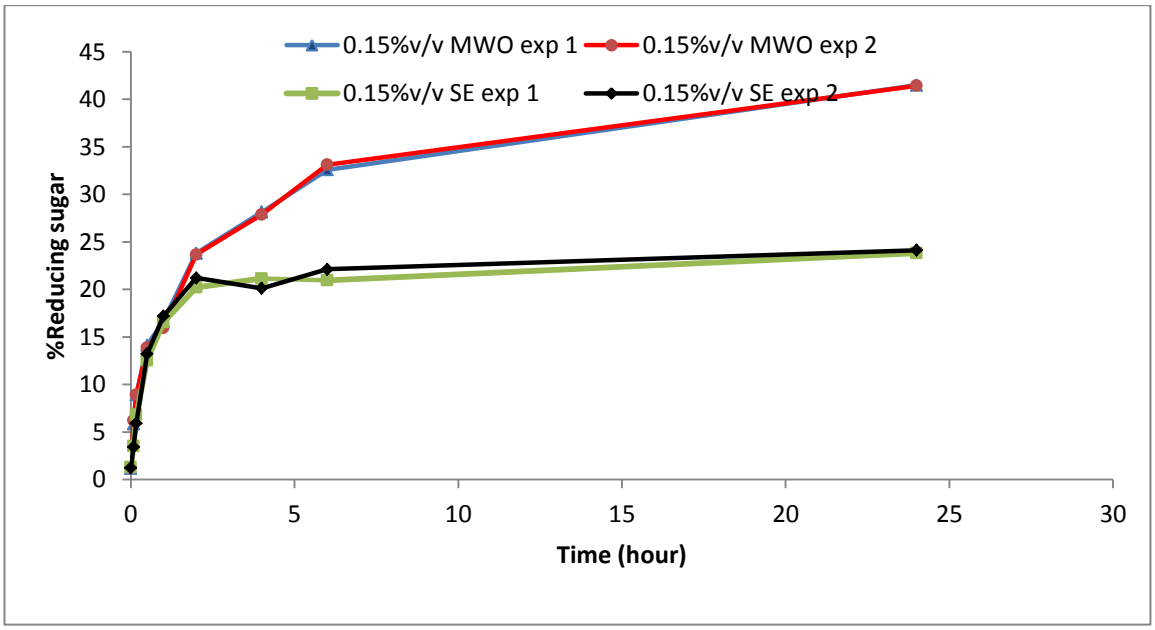


Fig 3.4: Comparison of duplicate experiments of the enzymatic hydrolysis of 10%w/v SE and 10%w/v MWO cassava peels in 0.05M sodium acetate buffer at pH 5 with 0.15%v/v of amylase and glucoamylase and at temperature 50^0C. Exp 1 and Exp 2 represents the two experiments carried out.

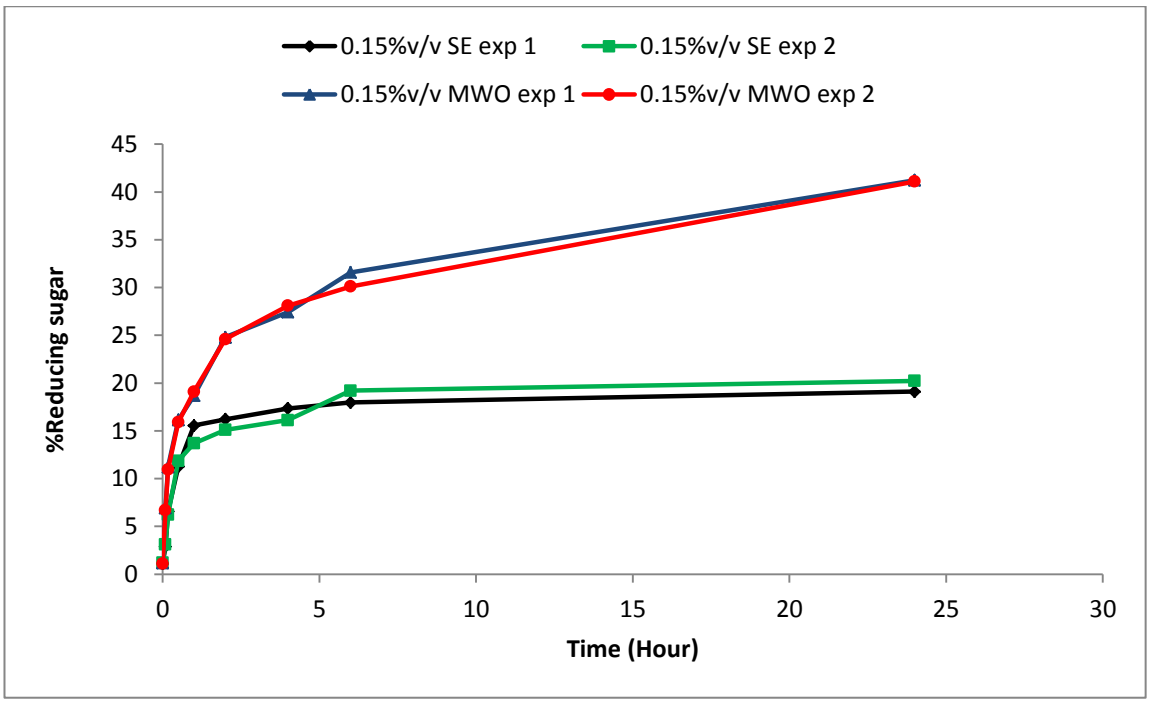


Fig 3.5 : Comparison of Duplicate experiments of the enzymatic hydrolysis of 14%w/v SE and MWO cassava peels in 0.05M sodium acetate buffer at pH 5 with 0.15%v/v of amylase and glucoamylase and at temperature 50^{0}C. Exp 1 and Exp 2 represents the two experiments carried out.

It was also observed that at longer reactions times greater than 48 hours, there is a decrease of final reducing sugar concentration in all reactions possibly as a result of microorganism contamination. Reactions with 10%w/v and 14%w/v cassava peels were mostly completed in 24 hours with very little difference between yields at 24 hours and 48 hours. However, for 5%w/v cassava peel concentration the reaction continues albeit more slowly although it would be difficult to assume the final reducing sugar yield to be reliable.

3.3.0 STARCH HYDROLYSIS: AMYLASE AND GLUCOAMYLASE TREATMENT

Digestion by amylolytic enzymes was carried out on sample prepared cassava peels (MWO; milled washed and oven dried sample), hot water pretreated (HW) and steam exploded (SE) samples. The enzyme hydrolysis was carried out at 10%w/v and 14%w/v cassava peels. The enzyme volume was varied between 0.05ml, 0.15ml and 0.3ml corresponding to 0.1%v/v, 0.3%v/v, 0.6v/v of both amylase and glucoamylase enzymes in 50ml 0.05M sodium acetate buffer solution at pH 5.

Although in theory, treatment by amylolytic enzymes would imply starch digestion, results presented in fig 3.7 and fig 3.8 below show that non starch component of

the peels were also hydrolysed. When the amylase and glucoamylase enzymes were used to treat whatman filter paper no 1, reducing sugar was produced showing that these enzymes had a hydrolysing effect on cellulose. Result from this experiment is shown in 3.6 below.

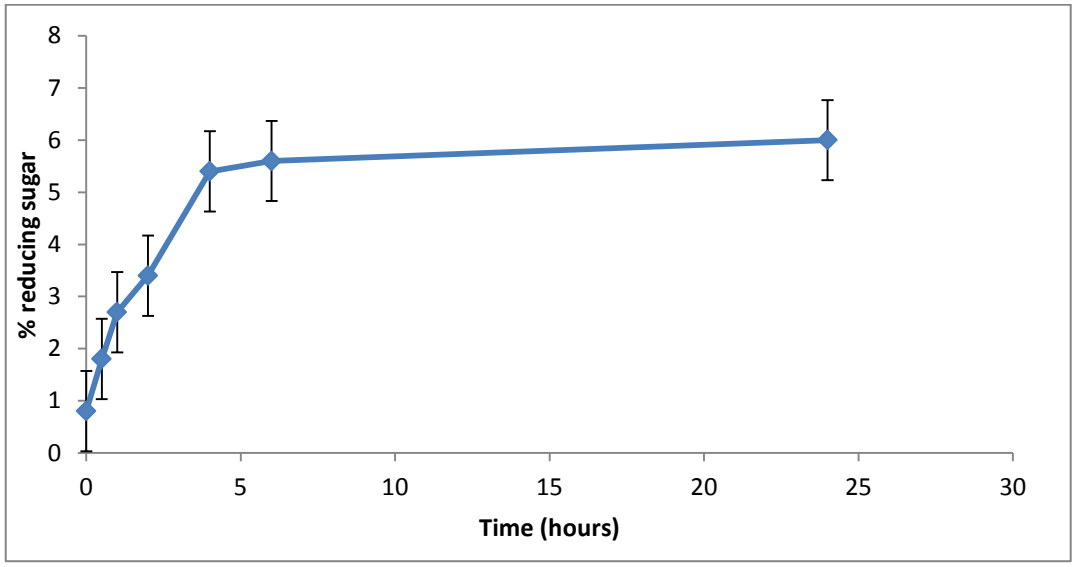


Fig 3.6: Enzyme hydrolysis of 5w/v (2g in 40ml) Whatman filter paper by 0.3%v/v amylase and 0.3%v/v glucoamylase in 0.05M sodium acetate buffer at pH 5 and 50^{0}C

The progress curves in fig 3.7 and fig 3.8 below show results from hydrolysis using amylase and glucoamylase enzymes on cassava peels.

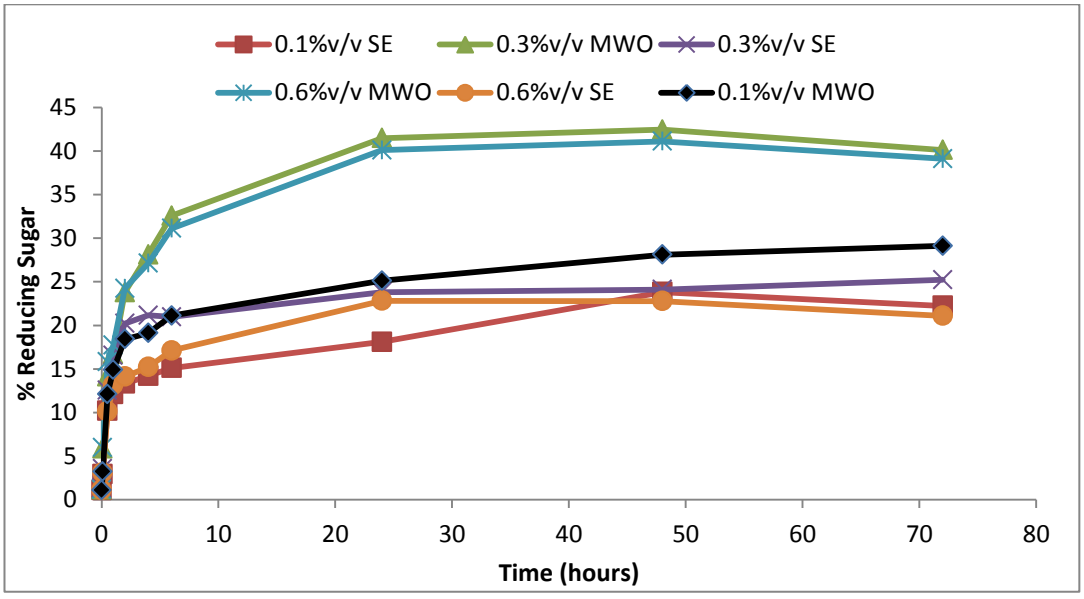


Fig 3.7: progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with amylase and glucoamylase and at temperature 50^{0}C

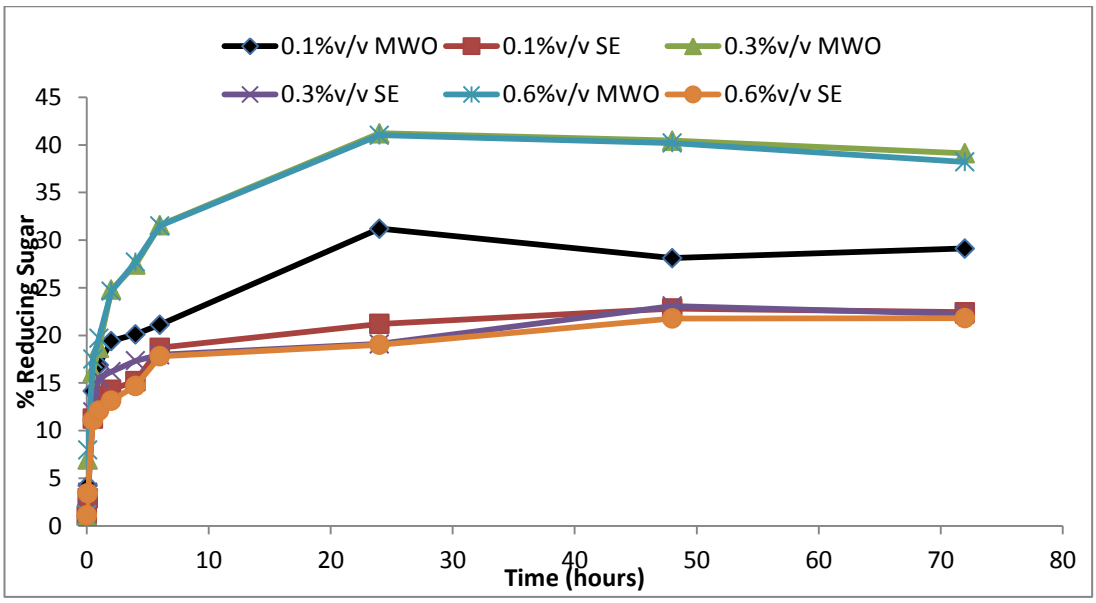


Fig 3.8: progress curve of the enzymatic hydrolysis of 14%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with amylase and glucoamylase and at temperature 50^{0}C

The progress curves show that the milled samples gave higher reducing sugar yield when compared to the steam exploded samples at both substrate concentrations. This suggest that steam explosion pretreatment must have caused a degradation of starch in the cassava peels by perhaps causing a further degradation of the monosaccharides formed by a partial hydrolysis of starch at 220^{0}C. Glucose is known to be decompose to hydroxymethylfurfural (HMF), 1,6-anhydroglucose, levulinic acid, and formic acid at high temperatures (Corredor et al 2007).

The highest hydrolysis yield was obtained with substrate concentrations of 10% w/v and 14%w/v which gave similar results with yields of 41.5% and 41.2% reducing sugar in 24 hours for milled samples. Also, steam exploded samples at 10% w/v and 14% w/v gave yields of 23.8% and 19.0% respectively. Both of these reactions were at 0.3%v/v of alpha amylase and glucoamylase. Both sets of reactions showed a decrease in reducing sugar after 48 hours as noted earlier and this might have been caused by microbial contamination. Results after 48 hours were therefore suspect and better ignored. Fig 3.7 and Fig 3.8 shows that 10%w/v and 14% w/v samples gave very high hydrolysis yield at 24 hours without much of an increase at 48 hours. The reducing sugar yield of about 41% for milled samples however contradicts the compositional analysis of cassava peels in table 3.2 which gives the starch content

of cassava peels as 28%. This would have translated to a theoretical value of 31.1% reducing sugar content. This is because for each mole of starch converted to glucose, there is an addition of a molecule of water to starch with an empirical formula $(C_6H_{10}O_5)n$ to glucose $C_6H_{12}O_6$ with a molecular weight of 180g/mol.

The amount of starch present as reported in the compositional analysis of cassava peels was determined by the Megazyme Total Starch assay procedure (amyloglucosidase/α-amylase method) listed as AOAC method 996.11 and uses HPLC for glucose analysis. However this method of analysis might depend on the sample size of the cassava peels as well as being subject to sampling error.

It is also possible that the standard assay may not digest all the starch in the peels. Another possibility is that non starch components of the cassava peels were also hydrolysed.

It can be assumed that the over 40% reducing sugar yield represents a complete starch digestion of the starch portion of the cassava peels.

Hot water pretreatment was also investigated. One advantage hot water pretreatment would be of interest is that for current industrial hydrolysis of starch, a gelatinization step at between 90^0C and 100^oC is used. For the purpose of determining optimum conditions of the amylolytic enzymes, experiments were also carried out at different pH to determine optimum pH for both amylase and glucoamylase enzymes. Results from these experiments are shown below.

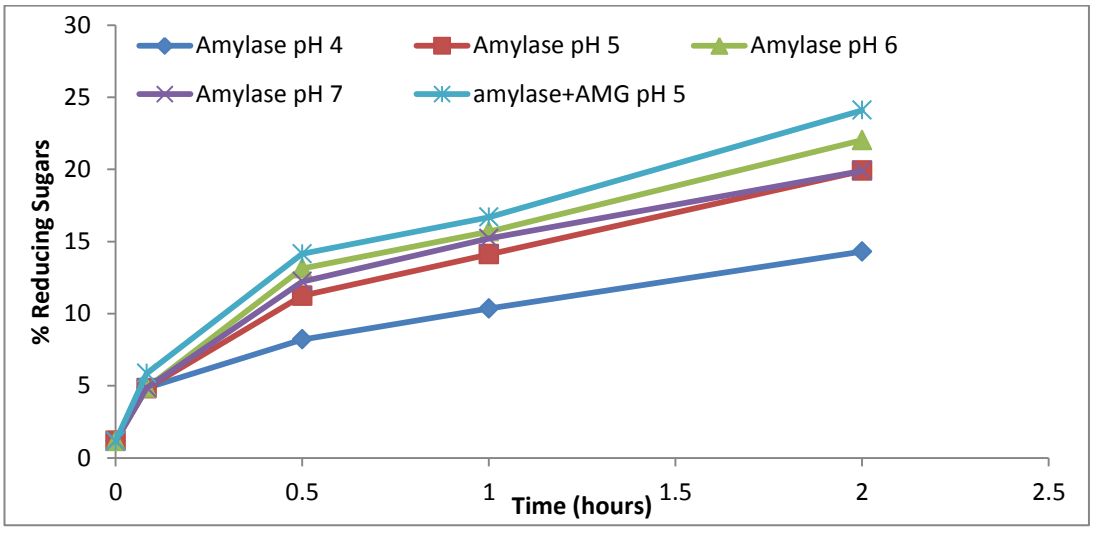


Fig 3.9: progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer with amylase at temperature 100^0C and different pH for 2 hours.

Results above show an optimum pH for amylase to be 6. Subsequent addition of glucoamylase after changing the pH to 4 however did not show any significant differences in overall reducing sugar yield after 24 hours as shown in fig 3.10 below.

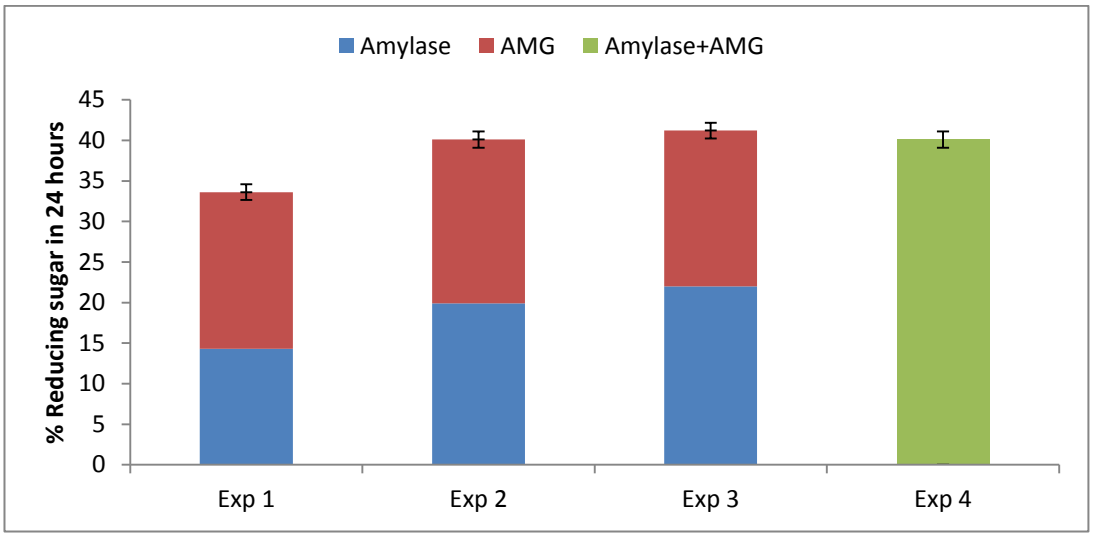


Fig 3.10: *Final reducing sugar yield after an Initial 2 hours of amylase treatment at different pH and subsequent treatment with glucoamylase for a further 22 hours Exp 1: HW +amylase +glucoamylase at pH 4, Exp 2:HW +amylase +glucoamylase pH 5 Exp 3:HW +amylase +glucoamylase at pH 6,Exp 4 amylase +glucoamylase at pH 5 without HW*

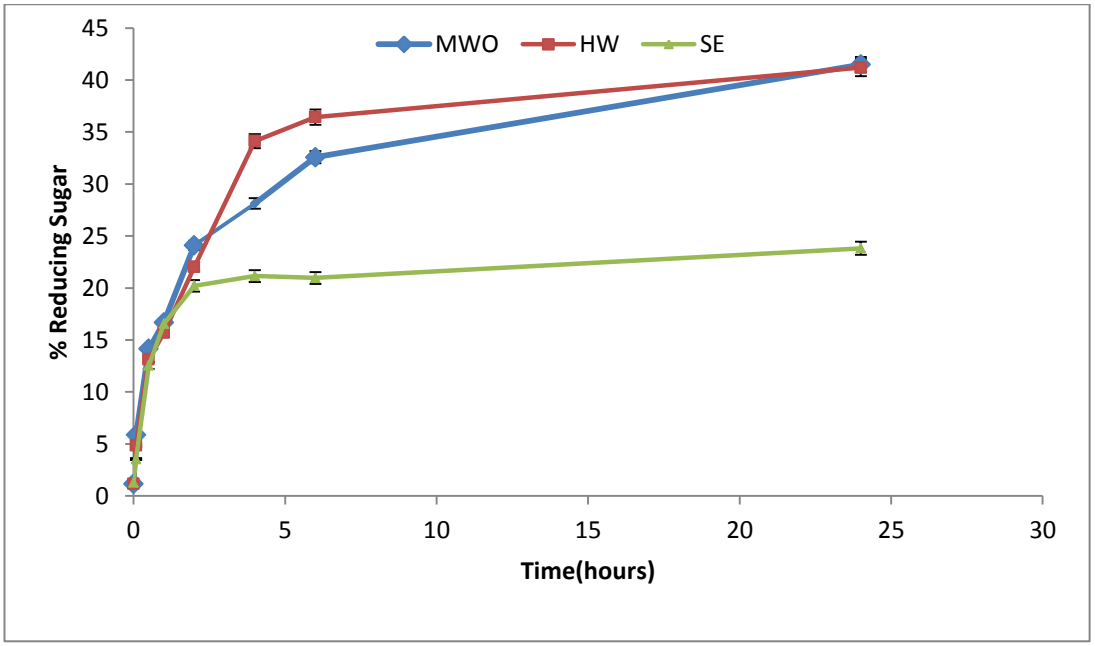


Fig 3.11: Comparison of the progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer with amylase and glucoamylase at 0.3v/v enzyme concentration using different pretreatment strategies. All reactions at pH 5. MWO and SE at 50°C and HW: hot water pretreatment at 100°C then a subsequent enzyme treatment at 50°C.

A comparison of the three pretreatment methods in fig 3.11 shows that although the total reducing sugar for MWO and HW cassava peels were identical, the experiment with hot water treatment shows a faster reaction. At 6 hours of reaction, more reducing sugar is produced compared to the milled peels without hot water treatment. A clear benefit of hot water pretreatment is discussed in chapter 5.

A further look at the first few hours of the enzyme hydrolysis of cassava peels to assess the changes in hydrolysis rate for the different treatments is discussed below. Hydrolysis rate estimate was done by drawing a line by eye and using a macro to estimate the hydrolysis rate for the different experiments.

Fig 3.12 illustrates how the lines were drawn and used to estimate the hydrolysis rate.

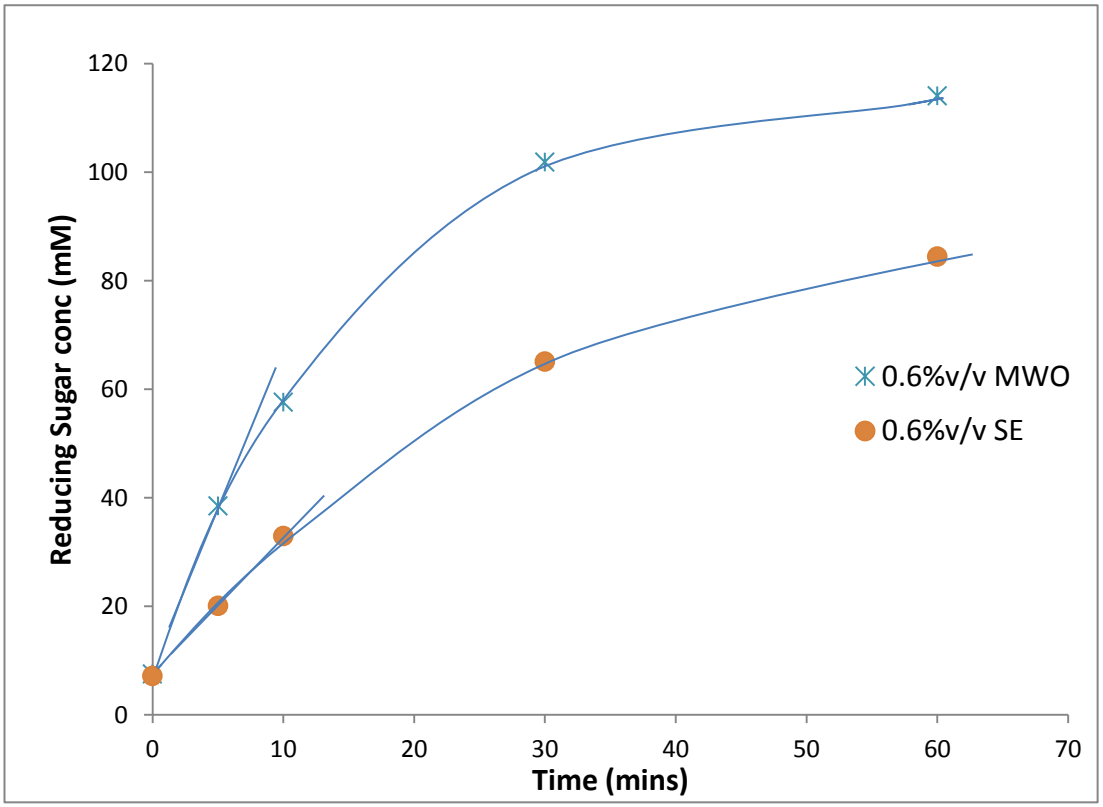


Figure 3.12: First hour of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with 0.6 %v/v amylase and glucoamylase and at temperature of 50^0C.

Sampling points were usually at 0, 5 and 10 minutes after enzymes are added to the substrate and considering additional time (taking samples, centrifuging and adding the DNS reagent to stop the reaction), hydrolysis rate was estimated within the first 10 minutes of the reactions. The first 10 minutes of the reaction represents the fastest phase of the entire reaction. Between 10 minutes and about two hours this rates slows down significantly and progresses slowly until the end of the reaction time. This is observed for reactions at 10%w/v and 14%w/v cassava peel concentrations.

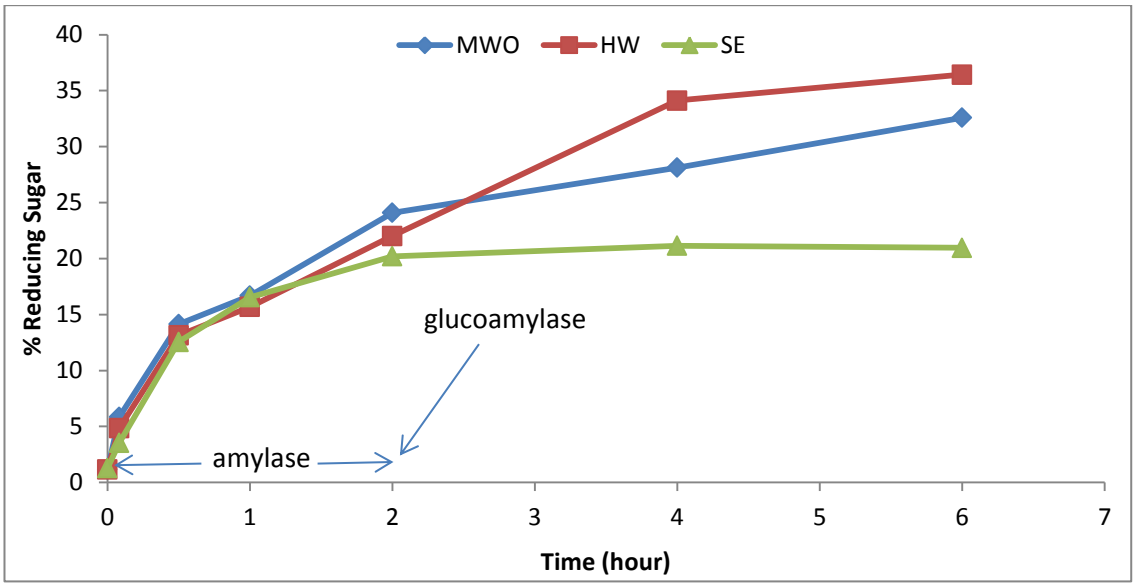


Fig 3.13 *Comparison of the progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer with amylase and glucoamylase at 0.3v/v enzyme concentration using different pretreatment strategies. All reactions at pH 5. MWO and SE at 50^{o}C and HW at 100^{o}C and represents a 2 hour treatment with amylase and subsequently at 50^{0}C with glucoamylase.*

Table 3.3 below presents the results for hydrolysis rate at different enzyme and substrate concentrations for SE and MWO cassava peels. The hydrolysis rate was expressed as mM of reducing sugar expressed as glucose per minute.

Hydrolysis Rate of Reaction (mM/min)						
Substrate concentration	*0.1%v/v amylase+AMG*		*0.3%v/v amylase+AMG*		*0.6%v/v amylase+AMG*	
	MWO	SE	MWO	SE	MWO	SE
10 % w/v	3.8	3.0	6.2	3.4	6.8	3.6
14 % w/v	4.0	3.2	6.8	3.4	7.0	3.4

Table 3.3: Hydrolysis rate estimate of enzyme hydrolysis of cassava peels at 10 and 14%w/v in 0.05M sodium acetate at 50^{0}C and pH 5 for 24 hrs at different enzyme concentration **MWO**-milled, washed and oven dried sample **SE**-*steam exploded sample. Standard deviation was ± 0.1 for all experiments*

Reaction rate measurements for milled samples show that an increase in enzyme and substrate concentration gave higher initial rates. The increase is more pronounced from 0.1%v/v to 0.3%v/v amylase and glucoamylase concentrations although it is less than a 3 fold increase that would have been expected. The increase in reaction rates between 0.3%v/v and 0.6%v/v enzyme concentration is not as large. An increase in cassava peel concentration also gave marginally higher reaction rates for milled samples although for enzyme concentration of 0.3%v/v there is a more significant difference as 0.3%v/v enzyme concentration gave a reaction rate of 6.20mM/min at 10%w/v and 6.80mM/min at 14%w/v. At 0.6%v/v enzyme concentration, the increase in initial rates is also observed however between 10%w/v and 14%w/v, the initial rates are almost the same at 6.80mM/min and 7.00mM/min respectively.

