Comparative analysis of sorghum and other South African grains for sustainable bioethanol production

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Submitted in partial fulfilment of the requirements for the degree of Masters in the

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November 2012

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Keywords

Sorghum

South Africa

Biofuels

Bioethanol

Grain crops

Starch

Hydrolysis

Glucose

Fermentation

Ethanologens

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ABSTRACT

Comparative analysis of sorghum and other South African grains for South African bioethanol production

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MSc thesis, Department of Biotechnology, University of the Western Cape

The depletion of oil reserves and the constant discharge of greenhouse gasses (GHG) that are associated with global warming have forced both political and scientific sectors to pursue alternative, renewable and sustainable fuels that will be blended with petrol and ultimately replace it as the fuel of choice. Bioethanol is a form of fuel that is obtained from natural materials such as biomass. Starch and sugar containing materials are the primary carbon sources for bioethanol production and a range of feedstocks are currently being exploited for this purpose worldwide.

This study was aimed at measuring, comparing and analyzing fermentable sugars liberated by sorghum and three other grain crops (maize, barley and wheat) that are grown in South Africa and subsequently analyze ethanol yield after fermentation. Starch was extracted from sorghum, maize, barley and wheat via hot water treatment and hydrolyzed by use of α-amylase, gluco-amylase and a cocktail of both enzymes under various conditions to determine optimum hydrolysis conditions. The resultant liberated soluble sugars were measured with a pocket refractometer and High Performance Liquid

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Chromatography (HPLC) respectively. Hydrolysates obtained under optimum conditions were fermented with various ethanol producing microbial strains and a high-performing strain was selected. The selected high-performing strain (*Saccharomyces cerevisiae* NT 53) was used to ferment different grain hydrolysates (sorghum, maize, barley and wheat). The working volumes of the solutions were increased ten-fold (small-scale) and experiments were performed using sorghum grains as substrates and alcohol content was measured with an Alcolyzer Wine M instrument.

The optimum hydrolysis conditions for the grain crops were determined and it was found that the enzymes performed well at 70°C and starch was hydrolyzed within the first hour. Sixty grams per litre (60 g/L) of grain solution produced a maximum of 50.8 g/L of glucose when treated with the cocktail treatment. However gluco-amylase facilitated a similar production, at 47.8 g/L glucose. Sorghum and maize produced high glucose amounts and subsequent ethanol amounts, and maximum fermentation efficiencies of 87 % and 98 % respectively when fermented with the high performing NT 53 strain. The NT 53 strain was compared with commercial baker's yeast and they yielded similar ethanol amounts across the grain types. Under small-scale conditions, sorghum retained the consistency of yielding similar glucose amounts compared to laboratory-scale (50ml) conditions and when analyzed with the Alcolyzer, sorghum yielded a maximum alcohol content of approximately 2 % v/v. This study also showed that gluco-amylase alone was sufficient for starch hydrolysis and sorghum a more favourable and less expensive crop for ethanol production in South Africa.

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List of abbreviations

ARC:	Agricultural Research Council
BC:	Before Christ
CGIAR:	Consultative Group on International Agricultural Research
DEDEA:	Department of Economic Development and Environmental Affairs
DME:	Department of Minerals and Energy
EU:	European Union
FAO:	Food and Agricultural Organisation
GBEP:	Global Bioenergy Partnership
GHG:	Greenhouse Gasses
HPLC:	High Performance Liquid Chromatography
IEA:	International Energy Agency
IMBM:	Institute of Microbial Biotechnology and Metagenomics
IPCC:	Intergovernmental Panel on Climate Change
LDH:	Lactate Dehydrogenase
Mha:	Million hectares
NI:	Nietvoorbij Isolate
NT:	Nietvoorbij Teeling (Breed)
OD:	Optimal Density
PDH:	Pyruvate Dehydrogenase
PFL:	Pyruvate Formate Lyase
PRG:	Proteomics Research Group
RI:	Refrective Index

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RuBisCO:	Ribulose -1,5- bisphosphate carboxylase oxygenase
SAGIS:	South African Grain Information Service
SBD:	Starch Binding Domain
SSA:	Sub-Saharan Africa
TMT:	Million Metric Tons
UNEP:	United Nations Environmental Programme
USA:	United States of America
USGC:	United States Grain Council
UWC:	University of the Western Cape
WBGU:	German Advisory Council on Global Change
WHO:	World Health Organisation
YPD:	Yeast Peptone Dextrone

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Declaration

I, Didi Xhanti Makaula, declare that the thesis entitled **"Comparative analysis of Sorghum and other South African grains for sustainable bioethanol production"** is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of references.

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Name: Didi Xhanti Makaula Date: November 2012

Signature…….

Acknowledgements

First and foremost I would love to thank my supervisor, **Prof B.K. Ndimba** for great guidance, supervision and a lesson to be independent. Secondly I would like to thank my co-supervisor, **Dr M.P. Taylor** for getting this project up running and his valuable input on the project.

I would like to thank my entire family, especially my mother, **Mrs N.L. Makaula (*MABHAYI)*** for allowing and encouraging me to further my studies. I also thank my small family, **Unathi** and **Hlomani** for their patience.

I thank the entire members of the Proteomics Research Unit based at UWC and the ARC for the academic and moral support, IMBM for allowing me to use their facilities.

I also thank **Dr N. Jolly** and **Mr R. Hart** of the Post - harvest and wine technology department, Agricultural Research Council (ARC Infruitec–Nietvoorbij), Stellenbosch, South Africa for providing the *Saccharomyces* strains. Secondly I would love to thank **Mr L. van Zyl,** who helped with HPLC experiments.

I wish to thank the **ARC Professional Development Programme (PDP)** in conjunction with the **National Research Foundation (NRF)** for their generous financial support for my entire studies.

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Chapter 1: Literature review

1.1 Introduction

In our modern society, development is always dependent on the availability of resources or lack thereof. Shortage or restriction of those resources directly tampers with sustainable social, industrial and economic development that eventually leads to lack of global growth and development. Energy is one of the essential resources in human life and a secure, accessible and abundant supply of energy is very crucial in sustaining expanding societies around the world. Development is often threatened by the fluctuations in conventional fuel costs, increase in prices of basic food products, socio-political instability in oil-rich countries, limited oil reserves and the increase in fuel demand relating to the increased consumption (Haber, 2007).

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Petroleum fuel is one of the key factors that contribute directly or indirectly to development. Today fossil fuels take up 80% of the essential energy consumed globally and 58 % of that energy is consumed by the transport sector (FAO, 2008b). Fossil fuels are being depleted and their combustion contributes significantly towards the accumulation of greenhouse gas (GHG) emissions (FAO, 2008b; GBEP, 2007), which eventually lead to the negative effects such as climate change, declining of glaciers, rise in sea levels, loss of biodiversity, etc. (WBGU, 2010).

Climate change is described by Poortinga *et al*., (2011) as "arguably one of the greatest challenges the world is facing in the 21st century and as a result the threats that climate change poses have forced the global community to drastically limit the emissions of

GHG's or else face the ultimate consequences associated with increase in global temperatures". Although climate change is a result of various factors including natural internal forcing mechanisms (e.g. atmosphere and hydrosphere) and external forcing mechanisms (e.g. volcanism and plate tectonics), human activities that result in emissions of carbon dioxide (CO_2), methane (CH_4) and halocarbons have been identified as major drivers of climate change (Pachauri and Reisinger, 2007). The constant depletion of conventional fossil fuels with increasing energy consumption and GHG emissions have focused both political and scientific attention to pursue alternative, renewable, sustainable, efficient and cost effective energy sources with minimized emissions (GBEP, 2007; FAO, 2008b; IEA, 2004).

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Biofuels are renewable sources of energy that are obtained from natural materials such as plant biomass and animal fat. These can be used as substitutes for petroleum fuels (Demirbas, 2009). Biofuels are referred to as solid, liquid or gaseous fuels that are obtained from biomass (Table 1.1; Demirbas, 2008a, 2008b; Balat, 2008, 2009; Kong *et al*., 2008). Common biofuels include ethanol and biodiesel, derived respectively from maize, wheat, sugarbeet and oil seeds (Demirbas, 2009). Ethanol is also a petrol additive that can be obtained from a variety of domestic, cellulosic biomass, agricultural and forestry residues and municipal and industrial waste streams (Keskin, 2009; Chhetri and Islam, 2008). Ethanol production from biomass is considered as a way of reducing consumption of crude oil and environmental pollution.

Table 1.1: Classification of biofuels based on their production technologies. *Summary of different biofuel generations including the main feedstock sources and end products. The table represents different commercially viable biofuel sources and their possible products once they are processed. Table adapted from (Demirbas et al., 2011)*

Generation	Feedstock	Example
First generation biofuels	Sugar, starch, vegetable oils, or animal fats	Bio-ethanol, biodiesel, biosyngas, biogas
Second generation biofuels	Non-food crops, cellulosic material, wheat straw, corn, wood, solid waste	Bio-ethanol, wood diesel, biohydrogen, bio-oil
Third generation biofuels	Algae	Biodiesel
Fourth generation biofuels	Vegetable oil waste, biodiesel	Biogasoline

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Biofuels will not only benefit urban city dwellers; rural households have used biofuels resources such as wood, dried manure and charcoal traditionally for cooking and heating for centuries. The International Energy Agency (IEA) reported that by 2006, 2.5 billion people around the world depended on traditional biomass such as wood, charcoal, crop residues and dung to combat their energy needs for cooking and heating (IEA, 2006). In 2002, the World Health Organisation (WHO) also reported that an estimated 80-90% people in Sub-Saharan Africa (SSA) depended on biomass fuels and that fuel-wood accounted for more than 75% of the household balance (WHO, 2002). Sub-Saharan Africa has the highest bio-energy potential in the world after considering food production (Smeets *et al*., 2007). The SSA region has a favourable climate to grow these crops; biomass production can be up to five times higher in tropical and sub-tropical regions in

terms of photosynthetic efficiency, compared to temperate regions (Bassam, 1998). In Africa alone, the Food and Agricultural Organisation (FAO) of the United Nations estimated that there are 379 million hectares of available arable land, but only 43 million hectares of this land is currently in use. This translates to only 11 % of the available, arable land being in use (Biopact, 2006; FAO, 2008c). A significant portion of the enormous remaining land could be designated to bio-energy crop cultivation. Globally, only 14 million hectares of land, or 1% of the world's currently available arable land, is being used to grow energy crops for biofuels (IEA, 2006). In South Africa, biofuel targets are estimated to require only about 1.4 % of national arable land to produce 2% of the countries liquid fuel needs, which is not a large percentage given that nearly 14% of arable land is currently under-utilized (Department of Energy, 2007). This leaves a large untapped land resource and it creates an opportunity for most people residing in developing countries such as South Africa to use biomass resources as an energy source of choice for the foreseeable future.

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Developments in the biofuels sector offer both promises and challenges for developing countries. It is cautioned that biofuels production will tamper with food supply for the poor. However, there are also assurances that, if well managed, biofuels can be produced profitably and stimulate rural economic growth in developing countries (Jumbe *et al*., 2009). In a report by von Braun and Pachauri (2006), it was noted that biofuel production could create demand for energy crops that are grown by rural farmers. In addition, it is suggested that farmers would increase their income by growing crops that

can adapt to marginal land that is normally not suitable for other crop production systems.

Biofuels are categorised into four different technologies that are represented by various biological materials. First generation biofuels are fuels that are obtained from food sources. First generation bioethanol feedstocks are divided into two main categories; sugar-based (e.g. sugarbeet, sugarcane, sweet sorghum) and starch-based (e.g. maize, sorghum, triticale, cassava, and potato) (Ruane *et al*., 2010). The processes for producing ethanol from these feedstocks include either direct fermentation of sugars or enzymatic conversion of starch-based crops such as maize and fermentation of the resultant carbohydrate (Mielenz, 2001). Second generation biofuels are fuels that are derived from non-food products such as lignocellulosic material (Timilisina and Shrestha, 2011).