Steam exploded samples showed very little differences at all substrate and enzyme concentrations as shown in the figure below. Reactions with steam exploded samples were also slower than those of milled samples at all substrate concentration as observed above.

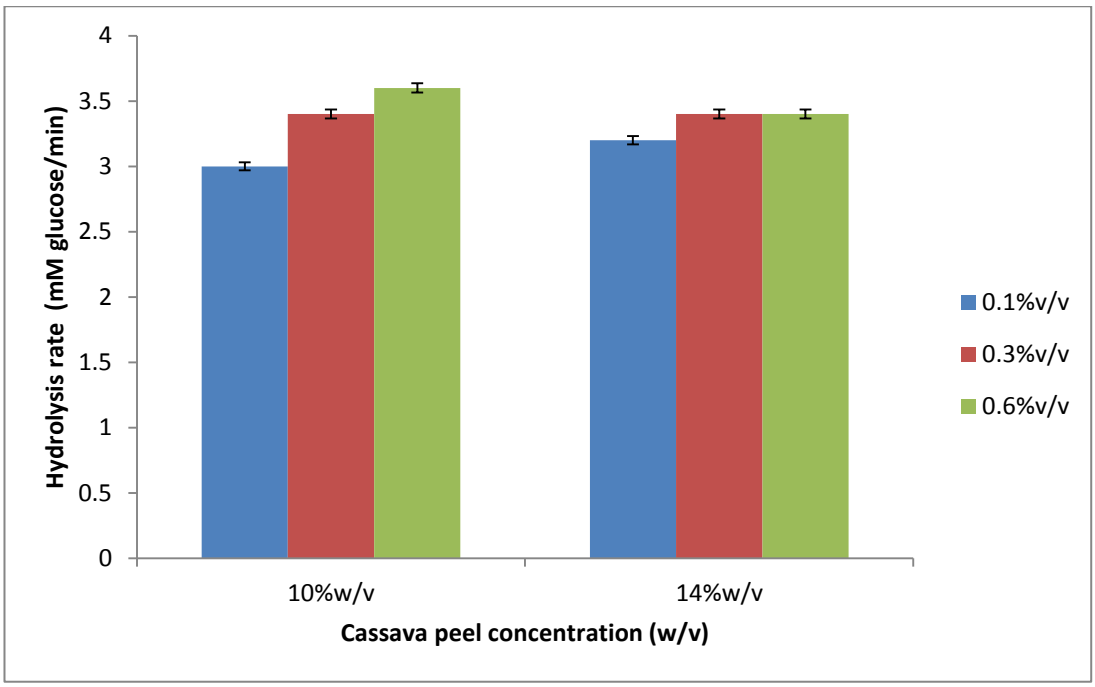


***Figure 3.14**: Comparison of the hydrolysis rates of 10%w/v and 14%w/v SE cassava peels in 0.05M sodium acetate buffer at pH 5 at different amylase and glucoamylase concentrations for milled samples in the first ten minutes of the reaction at temperature 50^{0}C. The reaction was carried out in 50ml.*

This suggest that not much starch was available for hydrolysis after the steam explosion pretreatment and so the addition of more cassava peels or enzyme didn't seem to have any significant difference on the hydrolysis rate or yield. Reaction rates can be limited by either available surface or enzyme available in normal enzyme reactions involving solid substrates. An increase in substrate or enzyme concentration should lead to an increase in hydrolysis rate. However, this is not the case with these samples. We can speculate that the presence of inhibitors from the steam explosion has an adverse effect on the hydrolysis rate and yield.

The progress curves show that over 70% of the final reducing sugar yield is obtained at 6 hours of reaction time for 10%w/v peels concentration (Fig 3.15 below). This is also the case at 14%w/v cassava peels concentration.

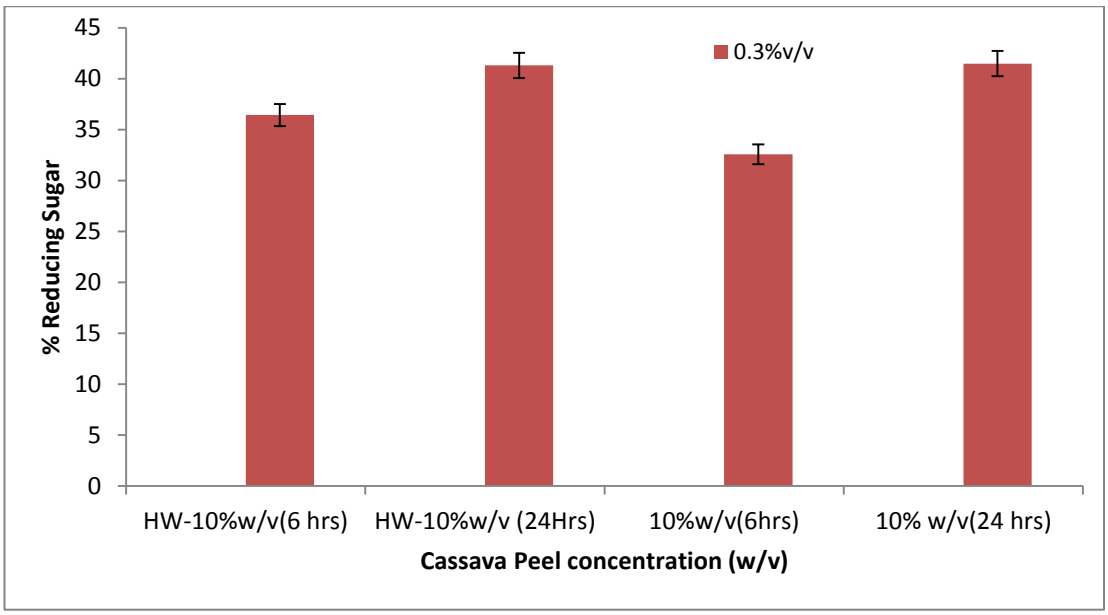


***Fig 3.15**: Reducing sugar concentration at 6 hours and 24 hours for 10%w/v cassava peel concentration in 0.05M sodium acetate buffer at enzyme concentrations of 0.3v/v for milled cassava peel. The reaction was at temperature 50^{0}C and pH of 5. 0.3%v/v represents concentration of 0.3%v/v of amylase and 0.3%v/v glucoamylase enzyme. HW was at 100^{0}C.*

Although reactions are faster at 0.6%v/v enzyme concentration, an enzyme concentration of 0.3%v/v produces the same amount of sugars in 24 hours as the reaction with 0.6%v/v enzyme concentration.

A comparison of final reducing sugar for milled sample is shown in the figure below.

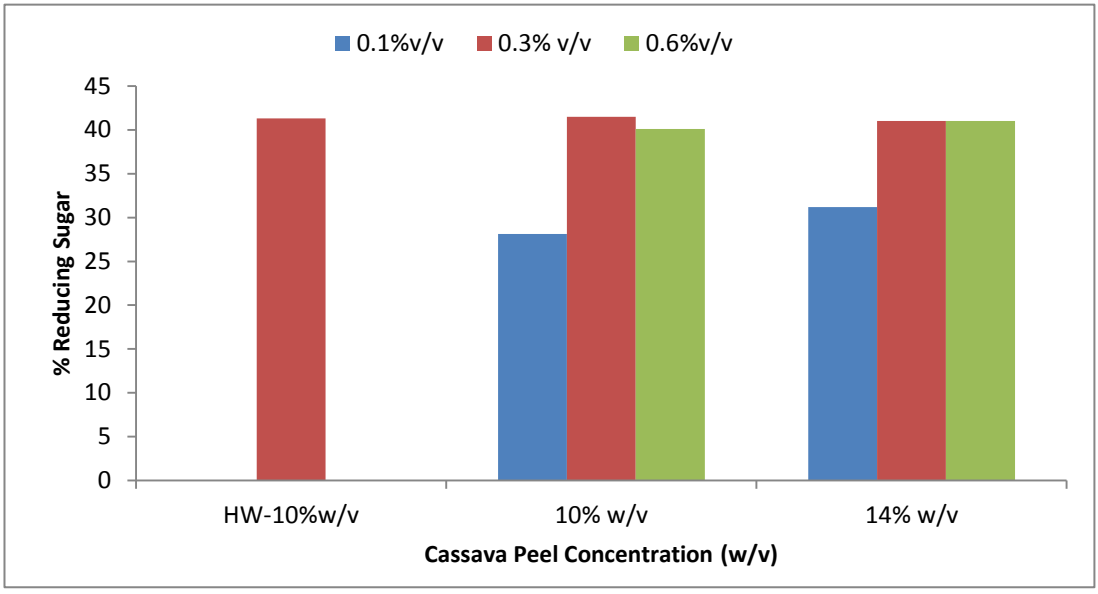


Figure 3.16: Comparison of the enzymatic hydrolysis of 10%w/v HW pretreated peels, 10%w/v MWO and 14%w/v MWO cassava peels in 0.05M sodium acetate buffer at pH 5 at different enzyme concentrations for milled samples at 48 hours and at temperature 50^{0}C. 0.1%v/v, 0.3%v/v and 0.6%v/v represents a combination of amylase and glucoamylase enzymes. HW was at 100^{0}C.

To monitor the release of oligosaccharides as the reaction progresses, size exclusion chromatography using shodex KS 801 column was also used and the HPLC results of the released oligosaccharides were also analysed and compared with results from the DNS assay as shown in fig 3.17a and 3.17b below.

Standards curves for glucose, maltose and cellobiose showed that the areas of chromatogram represented the mass concentration of standards and so for DP 3 and above, the mass concentration of cellotriose/maltotriose and cellotetrose/maltotetrose were estimated using glucose as a standard and then the molar concentrations calculated by assuming the molecular weight of the cellotriose/maltotriose and cellotetrose/maltotetroase each with molecular weights of 504.45 and 666.58g/mol respectively.

For DP greater than 4, oligosaccharides of DP 6 (cellohexose/maltohexose) with a molecular weight of 990.86g/mol was used to estimate the molar concentration of Dp greater than 4. This is because oligosaccharides of cellulose with DP of 2 to DP of 6 are soluble in water although cellulose of DP 7 to DP 12 are slightly soluble in hot water (Zhang and Lynd 2004). While this might not hold for maltodextrins as there are soluble in water up to DP 60 (Arantes and Sandler 2010). However, for this study, it is conceded that the estimated number of moles of maltodextrins present in the supernatant is grossly overestimated.

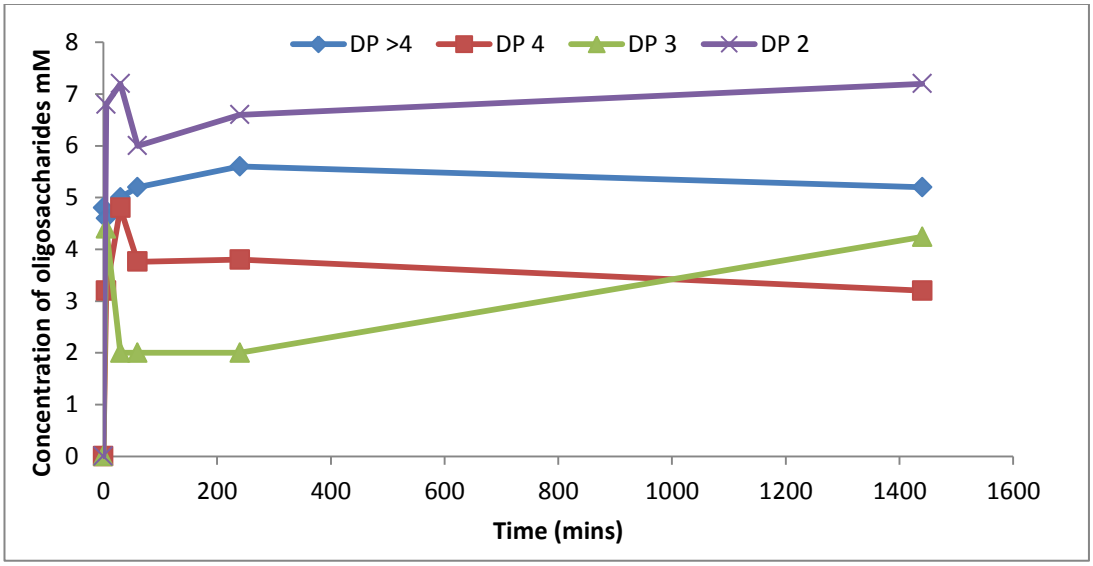


Fig 3.17a: Concentration of oligosaccharides during enzyme hydrolysis of 10%w/v MWO cassava peels by 0.3% v/v amylase and glucoamylase at pH 5 and at temperature 50^{0}C

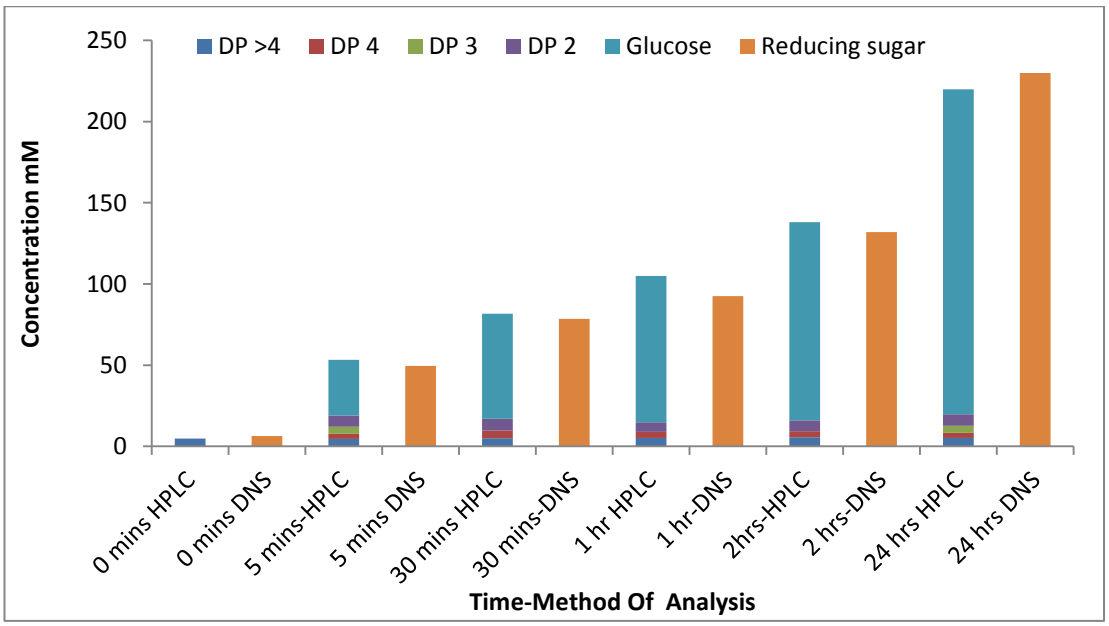


*Fig 3.17b: comparison of Hplc analysis with DNS assay for 10%w/v **MWO** cassava peels at 0.3%v/v enzyme concentration in 0.05M sodium acetate buffer at pH 5 and at temperature 50^0C*

The results from Fig 3.17b shows that glucose is produced immediately at a start of the reaction in large quantity. The presence of oligosaccharides with degree of polymerisation 2-4 are quite low throughout the reaction although between 5 minutes and 2 hours of the reaction, oligosaccharides with DP of 4 and above make up about 10 to 30% of the sugars present in the supernatant. The HPLC results also show consistent results with the DNS assay as shown in Fig 3.17b.

An example chromatogram of hydrolysis at 30 minutes is shown below

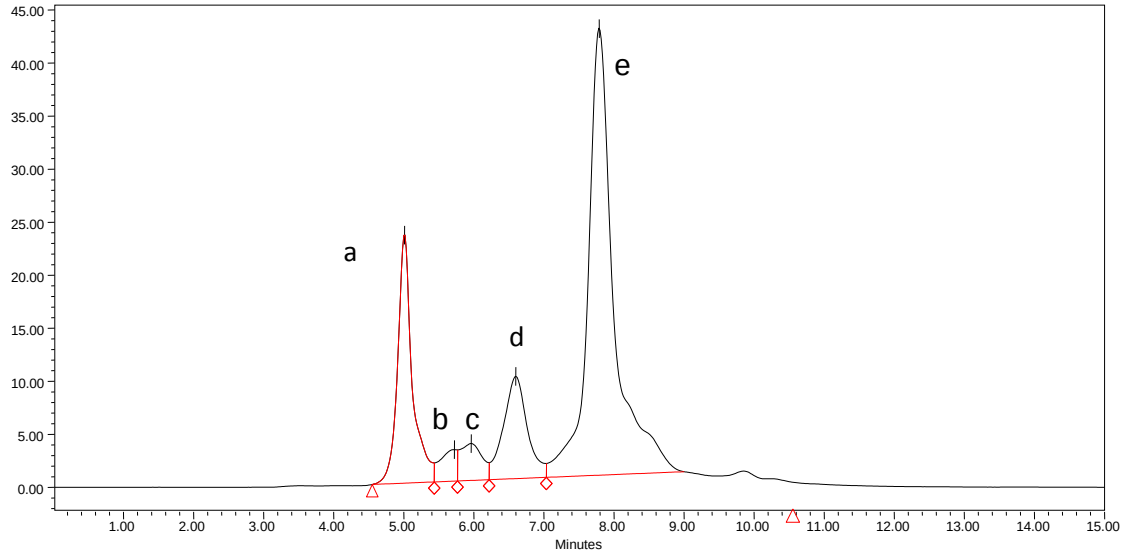


Fig3.18: An example chromatogram of enzyme hydrolysis of 10%w/v cassava peels using amylase and glucoamylase at 30 minutes. a- DP>4, b-DP 4 c- DP 3, d-DP 2, e- Glucose

3.4.0 CELLULOSE HYDROLYSIS: (CELLULOLYTIC TREATMENT) CASSAVA C AND CASSAVA R TREATMENT

Cassava C (cellulase enzyme of endo and exo glucanase) and cassava R (an enzyme mixture of xylanase, cellulase and hemicellulase) were used to hydrolyse the cellulosic component of the cassava peels. It is however significant to note that these cellulolytic enzymes also have hydrolysing effect on the starch component of the peels. This was confirmed by the production of reducing sugar when cassava C and cassava R were used to treat pure potato starch. This is shown in fig 3.19 below.

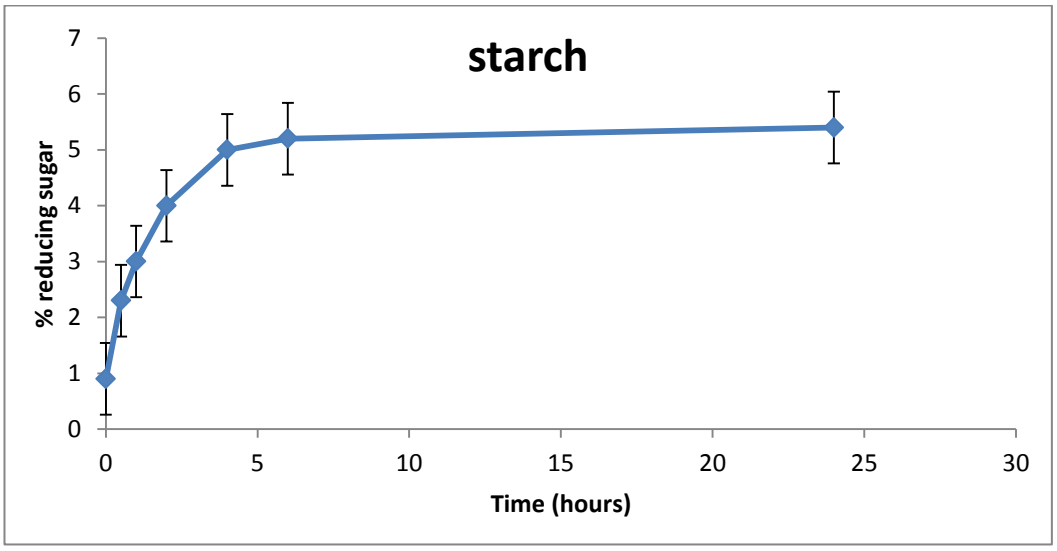


Fig 3.19: Enzyme hydrolysis of 10w/v potato starch by 0.3%v/v cassava C and cassava R in 0.05M sodium acetate buffer at pH 5 and 50^0C

The cellulase enzymes were combined with a mixture of 0.1% v/v of each enzyme. 0.3%v/v and 0.6%v/v of each enzyme were also used to treat cassava peels. Again 10%w/v and 14% w/v cassava peels were used. Both steam exploded samples and milled samples (MWO) were used. The effect of hot water pretreatment would be discussed in the next chapter. Progress curves of the reactions are shown in fig 3.20 and 3.21.

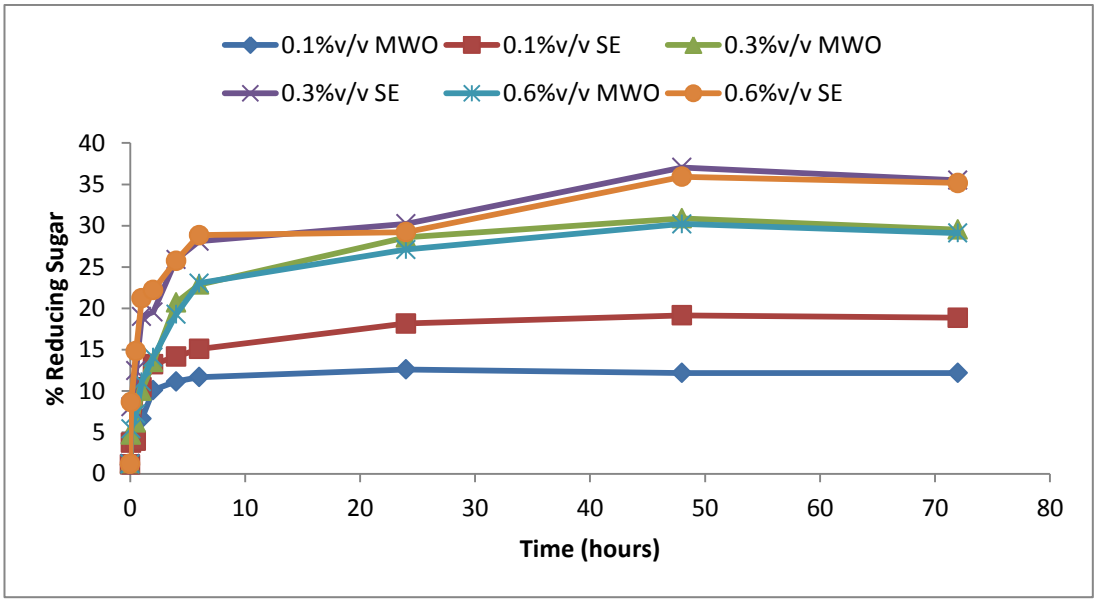


Fig 3.20: Progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with cassava C and cassava R at temperature 50^{0}C

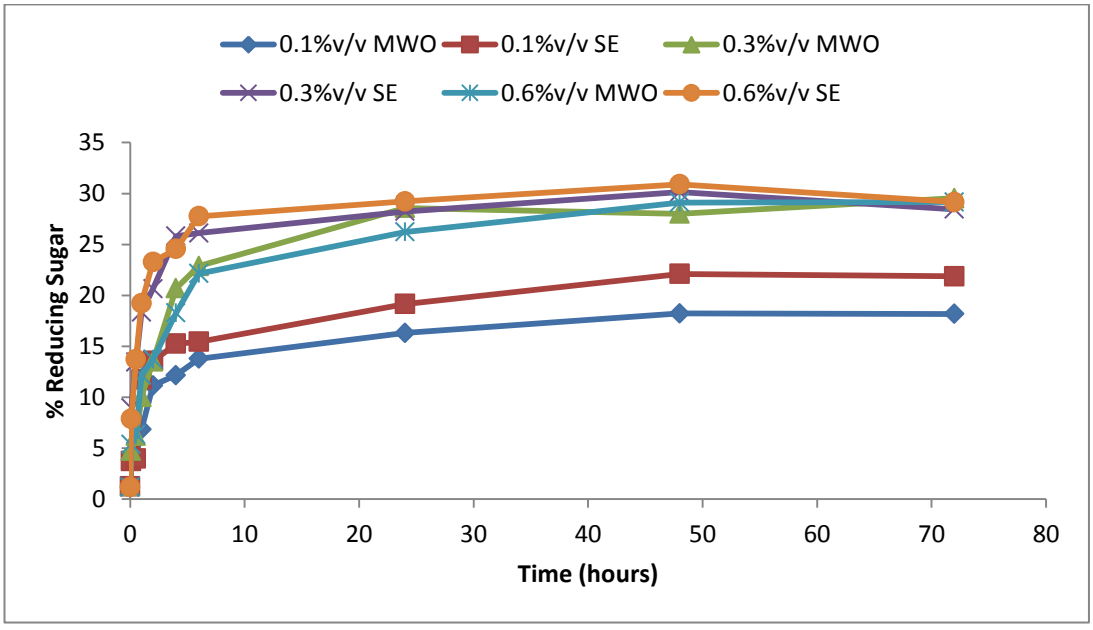


Fig 3.21: Progress curve of the enzymatic hydrolysis of 14%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with cassava C and cassava R at temperature 50^{0}C

Results from the progress curves show that both steam exploded samples and milled samples produced a significantly high reducing sugar yield at 10%w/v and 14%w/v cassava peels. Although steam exploded samples showed marginally higher yields when compared to milled samples especially at 10%w/v peels. At 10% w/v, the steam exploded sample produced 37.04% reducing sugar yield in 48 hours which is about 100% of reducing sugar expected from cellulose and hemicelluloses component of the cassava peels. This high hydrolysis yield might also be attributed to the partial hydrolysis of the starch component especially as steam explosion occurs at very high temperature which could gelatinise the starch. We also suspect that the hydrolysing effect of these cellulolytic enzymes on starch as stated earlier might have contributed. Milled samples produced 30.88% reducing sugar at the same cassava peel concentration. Both reactions were at 0.3%v/v enzyme concentration. At 14%w/v cassava peels, less reducing sugars are produced with steam exploded samples producing 30.13% and milled samples producing 28.0% reducing sugar. This might be due to the inhibition of cellulases by glucose and cellobiose at higher glucose concentration expected from higher substrate concentration. Cassava peels of 14% w/v also gave less reproducibility when compared to samples taken from 10%w/v cassava peels experiments. The results from hydrolysis using cassava C and cassava R suggest that steam explosion as a pretreatment is quite successful with hydrolysis as over 90% of sugars are released in 24 hours.

Most of the reaction is concluded in 48 hours for both steam exploded and milled samples at 10%w/v and 14%w/v however it might be important to note that 85.5% and 87% of the final reducing sugar is obtained in 24 hours for milled and steam exploded samples respectively at 10%w/v cassava peels. Similar results are obtained at 14%w/v cassava peels with over 80% of sugars obtained in 24 hours.

A further look at the first hour of the reaction shows how fast the steam exploded pretreated sample proceeds when compared to the milled sample as shown in fig 3.22 and fig 3.23.

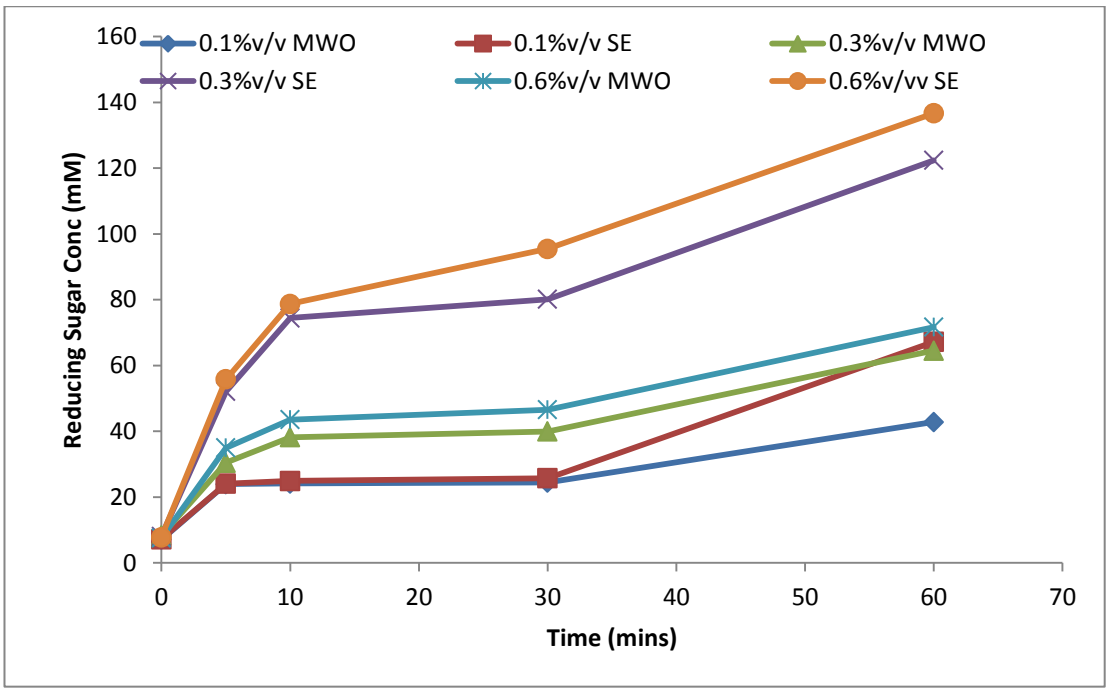


Fig 3.22: First hour of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 by cassava C and cassava R at temperature 50^0C

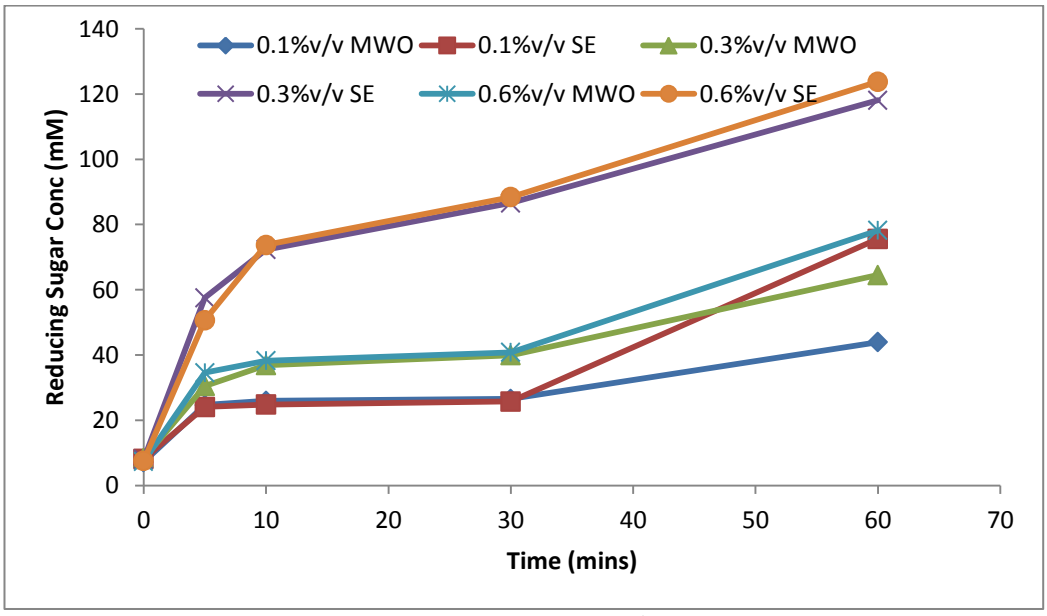


Fig 3.23: First hour of the enzymatic hydrolysis of 14%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 by cassava C and cassava R at temperature 50^0C

Hydrolysis rate was again estimated with the help of a macro as stated earlier.