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For the scope of this work, we will be examining the efficiency of classic energy crops to produce fermentable sugars. We will begin by giving short descriptions of first generation biofuel technologies. We will also briefly explain the conversion of various starches and sugar materials to ethanol and then describe the relevance of first generation biofuel feedstocks, in particular sorghum, as a sustainable energy crop for the South African biofuel industry and beyond.

1.2 First generation liquid biofuel technology

Modern bioenergy relies on efficient conversion technologies for application at the household, small business and industrial scale. Both solid and liquid biomass inputs can

be processed to be more convenient energy carriers (Ruane *et al*., 2010). Among the different segments of the bioenergy sector, the largest and most rapid growth has been seen in liquid biofuels, especially first generation liquid biofuels (FAO, 2008a). First generation fuels are generally obtained specifically from an edible portion of the plant (sugar, grains or seeds).

Production of these fuels has become substantially more efficient over the last three decades as Brazil and the United states have scaled up their industries. Ethanol fuel production in the USA has increased significantly (Figure 1.1) and, in recent years, ethanol imports have increased to fulfil production requirements (Taylor *et al*., 2009). The two main first generation liquid biofuels are currently biodiesel and bioethanol, representing about 15 and 85% of current global production, respectively (FAO, 2008). Biodiesel is derived from transesterification of vegetable oils and animal fats that are composed of saturated and unsaturated long-chain fatty acid alkyl esters, and common feedstocks for biodiesel include soybean oil, sunflower oil, cottonseed oil and rapeseed oil (Fazal *et al*., 2011; Canakci, 2007; Aydin and Iikilic, 2010; Nabi *et al*., 2009; Kegl, 2008).

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With estimates of land requirements for future biofuels varying widely and depending on the type of feedstock, geographical location, level of input and yield increase, it is estimated that about 118 to 508 million hectares (Mha) would be required to provide at least 10% of the global transport fuel demand with first generation biofuels in 2030 (this would be equivalent to 8%-36% of current cropland; UNEP, 2009). In the US, ethanol

from maize is now competitive with conventional petroleum fuel, while in countries such as Brazil, ethanol derived from sugarcane is far less expensive than petroleum fuel (Hunt, 2007; Taylor *et al*., 2009). Such incremental gains are likely to continue for years to come.

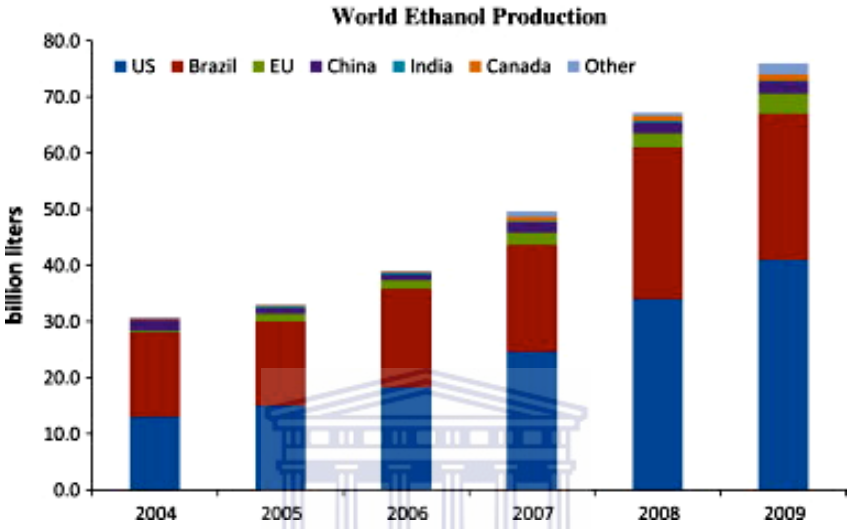

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Figure 1.1 Global production of fuel ethanol over the years. *This figure shows the consistent increase in bioethanol production in the world, especially in the ethanol powerhouses; the US and Brazil. Figure adapted from (REN 21, 2009)*

1.2.1 First generation bioethanol

Bioethanol is a type of liquid fuel that is derived from any biomass that contains significant amounts of sugar or materials that can be converted into sugar, such as starch. Sugarcane, sweet sorghum and sugar beet are typical examples of feedstocks that contain sugar. Maize, wheat and other cereals contain starch in their grains that can be converted into sugars (Ruane *et al*., 2010). During the process of ethanol production from sugar-based crops, they are first processed in order to extract the sugars. Subsequent to that, the

sugars are then fermented to yield ethanol (also known as bioethanol). This is a biochemical process by which sugars, such as glucose, fructose and sucrose, are converted/fermented into ethanol and carbon dioxide (CO_2) using yeast or other ethanol-producing microorganisms (ethanologens) (Mojovic *et al.,* 2006). Glucose and fructose are monosaccharides, whereas sucrose is a disaccharide consisting of glucose and fructose joined together. A final step purifies the ethanol (distillation) to the desired concentration and removes excess water to produce anhydrous ethanol that can be blended with petrol. In regions such as the USA and EU, first generation bioethanol is well established; with recent green legislation suggesting that it will play an important role in lowering petroleum use in transport fuels for the future (IEA, 2008).

The process of producing bioethanol from starch-based materials is, however, much more complex compared to sugar-based crops due to an additional step known as hydrolysis. Hydrolysis can either be enzymatic (using a mixture of enzymes such as amylases) or acid-based (Balat *et al*., 2008). Starch hydrolysis is traditionally carried out in a sequential manner with hydrolytic enzymes such as α-amylase and gluco-amylase (Figure 1.2; Zhao *et al*., 2009). The enzymatic treatment requires enzymes of high purity; particularly gluco-amylase has to be free of contaminating activities such as cellulase and catalase. Cellulase contamination results in detection of false increases in starch values due to cellulose hydrolysis, whereas catalase lowers the stability of the chromogen formed in glucose assay methods (McCleary *et al*., 1997).

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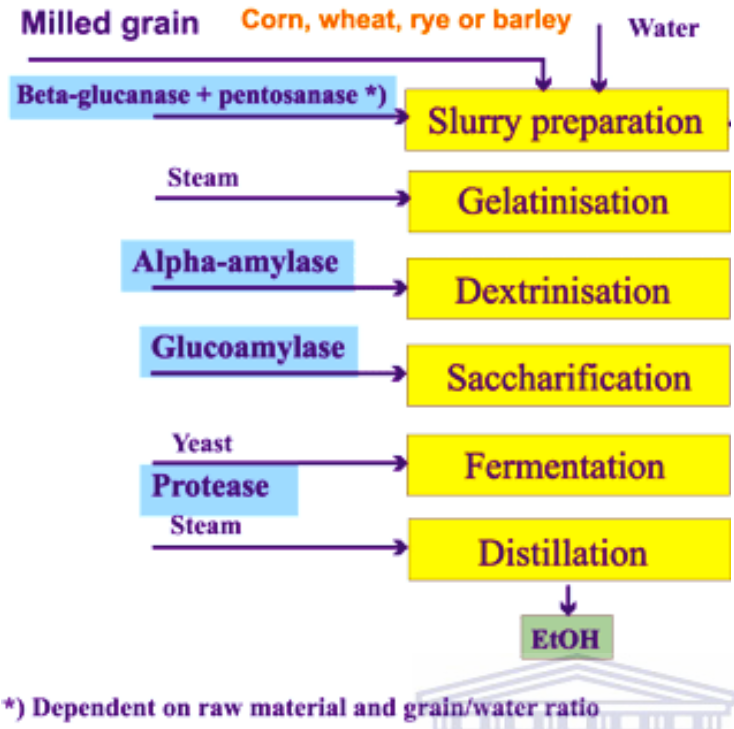

Figure 1.2: Schematic diagram illustrating starch hydrolysis and fermentation process. *This process is traditionally carried out in a sequential manner, using hydrolytic enzymes and fermentative organisms as indicated in the figure. Figure adapted from http://www.biokemi.org/biozoom/issues/515/articles/2295, accessed 24/10/2012*

At present, the cost of harvesting and processing sugar-based crops and starch by crushing stems to extract juice and milling grain followed by saccharification, respectively, is relatively low compared to the cost of harvesting and processing lignocellulosic biomass (Byrt *et al*., 2011). Processing of lignocellulose is expensive due to the energy (steam explosive treatments) and or enzymatic costs involved in separating cellulose from lignin, and the enzymatic cost of hydrolysing the cellulose (Byrt *et al*., 2011). This revelation puts emphasis on the immense relevance of first generation biofuel production around the world and particularly in budget restricted nations.

1.2.2 First generation bioethanol feedstock sources

1.2.2.1 Starch

Starch is composed of two different polysaccharide fractions; amylose and amylopectin polymers, which are made up of glucose monomers, but differ in size and shape (Stevnebo *et al*., 2006). It constitutes a major component of foods and also a raw material for use in the production of industrial products. Amylose is a linear chain of glucose bound together with α-(1,4)-linkages (Figure 1.3 C). Amylopectin is larger than amylose, highly branched and has an α-(1,6)-bond in the branching points in addition to the α-(1,4)-linkages in the linear chains (Figure 1.3 C; Stevnebo *et al*., 2006). Application of starch as a raw material usually requires disruption of the granule, which involves additional processing steps, collectively known as hydrolysis. Hydrolysis is usually achieved with application of enzymes; however the information that is available on starch hydrolysis and it's hydrolysing enzymes is still not well understood (Oates, 1997).

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Starch hydrolysis is a biochemical process that starts with the heating of starch above critical temperature, thus resulting in a multistage process known as gelatinization.

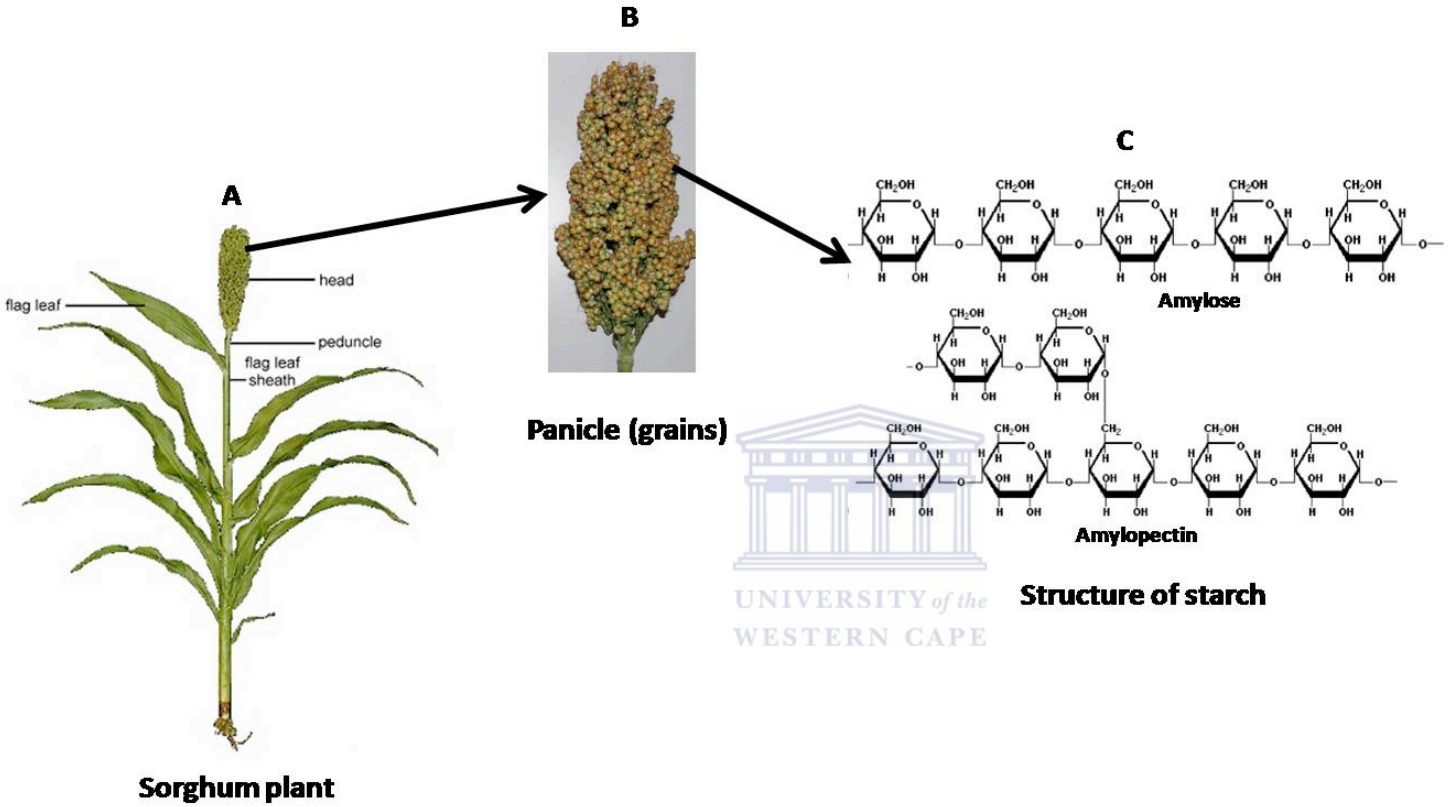

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Figure 1.3: The figure illustrates the position of starch source (grains) within the sorghum plant (A), how grains are packaged on the panicle (B) and it also demonstrates the animated structure of starch (C). *Starch has two forms; amylose that consist of linear linkages (1→4) and amylopectin that consist of linear linkages (1→4) and in addition α-(1→6) branches.*

The process includes disruption of the radially-ordered structure and eventual opening of crystal structures as the polymer chain becomes increasingly hydrated. This increases the chemical reactivity of inert starch granules towards amylolytic enzymes. The most important and common enzymes acting on starch are α-amylase, gluco-amylase and debranching enzymes such as pullulanase and isoamylase. Debranching enzymes attack α-(1,4) links of amylopectin to give mixture of dextrins plus few sugars (Hough, 1985).

α-Amylase is a metalloenzyme with an endo-action that randomly attacks starch molecules by hydrolyzing α-(1,4) links yielding shorter polysaccharide chains. When hydrolysing amylopectin, α-amylase produces a mixture of branched and unbranched starch molecules (dextrins) (Oates, 1997). Gluco-amylase on the other hand is an exo–enzyme that is traditionally utilized to hydrolyze the dextrins from the non–reducing end of a molecule, progressively releasing glucose. This method is an abundantly utilized method for enhancing starch hydrolysis (Oates, 1997). It is suggested that other structural features possessed by different crop grains also influences the susceptibility of granules to enzyme hydrolysis.

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1.2.2.2 Sugars

Sugars are a class of carbohydrates that are classified as monosaccharides, disaccharides, or oligosaccharides. One group of saccharides that is used for bioethanol production includes soluble (non-structural) sugars. Sugars are mainly derived from plants and sucrose is the primary product of carbon fixation during photosynthesis in the source leaves and the major transported form of carbohydrates to the rest of the plant (Kortschak *et al*., 1965). Triose-P exported from chloroplast is converted to hexose phosphates

(hexose-P), which are in turn converted to sucrose in the cytosol (Figure 1.4 A and B; Winter and Huber, 2000). In the cytosol, sucrose synthesis is regulated by various enzymes as illustrated in Figure 1.4 B. The dominant crop grown worldwide for sucrose production is sugarcane (Wu and Birch, 2007). In plants, sugars are basically formed through a process that converts CO_2 into organic compounds such as sugars. Plant tissues such as mature sweet fruits and sweet stems accumulate high concentrations of sugars that are readily fermentable and generate ethanol. Humans over the ages have taken advantage of this and derived foods, wines and beers, possibly since 5000BC (Cavalieri *et al*., 2003).

As previously mentioned, there is currently a wide range of sources to choose from for the production of first generation bioethanol and some of them being investigated include maize, wheat, cassava, and sorghum. We have identified sorghum as our preferred primary feedstock due to the advantages it possesses compared to other grain bearing feedstocks (Department of Energy, 2007). In the following sections, the positive attributes of sorghum crop will be described.

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1.3 The *Sorghum bicolor*

Sorghum [*Sorghum bicolor* (L) Moench] is a tropical plant belonging to the family of *Poaceae*, and is one of the most important crops in Africa, Asia and Latin America (Figure 1.3 A; Anglani, 1998).

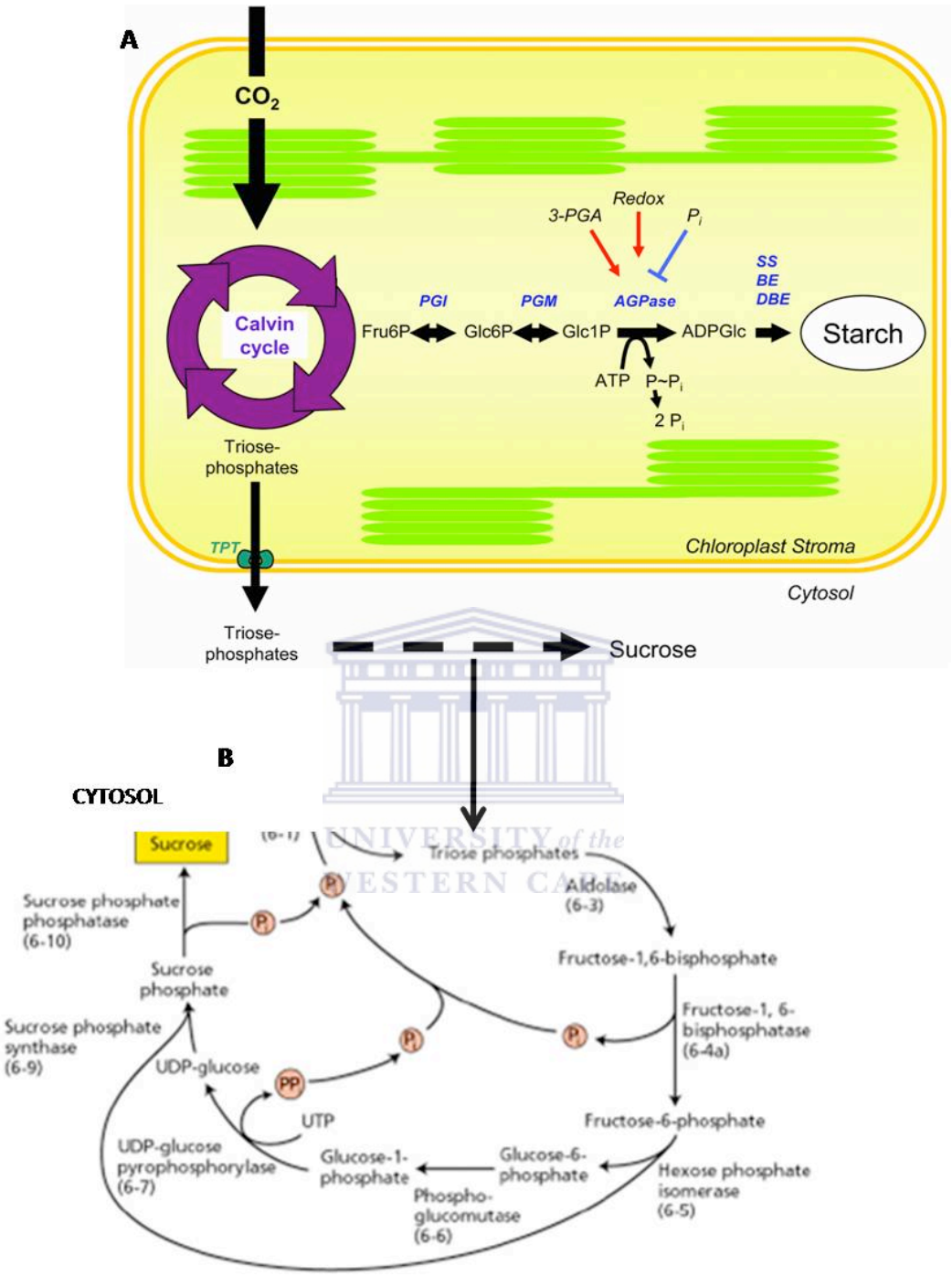

Figure 1.4: Pathway synthesis of starch and sucrose in chloroplast and cytosol. *(A) Carbon is absorbed through the Calvin cycle, is separated and a fraction is exported to the cytosol for sucrose synthesis. Another fraction retained in the chloroplast for starch synthesis. (B) Synthesis of sucrose regulated by various enzymes in the cytosol. Figure adapted from (Zeeman et al., 2007 A; Plant physiology., 2002 B)*

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Sorghum is a C_4 crop, together with other economically important crop species such as sugarcane (*Saccharum L.*) and maize (*Zea mays L.*; Hatch, 1987). Sorghum, an African native crop, is arguably believed to have originated from North East Africa, probably domesticated in Ethiopia between 5000 and 7000 years ago (Dicko *et al*., 2006). Sorghum then spread through trade and shipping routes to other African regions, Asia, Europe, Australia and the US (Gnansounou *et al*., 2005). The USA is among the largest producers of sorghum in the modern era (Figure 1.5). Sorghum is believed to have been introduced to the United States by West African slaves, who cultivated it in the Southern states for food purposes, but was re-introduced in the late 19th century for commercial cultivation (Dicko *et al*., 2006). It is a highly complex crop that can be utilized in many ways; including as food for human consumption, animal feed, brewing and recently as a source of carbohydrate for biofuels and sorghum fibers (fibers are used for biodegradable packaging materials and solvents).

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Sorghum is the only crop that provides both grain and a stem that can be used for the above-mentioned activities. It is relied upon as a principal energy source for more than 300 million people across semi-arid and tropic regions of the developing world (Dicko *et al*., 2006). It is a very complex crop that has been bred into four varieties including grain, sweet, fibre, and multi-purpose (Woods, 2001). Although the sorghum plant has the ability of growing above 4 m within a period of 3 to 5 months, many varieties selected for cultivation are dwarf breeds, specially designed for easy harvest (Lu, 1997).

1.3.1 Sorghum world production

Sorghum is cultivated in more than 100 countries throughout the world, covering areas in the North and South America, Africa, Asia, and the Pacific. World production of sorghum in 2010 was reported to be 59.5 million metric tons (TMT; Figure 1.5) which is a decline compared to production figures for 2007-2008 season, where a production of 64 TMT was reported (www.fas.usda.gov). Nigeria produced the most sorghum in 2010 (19.3%) followed by the US (16.3%) and India (11.7%; Figure 1.5), whereas in the 2007-2008 season the US was the leading sorghum producing country with 19.9% followed by Nigeria (15.5%), and India (11.3%; Shewale and Pandit, 2009).

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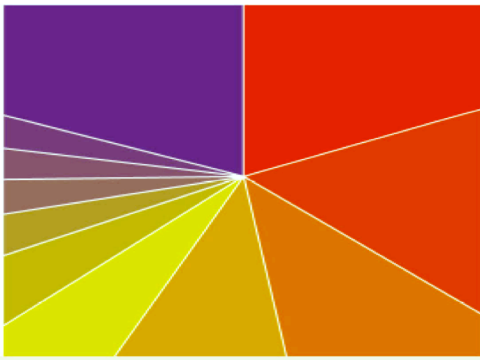
Despite the fact that sorghum has gained immense exposure in many countries, it still has a long way to go for it to be on the same production scale as crops like maize. Comparing the world production of maize of 812.4 TMT (www.grains.org) to that of sorghum in the year 2010, it is more than 13 times higher. Continentally, Africa is the largest sorghum producer (28.2% and more in 2010), accounting for more than 16.3 TMT from the total of 59.5 TMT produced worldwide (Figure 1.5). However, this is a significant decline from over 31.3% in 2007-2008.