Hydrolysis Rate of Reaction (mM/min)						
Substrate concentration	***0.1%v/v Cassava C+Cassava R***		***0.3%v/v Cassava C+Cassava R***		***0.6%v/v Cassava C+Cassava R***	
	MWO	**SE**	**MWO**	**SE**	**MWO**	**SE**
10 % w/v	2.8±0.5	3.0±0.4	3.4±0.1	7.6±0.1	5.0±0.1	7.6±0.1
14 % w/v	2.6±0.4	2.8±0.5	3.0±0.2	7.6±0.2	4.0±0.1	7.0±0.2

Table 3.4: enzyme hydrolysis of cassava peels at 10 and 14%w,v in 0.05M sodium acetate pH 5 at 50^{0}C for 24 hrs at different enzyme loading. **MWO**-milled, washed and oven dried sample **SE**-*steam exploded sample*

The reaction rate estimate and the progress curves shows that steam exploded samples react faster than the milled samples. Hydrolysis by these cellulolytic enzymes (cassava C and cassava R) also shows that hydrolysis is slowest at enzyme concentration of 0.1%v/v and highest at 0.6%w/v. At 14% w/v, there is a reduction in the rate of reaction from 7.60mM/min to 7.00mM/min for enzyme concentration of 0.3%v/v to 0.6%v/v. A closer look at the reaction rates table will also show a decrease in hydrolysis rate going from 10%w/v to 14%w/v cassava peel concentration. One possible reason would be cellulase inhibition by cellobiose and glucose as concentration increases although Lee and Fan (1983) also gave another possible reason as the role played by hydrodynamics of this hydrolysis system. They believed that a cellulose network with increase in substrate concentration could lead to entrapment of water from the cellulose suspension (in this case both cellulose and starch suspensions) making it thicker and less movable. This lack of movement of the aqueous phase is believed to hinder the hydrolysis reaction significantly.

The HPLC analysis of the released sugars shows that steam exploded samples had a large amount of oligosaccharides released into solution by the pretreatment at time zero (before enzymes are added). Figure 3.24- 3.28 show the HPLC results.

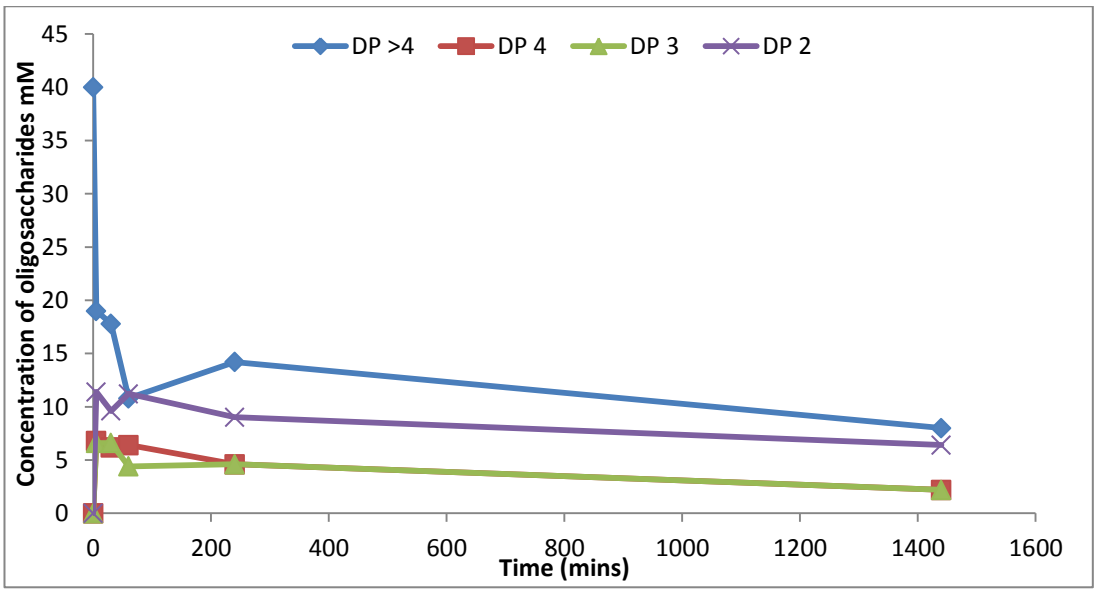


Fig 3.24: Concentration of oligosaccharides during enzyme hydrolysis of 10%w/v **SE** cassava peels by 0.3%v/v cassava C and cassava R in 0.05M sodium acetate buffer at pH 5 and at temperature 50^0C

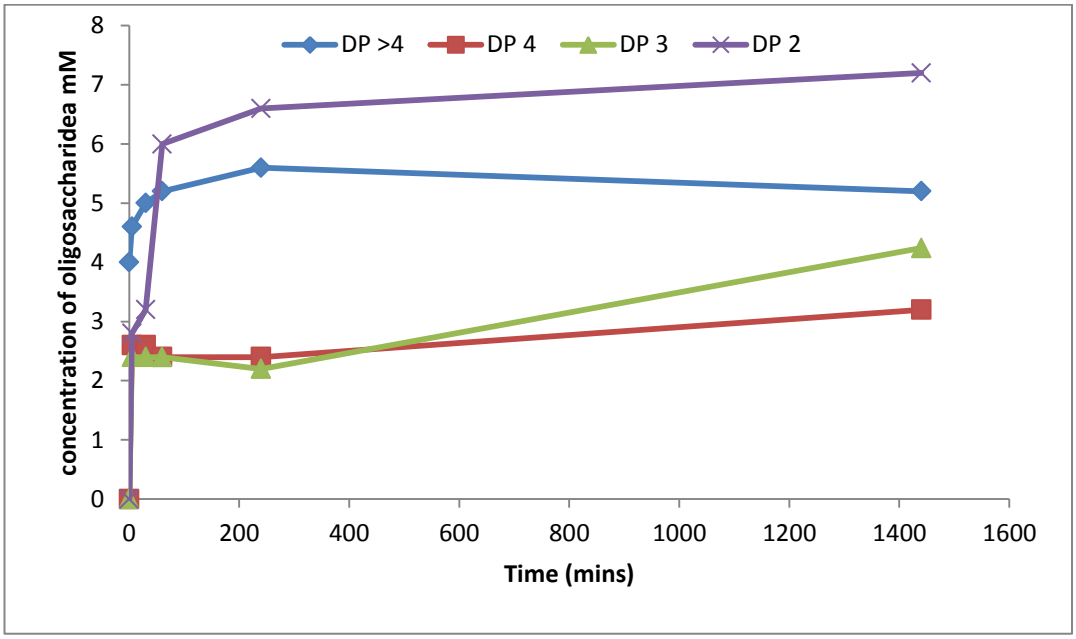


Fig 3.25: *Concentration of oligosaccharides during enzyme hydrolysis of 10%w/v **MWO** cassava peels by cassava C and 0.3%v/v cassava R in 0.05M sodium acetate buffer at pH 5 and at temperature 50^0C*

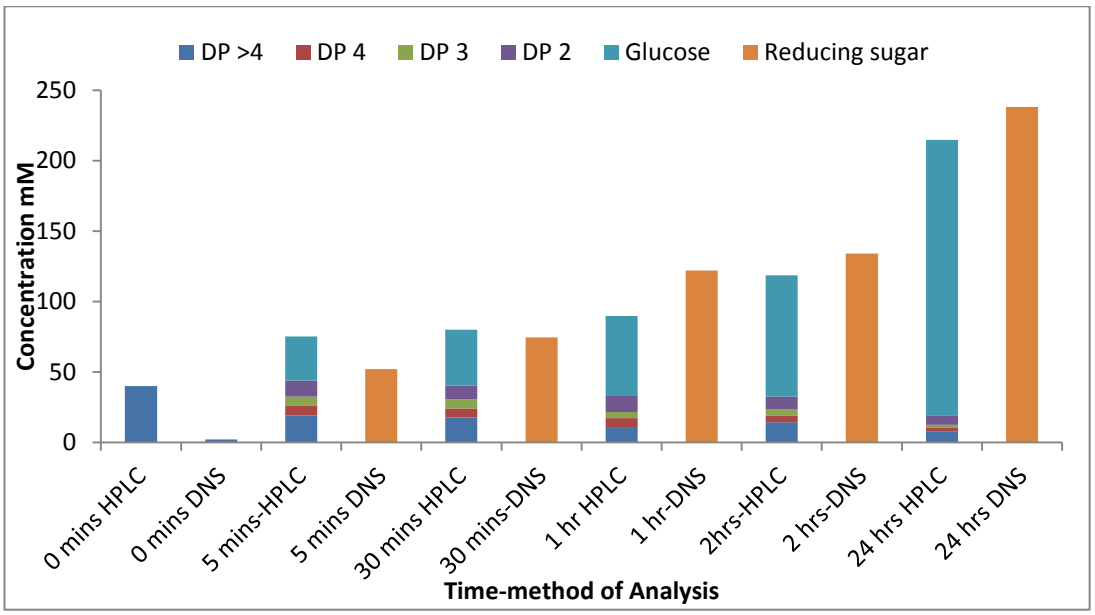


Fig 3.26 *Comparison of HPLC analysis with DNS assay for 10%w/v **SE** cassava peels at 0.3%v/v enzyme concentration in 0.05M sodium acetate buffer at pH 5 and at temperature 50^{0}C*

An observed anomaly in figure 3.26 above is that at time 0, the DNS assay shows very low concentration of reducing sugar when compared to the HPLC analysis. It is possible that reducing sugars present during steam explosion can be oxidized. Studies (Ramos 2003, Nas 2008) have reported the oxidation of oligosaccharides and monosaccharides being converted to aldonic acids especially in biological systems. This is represented by the reactions outlined below.

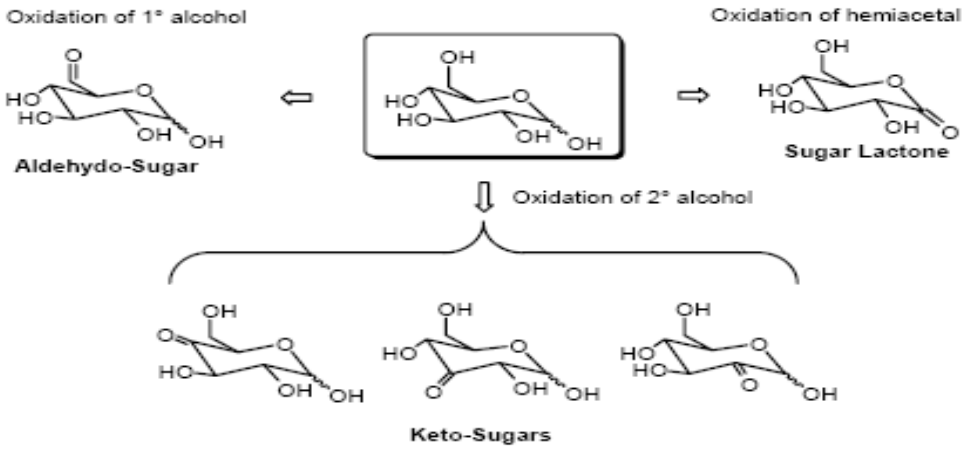


Fig 3.27; Possible oxidation products of glucopyranose.

A high temperature system like steam explosion in an aqueous environment can result an oxidation of sugar compounds. The reducing ends of the oligosaccharides may be oxidized during the steam explosion process making it impossible to be detected by DNS assay. However, as the cellulase enzymes act on the internal hydrolysable ends, More chain ends are then released for exo action as the reaction progresses. Another possible contribution might be the use of the molecular weight of 990g/mol (molecular weight of oligosaccharides with DP 6) to calculate the moles of oligosaccharides with DP greater than 4. It is possible that steam explosion produced oligosaccharides of higher DP from cassava peels. Therefore, the choice of the molecular weight of oligosaccharides with DP 6 to estimate the moles of DP greater than 4 might also be responsible for this anomaly.

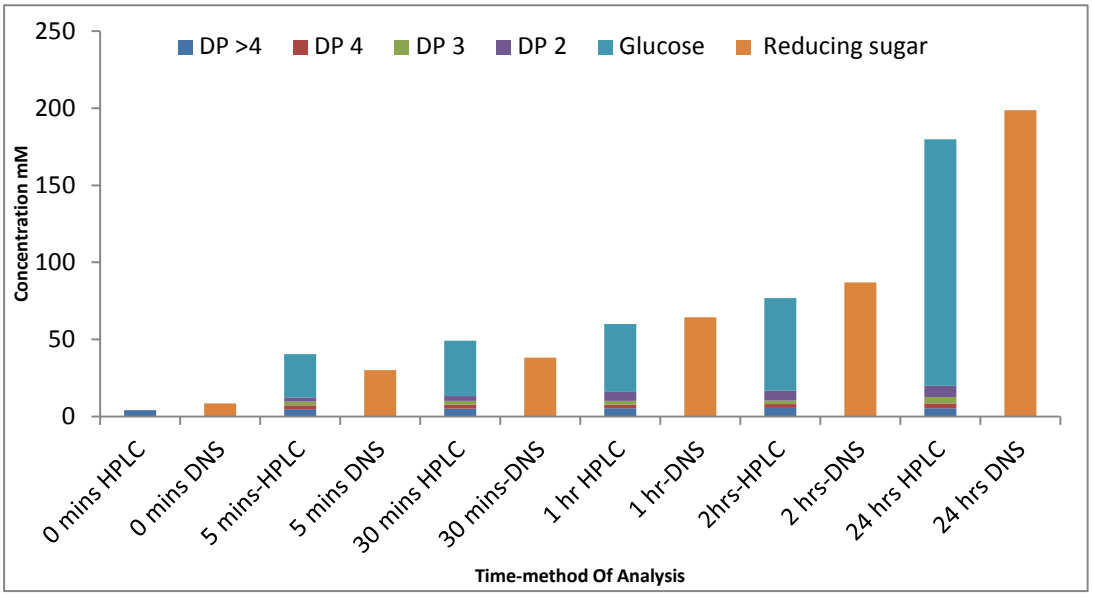


*Fig 3.28: Comparison of HPLC analysis with DNS assay for 10%w/v **MWO** cassava peels at 0.3%v/v enzyme concentration in 0.05M sodium acetate buffer at pH 5 and at temperature 50^{0}C*

The sugar release pattern of the enzyme hydrolysis shows that steam exploded samples had far more oligosaccharides with DP 2 and above present in the first 2 hours of the reaction when compared to the milled samples therefore facilitating a greater amount of glucose released into solution. Supernatant from the steam explosion process also shows a large amount of oligosaccharides already present before the reaction starts. Figure 3.29 a, b and c show chromatograms of steam exploded samples and milled samples.

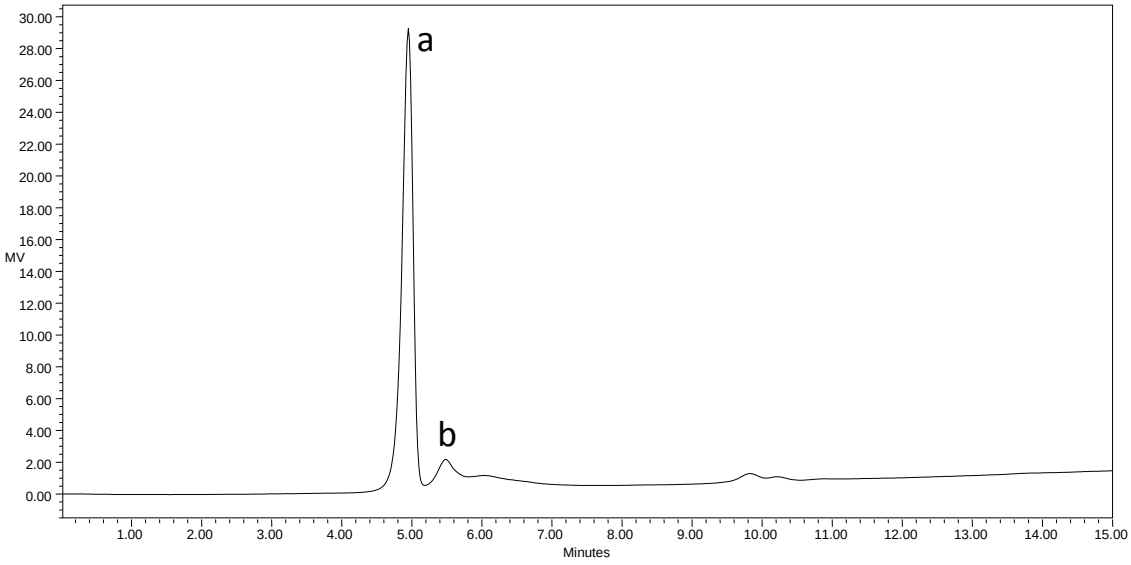


Fig 3.29a: Example chromatogram of the supernatant sample during the enzyme hydrolysis of 10%w/v SE cassava peels using 0.03%v/v cassava C and cassava R. SE sample at time 0 a; DP >4,b; DP 4

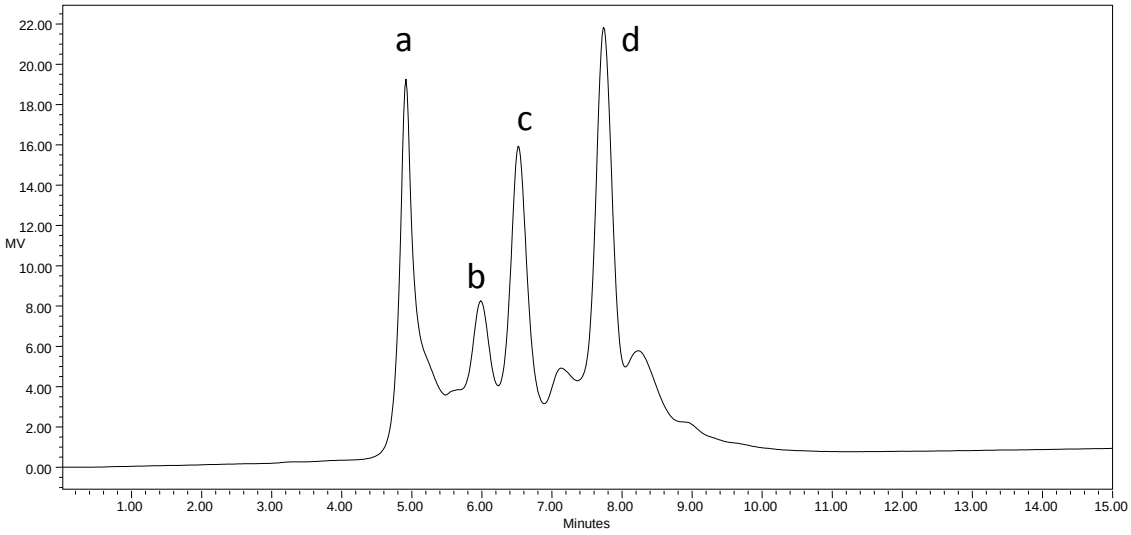


Fig 3.29b; Example chromatogram of the supernatant sample during the enzyme hydrolysis of 10%w/v SE cassava peels using 0.03%v/v cassava C and cassava R. SE sample at 30 mins. . Reactions carried out at 50^0C in 0.05M sodium acetate buffer at pH a; DP>4, b:DP3, c:DP2, d:glucose

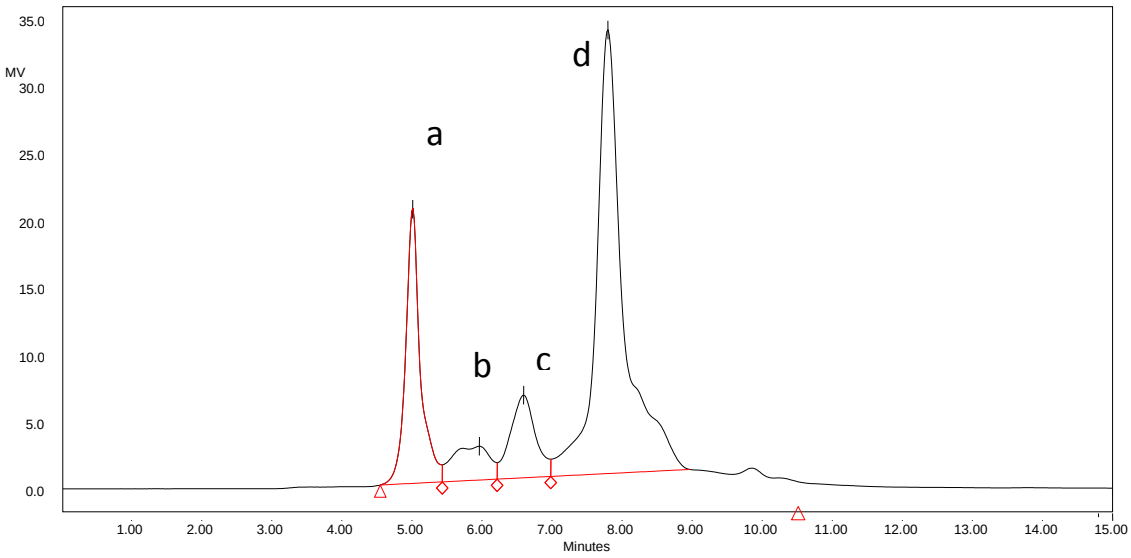


Fig 3.29c: Chromatogram of the supernatant sample during the enzyme hydrolysis of 10%w/v SE cassava peels using 0.03%v/v cassava C and cassava R. SE sample at 1 hour. . Reactions carried out at 50^0C in 0.05M sodium acetate buffer at pH. a:DP>4, b:DP 4 and DP 3, c; DP 2, d :glucose

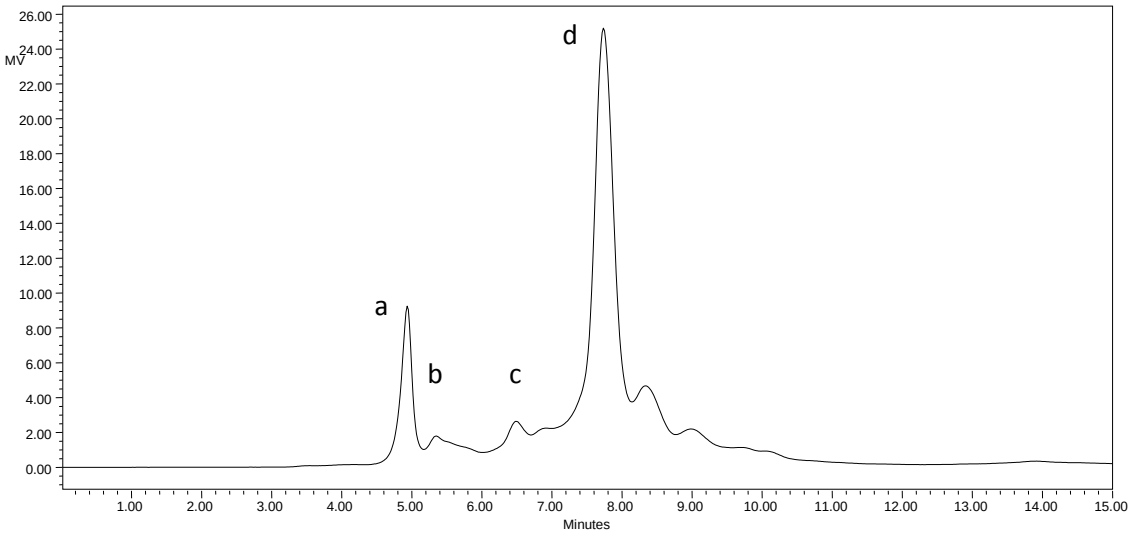


Fig 3.29d: Chromatogram of the supernatant sample during the enzyme hydrolysis of 10%w/v MWO cassava peels using 0.03%v/v cassava C and cassava R at time 30 minutes. Reactions carried out at 50^0C in 0.05M sodium acetate buffer at pH 5. a; DP>4, b:DP 4, c; DP 2, d :glucose

The resolution of the HPLC peaks is quite a challenge especially in the presence of a higher concentration of oligosaccharides of DP greater than 2. DP's of 3 and 4 often coalesce into one peak as shown in Fig 3.29 b and Fig 3.29 c. To estimate the true value of DP 2 and DP 3, the area is split proportionately between DP of 3 and DP of

4 as judged by eye. The presence of small peaks on either side of glucose which elutes at about 8-8.2 minutes is believed to be due to be caused by the presence of α and β anomers of the carbon atom of a terminal reducing group. These peaks were also present in the glucose standards as well. Terminal groups in sugars may have various types of cyclic forms in addition to the open-chain form. Under certain conditions where the rate of conversion of such diastereomers is low, α-anomer and β-anomer are separated as they pass through the column. This causes a undesirable splitting or broadening of the peak. It has been reported that anomer separation does not take place at high temperatures between 70^0C-80^0C (http://www.shodex.com/english/dc030202.html retrieved 02-05-2013)

Having made an attempt to analyse the effect of soluble oligosaccharides on the hydrolysis rate and yield and since cellulose is an insoluble and structured substrate, it is imperative to examine the possible role of insoluble solids in the overall kinetics of cellulose digestion. As can be observed from this section, upon the release of soluble oligosaccharides from insoluble solids, the sugars are acted upon by enzymes to produce glucose with little concentration of oligosaccharides of DP 2 and above. The rate limiting step of the hydrolysis of cellulose is most likely linked to hydrolysis of insoluble solid. As stated in the chapter 1, characterising cellulose insoluble solid and how it affects cellulose hydrolysis rate has been a subject of much research over the last two decades with varied interpretations on how exactly the different cellulose characteristics; assessable surface area, crystallinity and degree of polymerisation affect hydrolysis rate (Bommarius et al 2008). It is generally agreed that three main factors; namely the properties and mode of action of cellulase, structure of cellulose and mode of interaction between enzyme and cellulose molecules have the most effect on kinetics of cellulose hydrolysis.

For this study, insoluble solids of cassava peels were monitored as the reaction progresses with a modified DNS assay as discussed in chapter two. However, because of the unique composition of cassava peels (presence of both starch and cellulose), the assay was done after the peels were treated by amylolytic enzymes.

The results below show the reducing sugar concentration which is a measure of the chain end concentration.

Elsewhere in this thesis, reducing sugar yield (dextrose equivalent) has been measured based on the mass of cassava peels. However, for insoluble solid analysis, the Dextrose Equivalence (DE) in gram of glucose per gram of cellulose believed to be present in the peels used in calculating the percentage reducing sugar yield. The cellulose content in the cassava peels is estimated based on the cassava peel compositional analysis as presented in section 3.1. After amylase treatment, glucose is washed out and the residual cassava peels freeze dried. The residual solid is estimated to have roughly 50% cellulose and hemicellulose, 33% lignin and 17% other constituent material (moisture, acetyl groups and ash).

$$\text{Dextrose Equivalent (DE)} = \frac{\text{reducing sugar expressed as glucose in gram X 100}}{\text{0.50 X weight of residual cassava peels in gram}}$$

0.50 represents the fractional cellulose content after amylase treatment.

It is also known that DE is inversely proportional to the degree of polymerization (DP) of the cellulose molecule as it reflects the percentage of the cellulose molecule converted to glucose molecules i.e cellulose can be converted to glucose molecules with DE-values from 1 to 100 (pure cellulose having a DE of 0). To compensate for the addition of water to anhydroglucose to form glucose, the general formula below is used.

$$DP \times DE = 111$$

It should be noted however that The DE only gives an indication of the average degree of polymerisation (DP) of cellulose.

The results in Fig 3.30-3.33 below show the reducing sugar concentration which is a measure of the chain end concentration in the insoluble substrate. Fig 3.30 shows the reducing sugar concentration per gram of cellulose after amylase treatment. Fig 3.30a shows the reducing sugar yield per g of cellulose during the hydrolysis of cassava peels by cassava C enzyme and fig 3.30b shows results from the hydrolysis using cassava R.

It must be noted that the determination of chain end concentration by the DNS assay in insoluble solid has its limitations with regards to a possible incomplete accessibility of chain ends (Kongruang et al 2004). The drying of the substrate after hydrolysis also presents an added disadvantage as cell walls are known to collapse during the drying process. Freeze drying was used to minimise this effect as described in chapter 2.