1.3.2 Traditional uses of sorghum

Sorghum crops can be used efficiently and productively in various ways, ranging from human consumption, animal feed to biofuel production. In many parts of the world sorghum has traditionally been used in various food products such as porridge and

flatbread (Figure 1.6). Sorghum has unique properties that make it well suited for food uses. Some varieties are rich in antioxidants and all sorghum varieties are gluten-free, an attractive alternative for wheat allergy sufferers.



World Production

Nigeria	11,500	**19.3%**
US	9,728	**16.3%**
India	6,980	**11.7%**
Mexico	6,250	**10.5%**
Argentina	3,629	**6.1%**
Sudan	2,630	**4.4%**
Ethiopia	2,084	**3.5%**
Brazil	1,825	**3.0%**
China	1,650	**2.8%**
Australia	1,600	**2.7%**
Others	11,637	**19.6%**
WORLD	**59,513**	**TMT**

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Figure 1.5: World sorghum production in the year 2010. *The figure represents the production of sorghum globally, with Nigeria being the leading nation in sorghum production in 2010. Figure adapted from www.grains.org accessed 25/10/2012*

Figure 1.6: The figure above represents the different products that are obtained from sorghum. *These include porridges, energy drinks, alcoholic beverages and bread*

Sorghum has been an important staple food in the semi-arid tropics of Asia and Africa for centuries and is currently responsible for feeding millions worldwide. More than 35% of the global annual sorghum produce is produced in Asia and Africa for food purposes (FAO, 1996). The rest is primarily used for animal feed, alcohol and industrial products (Awika and Rooney, 2004). Sorghum crop is still one of the principal sources of energy, protein, vitamins and minerals for millions of the poorest people in these regions particularly in SSA where millions of humans rely on it as their staple food. However, in developed nations, sorghum is widely used for the production of forage and silage for animal feed. Sorghum crop residues are a major animal feed resource in many crop-livestock farming systems. They are very useful in bettering the problem of lack of feeds for ruminant livestock during the dry season (Sibanda and Said, 1991; Adu *et al*., 1992)

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1.3.3 Sorghum in the context of C_4 crops

Crop plants require characteristics that will assist them in utilizing the available resources on land, to adapt to the cultivation conditions, and eventually give rise to high production yield. The contribution of C_4 crops towards the sustainability of the world is huge, thus sustainability in many tropical and sub-tropical regions of Africa is largely based on C_4 plants. In hot, dry conditions (above 30°C), C_4 plants have increased CO_2 absorption rates compared with C_3 plants, therefore they adapt better photosynthetically to tropical habitats (Tarpley *et al*., 1994). C_4 crops are also economically important and their importance is due to their ability to produce high amounts of photoassimilates and accumulate these as carbohydrates such as sugars and starch. They pump CO_2 into specialized cells surrounding the vascular bundles, where ribulose -1,5- bisphosphate carboxylase oxygenase (RuBisCO) is excluvely localised, and CO_2 can accumulate to levels in excess of tenfold the atmospheric concentration, in these cells (Furbank, 1998). Sorghum, pearl millet and maize are responsible for ~ 95% of the world C_4 cereal production, with Africa and Asia being the leading producers of C_4 cereals.

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C_4 plants are shown to have biochemical advantages over C_3 plants (Ludlow, 1985). The water use efficiency of C_4 crop is approximately twice as high as that of C_3 species, due to the increased leaf photosynthesis rates and low transpiration of the crops (Byrt *et al*., 2011). Sorghum, being a C_4 crop has this trait that gives it an advantage to survive, develop and produce decent yields in hot conditions. This also allows sorghum to have high photosynthetic efficiency, which results in high production and fast accumulation of carbohydrates (including sugars) (Dajue, n.d).

1.3.4 Why is sorghum a potential feedstock for sustainable fuel production?

Water and climate fluctuations are among the reported main limiting factors of crop production in many areas worldwide. Salinity also causes great loss in agriculture by restricting yields of various crops (Vasilakoglou *et al*., 2011). Sorghum has the ability to grow in marginal areas because of its high tolerance to less favorable (saline and drought) conditions (Berenguer and Faci, 2001; Almodares and Hadi, 2009). Sorghum has higher water-use efficiency than other summer crops under both well-watered and water-stressed conditions (Steduto *et al*., 1997). From an agronomic point of view, sorghum is believed to be more environmentally friendly than maize because of its relatively low nitrogen (Barbanti *et al*., 2006) and water requirements (Mastrorilli *et al*., 1999). Almodares and Hadi (2009) suggested that sorghum used for biofuel production would be an appropriate alternative crop to maize in marginal irrigated areas where irrigation water is limited during crop development.

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As mentioned before, sorghum has been suggested to be a good source for ethanol production because of its rapid growth rate, early maturity and high total energy value (Smith and Buxton, 1993). Moreover, sorghum production is encouraged by new policies with regards to non-food crops in areas such as the European Union (Rexen, 1992). The potential of sorghum as an alternative energy crop has been emphasized (Smith and Buxton, 1993; Steduto *et al*., 1997); however, the ability of various sorghum cultivars to grow under soil salinity and water deficient field or greenhouse conditions has not been sufficiently determined. Vasilakoglou *et al*. (2011) demonstrated that sweet

sorghum provided sufficient yields even when grown under the stresses of soil salinity and reduced irrigation. This study revealed that sweet sorghum plants produce sufficient juice, total sugar and ethanol yields in fields with soil salinity up to 3.2 dS m^{-1}, even though the plants received 50–75% of the water regimes typically applied to sorghum. Although undesirable climate changes and the continuing decline in water availability have forced strict conservation of the available energy resources whilst trying to increase development. However, sorghum has evolved to be an attractive feedstock for sustainable energy production (such as bioethanol production) over its counterparts including sugarcane, sugarbeet, and maize (Geng *et al*., 1989). This is because of its adaptability in diverse conditions, high fermentable stem sugars ranging between 16–18% Brix° (Wu *et al.*, 2010), steep yield of green biomass (20-30 dry tons/hectare), relatively lower need for fertilizer and increased water use efficiency. This latter is only one third compared to sugarcane and half compared to maize.

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Options for expanding the production of ethanol have been considered and various crop plants have been studied and reviewed. One crop with promising potential in contributing to sustainable energy production is sorghum. Besides having rapid growth, high sugar accumulation, and high biomass production potential, sorghum also has a wide adaptability to various climate conditions (Reddy and Sanjana, 2003). Given that water availability is poised to become a major constraint to agricultural production in the coming years (Ryan and Spencer 2001), cultivation of crops such as maize and sugarcane for fuel production will be difficult. In contrast to maize that relies only on starch as a first generation bioethanol source, sorghum contains stem juices comprised of the three

main sugars (glucose, fructose, sucrose) that are readily fermentable into ethanol by yeast as well as the starch found in the grains. Sorghum also contains reducing sugars that prevents crystallization, therefore increasing the fermentation efficiency to ~90% (Ratnavathi *et al*. 2004). The lignocellulosic/ cellulosic waste resulting from the sorghum juice extraction also has several routes of utilization (Negro *et al.*, 1999). Enzymatic processes and pre-treatment steps can be applied to produce cellulose-based ethanol (Figure 1.7), heat and power (Gnansounou *et al.,* 2005).

1.3.5 Relevant sorghum carbohydrates

The main simple sugar in the stalk of sorghum is sucrose, which is the dominant form of carbohydrate transported in the plant, while starch is the main carbohydrate content in the grains (Somani *et al*., 1995). Subramanian *et al*. (1994) reported that cultivars with white or pale yellow seeds are the most suitable for starch production. The primary sugars present in grains of sorghum are fructose, glucose, raffinose, sucrose and maltose. In sorghum leaves, sucrose is produced and then translocated into developing grains where it is transformed into starch and stored. Together the grains and the stem of sorghum have been shown to yield more fermentable carbohydrates than any other fuel crop (Murray *et al*., 2008). In addition, the grain can be used for production of high fructose syrup and animal feed (Hosseini *et al*., 2003). Therefore, sorghum is an excellent crop for biomass production. The high sugar content of its vegetative biomass can be fermented to methane or ethanol (Almodares *et al*., 2008a). In stems, the extent of sucrose accumulation varies among cultivars. Sorghum sugar content is affected by temperature, time of day, maturity, cultivar, culm section, spacing and fertilization (Almodares *et al*.,

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2000).

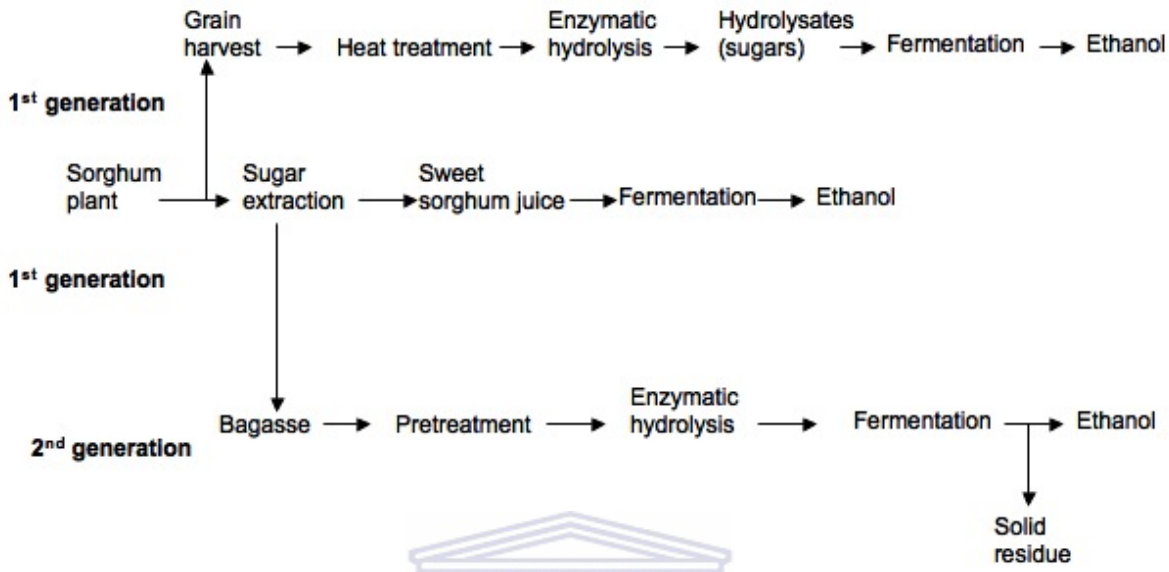

Figure 1.7: Use of sorghum for ethanol production. *The above figure demonstrates the process of ethanol production from grain, sweet sorghum juice, lignocellulosic biomass and various bioethanol generation routes. Adapted from Sipos et al., 2009*

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Environmental conditions such as water quality, growth stage and maturity are factors that affect carbohydrate content. In sweet sorghum, sucrose, glucose and fructose contents increase after the flowering stage. In stems, nonstructural carbohydrate contents increase after preboot and reach a maximum level near post flowering (Almodares *et al*., 2008b). Senescence (ageing) and nonsenescence affect levels of sugar accumulation in the culm of sorghum cultivars (McBee *et al.,* 1983). The nonsenescent cultivars contain more carbohydrates at all maturity stages than the senescent cultivars. Sugar production of sorghum was compared with sugarcane and sugarbeet and the results showed that sugar production from sorghum is cheaper than both sugarcane and sugarbeet (Blas *et al*., 2000). Therefore, it can be used as a supplementary sugar crop. So, it seems that through

cultural practices, breeding and physiological manipulation, the carbohydrate contents in sorghum plants can be increased. Sorghum also has high amount of sucrose, glucose and fructose that is readily fermentable to ethanol, with ethanologens such as *Saccharomyces cerevisiae*. These make sorghum a suitable crop for sustainable energy production.