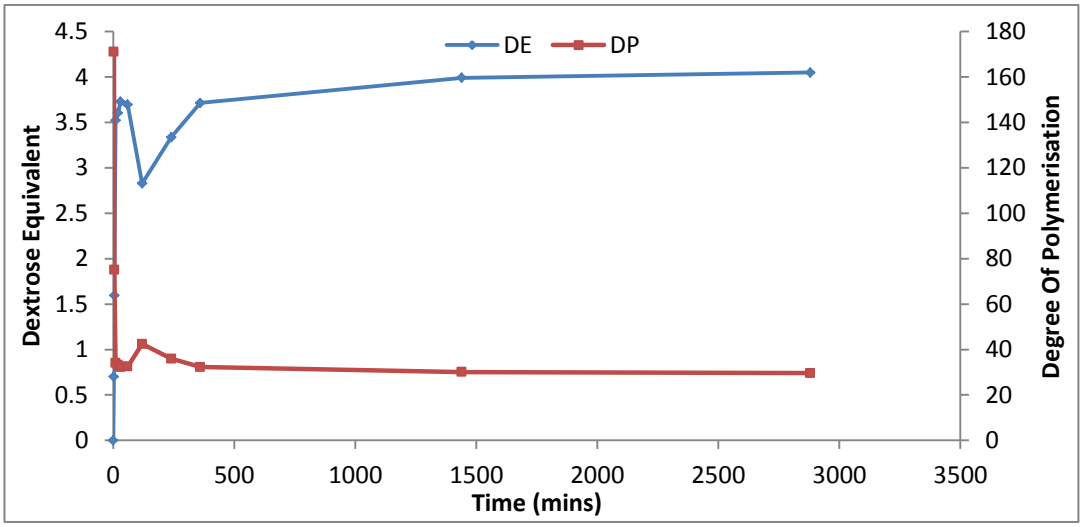


Fig 3.30a: Reducing sugar concentration (DE) and average degree of polymerisation (DP) of cellulose in residual solid phase of cassava peels. Reaction was with 10%w/v Cassava Peels and 0.3%v/v Cassava C in 0.05M sodium acetate buffer at pH 5 and 50^{0}C

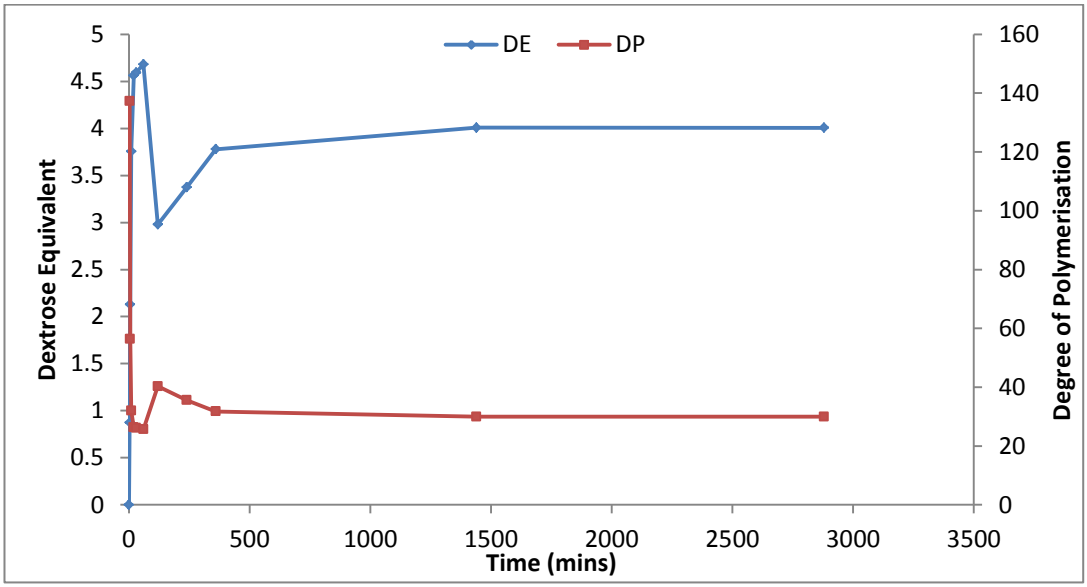


Fig 3.30b: Reducing sugar concentration (DE) and average degree of polymerisation (DP) of cellulose in residual solid phase of cassava peels. Reaction was with 10%w/v Cassava Peels and 0.3%v/v Cassava R in 0.05M sodium acetate buffer at pH 5 and 50^{0}C

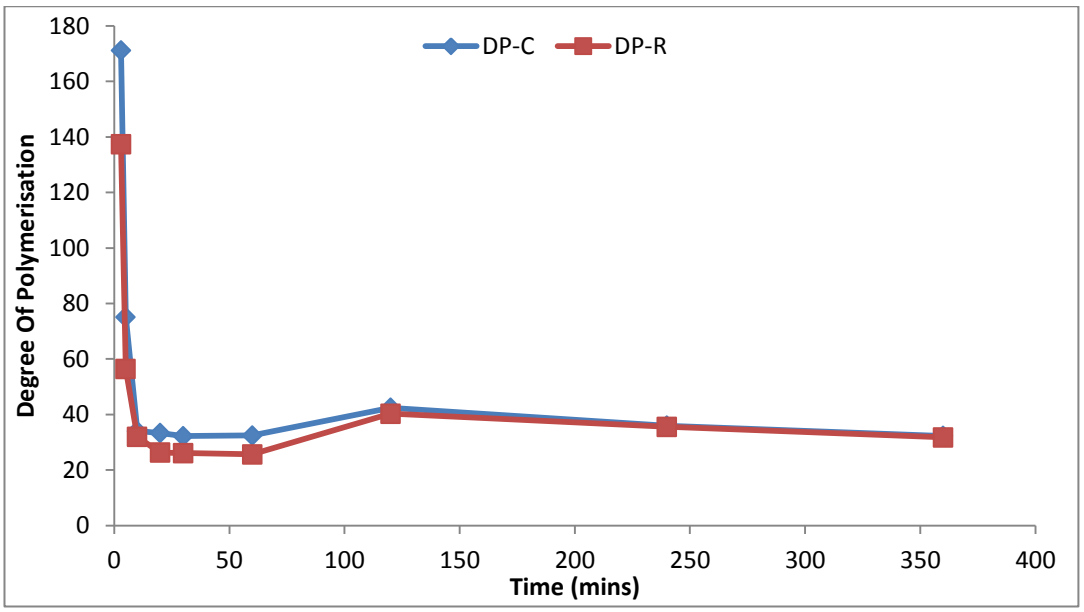


Fig 3.31: Average degree of polymerisation (DP) of residual cellulose within first 3 hours Reaction was with 10%w/v Cassava Peels and 0.3%v/v Cassava C and Cassava R in 0.05M sodium acetate buffer at pH 5 and 50^{0}C. DP- C shows DP of cellulose in cassava peels using cassava C enzyme and DP-R shows DP of cellulose in cassava peels using cassava R enzyme

The results above show that the degree of polymerisation of the residual cellulose drops very rapidly within 10 minutes of the reaction to a fairly constant value. The degree of polymerisation is constant for about two hours after which it increases slightly to approximately 40. There is then a gradual decrease noted after 2 hours of reaction in the degree of polymerisation and these continues until fairly constant value of 30 is noticed until 48 hours of reaction. Comparing the degree of polymerisation in the residual solid and the percentage reducing sugar observed in the liquid phase is shown in fig 3.32 and fig 3.33.

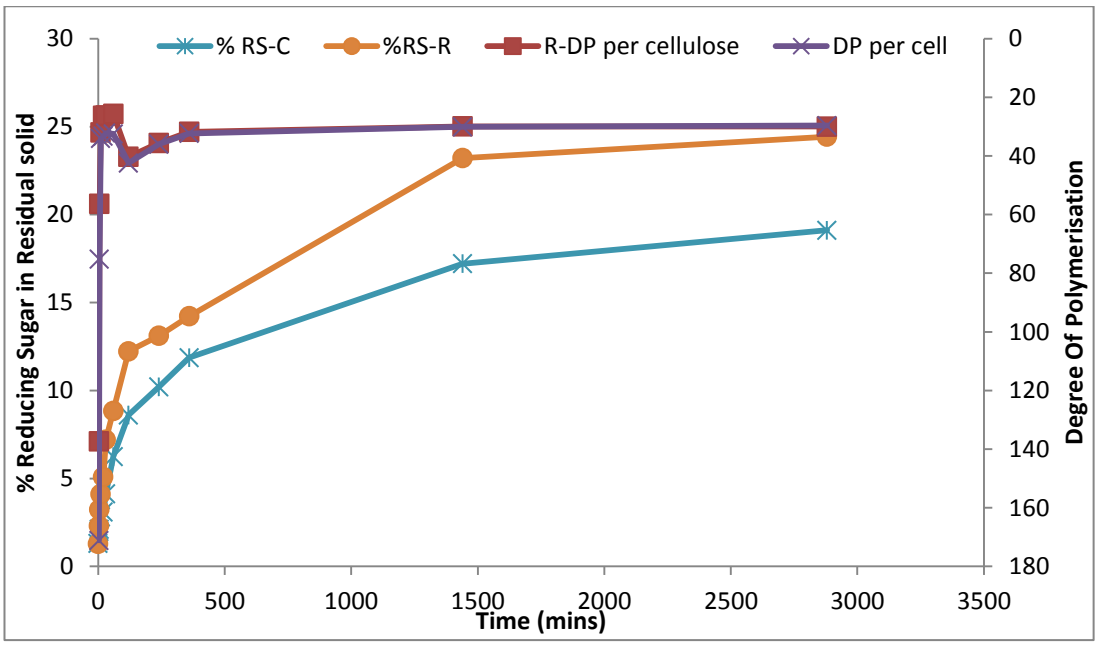


Fig 3.32 Comparison of the average degree of polymerisation of cellulose in cassava peels with reducing sugar concentration in solution. Reaction was carried out with 10%w/v Cassava Peels and 0.3%v/v Cassava C and Cassava R in 0.05M sodium acetate buffer at pH 5 and 50^{0}C. . DP- C shows DP of cellulose in cassava peels during hydrolysis using cassava C enzyme and DP-R shows DP of cellulose in cassava peels during hydrolysis using cassava R enzyme. %RS- C shows the percentage reducing sugar of cassava peels when using cassava C enzyme and %RS- R shows the percentage reducing sugar of cassava peels when using cassava R enzyme

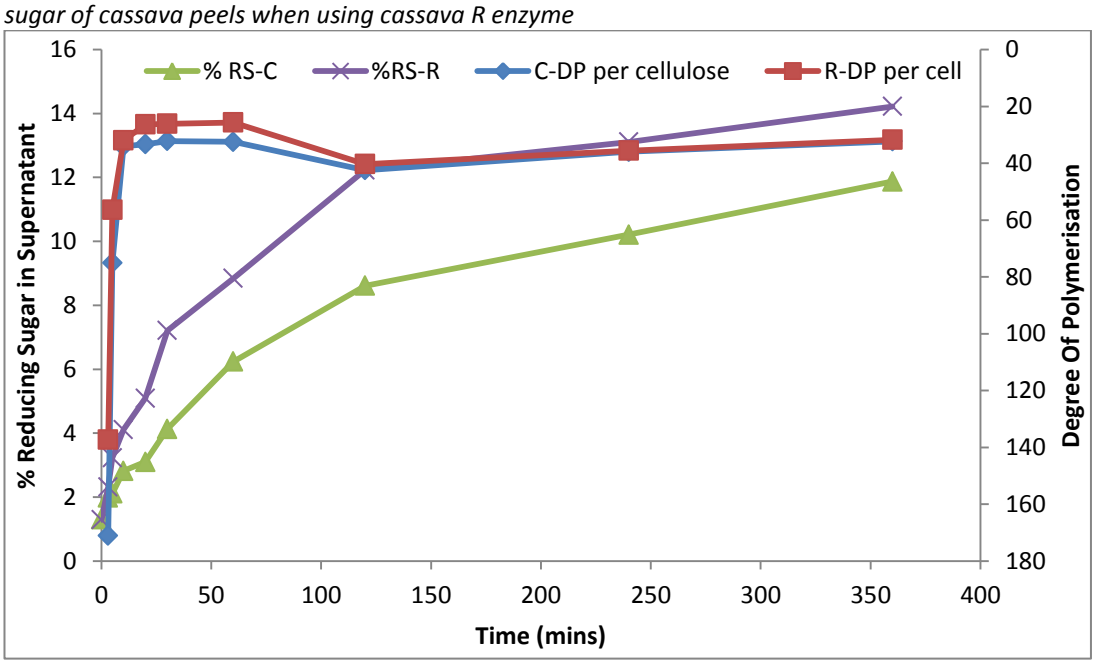


Fig 3.33 Comparison of the average degree of polymerisation of cellulose in cassava peels with reducing sugar concentration in solution within the first hour of the reaction. Reaction was carried out with 10%w/v cassava peels and 0.3%v/v Cassava C and Cassava R in 0.05M sodium acetate buffer at pH 5 and 50^{0}C. DP- C shows DP of cellulose in cassava peels during hydrolysis using cassava C enzyme and DP-R shows DP of cellulose in cassava peels during hydrolysis using cassava R enzyme. %RS- C shows the percentage reducing sugar of cassava peels when using cassava C enzyme and %RS- R shows the percentage reducing sugar of cassava peels when using cassava R

Fig 3.32 and fig 3.33 show a rapid increase in reducing sugar concentration in the solid residue within the first 10 minutes of the reaction. Previous experiments with cellulose hydrolysis elsewhere in this thesis also show a rapid increase in hydrolysis yield within the first 3 hours of the reaction. This rapid increase in hydrolysis observed in the supernatant is most likely related to the increase in chain end concentrations observed in the insoluble solid. The sharp decrease in the degree of polymerisation in the residual cellulose (calculated from the reducing sugar concentration) within the first 10 minutes is usually attributed to endoglucanase activity (Zhang and Lynd 2004). It is also noted that within the first 10 minutes of the reaction, over 90% of the glucoside bonds in the cellulose are still not hydrolysed. However, a steady increase in the extent of hydrolysis is observed until about 3 hours of the reaction. Also, over 47% reducing sugar per gram of cellulose is obtained at 6 hours of reaction for cassava peels treated with hemicellulase and cellulases (cassava R). The hydrolysis reaction using just cellulases (cassava C) yields about 40% reducing sugar in that time. The availability of a high amount of chain ends for acting by endoglucanase is generally believed to be the main cause of an increase in the hydrolysis rate in the early stages of cellulose hydrolysis (Zhang and Lynd 2004). The dramatic slowdown of the rate as the reaction progresses still remains unexplained. Several hypotheses exist to answer the questions. One possible explanation is that the accessible part of the cellulose is rapidly degraded by the exoglucanase enzyme producing low molecular chain carbohydrates as observed in the first 10 minutes and remaining constant until 2 hours. Subsequently, the activity of the endoglucanase is reduced as the more crystalline part of the peels becomes more difficult to break down. The effect of glucose and cellobiose inhibition on cellulase can also have an effect on hydrolysis rate. Thermal deactivation of enzymes and irreversible adsorption of cellulases on lignin have also being mentioned as contributing factors.

Several investigators have also implicated the available surface area as the most significant contribution to this observed slowdown of the rate and yield. Levine et al

(2010) published a paper on the mechanistic model of the enzyme hydrolysis of cellulose in which they proposed that the rate of hydrolysis is directly proportional to the cellulose surface area. They believed that as reaction progresses, the cellulose particles shrink with the release of soluble oligosaccharides which results in a reduced cellulose area. These ''fresh '' chains of cellulose become more inaccessible as the reaction progresses as the more amorphous part of the cellulose is hydrolysed leaving the more crystalline part of the cellulose with lower total surface area. They compared the fraction of sites available to endoglucanase II for hydrolysis when the enzyme acts alone or when it is mixed with cellobiohydrolase. They found these ''hydrolysis sites'' much lower in a mixed cellulase hydrolysis compared to endoglucanase alone. Each cellulase that adsorb unto a lattice of glucose chain occupies a certain number of sites described by its footprints. The enzyme footprints of the cellulases on these ''hydrolysis sites'' were estimated by small angle Xray scattering measurements. The decreased accessibility of cellulose is believed to limit the effectiveness of the endoglucanase within a cellulose mixture. The competition between cellobiohydrolases and endoglucanases for hydrolysable bonds on the cellulose monolayer as the surface area decreases limits hydrolysis rate. As smaller oligosaccharides are released from the cellulose surface, there is a decreased total surface area. The competition for adsorbable hydrolysable sites is believed to affect the endoglucanase enzyme more than the cellobiohydrolase enzymes. This is because there are more ''glycosidic bonds than chain ends''. The argument put forward by Levine et al is that as the reaction progresses with an expected increase in additional chain ends, lots of the exoglucanase enzyme is adsorbed on the chain ends leading to enzyme crowding on the cellulose surface. The presence of exoglucanase and beta glucosidase enzymes also contributes to this enzyme crowding on the cellulose surface. However, the endoglucanase enzymes are more affected from accessing most of the glycosidic bonds on the cellulose surface. This interference decreases the ability of the endoglucanase to increase the number of chain ends available for cellobiohydrolase to act upon. This leads to lower reaction rate.

These experiments were conducted on model avicel paper, a pure cellulose substrate having different surface areas both in the presence of single enzymes and mixed enzymes. How this result could be relevant to the hydrolysis of cassava peels in the presence of lignin and hemicellulose would be difficult to explain.

3.5.0 CONCLUSION

This chapter set out to determine the effect of a separate amylase treatment or cellulase treatment on cassava peels. The following conclusions can be drawn from results in this chapter.

An amylase and glucoamylase treatment of milled cassava peels releases very high yields of monosaccharides from the starchy part of cassava peels. The study also shows that steam explosion pretreatment is not a good pretreatment strategy for starch treatment as it destroys the starch in cassava peels or inhibits the amylolytic enzymes. An industrial process where only starch is degraded by amylase enzymes is an option as amylase enzymes are readily available. A potential reducing sugar yield of 0.41g/g of cassava peels can be produced from milled cassava peels. A 10%w/v cassava peel concentration is the optimum cassava peel concentration because it guarantees an easier mixing of the enzymes and substrate.

The results of experiments in this chapter also show that a separate cellulase treatment of cassava peels also gave high yields of sugars both for milled and steam exploded samples. However, steam exploded samples gave higher yields and faster hydrolysis rate when compared to milled samples. Steam explosion can be used to treat cassava peels if the treatment of peels is targeted at cellulose and hemicellulose part of the peels.

These findings would suggest that a separate amylolytic and cellulolytic treatment of cassava peels would give very high sugar yields when both treatments are carried out sequentially. This is investigated in chapter 5. The drawback to a sequential treatment of the peels is that it might be more expensive when considering cost of separate reaction vessels, separation of the sugars by filtration and centrifugation.

It is also time consuming. This separate hydrolysis would also prevent the possibility of using a simultaneous saccharification and fermentation process option.

It would also be interesting to investigate the effect of steam explosion on a sequential treatment of cassava peels. An experimental design where milled samples are treated with the amylase enzymes and the sugars separated and then the cassava residue treated with steam explosion and cellulase enzymes can be investigated. This would combine excellent results obtained from the milled cassava peels treated with amylases with the very high hydrolysis yield and rates obtained from steam exploded cassava peels treated with cellulase enzymes.

CHAPTER FOUR: RESULTS AND DISCUSSION-SIMULTANEOUS HYDROLYSIS

4.0.0: A COMBINATION OF AMYLOLYTIC AND CELLULOLYTIC ENZYMES (AMYLASE, GLUCOAMYLASE, CASSAVA C AND CASSAVA R) ON CASSAVA PEEL.

A combination of all enzymes at same temperature and pH represents the simplest process although these enzymes are known to function at different optimum conditions(temperature and pH). The optimal temperature for amylases is known to be about 90^0C, cellulases 50^0C and glucoamylase between 50^0C and 60^0C . 50^0C was selected for this reaction as a compromise between cellulases, glucoamylase and the cassava R which have lower temperature optima compared to amylases. A pH of 5 was also picked because the cellulases and glucoamylases used for these experiments perform best at pH between 4.5 and 5. Some interesting results were obtained as shown below figs 4.1-fig 4.3 below. **MWO** still represents milled, washed and oven dried cassava peel and **SE** represents steam exploded cassava peel. The enzyme concentration in the experiments is still in %v/v. For example, a representation of 0.15v/v MW0 would represent 0.15%v/v MWO enzyme concentration of an experiment carried out on milled cassava peels and 0.30%v/v SE would represent an enzyme concentration of 0.3%v/v on a steam exploded cassava peel substrate. The cassava peel concentration is always given in %w/v. % reducing sugar is still expressed as g of reducing sugar produced per gram of cassava peel.

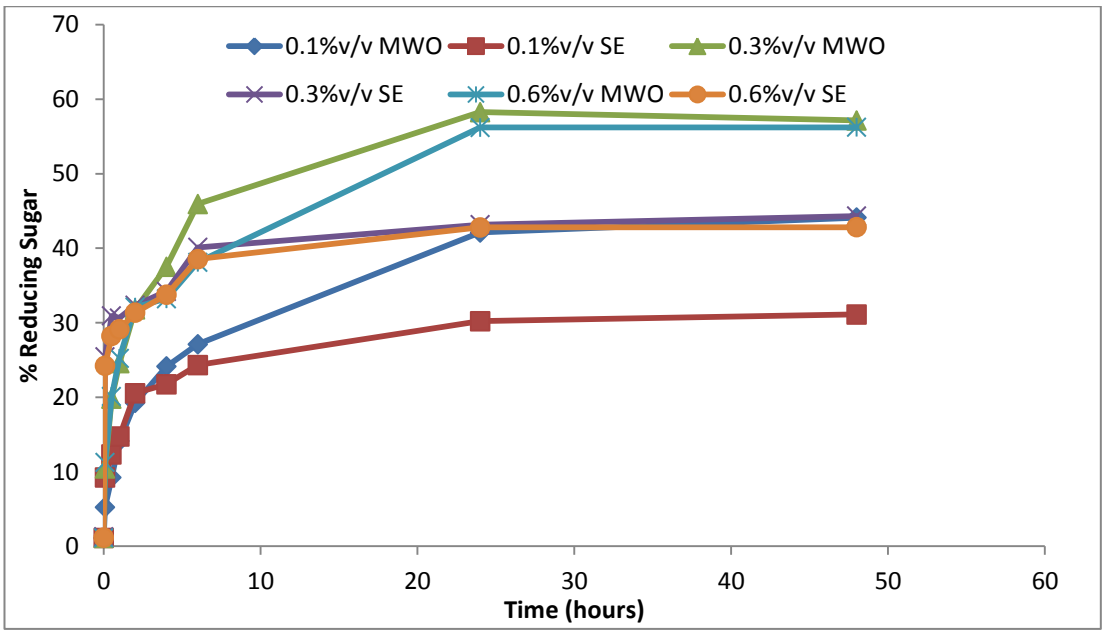


Fig 4.1: Progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with cassava C and cassava R, amylase and glucoamylase at temperature 50^{0}C

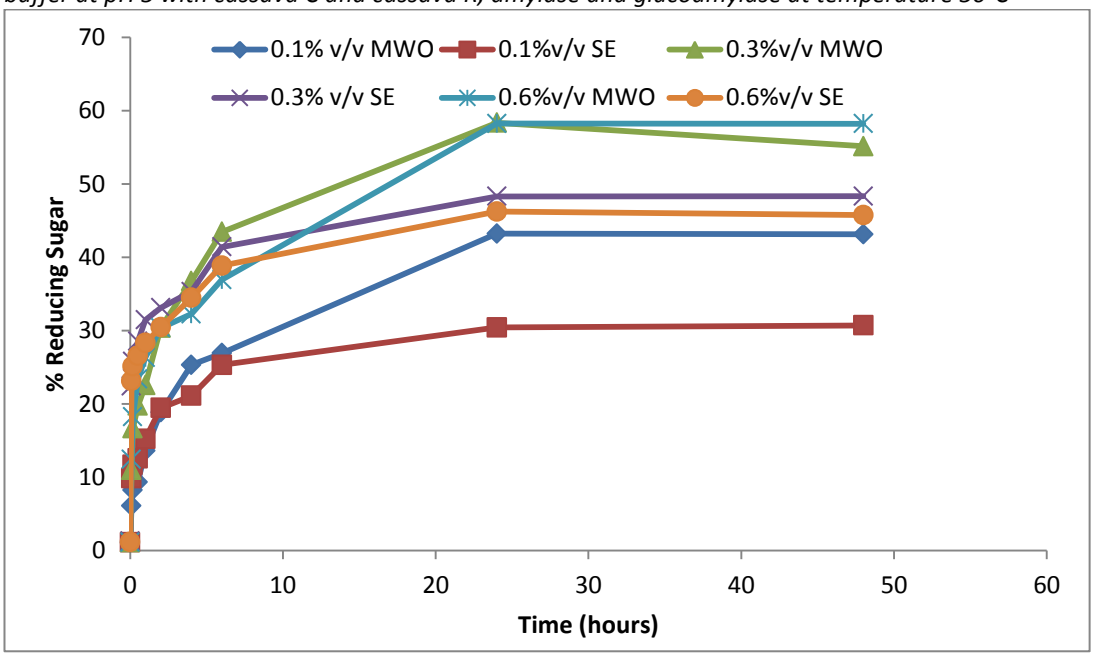


Fig 4.2: Progress curve of the enzymatic hydrolysis of 14%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with cassava C and cassava R, amylase and glucoamylase at temperature 50^{0}C

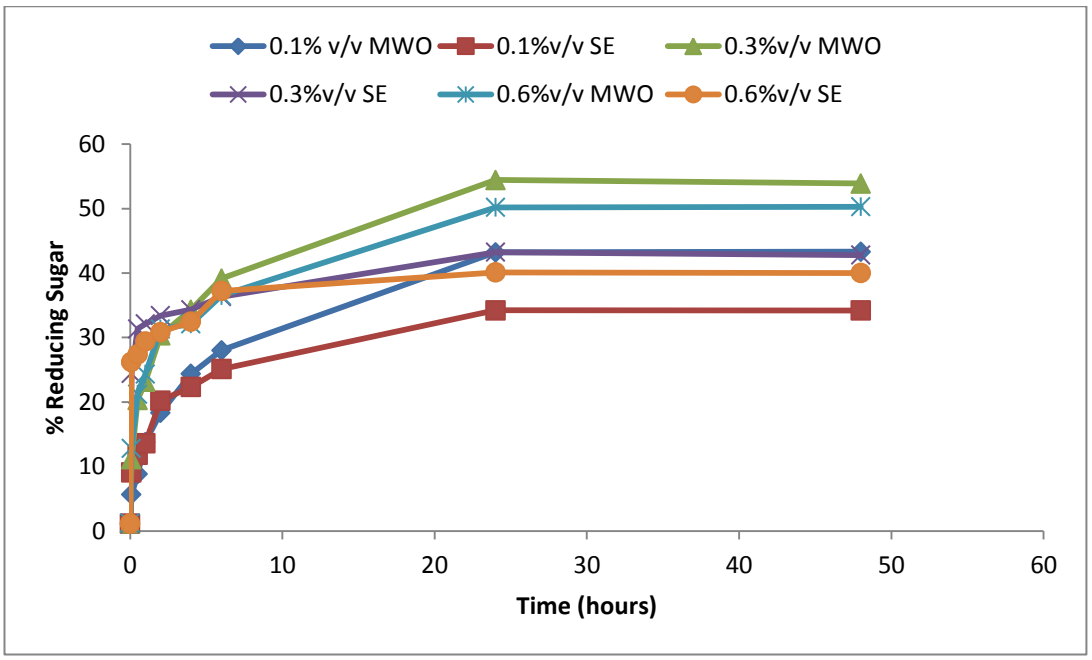


Fig 4.3: Progress curve of the enzymatic hydrolysis of 20%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 with cassava C and cassava R, amylase and glucoamylase at temperature 50^{0}C

The progress curves show that hydrolysis carried out at 10%w/v and 14%w/v cassava peels gave very similar hydrolysis yield. At 20% w/v a drop in the reducing sugar yield is observed perhaps due to inhibition of cellulase at high glucose concentration. Results also show that when all enzymes were used, most of the reactions were completed in 24 hours. However, in some cases where samples are taken in 48 hours, there was a small increase in the amount of reducing sugar produced perhaps due to experimental errors. For samples with an enzyme loading of 0.1%v/v, maximum glucose concentration was achieved at 48 hours. This reaction is slower than those carried out at 0.3%v/v and 0.6%v/v and progresses steadily until 48 hours.

0.3%v/v enzyme concentration gave slightly higher reducing sugar than 0.6%v/v enzyme concentration at all substrate concentrations although as will be shown later, reactions with 0.6%v/v gave higher initial rates.

The first hour of the reactions are shown below.

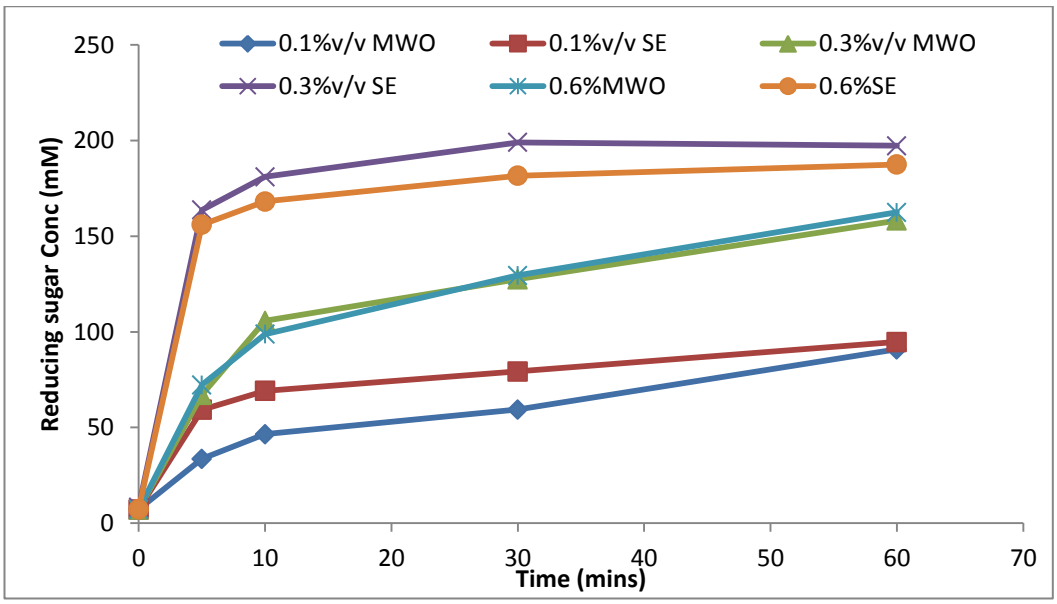


Fig 4.4: First hour of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 by cassava C and cassava R , amylase and glucoamylase at temperature 50^{0}C

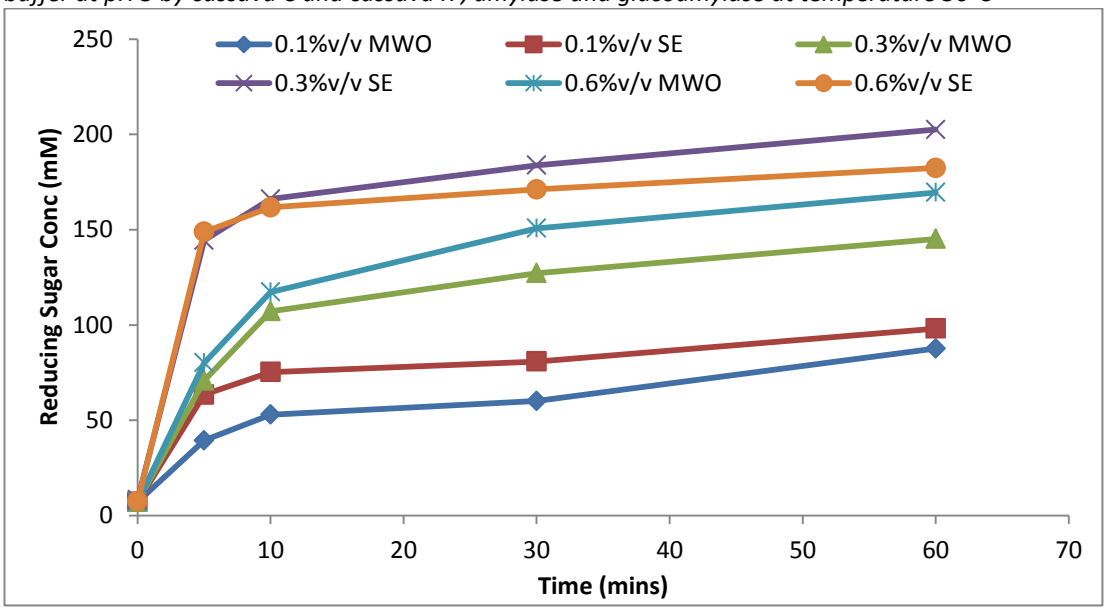


Fig 4.5: First hour of the enzymatic hydrolysis of 14%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 by cassava C, cassava R , amylase and glucoamylase at temperature 50^{0}C

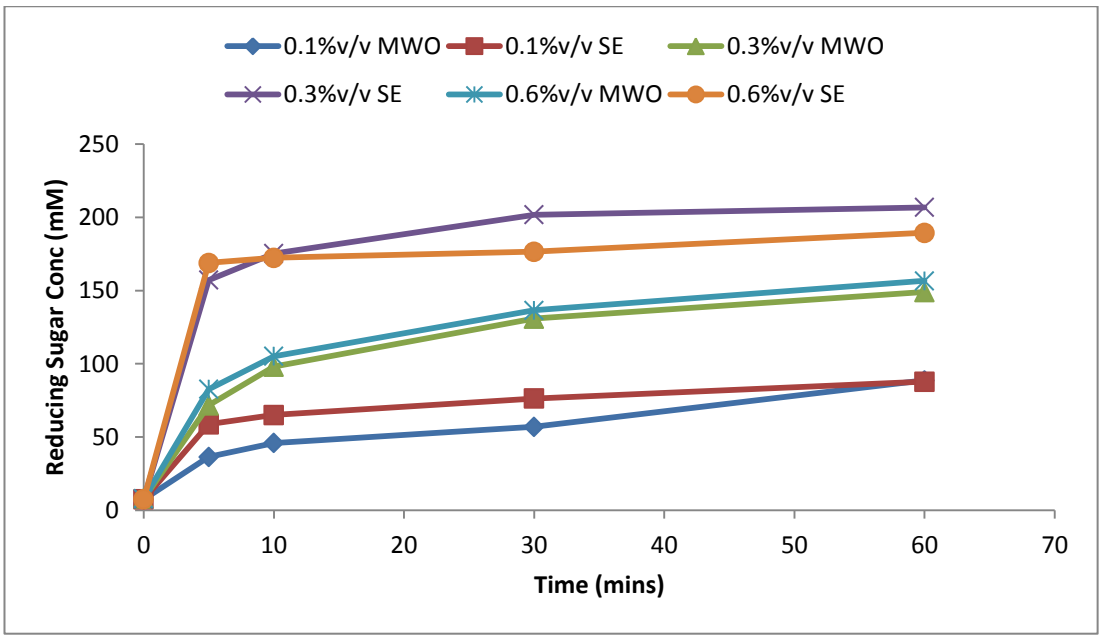


Fig 4.6: First hour of the enzymatic hydrolysis of 20%w/v cassava peels in 0.05M sodium acetate buffer at pH 5 by cassava C and cassava R, amylase and glucoamylase at temperature 50^0C

A further look at the first hour of hydrolysis as shown in fig 4.4- fig 4.5 above shows that the steam exploded sample reacts faster than the milled sample at all substrate concentrations. This suggests that perhaps steam explosion is effective in dislocating the hemicelluloses and cellulose component of the peels to make hydrolysis faster.

Initial Rates of Reaction (mM/min)						
Substrate concentration	***0.1%v/v amylase+AMG+ Cassava C +Cassava R***		***0.3%v/v amylase+AMG+ Cassava C +Cassava R***		***0.6%v/v amylase+AMG+ Cassava C +Cassava R***	
	MWO	**SE**	**MWO**	**SE**	**MWO**	**SE**
10% w/v	2.2±0.2	8.4±0.3	11.0±0.2	22.0±0.3	10.4±0.1	22.0±0.2
14 % w/v	5.2±0.3	8.4±0.2	11.4±0.2	22.0±0.2	12.0±0.1	19.6±0.2
20 % w/v	4.4±0.4	7.4±0.5	10.4±0.2	20.4±0.2	11.6±0.1	22.0±0.2

Table 4.1: Initial rate estimate of enzyme hydrolysis of cassava peels at different enzyme concentrations

The initial rates data also shows that steam exploded samples show far higher initial rates when compared to milled samples although the final yield of the milled samples were still greater than the steam exploded samples. HPLC analysis of the reactions also show the presence of shorter chain oligosaccharides from steam exploded pretreated sample at time zero. Samples taken at time zero and 5 minutes of the reaction as shown in fig 4.7 and fig 4.10 below for steam exploded samples also show a significant amount of reducing sugar yields. If there is a breakdown of cellulose to shorter chain oligosaccharides by the steam explosion process, this would cause the cellulase enzyme molecules to act on these short chain oligosaccharides and produce such a high amount of glucose in such a short time as shown below (fig 4.10). The same pattern is not observed for milled sample in fig 4.8 and fig 4.9.

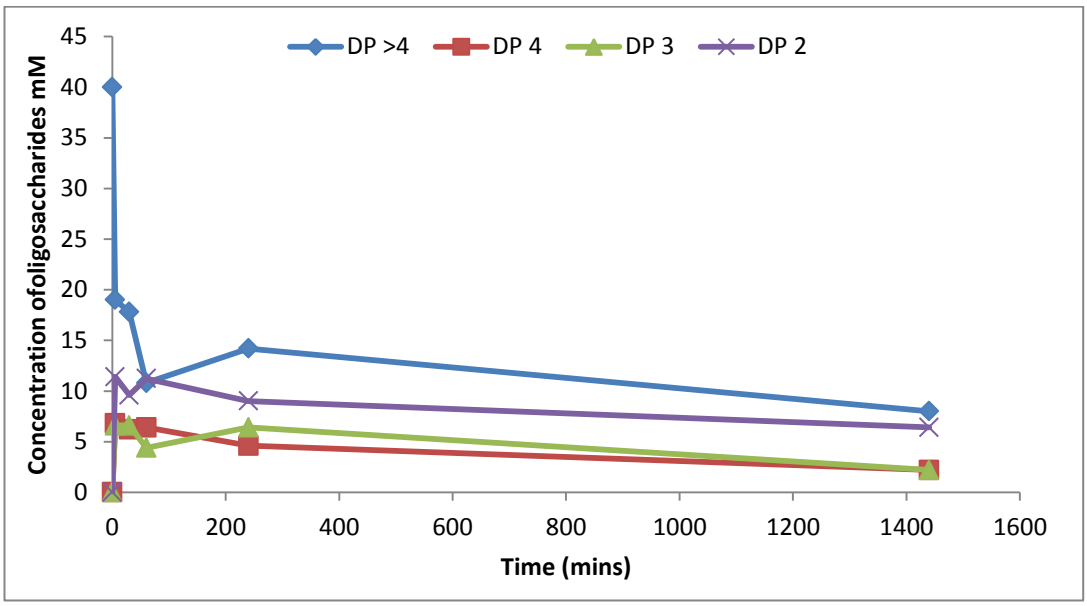


Fig 4.7: Concentration of oligosaccharides during enzyme hydrolysis of 10%w/v SE cassava peels by cassava C, cassava R, amylase and glucoamylase.

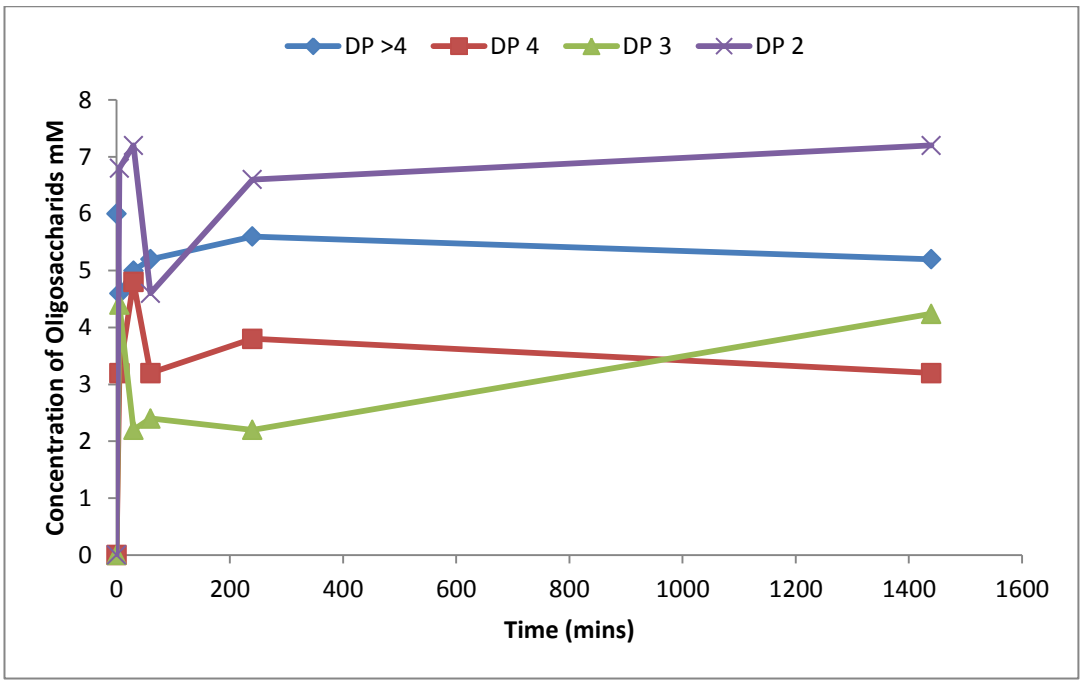


*Fig 4.8: Concentration of oligosaccharides during enzyme hydrolysis of 10%w/v **MWO** cassava peels by 0.3%v/v cassava C and cassava R, amylase and glucoamylase in 0.05M sodium acetate buffer at pH 5 and temperature of 50^{0}C*

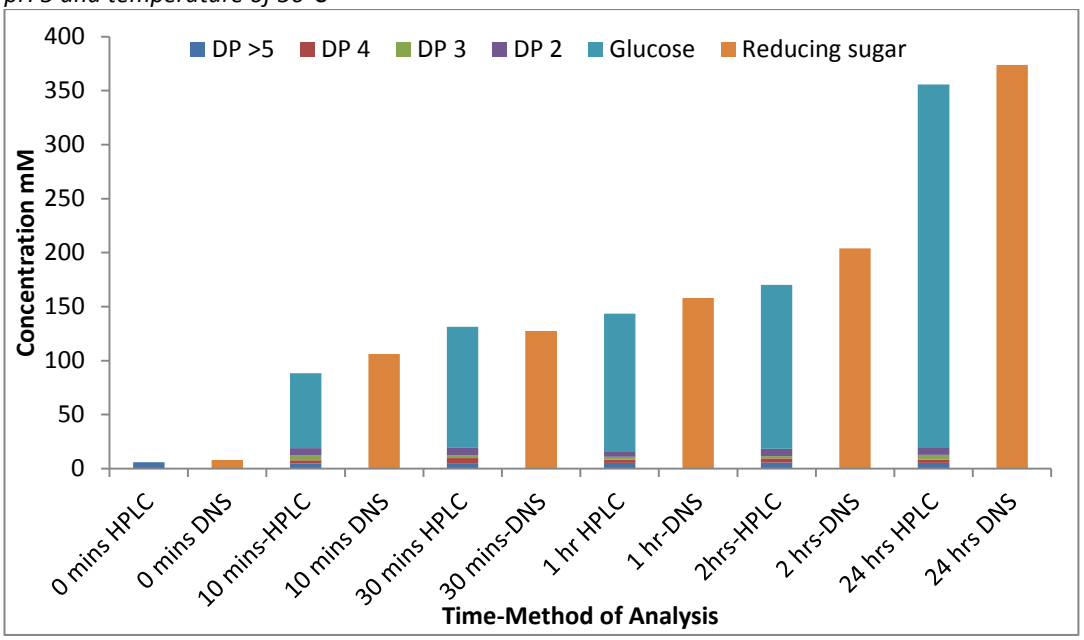


Fig 4.9: Comparison of HPLC analysis with DNS assay for 10%w/v MWO cassava peels at 0.3%v/v enzyme concentration in 0.05M sodium acetate buffer at pH 5 and temperature of 50^{0}C

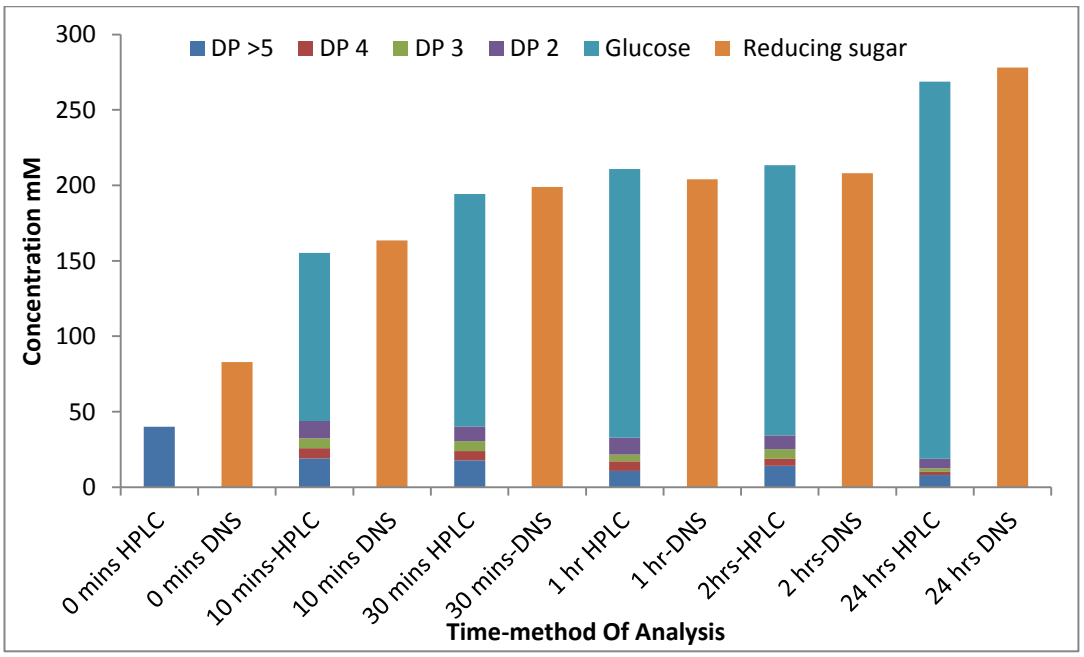


Fig 4.10: Comparison of Hplc analysis with DNS assay for 10%w/v SE cassava peels at 0.3%v/v enzyme concentration in 0.05M sodium acetate buffer at pH 5 and temperature of 50^0C

A comparison of the HPLC analysis of the oligosaccharides released into solution with the DNS assay shows some agreement as shown in fig 4.9 for milled sample. Fig 4.10 above also shows some consistency although it gives slightly lower yield for steam exploded sample for HPLC results when compared to the DNS assay.

Again, the oligosaccharides with DP 2-5 were low in concentration for the milled samples with glucose formed at high concentration immediately the reaction starts. The steam exploded sample again shows a higher amount of oligosaccharide of DP 2 to 5 within the first 2 hours of the hydrolysis reaction as stated earlier.

The final reducing sugar from the DNS assay is given below.

% Reducing Sugar at 24 hrs						
Substrate concentration	**0.1% v/v** Amylase+AMG+cassava C +Cassava R		**0.3%v/v** Amylase+AMG+cassava C +Cassava R		**0.6%v/v** Amylase+AMG+cassava C +Cassava R	
	MWO	**SE**	**MWO**	**SE**	**MWO**	**SE**
10% w/v	42.1±1.0	30.2±0.1	58.3±0.2	47.7±0.1	56.2±0.9	42.8±0.7
14 % w/v	43.2±0.9	30.4±0.3	58.4±0.4	48.3±0.2	58.2±0.5	46.2±0.4
20 % w/v	43.2±0.2	34.2±0.8	54.4±0.6	43.2±1.3	50.2±1.4	40.1±1.2

*Table 4.2 Enzyme hydrolysis of cassava peels at 10 and 14 and 20%w,v in 0.05M sodium acetate pH 5 at 50^0C for 24 hrs at different enzyme loading **MWO**-milled, washed and oven dried sample **SE**-steam exploded sample.*

4.1.0: EFFECT OF DIFFERENT ENZYME COMBINATION ON HYDROLYSIS

In this section, the effect of combining all the enzymes on cassava peels (simultaneous hydrolysis) is compared with experiments where the cassava peels are treated with either amylolytic or cellulolytic enzymes (separate hydrolysis). This section also compares the effect of the absence of cassava C and cassava R on hydrolysis.

Fig 4.11 shows the comparison of milled cassava peels at 10%w/v and Fig 4.12 shows steam exploded samples.

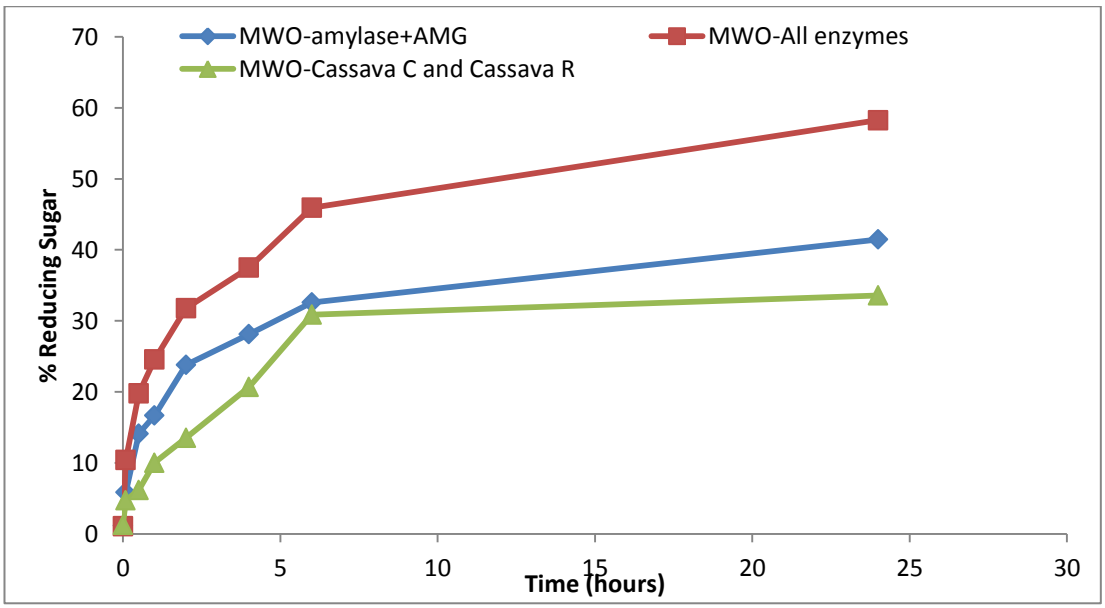


*Fig 4.11: Comparison of different treatment of 10%w/v **MWO** cassava peels by amylolytic enzymes, cellulolytic enzymes and a combination of all enzymes(amylases and cellulases) in 0.05M sodium acetate buffer at pH 5 and temperature of 50°c. (**MWO-amylase+AMG(0.3%v/v amylase and glucoamylase) MWO-cassava C and cassava R(0.3%v/v of cassava C and cassava R), all enzymes; amylase+glucoamylase+cassava C+cassava R ;each enzyme with concentration of 0.3%v/v.**)*

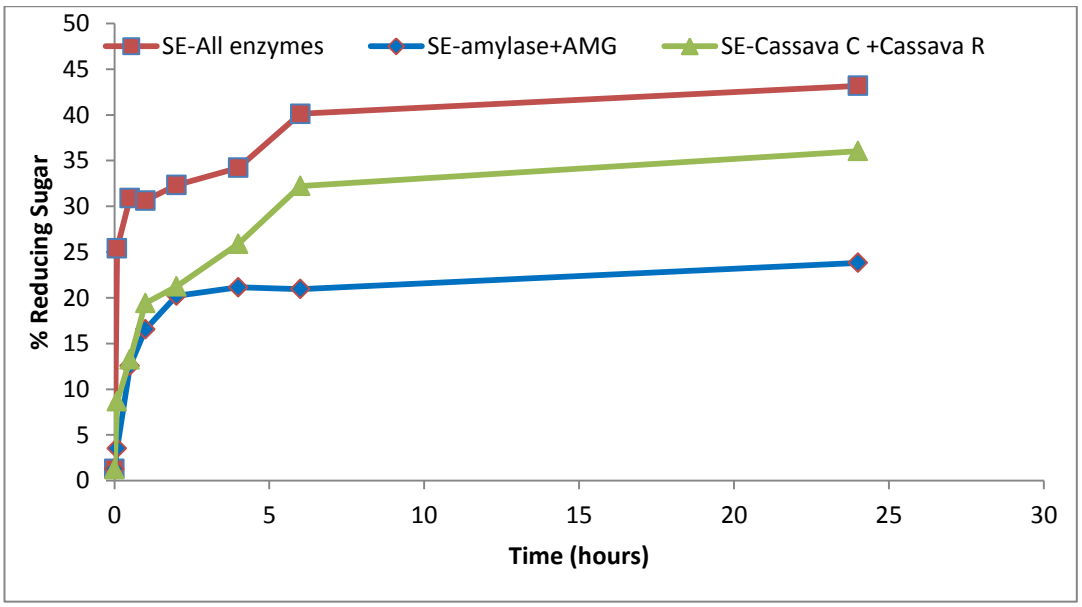


*Fig 4.12: Comparison of different treatment of 10%w/v **SE** cassava peels by amylolytic enzymes, cellulolytic enzymes and a combination of all enzymes(amylases and cellulases) in 0.05M sodium acetate buffer at pH 5 and temperature of 50°c. (**MWO-amylase+AMG(0.3%v/v amylase and glucoamylase) MWO-cassava C and cassava R(0.3%v/v of cassava C and cassava R), all enzymes; amylase+glucoamylase+cassava C+cassava R ;each enzyme with concentration of 0.3%v/v.**)*

Fig 4.11 and Fig 4.12 shows that the reactions with all enzymes were faster and yielded more reducing sugars compared to experiments where amylolytic enzymes

or cellulolytic enzymes are used. Milled samples combining amylase, glucoamylase, cassava C and cassava R yields a total reducing sugar of 58% compared to 41% reducing sugar produced from treatment with amylolytic enzymes(amylase and glucoamylase treatment) and 34% reducing sugar produced from cellulolytic treatment(cassava C and cassava R). Here, the total reducing sugar of 58% when all the enzymes are used is less than the total reducing sugar from the separate hydrolysis of using amylolytic and cellulolytic enzymes (41 + 34=75% reducing sugar. It must however be noted that the amylolytic enzymes produce some hydrolysing effect on the cellulose part of the peels while the cellulase enzymes also hydrolysed the starchy part of the peels. These enzymes would therefore be overestimating the total reducing sugars of 75% produced by both sets of enzymes. Further experiments to see the effect of using both sets of enzymes sequentially is presented in the next chapter. The 58% reducing sugar produced by the combination of all enzymes is about 85% of reducing sugar expected from the carbohydrate content of the peels (maximum reducing sugar expected is 68%).

For steam exploded samples, final reducing sugar yield was lower than for milled samples for all enzymes combined. The total reducing sugar when amylase, glucoamylase, cassava C and cassava R as used was 43% compared to 36% reducing sugar for cellulolytic enzymes and 24% reducing sugar for amylolytic enzyme treatments.

Hydrolysis was also carried out on 10% w/v milled cassava peels to investigate the effect of the absence of glucoamylase and cassava R respectively. The sugar release patterns were monitored by the DNS assay and Hplc. Fig 4.13 and 4.14 show results below.

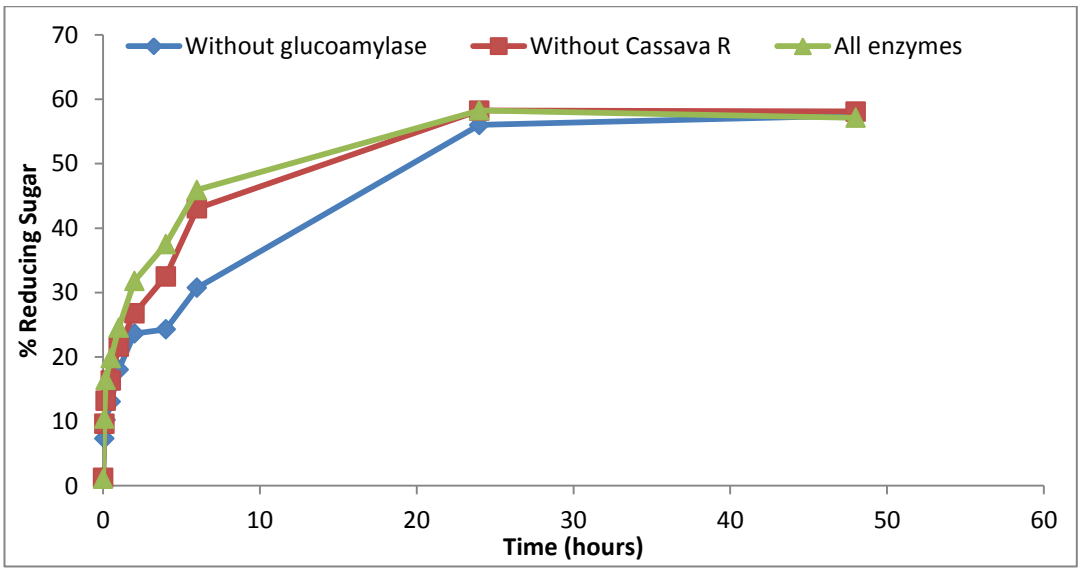


Fig 4.13: Progress curve of the enzymatic hydrolysis of 10 %w/v MWO cassava peels in 0.05M sodium acetate buffer at pH 5 and temperature of 50^{0}C. Comparison of the effect of the absence of glucoamylase and cassava R on hydrolysis.

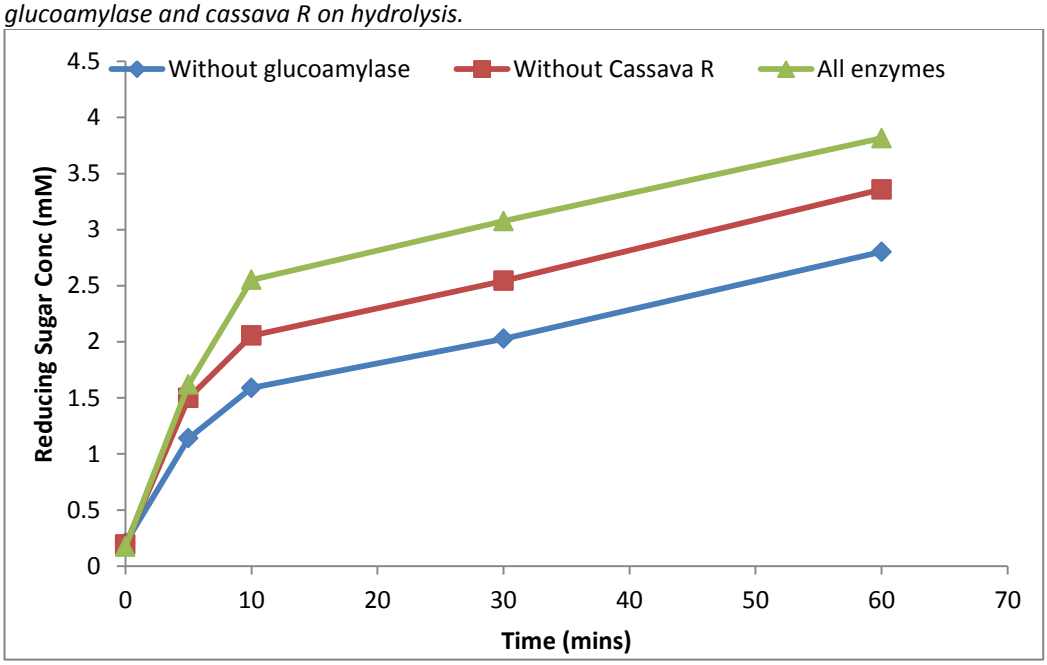


Fig 4.14: First hour of the enzymatic hydrolysis of 10%w/v MWO cassava peels in 0.05M sodium acetate buffer at pH 5 and temperature of 50^{0}C. Comparison of the effect of the absence of glucoamylase and cassava R on hydrolysis.

From the results above, hydrolysis carried out with 0.3%v/v amylase, cassava C and cassava R(in the absence of glucoamylase) and 0.3%v/v amylase, cassava C and glucoamylase(in the absence of cassava R) were slower than hydrolysis in the presence of all enzymes. The absence of cassava R only results in a slower reaction as the final reducing sugar was approximately the same as that observed for the reaction in the absence of glucoamylase. This is expected as the presence of

glucoamylase causes a greater conversion of smaller oligosaccharides to glucose at the start of the experiment.

Also, the absence of glucoamylase enzyme and cassava R enzymes did not show any significant difference in hydrolysis yield when compared to hydrolysis with all enzymes. Although the initial rates of hydrolyis is observed to be slower as reported below, the final reducing sugar was approximately in the same range. The final percentage reducing sugar for the hydrolysis at 24 hours was 56.0±0.3 for the reaction in the absense of glucoamylase enzyme and 58.3±0.4% in the absense of cassava R. When all the enzymes are used 58.3±0.4% reducing sugar was obtained in 24 hours. The results below shows the initial rates comparison. Again, the initial rate was estimated by taking a slope of each reaction in the first 10 minutes as described earlier.

	Hydrolysis rate(mM/ min)		
Substrate concentration (w/v)	**0.3%v/v** **Amylase+cassava C +AMG**	**0.3%v/v** **Amylase+cassava C +Cassava R**	**0.3%v/v** **Amylase+AMG+ cassava C +Cassava R**
10 %w/v	8.60	6.60	11.00

Table 4.3: Initial rate estimates of the enzyme reaction of 10%w/v MWO cassava peels at 0.3%v/v enzyme concentration

The HPLC analysis shows that maltose accumulates as expected in the absence of glucoamylase enzyme as shown in figure 4.15 below.

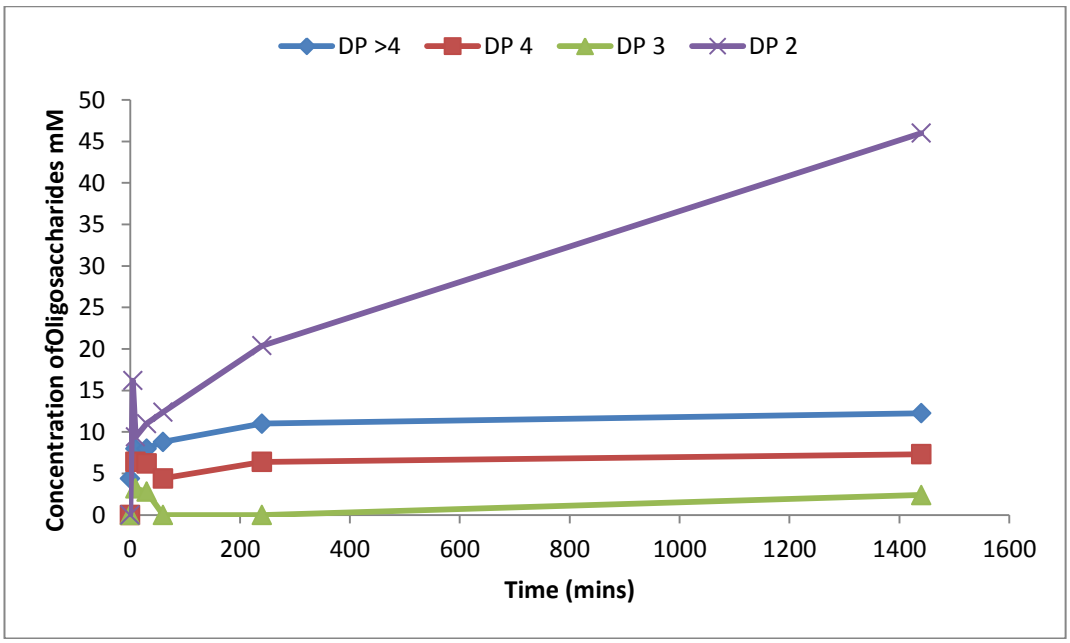


fig 4.15: Concentration of oligosaccharides during enzyme hydrolysis of 10% w/v ***MWO*** *cassava peels by 0.03%v/v amylase, cassava C and glucoamylase enzymes in 0.05M sodium acetate buffer at pH 5 and temperature of 50°C(absence of cassava R).*

In the presence of glucoamylase, there is less maltose generated compared with reactions where all four enzymes were used or reactions where glucoamylase was present as shown in figure 4.16 and 4.17 below.