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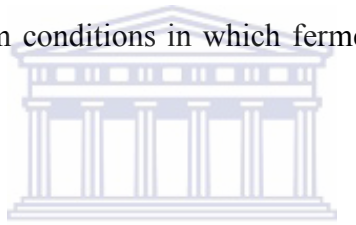
Aims and objectives of the study

This study is designed in order to examine and compare a limited range of South African Sorghum varieties that may be used as source of feedstocks, which may then be applied across a series of processes of first to second generation bioethanol production. In this particular study, we compared the sugar (glucose) content in four grains (Sorghum, Maize, Barley and Wheat) that are commonly found in South Africa. This was done through enzymatic hydrolysis and analysis of ethanol obtained through fermentation.

More specifically this work aimed to:

- Determine the optimum conditions in which fermentable sugars are released by the cereal grains



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- Analyze and compare the fermentable sugars liberated by the cereal grains

- Screen various ethanologen strains and select the best performing strain in fermenting the grain hydrolysates

- Compare fermentation ability of selected ARC experimental strain with commercial baker's yeast (Anchor Yeast)

- Measure alcohol volume (% v/v) yielded by the grains

Chapter 2: General Materials and Methods

This chapter outlines the general methodology followed in the whole thesis. Chapter specific materials and methods are included in the respective chapters.

2.1 Preparation of grain material

Sorghum and Maize grains were obtained from (Agricol – Brackenfell, South Africa) and maize (Pannar, Greytown South Africa), respectively, whereas barley (IMBO, Pioneer foods, Huguenot South Africa) together with wheat (Lion, Tiger food brands, Bryanston South Africa) grains were obtained from Dr Mark Taylor of the Institute of Microbial Biotechnology and Metagenomics (IMBM, UWC) South Africa. Grains were milled with a commercial blender (Russel Hobbs, Amalgamated Appliances Pty. Ltd., Booysens), transferred to 50 ml Falcon tubes and stored at –20°C until use.

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2.2 Grain starch extraction method

Three grams (3 g) from each grain source were weighed and transferred into 50 ml Falcon tubes (6% w/v). Grains were split to three respective experiments (Table 2.1), with the first being α - amylase treatment, second being gluco-amylase treatment and the third one being a cocktail of both enzymes. For starch extraction, the milled grains were cooked in boiling water by adding to each 3 g of grain into 30 ml of pre-boiled distilled water (dH_2O) and kept boiling for 30 minutes. After the cooking step, a further 20 ml boiling dH_2O was added to the respective solutions (sorghum, maize, barley and wheat).

Solutions were vortexed and thereafter centrifuged at 4400 *g* for 10 minutes. Solutions were stored at 4°C until further use.

2.3 pH determination

The natural pH of the boiled grain solutions (which acted as substrates for enzyme degradation) was recorded, using Crison Basic 20 pH meter (Crison Instruments, Spain). The pH measurements were recorded to observe changes in pH values of the solutions after each treatment.

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2.4 Starch presence determination (The iodine test)

Subsequent to the extraction of grain starch with hot water, presence of starch in the cooked grain solutions was confirmed by employing a basic biochemical test. Three millilitres (3 ml) of cooked grain solution was pipetted onto a Petri dish and drops of iodine were added to the solution to test for the presence of starch. A deep purple/blue color would be an indication of the presence of starch and light brown to bronze color indicates complete hydrolysis of the starch. This method was used to test the presence of starch after hot water treatment and after enzyme treatment.

2.5 Starch enzymatic hydrolysis

2.5.1 Alpha–amylase treatment

For starch deconstruction, alpha–amylase (α-amylase) solution from *Bacillus licheniformis* (Sigma Aldrich Corp, Missouri US) was used. The enzyme (133 KNU/g, the amount of enzyme which breaks down 5.26 g of starch in 1 hour) was in a liquid form having an activity of 500 units per mg protein. α-Amylase is generally known for cleaving starch chains randomly, thus producing mono, di, tri, or oligosaccharides. To the hot water treated samples, 0.5 ml (250U) of α-amylase (see Table 2.1) was added to the 50 ml substrate + dH_2O and the solution was vigorously shaken to distribute the enzyme evenly throughout the solution. The solution was incubated at various temperatures (45°C – 70°C) with constant shaking at 200 rpm on an orbital shaking incubator (Cape Scientific, South Africa). The samples glucose levels were analyzed using a pocket refractometer (Atago Co., Ltd, Tokyo) and HPLC (Dionex™ Ultimate 3000) and the amounts at different times (1–5 hours) incubation periods were recorded. Immediately after incubation, solutions were placed on ice in order to halt the enzyme reaction. Solutions were centrifuged at 4400 *g* for 10 minutes and thereafter the supernatant was transferred into clean 50 ml Falcon tubes. Hydrolysates were stored at 4°C until they were required.

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Table 2.1: Summary of grain starch hydrolysis experiments. *The table shows different enzymes that were used to hydrolyze various grain starches.*

Treatments		**Grains**		
Control (no enzyme added)	Barley	Maize	Sorghum	Wheat
Alpha-amylase (250 U)	Barley	Maize	Sorghum	Wheat
Gluco-amylase (150 U)	Barley	Maize	Sorghum	Wheat
Cocktail (alpha-amylase 250U and gluco-amylase 150 U)	Barley	Maize	Sorghum	Wheat

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2.5.2 Gluco-amylase treatment

Gluco-amylase solution from *Aspergillus niger* (Sigma Aldrich Corp, Missouri, US) was used. The enzyme (AMG 300 L; AGU/g 437, the amount of enzyme which hydrolyses 1 μmol of maltose per minute) was in liquid form, having an activity of 300 units per ml. Gluco-amylase is famous for its precision in starch deconstruction processes; it cleaves starch directly at the bonds connecting glucose units and thus produces glucose monomers. To the hot water treated samples, 0.5 ml (150 U) of gluco-amylase solution (see Table 2.1) was added to 50ml of substrate + dH_2O and the solutions were vigorously shaken to distribute the enzyme evenly throughout the solution. Treatments were incubated at various temperatures (45°C–70°C) with constant shaking at 200 rpm on an orbital shaking incubator (Cape Scientific, South Africa). The samples glucose levels were analyzed using a pocket refractometer (Atago Co., Ltd, Tokyo) and HPLC

(Dionex™ Ultimate 3000) and the amounts at different times (1–5 hours) incubation periods were recorded. Immediately after incubation, solutions were placed on ice in order to halt the enzyme reaction. Solutions were centrifuged at 4400 *g* for 10 minutes and thereafter the supernatant was transferred to clean 50 ml Falcon tubes. Hydrolysates were stored at 4°C until they were required.

2.5.3 Cocktail treatment

Both enzymes were used for the third set of treatments. To the hot water treated samples, 0.5 ml (250 U) of α-amylase and 0.5 ml (150 U) of gluco-amylase (see Table 2.1) were added. Solutions were vigorously shaken to distribute the enzyme evenly throughout the solution. Treatments were incubated at various temperatures (45°C–70°C) with constant shaking at 200 rpm on an orbital shaking incubator (Cape Scientific, South Africa). The samples glucose levels were analyzed using a pocket refractometer (Atago Co., Ltd, Tokyo) and HPLC (Dionex™ Ultimate 3000) and the amounts at different times (1–5 hours) incubation periods were recorded. Immediately after incubation, solutions were placed on ice in order to halt the enzyme reaction. Solutions were centrifuged at 4400 *g* for 10 minutes and thereafter the supernatant was transferred to clean 50 ml Falcon tubes. Hydrolysates were stored at 4°C until they were required.

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2.6 Determination of soluble sugars (Brix %)

For soluble sugar measurements, a portable "pocket" refractometer (Atago Co., Ltd, Tokyo) device was used. Refractometers are designed to measure the refractive index of a

solution. The brix scale used in this experiment is based on measuring the amount of sugar in a solution. The scale is known to be biased towards sucrose and that was practically proven by measuring three pure sugars (fructose, glucose, sucrose). Eighty millimolar (mM) of each sugar was measured in order to determine the distinction in their Brix %. Two hundred microlitres of supernatants were pipetted onto the device prism surface and the Brix % of soluble sugars present in the solution was determined. The device was calibrated with dH_2O (See Appendix 1).

2.7 Preparation of standards for High Performance Liquid Chromatography (HPLC)

Glucose and ethanol standard solutions were prepared at stock concentrations of 80 mM. Further dilutions were prepared using dH_2O to give concentrations in the range of 5 mM to 80 mM for standard calibration. From each of the diluted standards, 1 ml was transferred into 2 ml vials (Supelco Analytical, Bellefonte USA) and thereafter loaded onto the High Performance Liquid Chromatography (HPLC) for analysis. Detection was by Refractive Index detector (Shodex RI 101) column; Phenomenex Rezex™ RHM-Monosaccharide, flow rate 0.6 ml/min, temperature 48 °C, mobile phase 5 mM H_2SO_4, 20 µl injection, run time 30 minutes. Chromatograms and standard curves of glucose and ethanol pure samples are illustrated in Appendices 2 and 3 respectively.

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2.8 HPLC sugar analysis

Sample sugar composition was determined after the different enzyme treatments. The

hydrolysates were analysed for sugar composition by HPLC. Two millilitres of each sample were transferred into a clean tube and centrifuged at 13,200 *g* for 10 minutes. Supernatants were diluted ten times with dH_2O. From the diluted samples, 1 ml was transferred into 2 ml vials (Supelco Analytical, Bellefonte USA) and thereafter loaded into the HPLC. Samples were analysed by HPLC (Dionex™ Ultimate 3000) on a Phenomenex Rezex™ RHM monosaccharide column 00H 0132 KO at 48 °C with a 5 mM H_2SO_4 mobile phase at a flow rate of 0.6 ml min^{-1}. From each diluted sample, 20 µl was injected by an Ultimate 3000 autosampler and the sugar components were detected using a refractive index detector (Shodex RI 101). The concentration in g/L was calculated from a standard calibration graph with standards ranging from 5 to 80 mM of pure glucose.

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2.9 Fermentation system

2.9.1 Bacterial cultures

Geobacillus thermoglucosidasius strains (NCIMB 11955 and M10) were supplied by Dr Mark Taylor of the Institute of Microbial Biotechnology and Metagenomics (IMBM, UWC) South Africa. The strains were supplied in 80% glycerol stocks and they were streaked onto 2TY agar plates (10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl and 15 g/Lagar). They were then inoculated in 2TY media and split into vials with sterile 80% glycerol and stored at –80°C.

2.9.2 Yeast cultures

Saccharomyces cerevisiae strains (NI 5, NI 6, NT 2, NT 51 and NT 53) were obtained from Dr Niel Jolly's laboratory, Post - harvest and Wine Technology Department, Agricultural Research Council (ARC Infruitec–Nietvoorbij), Stellenbosch, South Africa. The hybrid strains were obtained from a series of breeding between wild types and they formed part of the ARC culture collection. Strains were received in Parafilm sealed yeast extract-peptone-dextrose (YPD) plates. These were inoculated in YPD media (5 g/L yeast extract, 10 g/L peptone, 10 g/L). Inoculated cultures were prepared into 1.5 ml Eppendorf tubes in a sterile 80% glycerol stock and maintained at -80 °C. These stocks were sustained by routine sub-culturing on YPD plates (5 g/L yeast extract, 10 g/L peptone, 10 g/L glucose and 10 g/L agar) by re-streaking a single colony and storing at 4°C.