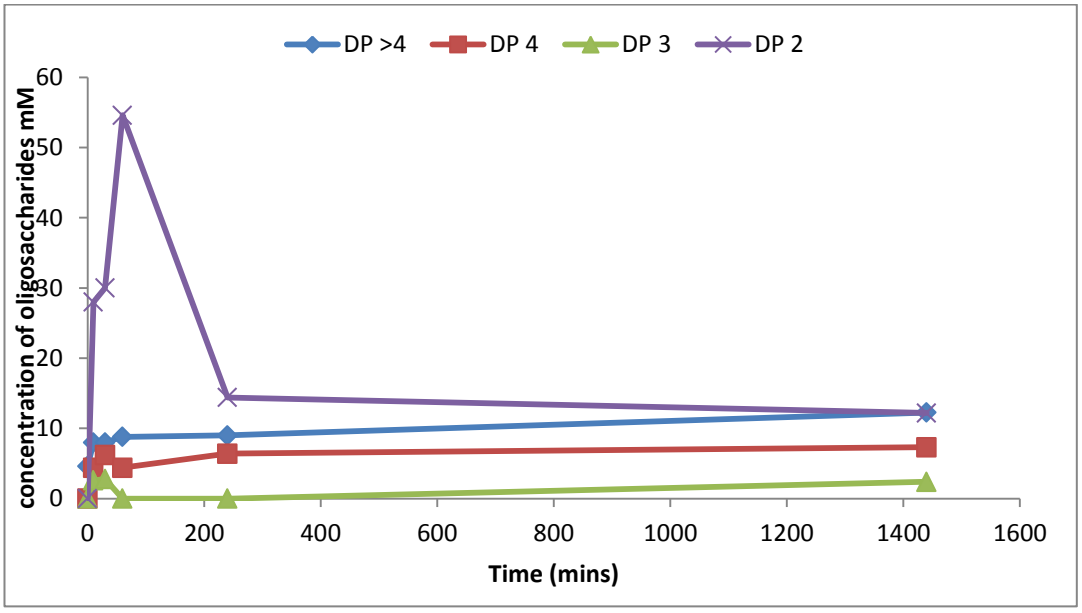


fig 4.16: Concentration of oligosaccharides during enzyme hydrolysis of 10% w/v ***MWO*** *cassava peels by 0.03%v/v amylase, cassava C and cassava R enzyme in 0.05M sodium acetate buffer at pH 5 and temperature of 50°c (absence of glucoamylase).*

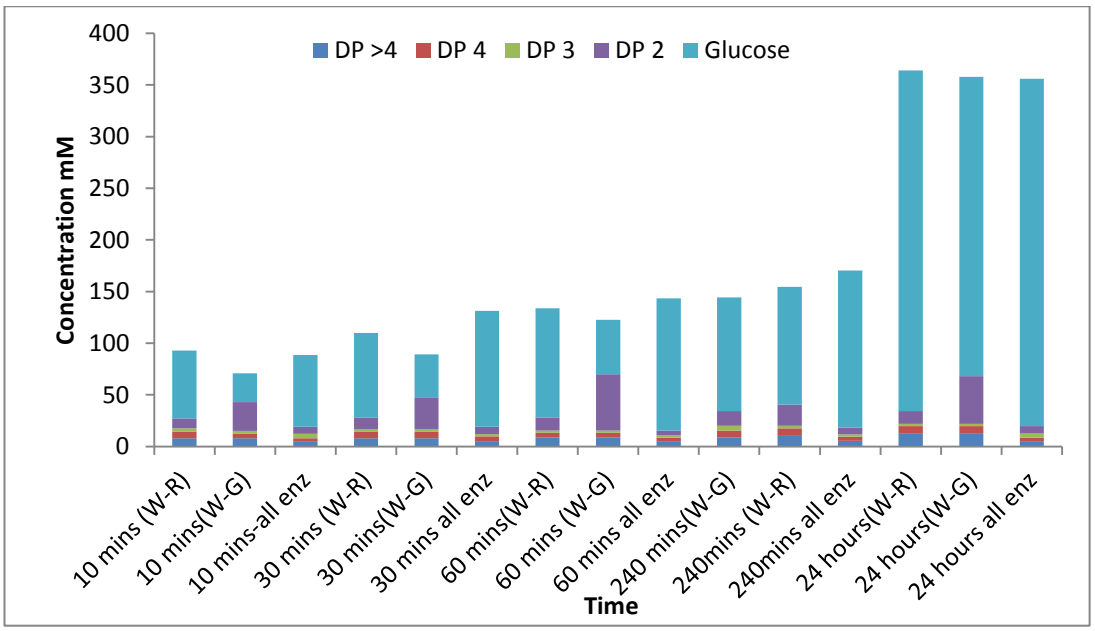


Fig 4.17: comparison of concentration of oligosaccharides during enzyme hydrolysis of 10%w/v ***MWO*** *cassava peels in the absence of Cassava R and Glucoamylase in 0.05M sodium acetate buffer at pH 5 and temperature of 50°c (****W-G;Without Glucoamylasse, W-R; Without cassava R all enz; amylase+glucoamylase+cassava C+cassava R****)*

4.2.0 CONCLUSION

In this chapter, the aim was to assess the effect of combining both cellulolytic and amylolytic enzymes in one reaction vessel on the hydrolysis yield and rates.

Experiments showed that a maximum reducing sugar yield of 0.58g/g of cassava peels was obtained with 10%w/v milled cassava peels and 0.3%v/v enzyme concentration(of amylase , glucoamylase, cassava C and cassava R). Experiments also showed that a cassava peel concentration 0f 20%w/v was too viscous with difficulty in proper mixing experienced.

The relevance of the simultaneous hydrolysis is that the hydrolysis of both starch and cellulose can be combined with a fermentation step if the hydrolysis yield is good enough. Results obtained at 50^0C showed that the amylolytic enzymes and cellulolytic enzymes can be used together with a reasonably high amount of sugars produced.

Further experiments in the absence of glucoamylase and cassava R showed that the hydrolysis yield did not change when compared to the experiments where all four

enzymes were used for the hydrolysis. However, the reactions were slower as would be expected.

Here, analysis of reaction rates showed that the highest hydrolysis rates were observed for steam exploded samples when compared to milled samples. Although, milled samples gave higher reducing sugar yield at all cassava peel concentration and enzyme concentration.

CHAPTER FIVE: RESULTS AND DISCUSSION - CONSECUTIVE HYDROLYSIS

5.0.0: OVERVIEW

The separate treatment of cassava peels with amylolytic enzymes or the cellulolytic enzymes with experiments on steam exploded samples and milled sample was discussed in chapter three. Hot water was however used to gelatinise starch for amylase treatment. In this chapter, a subsequent treatment after the hydrolysis of either starch or cellulose will be discussed.

In this chapter, the effect of amylolytic enzyme treatment on subsequent cellulolytic treatment of the peels is assessed. An alternative treatment method where cellulases are used to first treat the cassava peels before the amylase treatment is applied on the peels is reported. The experiments were carried out at 10%w/v cassava peels. Hot water pretreatment was also used to assess its impact on hydrolysis rate and yield. Figure 5.1 represents the flow chart for the amylase treatment followed by a subsequent cellulase treatment with and without hotwater pretreatment.

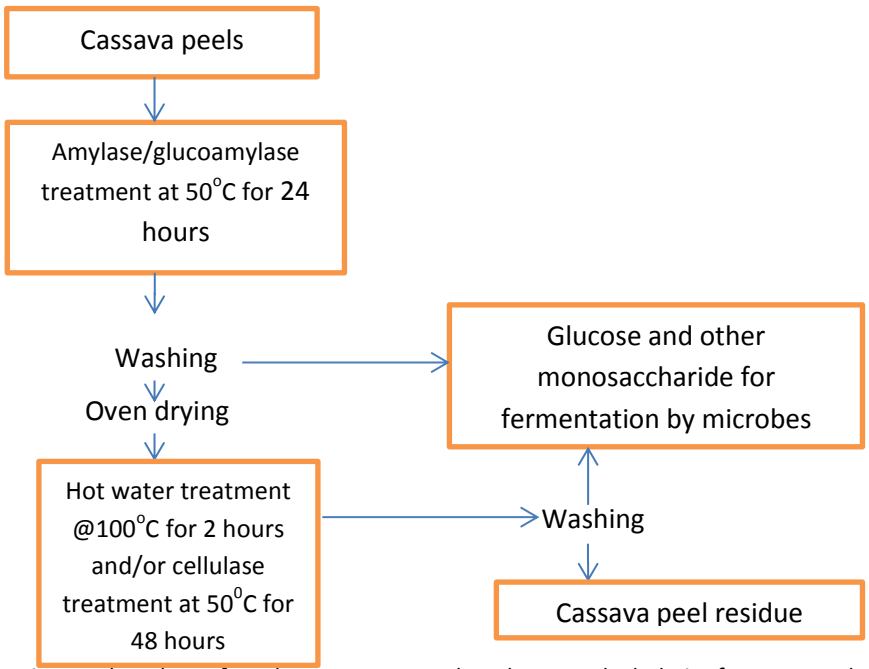


Fig 5.1: Flow chart of amylase treatment and a subsequent hydrolysis of cassava peels with cellulolytic enzymes. The cellulase treatment was done in the presence and absence of hot water pretreatment. Hotwater pretreatment was at 100°C.

Figures 5.2 and 5.3 presents the results obtained from the treatment of cassava peels with amylase and glucoamylase enzymes for 24 hours as shown in the flow chart above. All experiments in this chapter were carried out on 10%w/v milled, washed and oven dried cassava peel. Enzyme concentration is in 0.3%v/v.

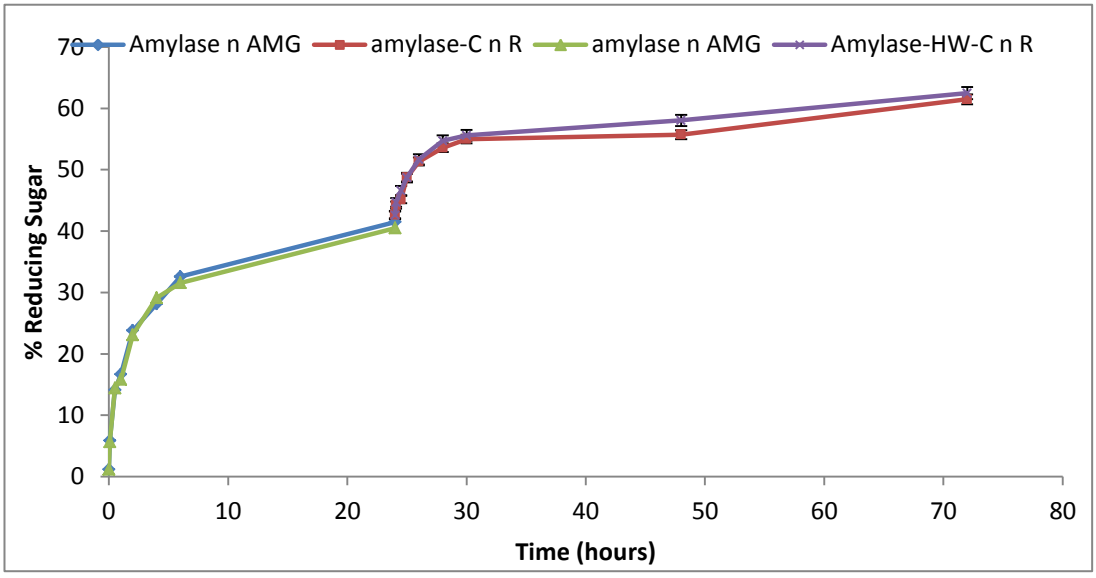


Fig 5.2: Progress curve of the overall enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer. The cassava peels were first treated with 0.3%v/v Amylase and glucoamylase for 24 hours at temperature 50^{0}C. Sugars were then washed out and the residue resuspended in 0.05M sodium acetate buffer after which a second treatment involved treating the peels with 0.3%v/v of cassava C and cassava R. (Amylase-C n R) and with hot water (Amylase-Hw-C n R) at 50^{o}C for a further 48 hours. All at pH 5

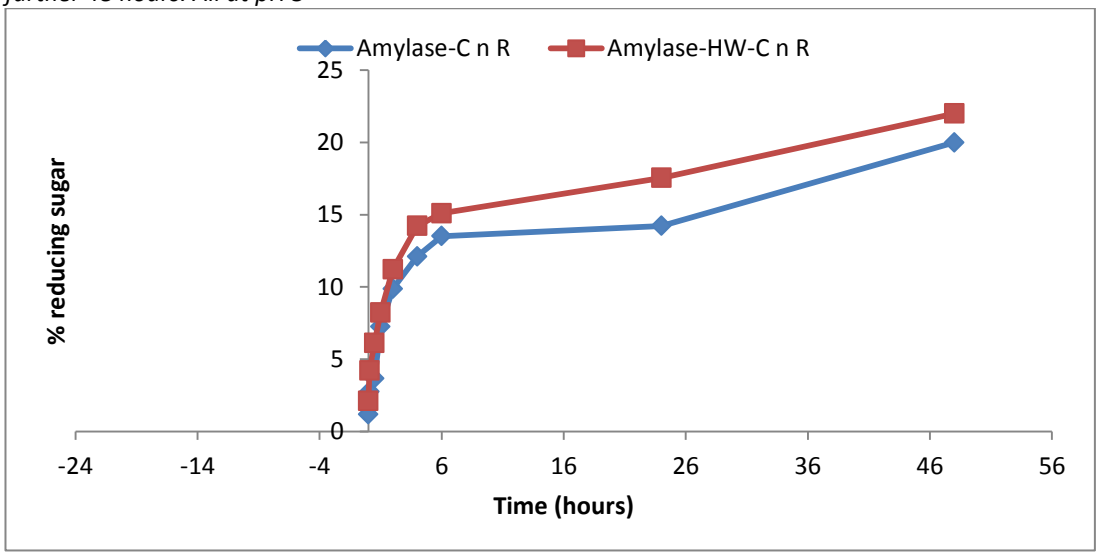


Fig 5.2: Progress curve of the enzymatic hydrolysis of 10%w/v resuspended cassava peels in 0.05M sodium acetate buffer. The cassava peels were first treated with 0.3%v/v Amylase and glucoamylase for 24 hours at temperature 50^{0}C. The residue was then washed and oven dried after which a second treatment involved treating the peels with 0.3%v/v of cassava C and cassava R. (Amylase-C n R) and with hot water (Amylase-Hw-C n R) at 50^{o}C for a further 48 hours. All at pH 5. % reducing sugar is based on original mass of cassava peels.

The final % reducing sugar yield obtained is 61.48 for amylase treatment followed by a subsequent treatment with cellulases and 62.49% for amylase treatment followed by a hot water treatment and cellulase enzyme treatment (amylase-HW-C n R). Since this method involves washing the peels with 0.05M sodium acetate after the first amylase treatment and then drying the peels in an oven at 40^{o}C for 24 hours, this resulted in a slowing down of the cellulolytic reaction as it is believed that drying substrates causes a collapse of the walls making adsorption of enzymes more difficult. However what is interesting to note is that 62% of reducing sugar yield represents about 94% of carbohydrate conversion of the peels.

Fig 5.4a shows a flow chart of an alternative treatment strategy with the treatment of the peels first with cellulolytic enzymes (either with hot water or without hot water pretreatment) followed by a subsequent hydrolysis by amylolytic enzymes. fig 5.4b shows the progress curve of the experiment.

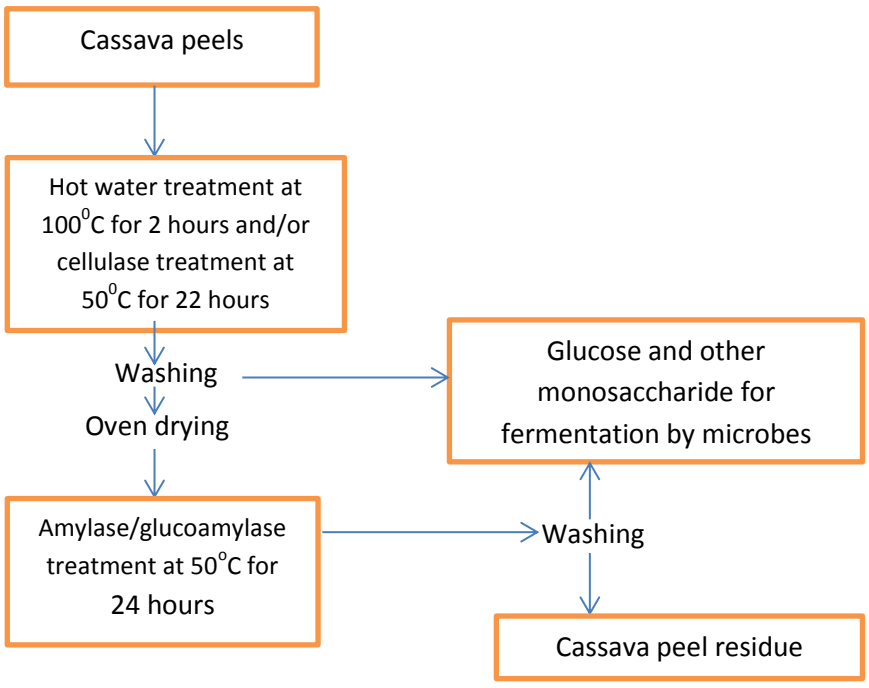


Fig 5.4a: Flow chart of the cellulolytic digestion of cassava peels (with and without hot water pretreatment) and a subsequent hydrolysis of cassava peels with amylolytic enzymes.

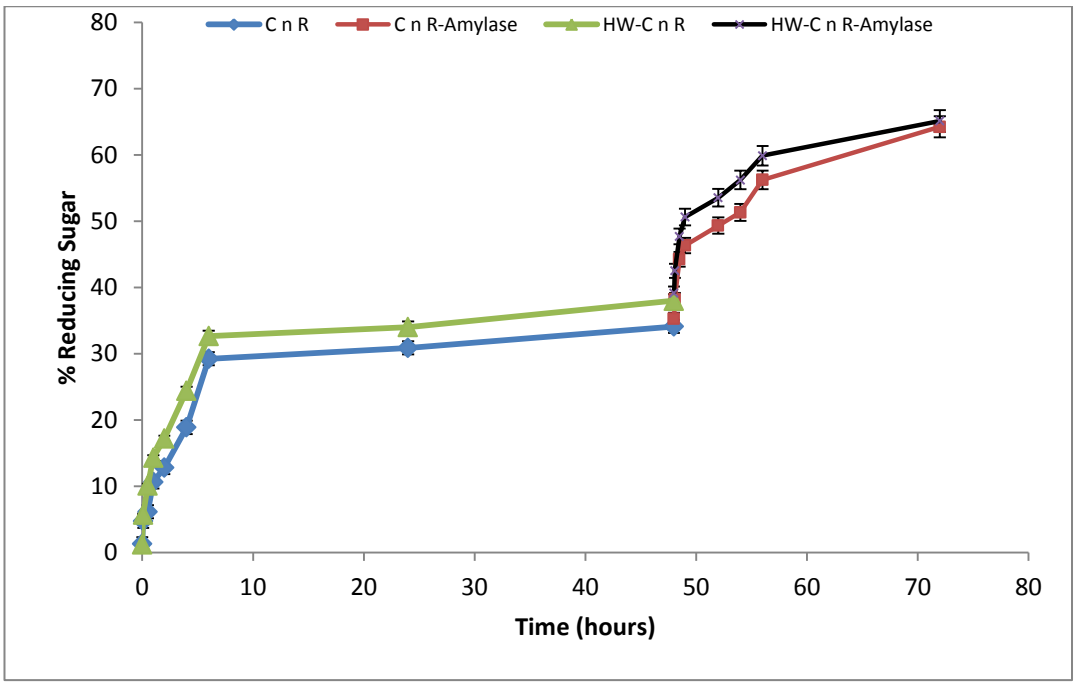


Fig 5.4b: Progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer. The cassava peels were first treated with 0.3%v/v cassava C and cassava R (C n R) and with hot water (Hw-C n R) at 50^{o}C for 48 hours. Second treatment involved treating the peels with 0.3%v/v Amylase and glucoamylase for 24 hours at temperature 50^{0}C . All at pH 5.

This treatment option gave the highest hydrolysis yield of 64.23% for treatment without hot water and 65.12% with hot water pretreatment. Although after treatment with cellulolytic enzymes was carried out with washing of the peels with buffer followed by oven drying at 40^{o}C for 24 hours, this didn't seem to have effect on the final reducing sugar yield. Samples pretreated with hot water had faster hydrolysis rate when compared with reactions in without hot water treatment as shown in fig 5.5 below. However, there was no significant difference in the final reducing sugar. The high reducing sugar yield might also be as a result of lower inhibition of cellulases by glucose.

The estimate of the hydrolysis rate was again done by drawing a line and then estimating the hydrolysis rate using a macro. However, it must be stated that the reaction rate estimate was based on two sample points and therefore subject to errors.

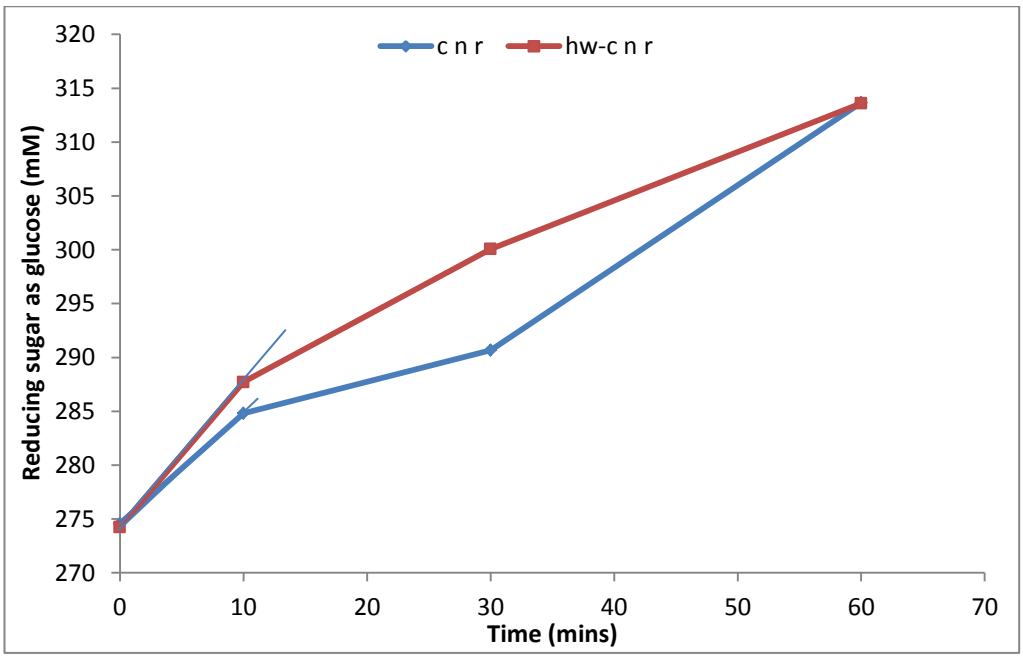


Fig 5.5 First hour of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer with 0.3%v/v cassava C and cassava R (C n R) and with hot water (hw-C n R) at 50°C and pH 5

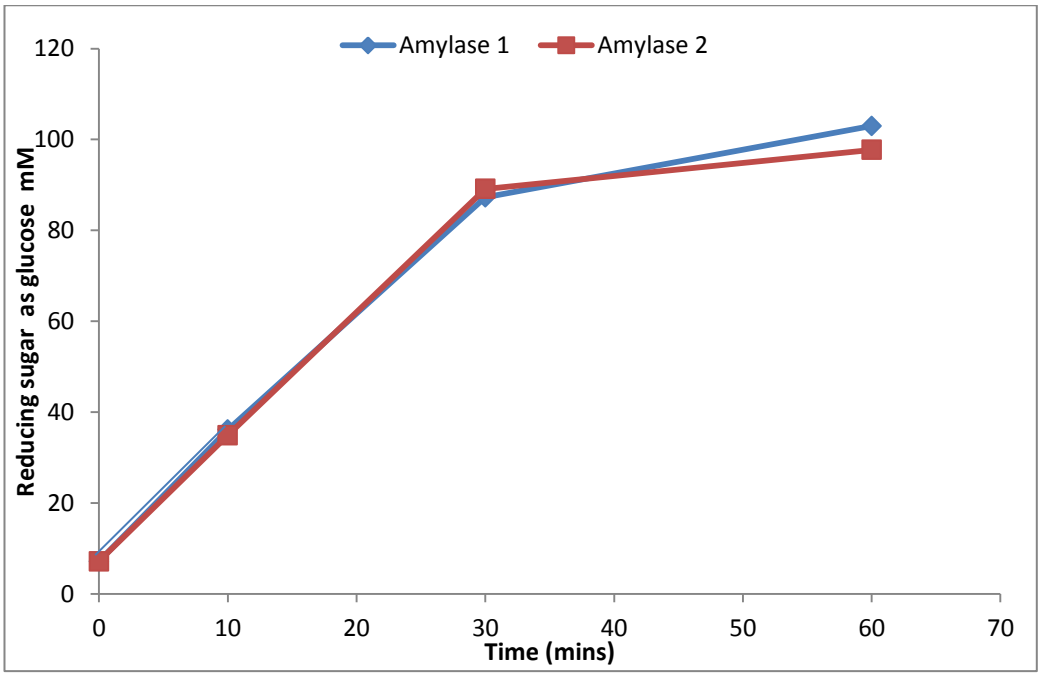


Fig 5.6 First hour of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer with 0.3%v/v amylase enzymes at 50°C and pH 5. Amylase 1 and 2 represents 2 different experiments but with same enzyme and substrate concentrations.

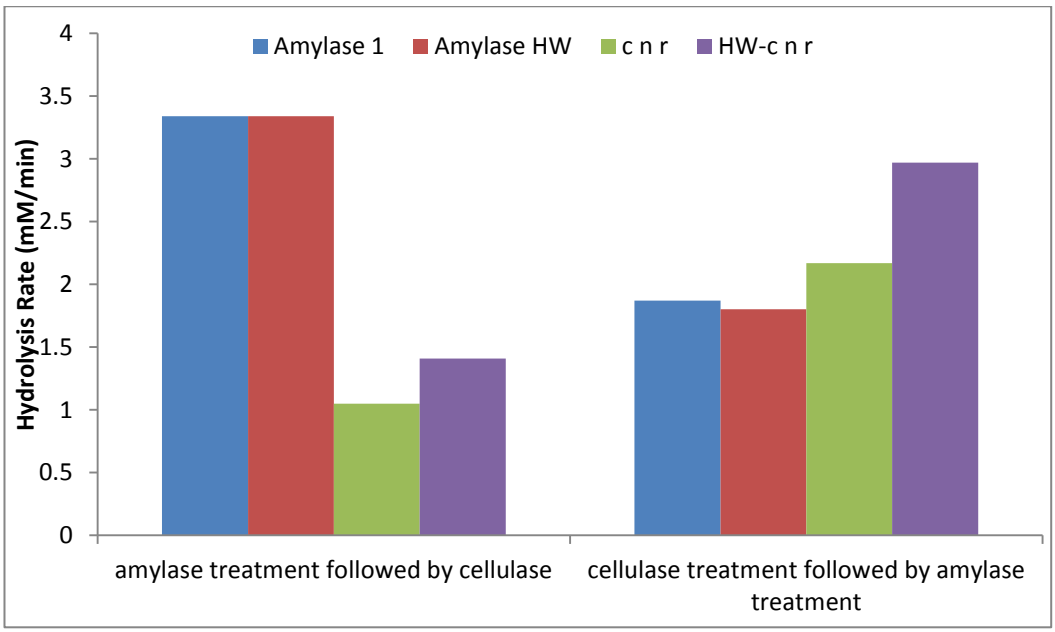


Fig 5.7: Initial rates of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer pH 5. Amylase 1 represents 0.3%v/v Amylase and glucoamylase for 24 hours at 50^{0}C. Amylase-HW represents 0.3%v/v Amylase treatment with hot water at 100^{0}C for 2 hours and addition of 0.3%v/v glucoamylase for 22 hour at 50^{0}C . c n r represents 0.3% v/v cassava C and cassava R at 50^{o}C for 48 hours and Hw-C n R represents 0.3% v/v cassava C and cassava R at 50^{o}C for 48 hours.

The reaction rate studies show that hot water pretreatment results in an increased hydrolysis rate for cellulase treatment although the difference is more pronounced where cellulose treatment was the first treatment carried out on the peels. The decreased rate observed when starch is first hydrolysed might be as a result of the lower accessibility of the substrate as a result of the oven drying which causes cellulose walls to collapse. Amylase 1 and Amylase HW also show a reduction when cellulases are first used to hydrolyse cassava peels. This might be a caused by the lower accessibility of the substrate by the enzymes. Another plausible contribution to the lower rates observed might be these cellulolytic enzymes have amylolytic activity on the peels and vice versa (amylolytic enzymes having cellulolytic activity).

Although the consecutive treatment of cassava peels has resulted in a higher yield of sugars as shown in these results above, one disadvantage this process might have when compared to other processes like the simultaneous hydrolysis method is that it might be more expensive when the cost of the various separation of the sugars are inputted (the centrifuging and possibly filtration of the liquid hydrolysate) before a subsequent hydrolysis.

This consecutive method also makes impossible the combination of the fermentation step in the same vessel. Simultaneous saccharification and fermentation is one method currently being widely researched to reduce cost of cellulose to bioethanol conversion.

The next section seeks to modify the consecutive hydrolysis strategy to accommodate for a possible process option where saccharification and fermentation is carried out in the same vessel thereby eliminating the need for a separation step for the sugars released. Hot water pretreatment was also incorporated in this step.