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2.9.3 Media and strain preparation

Two bacterial (*Geobacillus thermoglucosidasius* NCIMB 11955 and M10) and five yeast strains (*Saccharomyces cerevisiae* NI 5, NI 6, NT 2, NT 51 and NT 53) were used in this study to convert the available sugars within the hydrolysates to ethanol. The strains are shown in Table 2.2

2.9.3.1 Bacterial growth conditions

The two *Geobacillus thermoglucosidasius* strains (NCIMB 11955 and M10) were grown on 2TY media (10 g/L yeast extract, 5 g/L NaCl, 20 g/L tryptone and 15 g/L agar, final pH 7.0) overnight or until their absorbance at 600nm (OD_{600}) was between 0.7–1.3. Broths were incubated in a constantly shaking incubator (200 rpm) at 60°C.

Table 2.2: Strains used in this study. *The table shows the various Geobacillus and Saccharomyces strains that were used in this study*

Name	Relevant characteristics
G. thermoglucosidasius NCIMB 11955	Wild type isolate
G. thermoglucosidasius M 10	Wild type isolate
S. cerevisiae NI 5	Wild type isolate
S. cerevisiae NI 6	Wild type isolate
S. cerevisiae NT 2	Hybrid
S. cerevisiae NT 51	Hybrid
S. cerevisiae NT 53	Hybrid

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2.9.3.2 *Geobacillus* fermentation

The micro-aerobically grown broth cultures served as inoculums (10%) for fermentative growth on Urea Sulphates Media supplemented with yeast extract or USMYE (10% total of hydrolysate samples, 0.42 g/L citric acid, 0.31 g/L $MgSO_4$, 3.1 g/L NaH_2PO_4, 3.5 g/L K_2SO_4, 3 g/L urea, 2.2 mg/L $CaCl_2$, 0.4mg/L Na_2MoO_4, 1 g/L yeast extract, 1g/L tryptone, 1ml/L and 5 ml/L trace elements solution). The trace element solution contained 1.44 g/L $ZnSO_4.7H_2O$; 0.56 g/L; $CoSO_4.6H_2O$; 0.25 g/L $CuSO_4.5H_2O$; 5.56 g/L $FeSO_4.6H_2O$; 0.89 g/L $NiSO_4.6H_2O$; 1.69 g/L $MnSO_4$ and 5.0 ml/L 12M H_2SO_4. USM was used at a final pH of 7. The media was autoclaved and after autoclaving it, yeast extract was then added. Fermentations were carried out in 40 ml volume of pre-warmed

USMYE with 10% hydrolysates and 10% inoculums contained within 50 ml sealed Falcon tubes. This encouraged microaerobic and fermentative growth. Cultures were incubated at 60°C overnight and subsequently harvested by centrifugation (4400 *g*, Eppendorf centrifuge 5810R) for 10 minutes. The supernatant was collected by centrifugation and analyzed by HPLC, applying a similar regime as in Section 2.8.

2.9.3.3 Yeast growth conditions

Two wild type (NI 5 and NI 6) and three hybrid (NT 2, NT 51 and NT 53) strains of *Saccharomyces cerevisiae* were grown on YPD media overnight or until OD_{600} was approximately 1.5. Broths were incubated at 200 rpm and 30°C.

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2.9.3.4 Yeast fermentations

The yeasts served as inoculums (10%) for fermentative growth on yeast fermentation media (5 g/L yeast extract, 10 g/L peptone, 0.25 g/L $MgCl_2$, 2.5 g/L KH_2PO_4, 0.25 g/L $CaCl_2$). The media was autoclaved. Fermentations were carried out in 10 ml volume of pre-warmed yeast fermentation media with 10% hydrolysates and 10% inoculums contained within 15 ml Parafilm-sealed Falcon tubes. This encouraged fermentative growth. Cultures were incubated at 30°C for 48 hrs and harvested by centrifugation (4400 *g*, Eppendorf centrifuge 5810R) for 10 minutes. The supernatant was collected by centrifugation and analyzed by HPLC, applying a similar regime as in Section 2.8. The concentration in g/l was calculated as described in Section 2.7 but using absolute ethanol as standard.

2.10 Preparation of baker's yeast

Commercial baker's yeast (Anchor bakers yeast) was purchased at a local supermarket and was activated for fermentation purposes. Yeast grains were sprinkled onto YPD broth (5 g/L Yeast extract, 10 g/L peptone, 10 g/L glucose) and incubated at 30°C until they reached an absorbance of OD_{600} was 1-1.5. After activation, yeast was plated on YPD agar (5 g/L yeast extract, 10 g/L peptone, 10 g/L glucose and 10 g/L agar) and incubated at 30°C for 48 hours. Another portion of the broth was used for fermentation experiments in a similar manner to that outlined in Section 2.9.3.4

2.11 Determination of alcohol in volume per volume percentage

Concentrations of alcohol (% v/v) were measured from the fermentation products of different hydrolysates using a specialised alcohol detecting and analyzing instrument. Thirty millilitres (30 ml diluted one times with dH_2O) of fermentation product was injected into an Anton Paar alcolyzer wine M (www.anton-paar.com, USA; Figure 2.3) and the detected amounts of alcohol in the solution were displayed on the monitor. This was done in triplicates.

2.12 Statistical analysis

Statistical analysis was done using excel based two tailed student T test with a chosen threshold of 0.05 level p-value (95 % confidence) for statistical analysis. The values of the controls were compared against treated samples to achieve statistical significant values.

Figure 2.1: This figure demonstrates the sequential events of alcohol analysis by the alcolyzer wine M. *(A) Is the inlet where the sample is suctioned and flows through the duct for alcohol detection. (B) The monitor that displays the amount of alcohol in the solution after detection. (C) Is the outlet; post analysis the sample is discarded as waste through this channel.*

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Chapter 3: Evaluation of enzyme-facilitated grain starch hydrolysis and selection of high performing ethanologen

Abstract

Hydrolases catalyze the formation of simple products from specific complex substrates. Enzymes that are implicated in starch degradation are classified as E.C 3.2.1 and they catalyze the release of simple sugars such as mono, di, tri, and oligosaccharides from starch. Here we have used two commercial starch-hydrolyzing enzymes to degrade starches that were extracted from cereal grains in order to produce fermentable sugars. These enzymes were tested in a range of conditions using the substrates sources with their natural pH. The effect of temperature, time and the type of enzyme was observed on the four different substrates over five hours. Results obtained in this section suggested that both enzymes performed well under high temperatures (70°C) and they degraded most of the starch within the first hour. The α-amylase and gluco-amylase cocktail facilitated high production of fermentable sugars. The maximum glucose yield at optimum conditions was 50.8 g/L. This translates to 60 g/L of grain material having the ability to generate ± 50.8 g/L glucose (84.7%) after cocktail hydrolysis. Different types of organisms were further used to convert the available carbon source (glucose) in sorghum hydrolysates to ethanol through fermentation. *Geobacillus thermoglucosidasius*, which primarily produces lactic acid and a range of organic acids, managed to produce small amounts of ethanol in grains treated with gluco-amylase and the cocktail. Although *Geobacillus* wild type strains were used in this study, engineered strains exist and could be used to improve ethanol yield. *Saccharomyces cerevisiae*, a

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traditional organism used in ethanol fermentation, yielded relatively high ethanol 0.39 g/g (gram of ethanol per gram of glucose) content.

3.1 Introduction

Bio-ethanol can be produced from a variety of biomass that contain cellulose, starch and sugars (Demirbas, 2005; Dawson and Boopathy, 2008). Crops such as maize, cassava, potatoes and wheat are some commonly used starch sources (Moore *et al*., 2005; Jamai *et al*., 2007; Mohammad and Keikhosro, 2008, Ocloo and Ayenor, 2008). Over the years, starch has gained recognition in the fuel industry as a raw material for bio-ethanol production (Öhgren *et al*., 2006). Starch is considered to be a clean, non-toxic source of carbon for bio-ethanol production (Moore *et al*., 2005; Chen *et al*., 2008). From the different sources of starch, maize starch is the primary source for bioethanol production in well-developed countries such as the United States of America (USA) (Mielenz, 2001; Torney *et al*., 2007).

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There are two feasible methods of producing bio-ethanol from grain starch, namely through dry milling or wet milling processes (Sanchez and Cardona, 2008; Gnansounou, 2009). The dry milling process is the most commonly adopted technique in the United States, accounting for almost 80% of the production (Kim *et al*., 2008; Murthy *et al*., 2009). The smallest particle size of the grain is recommended for the optimum penetration of water into the starch granules in preparation of starch for the hydrolysis process. The grain powder formed from the milling step is gelatinized, followed by hydrolysis and subsequently fermentation. Gelatinization is a process for dissolving

starch into water at high temperatures. This step assists with starch extraction as well as the reduction of bacterial contamination / infection or inhibition (Torney *et al*., 2007; Mojović *et al*., 2006; Franceschin *et al*., 2008). The degradation of the two forms of starch is performed immediately after gelatinization during the hydrolysis process that is considered to be a crucial step in bio-ethanol production.

The hydrolysis process involves the breaking down of amylose and amylopectin over a range of temperatures and pH to produce glucose. Temperature, time, pH, and enzymes are the essential determinants associated with the hydrolysis processes (Shanavas *et al*., 2011). Glucose units produced during hydrolysis process are further converted to bioethanol in the presence of the ethanologen through a process called fermentation (Mojović *et al*., 2006). Fermentation is the most commonly used method of producing bioethanol from sugars such as sucrose, glucose and fructose using a traditional organism, *Saccharomyces cerevisiae* (Khaw *et al*., 2007). Here we showed that both enzymes tested, namely α-amylase and gluco-amylase, performed well under high temperatures and that they consumed all starch under the substrates natural pH. Secondly we showed that *Geobacillus thermoglucosidasius* has potential for being an ethanol producer and lastly we have showed that these different grains have the ability to produce substantial ethanol without any hindrance once they are fermented. The grains produced almost identical amounts of ethanol (to see the rest of the figures that represent other grain results, refer to Appendix 4).

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3.2 Materials and Methods

This section outlines the materials and methods that are specific to this chapter.

3.2.1 Determination of pH after grain starch extraction

α-Amylases obtained from different *Bacillus* species are generally active at slightly acidic to neutral pH conditions (Sajedi *et al.*, 2005). In this study, after starch extraction via hot water treatments, the solutions were cooled down to room temperature and their natural pH was recorded.

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3.2.2 Effect of enzymes on starch breakdown and glucose yield

Two enzymes were used and their effect on starch deconstruction and glucose yield from the hydrolysis processes was tested. The enzymes used in these processes were α-amylase, gluco-amylase and the combination of both enzymes. We used the combined enzymes to observe whether the simultaneous application of α-amylase and gluco-amylase would make a significant difference in glucose yield.

3.2.3 Effect of temperature and time on glucose yield

Different types of enzymes require specific temperature ranges in order to achieve their maximal rate of reaction or optimal performance. In this study, the effect of temperature on starch breakdown and glucose yield during liquefaction, saccharification and simultaneous application was investigated on heat-treated sorghum grains. Immediately after adding the respective enzymes to the starch solutions they were incubated at

different temperatures to establish an optimum temperature for starch hydrolysis. The solutions were incubated at temperatures of 45, 50, 60 and 70 °C and their natural pH was not altered. Solutions were incubated in a shaking incubator at 200 rpm.

The incubation period during starch breakdown into glucose depends on various factors including temperature, amount of substrate and concentration of enzyme that is used. Glucose concentration was investigated using 6% w/v (3g sorghum grains + 50 ml dH_2O) with their natural pH in all processes. The same concentration of α-amylase (250 U) and gluco-amylase (150 U) was used throughout the experiment. The solutions were incubated for different periods to establish the rate of starch hydrolysis and optimum period for complete starch hydrolysis. Solutions were incubated on an hourly basis ranging from 1 to 5 hours in a shaking incubator at 200 rpm.

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3.2.4 Screening and selection of ethanologens

α-Amylase and gluco-amylase (250 U and 150 U) were used in liquefaction and saccharification processes respectively. In this section, two organism species, *Geobacillus thermoglucosidasius* and *Saccharomyces cerevisiae*, were used for fermentation experiments. The different strains (Table 2.3 of Chapter 2) were screened with the purpose of selecting high ethanol producing strain(s). The strains were prepared as stipulated in Chapter 2, ten percent (10 %, v/v) of strain inoculums were added to the hydrolysates for the fermentation process. Sorghum hydrolysates from all samples obtained from the various enzyme treatments were subjected to ethanol fermentation by the various strains under anaerobic conditions. Fermentations were carried out in small

falcon tubes at 30°C and for 48 hours in a shaker at 150 rpm for yeast strains and 55°C and 24 hours for *Geobacillus* strains, all experiments were done in triplicates. Samples of the fermented broth were collected after specified fermentation times for the organisms and analyzed with the HPLC, as indicated in Section 2.8.

3.3 Results

3.3.1 Determination of pH after grain starch extraction

The enzymes used in this study have defined pH ranges in which they function optimally. Grain pH values were recorded to observe pH ranges suitable for enzyme functioning (see Table 3.1).

Table 3. 1: The table represents pH values of the four different grain starch solutions before hydrolysis. *The obtained pH is within the favorable range for the enzymes (α-amylase, gluco-amylase and cocktail) optimal activity.*

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Grain	pH
Barley	4.89 ± 0
Maize	6.05 ± 0.14
Sorghum	6.46 ± 0
Wheat	5.98 ± 0.02

α-Amylase from *Bacillus licheniformis* is reported to have a pH range for activity of 5-9 whereas for gluco-amylase from *Aspergillus niger* the optimum pH for activity ranges

from 4.5-5 but has been reported to also be stable at pH range of 5-7 (Morgan and Priest, 1981; Slivinski *et al*., 2011). The pH values recorded from the substrates are well within the stable ranges of the enzymes, thus not necessitating external chemicals to adjust the pH values.

3.3.2 Effect of enzymes on starch breakdown and glucose yield

The enzymes used in this study had different biochemical reactions and they resulted in different peak profiles and glucose concentration. The peak profiles were observed after analyzing the hydrolysates in the HPLC.

In all instances, 250 U of α-amylase and 150 U of gluco-amylase were used for hydrolysis experiments. α-Amylase treated samples portrayed a series of peaks and among them glucose was also identified (Figure 3.1). Both gluco-amylase and the cocktail treatment showed similar chromatogram profiles with both portraying glucose as the sole product obtained after starch hydrolysis (Figures 3.2 and 3.3). This information suggests and also confirms the fact that α- amylase is not a specific cleaver. α-Amylase is classified under debranching enzymes and these enzymes attack the α-(1,4) links of amylopectin randomly to give a mixture of unbranched starch molecules (dextrins) plus a few sugars, this hydrolysis process is known liquefaction (Hough *et al*., 1985). A similar phenomenon was observed in this study when dextrins and glucose were detected in α-amylase-treated samples.

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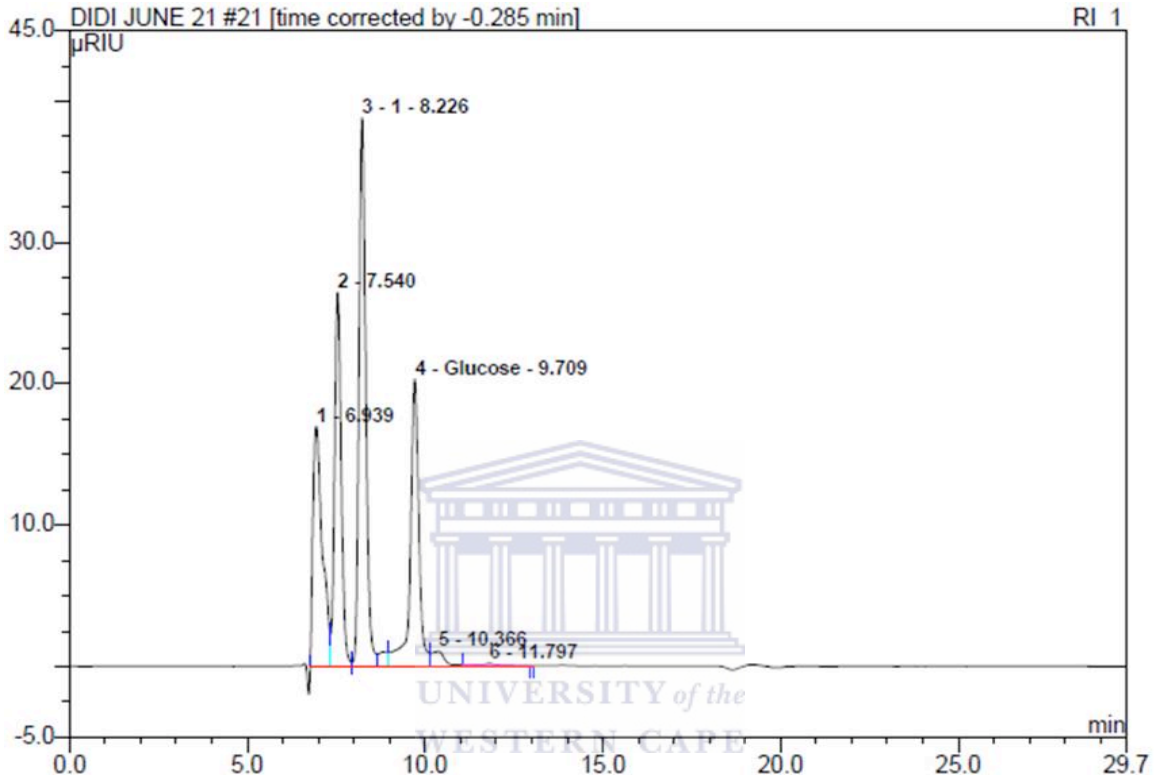

Figure 3.1: HPLC chromatogram showing the effect of α-amylase (liquefaction) in the breakdown of starch and glucose yield. *The above chromatogram shows multiple peaks portrayed by α-amylase treated grain starch. The 9.7 retained peak was identified as glucose.*

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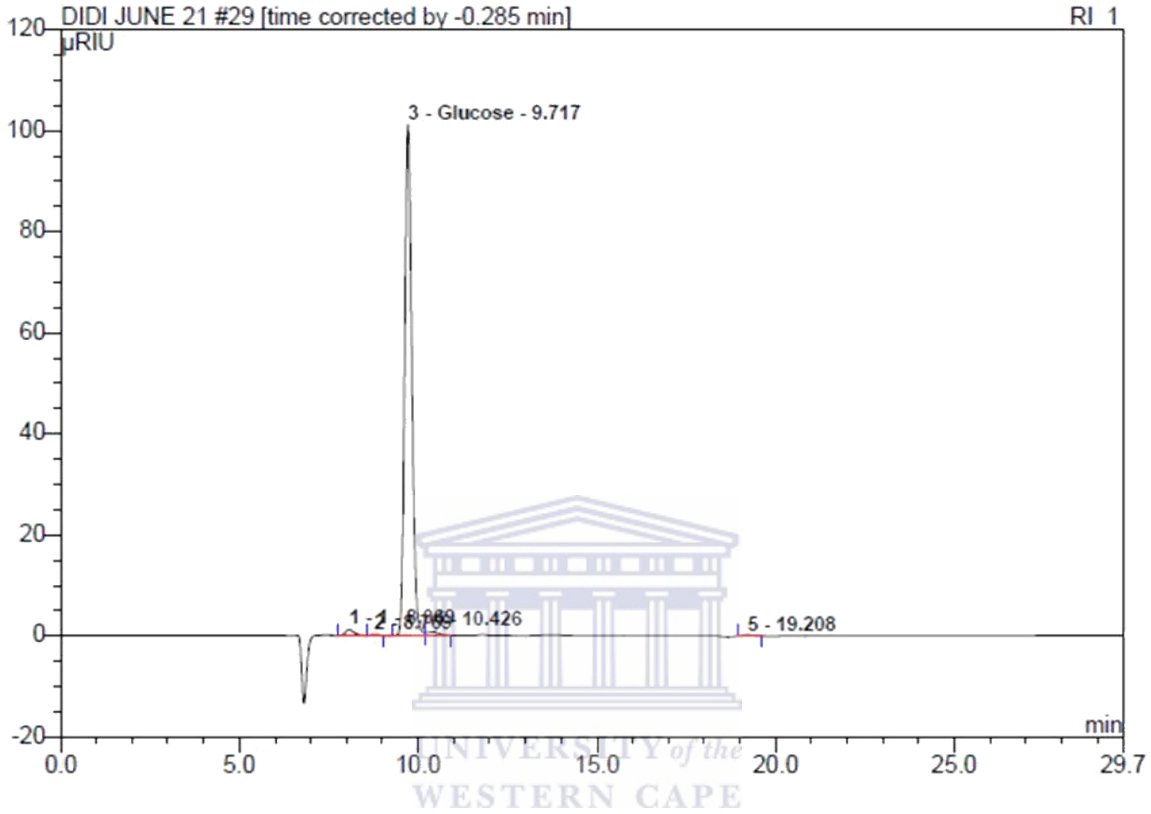

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Figure 3.2: HPLC chromatogram showing the effect of gluco-amylase (saccharifaction) in the breakdown of starch and glucose yield. *The chromatogram shows a single peak that resulted from gluco-amylase treated grain starch. The peak was retained at 9.7 minutes and was identified as glucose.*

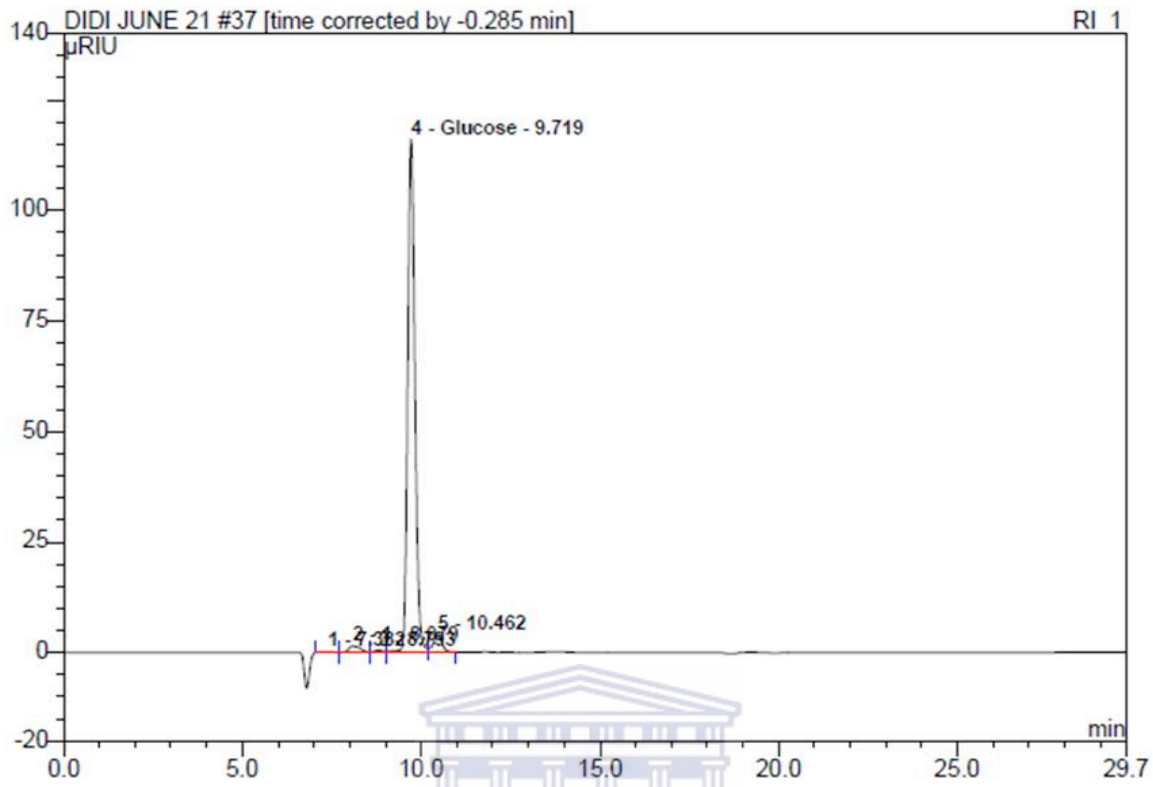

Figure 3. 3: HPLC chromatogram showing the effect of α-amylase and gluco-amylase (simultaneous liquefaction and saccharifaction) in the breakdown of starch and glucose yield. *The chromatogram shows a single peak that resulted from cocktail treated grain starch. The peak was retained at 9.7 minutes and was identified as glucose.*

Gluco-amylase, however, does not only cleave 1,4 links of amylopectin, it also has the ability to cleave 1,6 links from non-reducing ends to successfully produce glucose, this process is known as saccarification (as reviewed by Tanriseven *et al*., 2002). Similar results were also observed in this study when the gluco-amylase-treated sample showed only glucose as the sole hydrolysis product. In the samples treated with both enzymes in a process known as simultaneous liquefaction and saccharifaction, glucose was also the sole product after hydrolysis (Figure 3.3). These results demonstrate that α-amylase is not a major factor in the biochemical process of starch degradation, as it was shown that

gluco-amylase has the capacity of breaking down starch and produce glucose on its own.