5.1.0 HOT WATER PRETREATMENT: EFFECT OF TEMPERATURE AND pH On HYDROLYSIS YIELD AND RATE.

A subtle change in consecutive hydrolysis that combines hot water pretreatment and consecutive hydrolysis treatment was also experimented. This involves a pretreatment step with hot water and amylase enzymes at 95^{o}C-100 ^{o}C after which glucoamylase and the cellulolytic enzymes were added after the temperature is reduced to 50^{o}C. Several changes in pH and temperature were also investigated.

The results are shown in the figures below.

5.1.1 HOT WATER PRETREATMENT AND ENZYME HYDROLYSIS AT 50^{0}C

Figure 5.8 shows progress curves at different pH for reactions at 50^{0}C while Fig 5.9 shows a chart comparing hydrolysis yield at different pH.

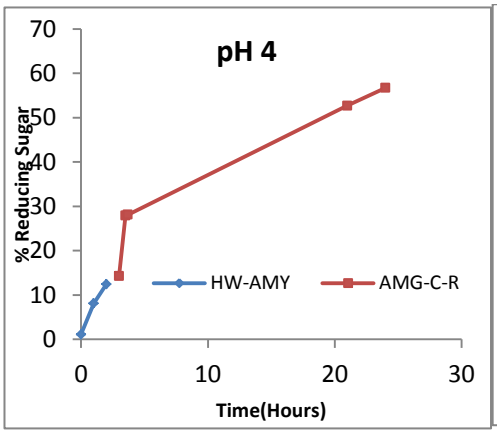


Fig 5.8a

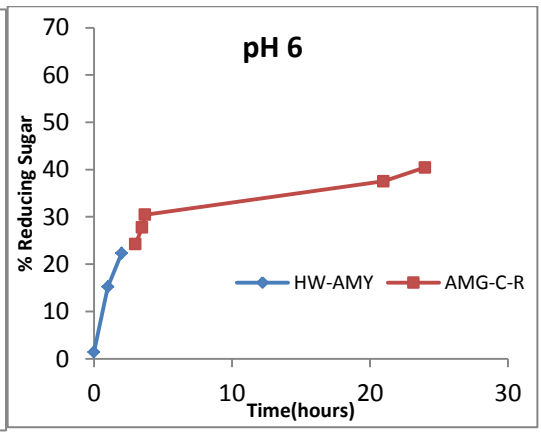


Fig 5.8b

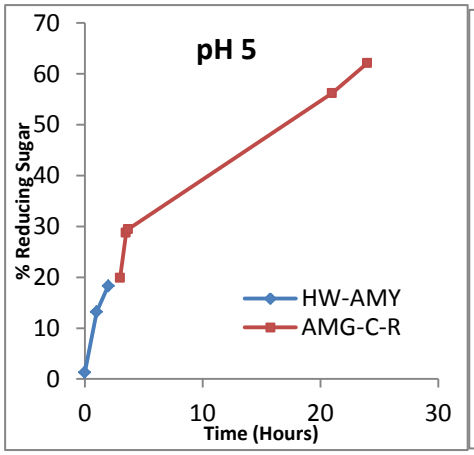


Fig 5.8c

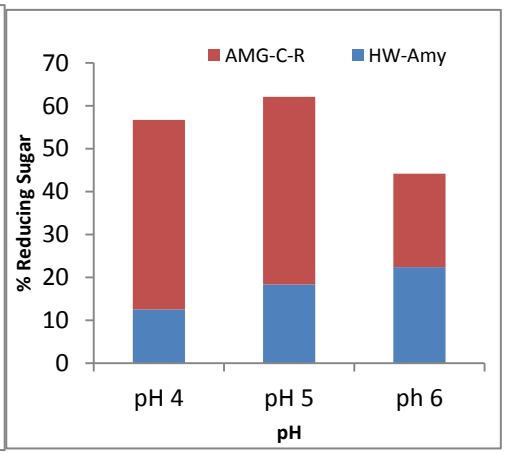


Fig 5.9

Fig 5.8: progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at temperature 50^{0}C. HW-AMY: Hot water pretreated peels with 0.3%v/v Amylase for 2 hours AMG-C-R: Further Treatment with 0.3%v/v of glucoamylase, cassava C and cassava R (a) Reaction at pH 4 (b) Reaction at pH 6 (c) Reaction at pH 5

Fig 5.9: Chart showing Summary of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at temperature 50^{0}C at different pH.

It is apparent from Fig 5.8 and Fig 5.9 that pH of 6 gives the highest hydrolysis yield for amylases (fig 5.8) however when all the other enzymes are added after the hot water/amylase treatment, pH 5 becomes the optimal pH for the combination of enzymes with a final reducing sugar yield of 62% and pH 4 also shows very good results with hydrolysis yield of 57% while pH 6 lags far behind in terms of final extent of hydrolysis with 40.43% reducing sugar in 24 hours. The treatment of the peels with hot water and amylase for 2 hours also resulted in a sharp increase in hydrolysis rate when the other enzymes are added after the reaction is cooled to 50^{0}C from 100^{0}C. A comparison of the hydrolysis rate is shown later in fig 5.14.

5.1.2 HOT WATER PRETREATMENT AND ENZYME HYDROLYSIS AT 40^{0}C

Simultaneous saccharification and fermentation is meant to be carried out at temperatures that favour both hydrolysis and fermentation microorganisms and so experiments were also carried out at 40^{0}C after an initial pretreatment with hot water and amylase treatment for 2 hours at 100^{0}C. The following progress curves gives results obtained.

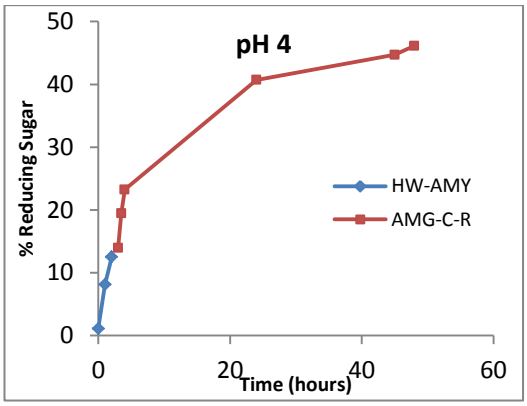


Fig 5.10a

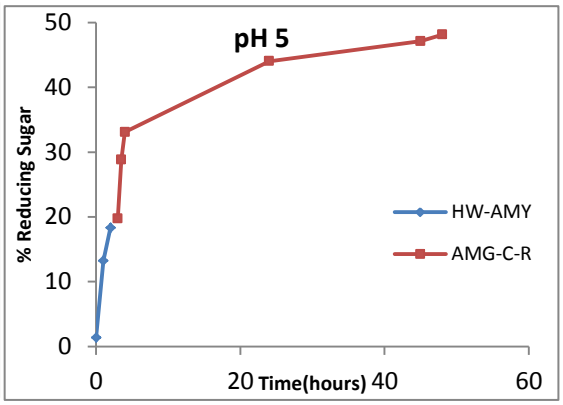


Fig 5.10b

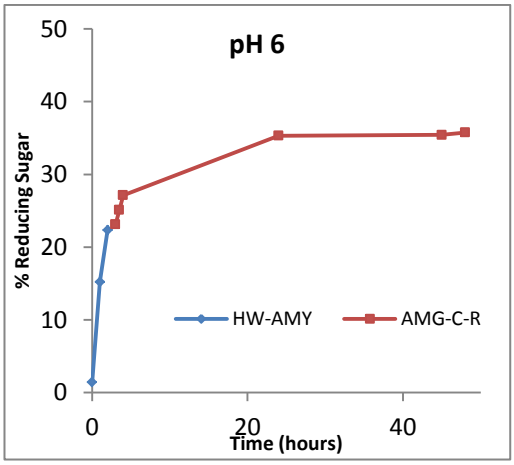


Fig 5.10

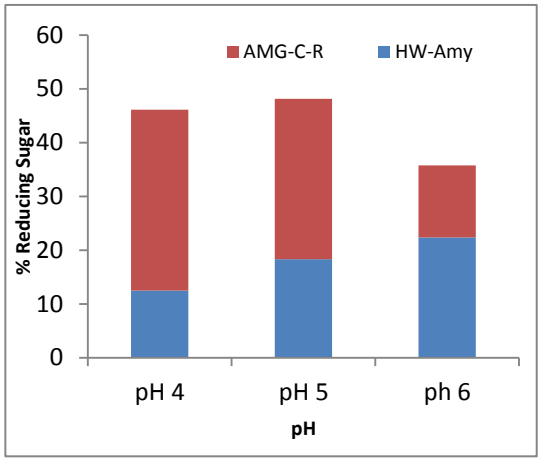


Fig 5.11

Fig 5.10: progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at temperature 40^{0}C. HW-AMY: Hot water pretreated peels with 0.3%v/v Amylase for 2 hours AMG-C-R: Further Treatment with 0.3%v/v of glucoamylase, cassava C and cassava R (a) Reaction at pH 4 (b) Reaction at pH 5 (c) Reaction at pH 6

Fig 5.11: Chart showing Summary of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at temperature 40^{0}C at different pH.

Data from fig 5.10 and fig 5.11 again show that amylases give highest hydrolysis yield at pH of 6 while on addition of glucoamylase and the cellulolytic enzymes after a 2 hour treatment with hot water and amylase enzymes, pH 5 still gives a better

hydrolysis yield (48% reducing sugar) compared to reactions at pH 4 (46% reducing sugar). Reactions at pH 6 give 36% reducing sugar yield.

5.1.3 HOT WATER PRETREATMENT AND ENZYME HYDROLYSIS AT 60^{0}C

Hydrolysis reaction carried out at 60^{o}C after hot water pretreatment at 100^{0}C with amylase enzymes are also presented below

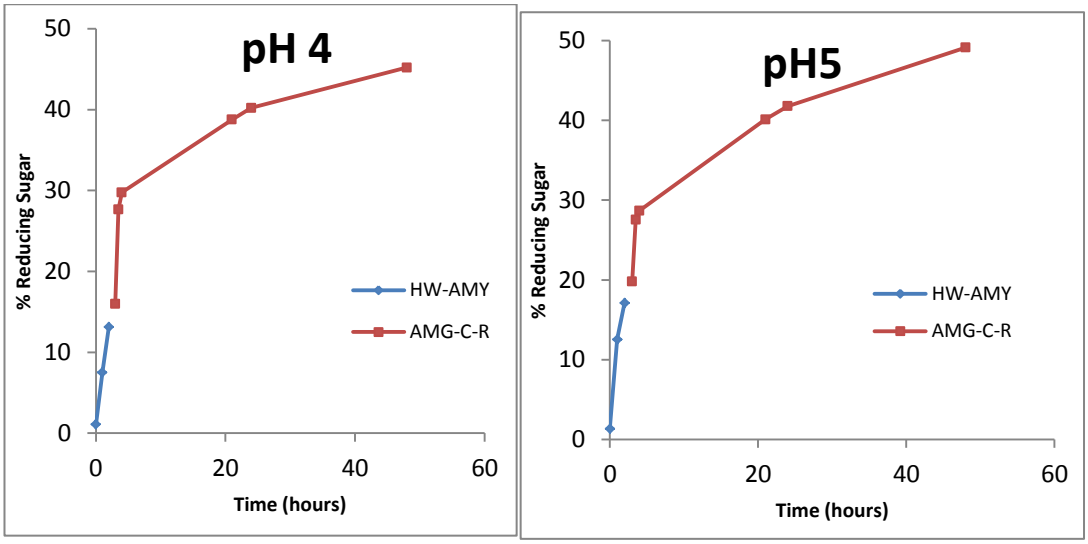


Fig 5.12: Progress curve of the enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at temperature 60^{0}C. HW-AMY: Hot water pretreated peels with 0.3%v/v Amylase for 2 hours AMG-C-R: Further treatment with 0.3%v/v of glucoamylase, cassava C and cassava R (a) Reaction at pH 4 (b) Reaction at pH 5

As can be seen from the progress curves above, Final reducing sugar yield for reactions carried out at 60^{0}C is 45% and 49% for pH 4 and pH 5 respectively.

Fig 5.13 below compares the hydrolysis yield at 40^{0}C, 50^0 C and 60^{0}C

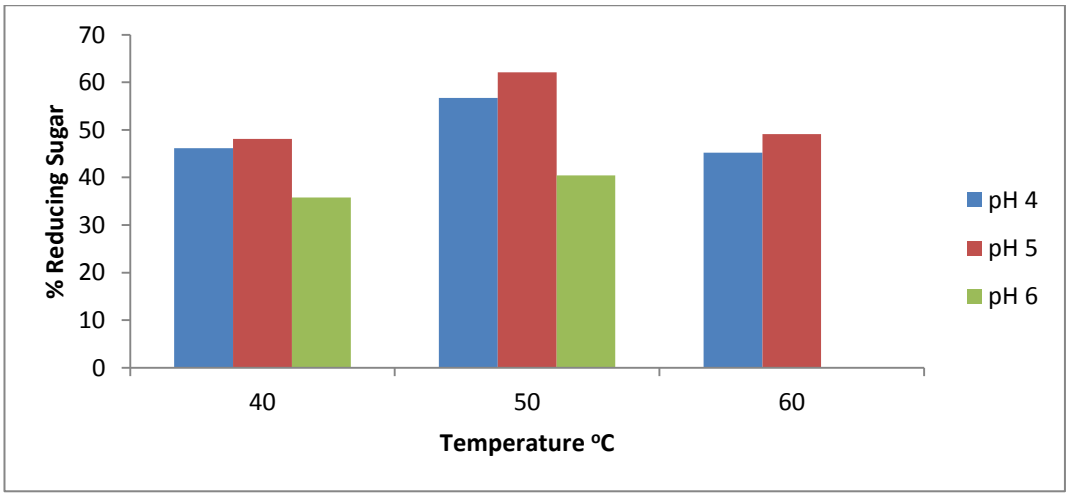


Fig 5.13 Comparison of the final hydrolysis yield of enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at different temperatures and pH.

The results above show clearly that reactions at 50^{0}C gave highest hydrolysis yield compared to reactions carried out at 40^{0}C and 60^{0}C. pH 5 also shows the most ideal

pH to combine glucoamylase and the cellulolytic enzymes (cassava C and cassava R) after hot water pretreatment. It has also been established that pH 6 suits the amylase enzyme better. Although lower yields of reducing sugar are observed for 40^0C, the amount of sugar formed is good enough for simultaneous saccharification and fermentation experiments. One of the advantages of these experiments at pH and temperature is to enable the correct choice of microorganism for fermentation.

Turning to the effect of these changes in pH and temperature on the hydrolysis rate, fig 5.14 shows the effect. Again, the hydrolysis rate was estimated with the help of a macro. Fig 5.15 shows how the line was drawn to estimate the initial rates after the addition of the glucoamylase enzymes.

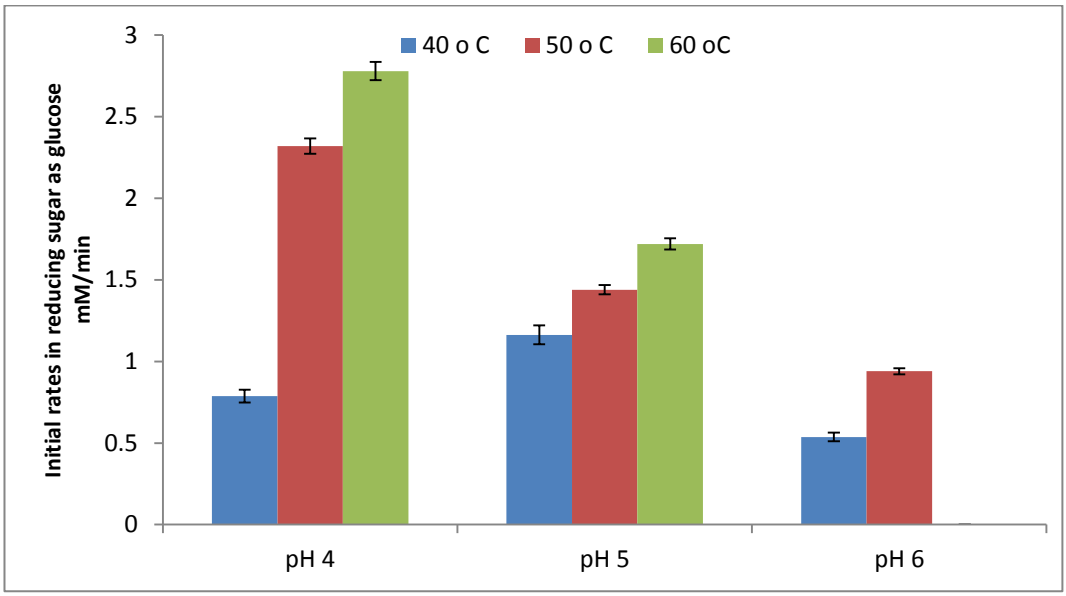


Fig 5.14 Initial rates after addition of 0.3%v/v of glucoamylase, cassava C and cassava R after hot water pretreatment/ amylase treatment. Reaction was with 10%w/v cassava peels in 0.05M sodium acetate buffer at diff pH at 50^0C

This result shows that although the reaction is fastest after the addition of these enzymes at 60^0C, it dramatically slows down as shown in fig 5.15a below. Reactions at 40^0C show the slowest reaction rate while 50^0C show a higher rate after addition of the three enzymes.

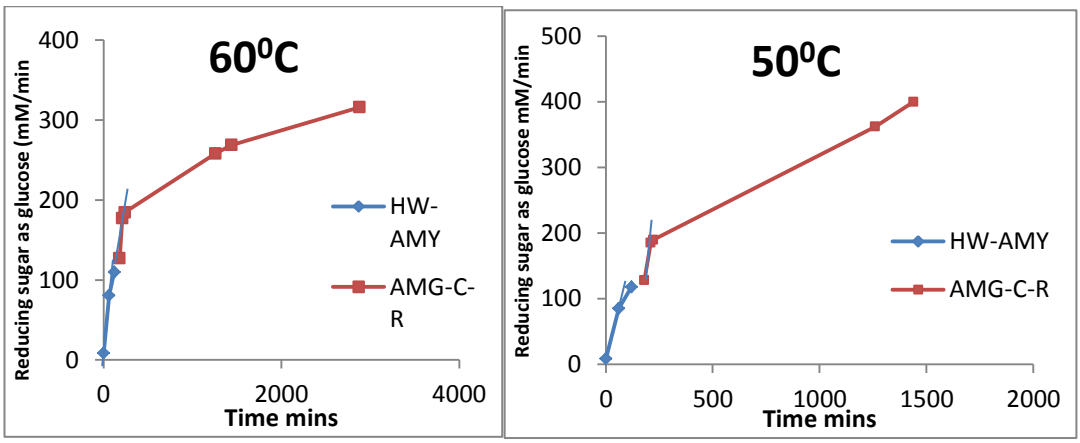


Fig 5.15: Comparison of progress curves of enzymatic hydrolysis of 10%w/v cassava peels in 0.05M sodium acetate buffer at temperatures 60^{0}C and 50^{0}C). Both at pH 5. The progress curve shows that although hydrolysis rate at 60^{0}C is faster, it slows down significantly.

These hydrolysis rate estimates might be subject to errors as not many sample points were taken. However, there are just being used to compare these different reactions since similar sample points were taken for these experiments.

Fig 5.16 compares the hydrolysis rate estimate for the first part of the reaction where hotwater pretreatment was combined with amylase treatment.

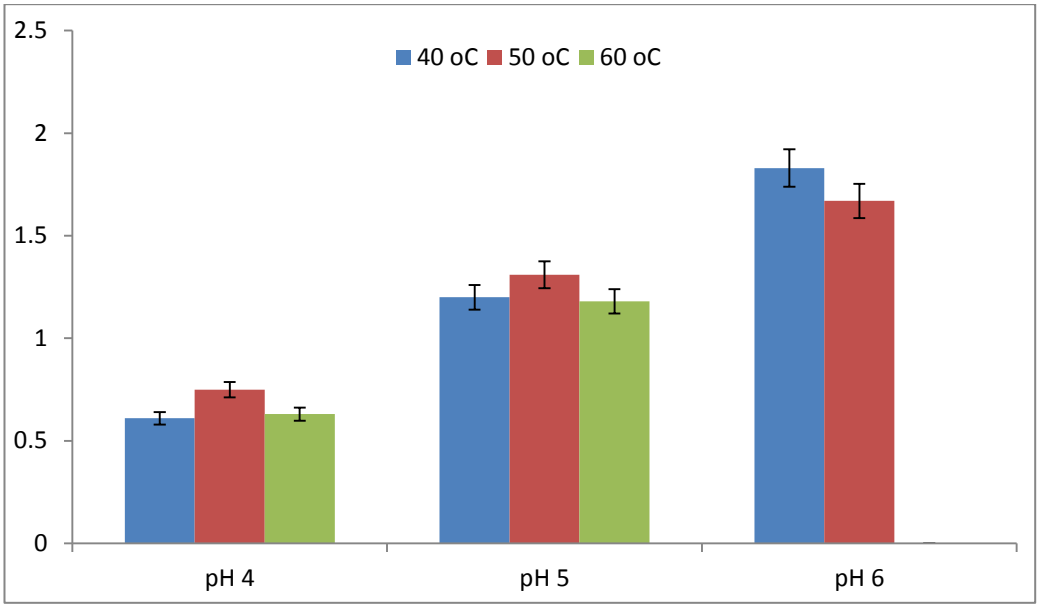


Fig 5.16 Initial rates of Hw pretreatment and amylase treatment. Reaction was with 10%w/v cassava peels in 0.05M sodium acetate buffer at diff pH using 0.3%v/v amylase at 100^{0}C

Here, the initial rates were almost the same for all the reactions and any difference attributed to experimental errors because the reactions were simply carried out at

the same temperature of 100^{o}C for 2 hours. pH 6 shows the highest initial rates observed which agrees with literature that amylase function best between pH 6 and 7. pH 4 reflects the lowest initial rates observed.

It is suggested that experiments can be modified such that the hot water/amylase treatment is carried out at pH 6 after which the pH can be adjusted to 5 for the subsequent step when glucoamylase and the cellulolytic enzymes are added.

5.2.0 CONCLUSION

This chapter explored the possibility of combining hot water pretreatment and enzyme treatment. It also investigated the consecutive or sequential treatment with enzymes as a follow up of the separate hydrolysis already discussed in chapter 3. The following conclusions can be drawn from this chapter.

A consecutive treatment with amylolytic enzymes and cellulolytic enzymes where the sugars are washed and separated after a first treatment and the cassava peel resuspended for subsequent hydrolysis by another set of enzymes gave the highest yields of reducing sugars of between 0.61-0.65g/g of cassava peels. However, to maximize the sugar yield, a minimum of 24 hours is required for starch degradation and 48 hours for cellulose/hemicellulose degradation. Separate hydrolysis vessels would also be required as well as equipment cost for filtration and centrifuging the sugars for further experiments making this process cumbersome and a hotwater pretreatment combined with amylase treatment for 2 hours before a subsequent treatment with a combination of glucoamylase, cassava C and cassava R for another 24 hours gave very high sugar yields of about 0.62g/g of cassava peels.

Experiments carried out at 40^{0}C, 50^{0}C and 60^{0}C showed that 50^{0}C gave the highest sugar yields. 40^{0}C also gave good sugar yields

Experiments at different pH showed that an enzyme treatment at pH 5 gave the highest hydrolysis yield and rate when these enzymes were combined. However pH 6 gave a better yield for the initial amylase treatment.

The recommended industrial processing option would be a hot water pretreatment combined with amylase treatment at pH 5 for 2 hours followed by a subsequent treatment with glucoamylase and the cellulase enzymes for a further 22 hours at 50^0C.

CHAPTER SIX: RESULTS AND DISCUSSSION FERMENTATION

6.0.0 FERMENTATION: OVERVIEW

The biomass to bioethanol process which begins with the hydrolysis of polysaccharides to simple sugars terminates with the fermentation reaction of these simple sugars to ethanol.

The fermentation of these simple sugars by microorganisms like yeast and other bacteria to ethanol is usually carried out by the breakdown of each molecule of glucose into two pyruvate molecules in a process called glycolysis.

This is summarised below;

$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ NAD^+ \rightarrow 2\ CH_3COCOO^- + 2\ ATP + 2\ NADH + 2\ H_2O + 2\ H^+$

Atom balance is maintained by the two phosphate (P_i) groups

This glucose to pyruvate (CH_3COCOO^-) reaction takes place in 10 reaction steps. Fermentation of pyruvate to ethanol which is a pyruvate derivative is mostly carried out by the enzymes pyruvate decarboxylase and alcohol dehydrogenase present in the fermentative microbes.

The objective of this chapter is to ferment the sugars released from the enzyme hydrolysis of cassava peels at a higher temperature than the normal temperature fermentation reactions are carried out in industries. The combination of the saccharification and fermentation reactions (SSF) was carried out at 40^oC. A separate hydrolysis and fermentation (SHF) was also carried out as well at the same temperature.

6.1.0 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

The advantage of using the Simultaneous Saccharification and Fermentation process is also believed to limit the effect of microbial contamination as ethanol concentration increases in the reaction vessel. This is besides saving cost in investment as one vessel is used for both fermentation and hydrolysis. Experiments were conducted using 5.5 %w/v (3%w/w carbohydrate content) cassava peels as recommended by the NREL lap protocols (www.nrel.com/lap008). 10 w/v cassava peels were also used in these experiments. Experiments were also carried out by a

simple modification of the simultaneous saccharification and fermentation process by introducing the microorganism 24 hours after hydrolysis by amylase and cellulase enzymes. Results from these experiments are presented in figures 6.1, 6.2 and 6.3.

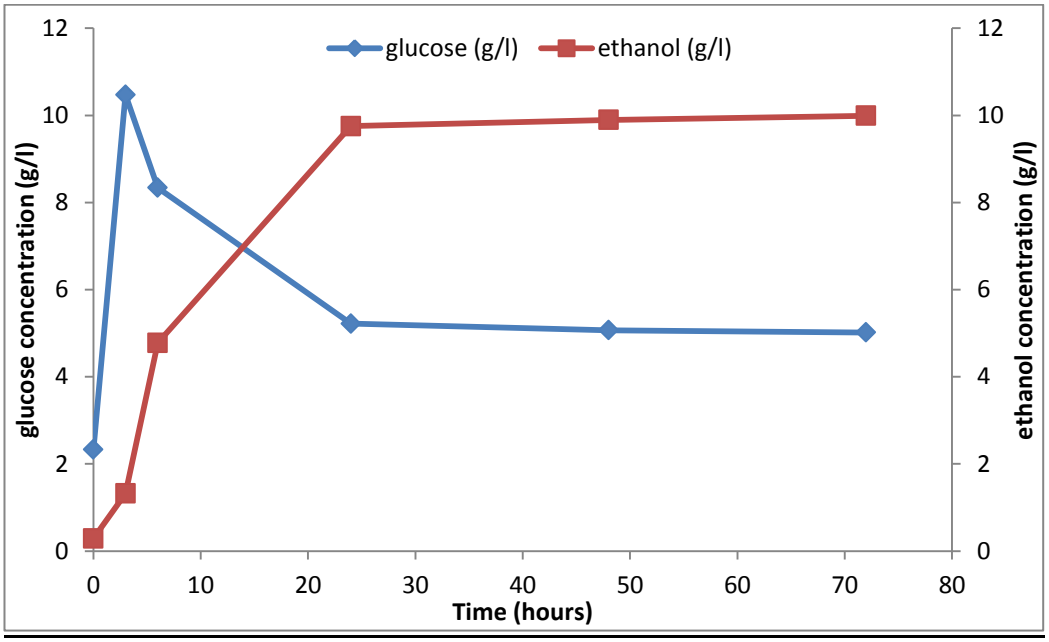


Fig 6.1: Progress curve of the simultaneous saccharification and fermentation of 5.5%w/v cassava peels using 0.3%v/v of amylase, glucoamylase, cassava C and cassava R and K. marxianus. Total volume of reaction was 0.6L at 40^{0}C and pH 5.

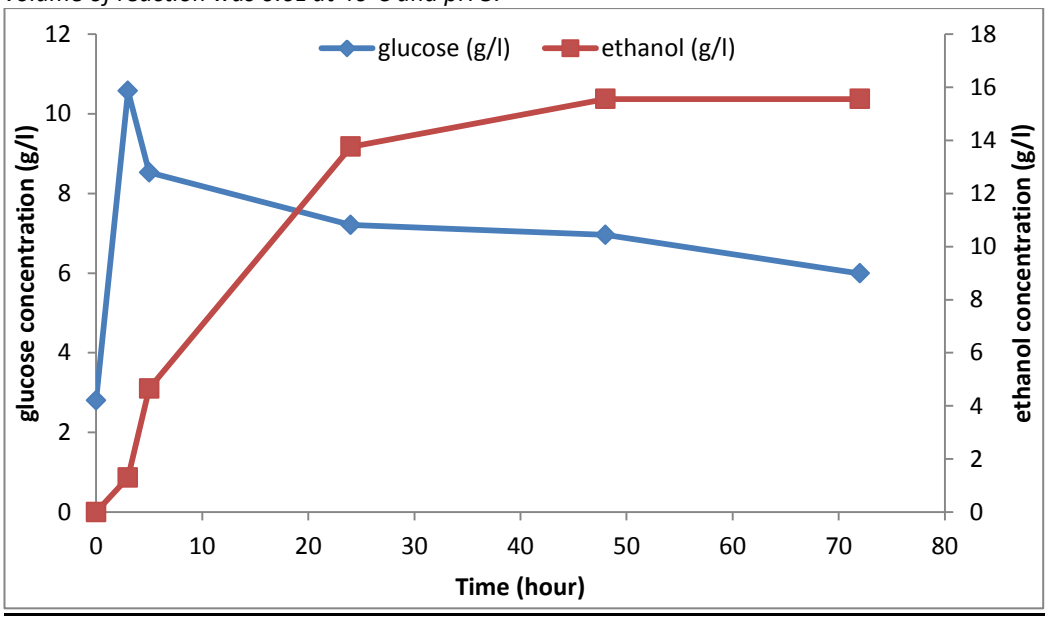


Fig 6.2 Progress curve of the simultaneous saccharification and fermentation of 10%w/v cassava peels using 0.3%v/v of amylase, glucoamylase, cassava C and cassava R and *K. marxianus* at pH 5. Total volume of reaction was 0.6L with temperature *at 40^{0}C and pH 5.*

Samples taken at time zero in the experiments here represent time immediately after enzymes and microbes have been added to the substrate. The glucose concentration at time zero is a bit higher than usual. This might be as a result of a possible hydrolysis during the autoclaving of the substrate. Results show that the SSF process favours a higher yield of ethanol with reactions at 5.5%w/v when compared to reactions at 10%w/v cassava peels. Final ethanol concentration at 9.99g/l for 5%w/v cassava peels at 48hours although the reaction was more or less complete in 24 hours. 10%w/v cassava peels gave a final ethanol concentration of 15.6g/l from 60g of cassava peels. These results translated to 53% of theoretical yield for 5.5%w/v peels and 44.8% of theoretical yield for 10%w/v peels based on total carbohydrate content of the peels. The lower theoretical yield associated with 10%w/v cassava peels might have been as a result of difficulty associated with mixing in the fermenter. The higher concentration of ethanol might have had an effect on the yeast strain. It was also observed that very little ethanol is produced within the first 3 hours of the experiments. More significant ethanol is produced as from 6 hours of the reaction. Over 95% of the final ethanol concentration was also produced in 24hours of reaction for the 5.5%w/v cassava peels as against about 88% of the final concentration in the same time for 10 %w/v.

Glucose concentration rises within the first 3 hours after which it drops gradually from about 10g/l to between 5-7.5g/l at 24 hours. From 24 hours, the glucose is fairly constant for in Fig 6.1. However, in Fig 6.2, it continues to drop from 7.2g/l in 24 hours to 5.9g/l in 72 hours.

A modification of the SSF which is sometimes called the simultaneous hydrolysis and co-fermentation process was also carried out with result shown below

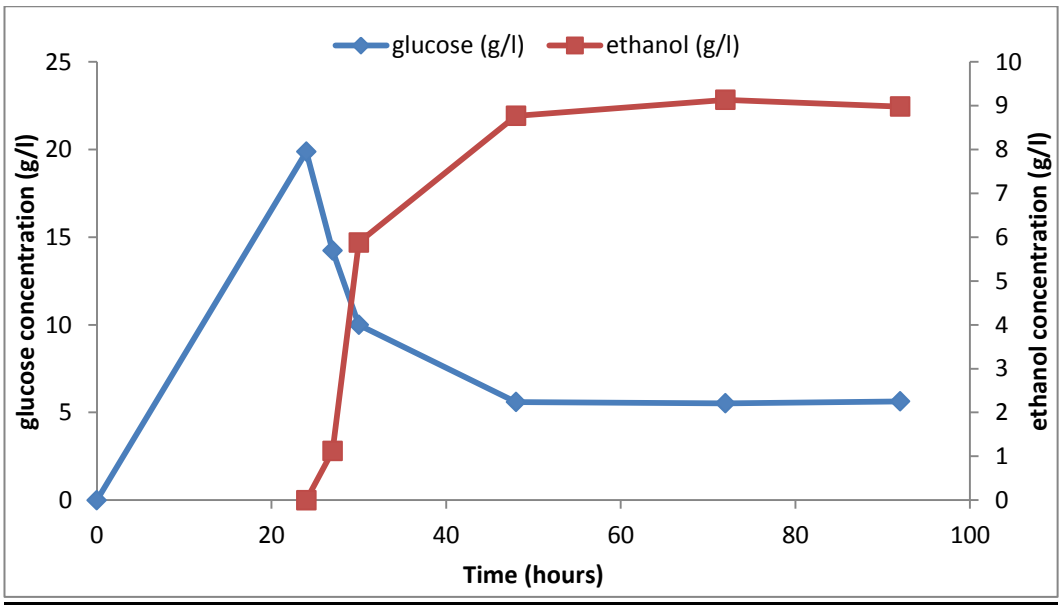


Fig 6.3: Progress curve of the simultaneous saccharification and co-fermentation of 5.5%w/v cassava peels using 0.3%v/v of amylase, glucoamylase, cassava C and cassava R. The microorganism was introduced after 24 hours of enzyme hydrolysis. Total volume of reaction was 0.6L with temperature *at 40^{0}C and pH 5.*

This experiment did not show any improvement in ethanol fermentation yield over the SSF process. The final ethanol yield at 48 hours corresponds to 47.9% of theoretical yield based on total carbohydrate content. This process was meant to have an advantage over SSF due to the higher amount of sugars available for the microorganism to metabolise while at the same time, the excessive glucose and cellobiose that is believed to inhibit cellulase enzymes would also be reduced.

6.2.0 SEPARATE HYDROLYSIS AND FERMENTATION

A separate hydrolysis and fermentation (SHF) was also carried out. 10%w/v peels were hydrolysed by 0.3%v/v of amylases and cellulases for 24 hours after which it was decanted, centrifuged filtered and separated. The filtrate was then diluted and fermented with *K.marxianus*. Results from this experiment are shown in Fig 6.4 below.

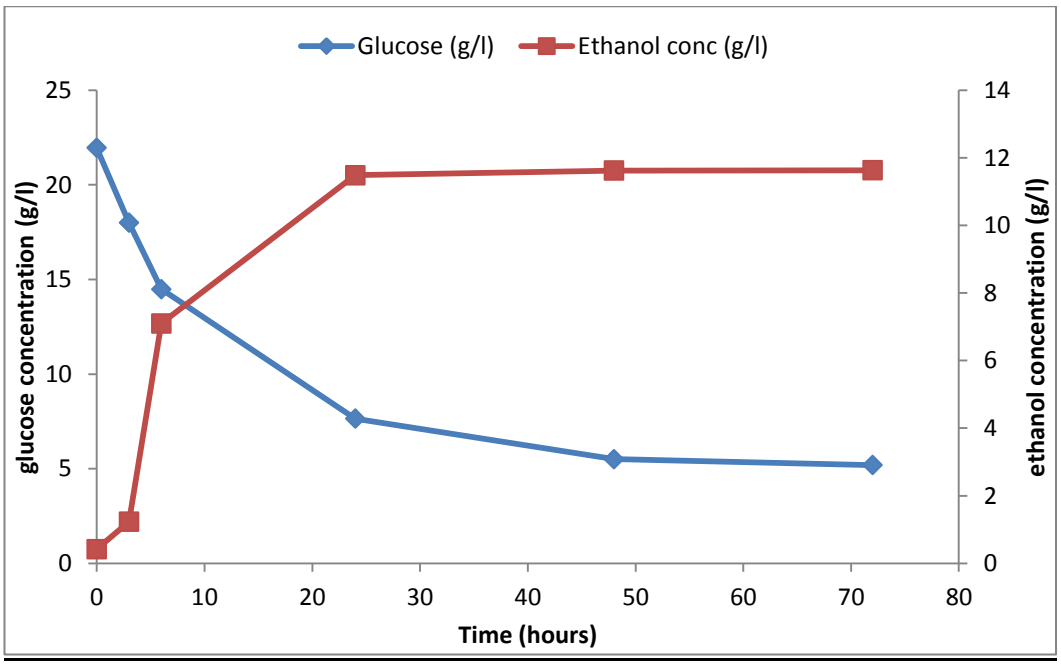


Fig 6.4: Separate Hydrolysis and Fermentation of cassava peels. Enzyme hydrolysis was carried out using 0.3%v/v of amylase, glucoamylase, cassava C and cassava R for 24hours. Fermentation was at 40^{0}C and pH 5 after separation of supernatant from cassava residues. Total volume of reaction was 0.5L

Results also show that the percentage theoretical yield of ethanol based on the concentration of the initial cassava peel is 41%. However, theortical yield based on glucose concentration at the start of the fermentation is 53%. Again the total yield is lower than the yields from SSF experiments. A comparison of the total percentage theoretical yield of ethanol is shown in table 6.1.

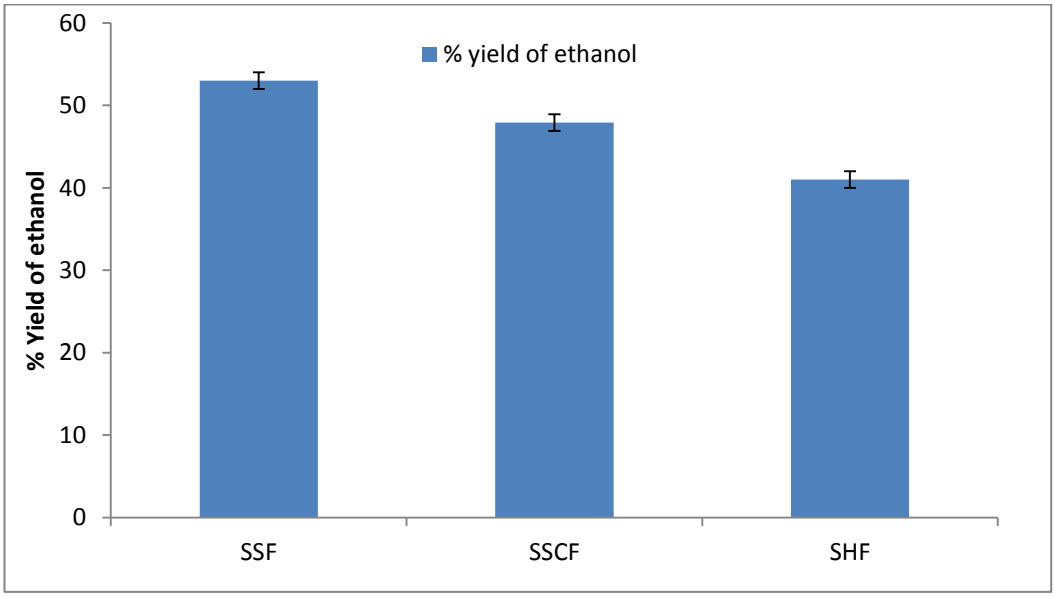


Table 6.1: Comparison of percentage theoretical yield of ethanol from cassava peels. Reactions carried out at 40^{0}C and pH 5.

Although overall yield of ethanol is significantly lower than what is usually obtained in industrial processes, it must be noted that results agree with a few quoted figures in literature. Oliva et al. (2004) reported ethanol yields from the simultaneous saccharification of wheat straw, sorghum bagasse and poplar biomass to be between 50-72% of maximum theoretical yield. They carried out their experiments at 42^{o}C. Kadar et al (2003) also reported maximum theoretical yields of between 55-60% from solca floc and paper sludge at 40^{o}C. There have been reports of ethanol yields of 65-67% of theoretical yield from the separate hydrolysis and fermentation of glucose (Vagar-Eldei, 2011) by *Kluveromyces marxianus* CBS712 at 44^{0}C. When the fermentation was carried out at 47^{0}C, the maximum yield was reduced to 27% and 42% for a thermotolerant mutant strain of *Kluveromyces marxianus* CBS712. A study by Reeves, (2004) on the kinetics of the growth of *K.marxianus* reveals that growth of the microorganism is limited severely under anaerobic conditions. The study also reported that this microbe has very low ethanol tolerance during its exponential growth phase. The study suggested a two-stage fermenter in series in which the first would be an aerobic process that retains the biomass and the second stage would be an anaerobic process where the yeast cells would be able to maximise ethanol production (Reeves, 2004).

6.3.0 CONCLUSION

One obvious feature of the experiments was the significant concentration of glucose left after fermentation. Further experiments using different substrate concentrations and varying other process variables like temperature and pH is required so as to maximise the sugars produced from the cassava peels. Further experiments to ascertain the viability of high temperature fermentation process in industry is much needed.

The use of a larger bore sample tube would also make sampling easier as it was quite a challenge getting samples at intervals. Perhaps sampling from the drain would be a better reactor design for solid substrate fermentation processes.

CHAPTER SEVEN: CONCLUSIONS AND RECOMMENDATION

Cassava peels can be characterised as an excellent source of carbohydrate for biofuel production. This dissertation has investigated several process strategies of different enzyme treatments with an objective of suggesting an appropriate treatment option which can also be combined with a pre-treatment method. Another consideration was to consider that the process suggested can also be easily streamlined with the fermentation process.

From the results of experiments conducted, it can be seen that a separate hydrolysis of either the starch or cellulosic component of the peels followed by a washing and separation of that component and a subsequent hydrolysis of the peels by another set of hydrolytic enzymes gave the highest yield of sugars. To incorporate fermentation into this separate hydrolysis, each of the sugars from the hydrolysis of both starch and cellulose would need to be separated and filtered and then fed into a different vessel for fermentation. However, this process is a lot more cumbersome and would involve different reaction vessels besides the cost of separating the simple sugars from the cassava peel residue.

A simultaneous saccharification of both starch and cellulose represents a simpler alternative process option. Both starchy and cellulosic parts of the peels are treated at the same time in the same vessel. A pretreatment process as well as the fermentation process can also be incorporated into this treatment strategy. It provides a cheaper option in terms of equipment cost and the possibility of using the SSF process for the hydrolysis and fermentation. However, hydrolysis experiments showed lower yield of sugars when compared to the separate hydrolysis of starch and cellulose.

A slight modification of the simultaneous saccharification of starch and cellulosic components that incorporates the hot water treatment is however recommended

for the treatment of cassava peels. The hot water pretreatment would be combined with the amylase treatment since these amylases are known to function well at temperatures above 90^{o}C. Subsequently, cellulases, hemicellulases and glucoamylase could then be used to further treat the peels within the same vessel at a lower temperature of between 40^{0}C-50^{0}C. This process option would take advantage of the effect of hot water on the substrate properties of the peels as well as create an optimal operating condition for amylase enzymes. This process strategy could also allow for the subsequent fermentation of the sugars generated from the enzyme hydrolysis either simultaneously or after separation from the residue. A likely disadvantage of this process might be the inhibition of cellulases by glucose and cellobiose generated from the amylase treatment. The choice of a simultaneous saccharification and fermentation process after the amylase and hot water pretreatment should reduce the impact of the sugar inhibition on the cellulase enzymes. This treatment option would also leave room for further research on high temperature fermentation to be incorporated in this same step.

The studies also suggest that starch is easily hydrolysed from the peels and so might not be chemically bonded to the cellulose and hemicellulose component of the peels. The removal of starch also seems to have a better effect on the enzyme hydrolysis of cellulose possibly as a result of increased accessibility of enzymes in the peels.

Further work is needed to ascertain the effect of substrate properties on the overall kinetics of enzyme hydrolysis of cellulose with regards to the contributions of the assessable surface area, degree of polymerisation and crystallinity. However, this is still limited by the unreliable assays for measuring these properties especially in lignocellulose materials.

The difficulty in measuring these properties in the presence of lignin and in the case of cassava peels (starch and lignin) remains a disadvantage. One of the limitations of this study in proposing a kinetic model that describes the enzyme hydrolysis of cellulose in cassava peels was the use of cellulase enzymes that are not well defined. A mechanistic model that describes the action of the individual enzymes

that make up on the cellulase system and how these enzymes are affected by the presence of starch, lignin and substrate properties in rich lignocellulose biomass would be of tremendous help to understanding similar biomass. Although an attempt was made in this study to follow the degree of polymerisation in both the solid residue and sugars released in solutions, it is still difficult to ascertain its overall effect on the overall kinetics of this reaction. Another interesting recommendation would be to investigate the possible contribution the removal of non-cellulosic (hemicellulose linkages, pectins) to the fast initial rates within the first few hours of the enzyme hydrolysis of starch rich cassava peels.

Further work should also be carried out on streamlining the fermentation process with the enzyme hydrolysis of the peels and pretreatment process especially at elevated temperature. A two stage aerobic-anaerobic fermentation process could be explored to ascertain if ethanol yield can be increased as has been suggested in literature using *Kluyveromyces marxianus* (Reeves, 2004). Further work on the limiting potentials of these yeast species with different substrate concentration, temperature, process options and likely inhibitors of *K*. *marxianus* should be investigated.

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APPENDIX A

LIST OF EQUIPMENTS USED

(a) Eppendorf 5415 D Bench Top Centrifuge

(b) Grant GLS 400 Water bath incubator with shaker

(c) Waters 2695 HPLC equipped with Waters 410 refractive Index Detector and Shodex KS-801 size exclusion column

(d) Techno Block Heater

(e) Beckman Coulter Allegra Centrifuge

(f) Stuart Heat Stir CC162

(g) Beckman Coulter DU 800 Uv-Vis Spectrophotometer

(h) Seven Go pH meter SG2

(i) Kenwood BL 450 Kitchen Blender with grinder

(j) Gallenkamp Oven

(k) Gallenkamp incubator

(l) Gilson 234 Autoinjector Hplc equipped with REZEK ROA column and connected to Viscotek VE 3580 RI detector

(m) Thermo Electron Corporation Biomates Uv detector

(n) Infors AG CH-4103 incubator

(o) Olympus CH-B143-3 microscope

(p) Virtis benchtop K freeze dryer

APPENDIX B

CALIBRATION CURVES

1.CALIBRATION CURVE FOR GLUCOSE FROM THE DNS ASSAY

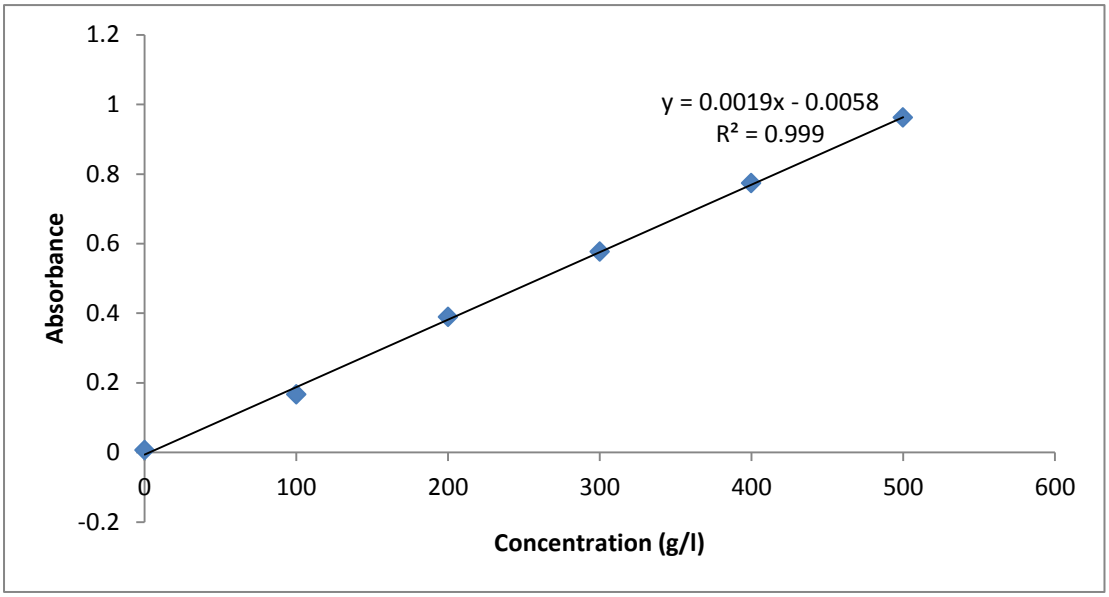
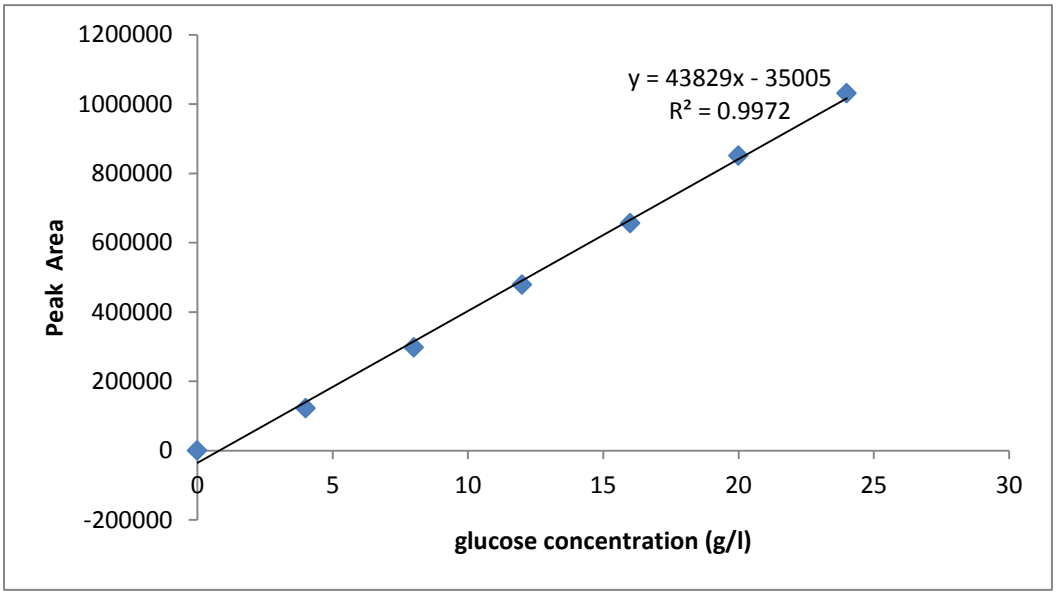
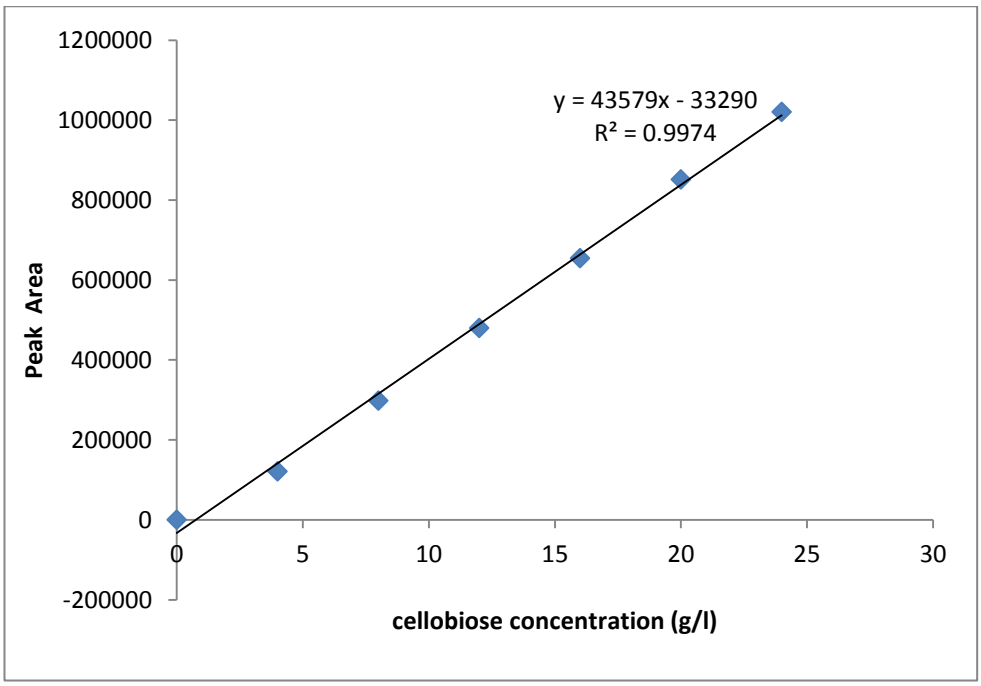


Fig 3.1: An example UV/Vis standard curve with absorbance at 575nm for a range of glucose concentrations for the DNS assay in which glucose is used as standard.

2. CALIBRATION CURVES FOR THE HPLC ASSAY

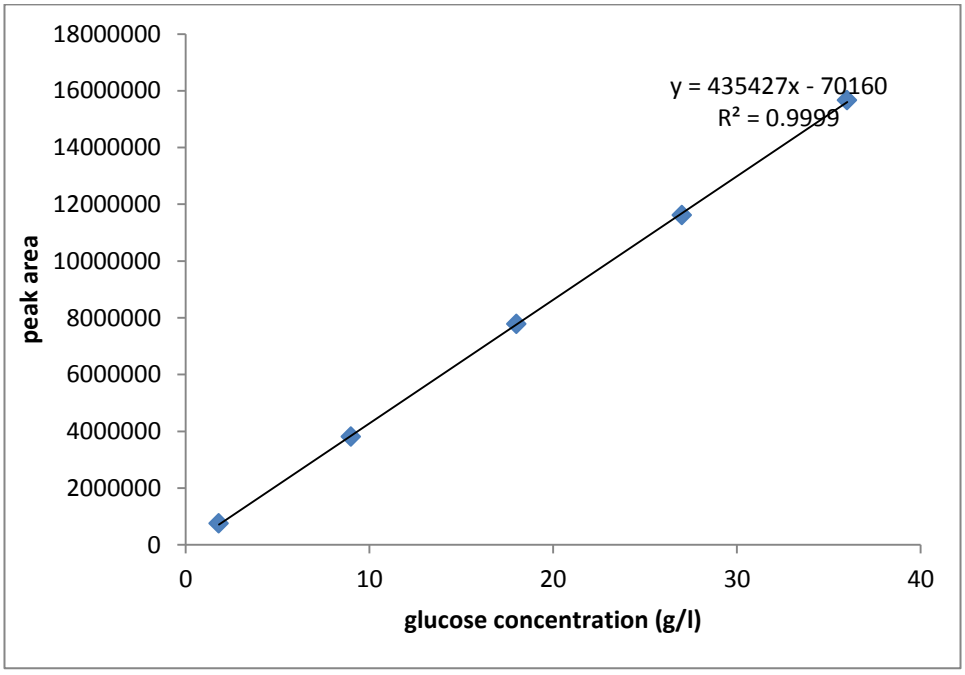


An example HPLC standard curve for a range of glucose concentrations

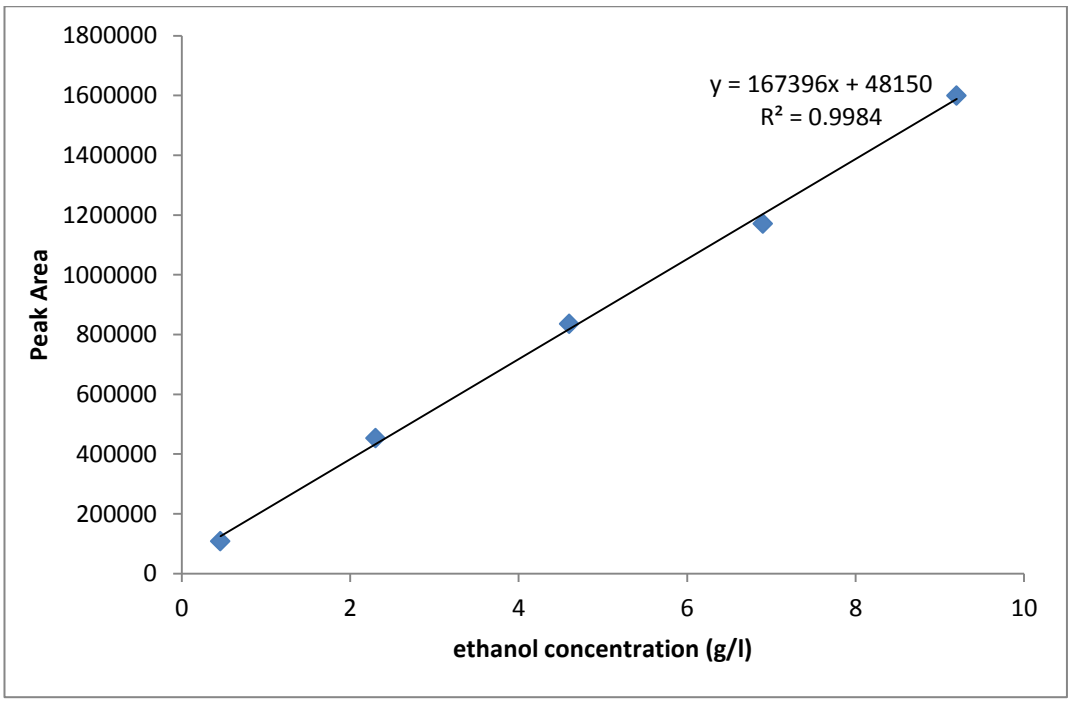


An example HPLC standard curve for a range of cellobiose concentrations.

3. CALIBRATION CURVES FOR HPLC FERMENTATION ASSAY

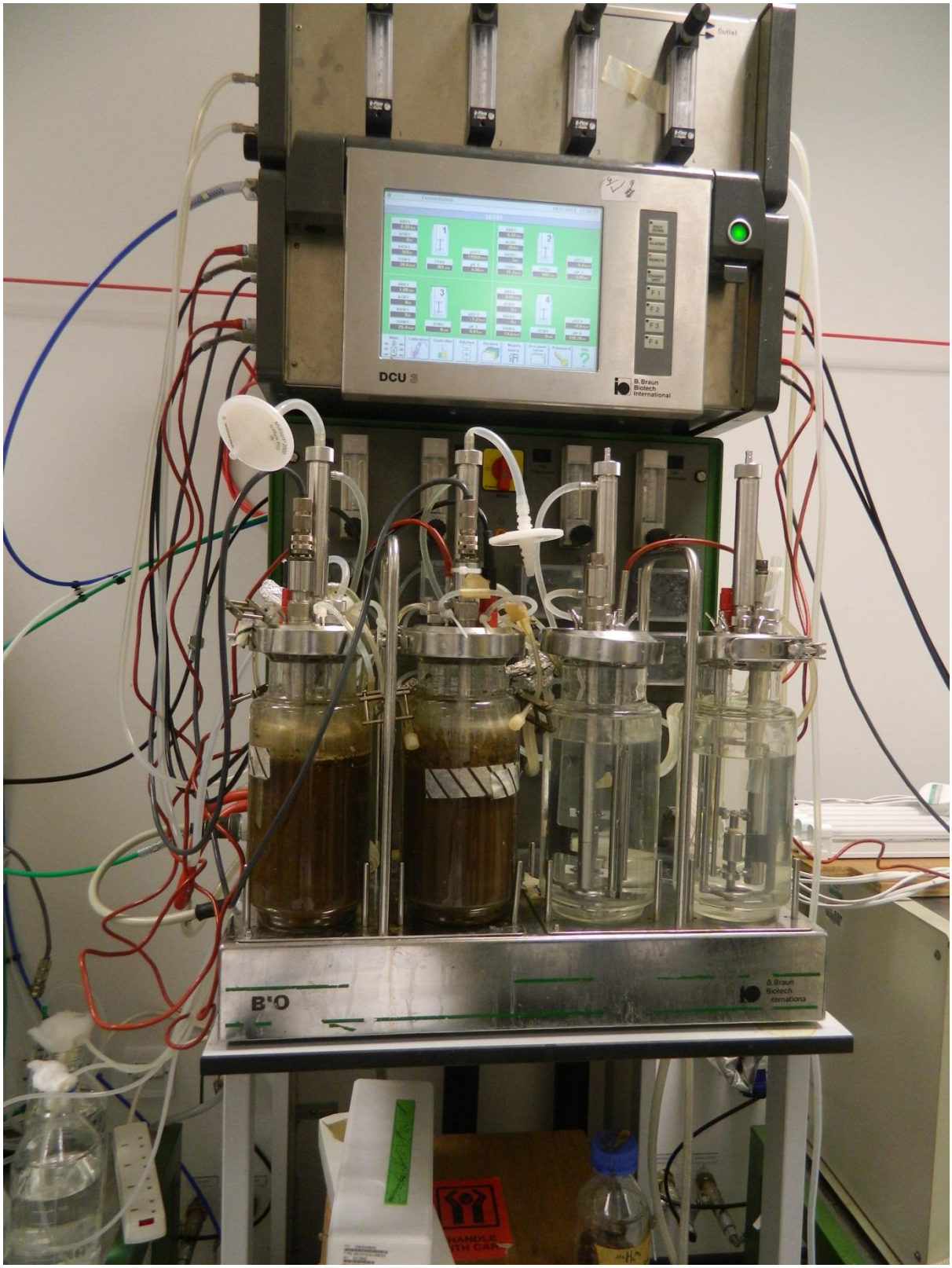


An example HPLC standard curve for a range of glucose concentrations



An example HPLC standard curve for a range of ethanol concentrations

APPENDIX C



Braun Biostat Q Bioreactor used for fermentation reactions