3.3.3 Effect of temperature and time on glucose yield

Solutions were incubated at different temperatures for different times as stipulated in Sections 3.2.3 and 3.2.4. Presented below are the figures displaying glucose production under different temperatures and time frames. These experiments were performed with all the different grains but only sorghum was chosen to represent these results in order to avoid unnecessary repetition since the grains produced almost identical results (See Appendix 4).

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α-Amylase enzyme solution is thermostable, thus the liquefaction step is performed at high temperatures and in some cases between 85°C and 100°C, usually for an hour (Sanchez and Cardona, 2008). On the other hand gluco-amylase is traditionally added after liquefaction to further break down the dextrins that result from α-amylase hydrolysis and release glucose as the end product. However, Kumamneni and Singh (2005) reported that unlike α-amylase, gluco-amylase generally performs optimally between 55°C and 65°C and usually over four hours. In this experimental set up we tested starch hydrolysis between 45°C and 70°C over a period of five hours due to the fact that 100°C is the boiling point of water.

This was to test starch hydrolysis and glucose conversion efficiency in these conditions and compare the obtained results with the traditional literature-based conditions. The obtained results revealed that both α-amylase and gluco-amylase enzymes performed

well at increased temperatures and that a large amount of starch was depleted within the first hour. It was seen that at 45°C and 50°C (Figures 3.4 and 3.5), gluco-amylase was the most efficient enzyme in facilitating glucose production whereas at 60 and 70°C (Figures 3.6 B and 3.7 B), the cocktail treatment was more efficient and resulted in higher glucose production. At 45°C (Figure 3.4) gluco-amylase yielded 37.2 g/L that was the highest at that particular temperature. However, as the temperatures arose, the cocktail of enzymes became dominant and at 70°C saw a maximum Brix amount of 6% (Figure 3.7 A) and glucose concentration of 50.8 g/L from a starting grain material of 60 g/L. Although gluco-amylase was dominant at 45 and 50°C (Figures 3.4 and 3.5) it produced high glucose amounts at 70°C (47.8 g/L) but overall the enzymes cocktail produced the most concentrated glucose amounts at this temperature (Figure 3.7). Although the optimum temperature for maximum glucose production using the various treatments was observed at 70°C (Figure 3.7), there was no remarkable difference between glucose production at 60°C and 70°C (Figures 3.6 and 3.7). This demonstrates that a slight variation in the temperature would not affect glucose production during hydrolysis.

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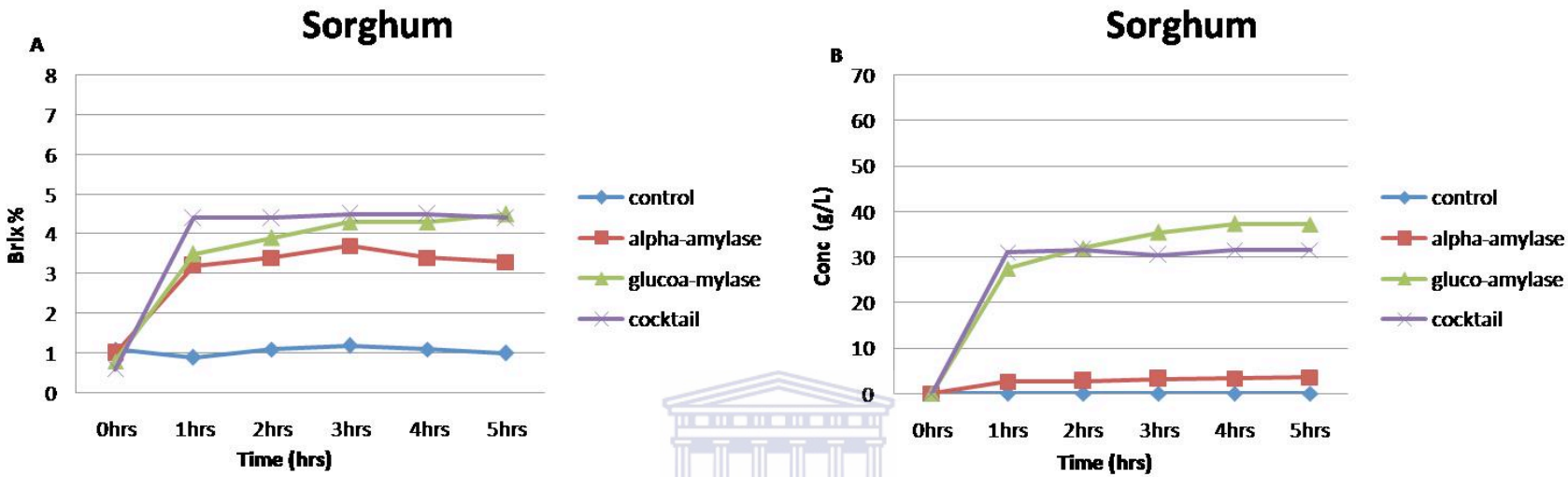

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Figure 3. 4: This figure represents hydrolysis of sorghum starch at 45° C and different time-frame. *(A) Is the Brix % results which shows total soluble sugars in a solution and (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.*

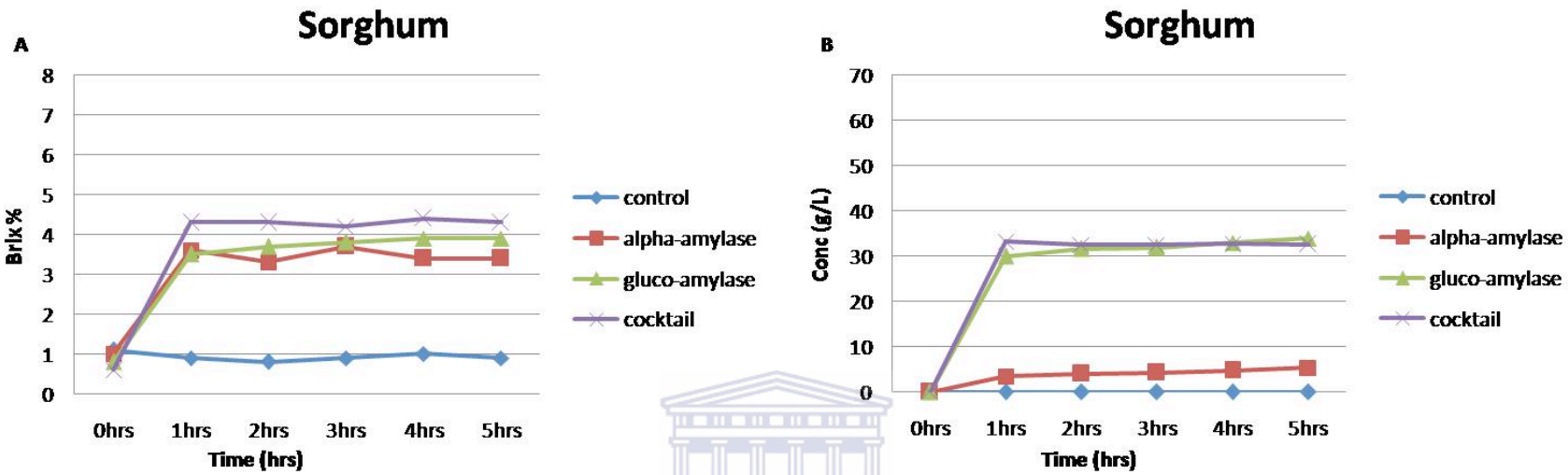

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Figure 3. 5: This figure represents hydrolysis of sorghum starch at 50° C and different time-frame. *(A) Is the Brix % results which shows total soluble sugars in a solution and (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.*

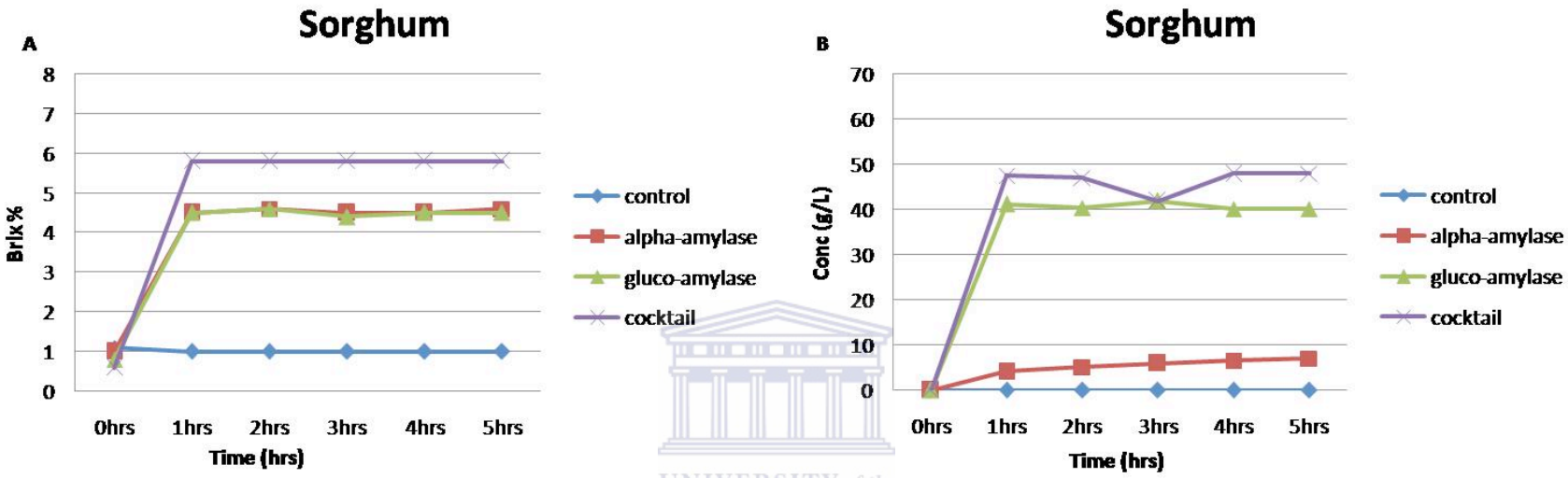

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Figure 3. 6: This figure represents hydrolysis of sorghum starch at 60°C and different time-frame. *(A) Is the Brix % results which shows total soluble sugars in a solution and (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.*

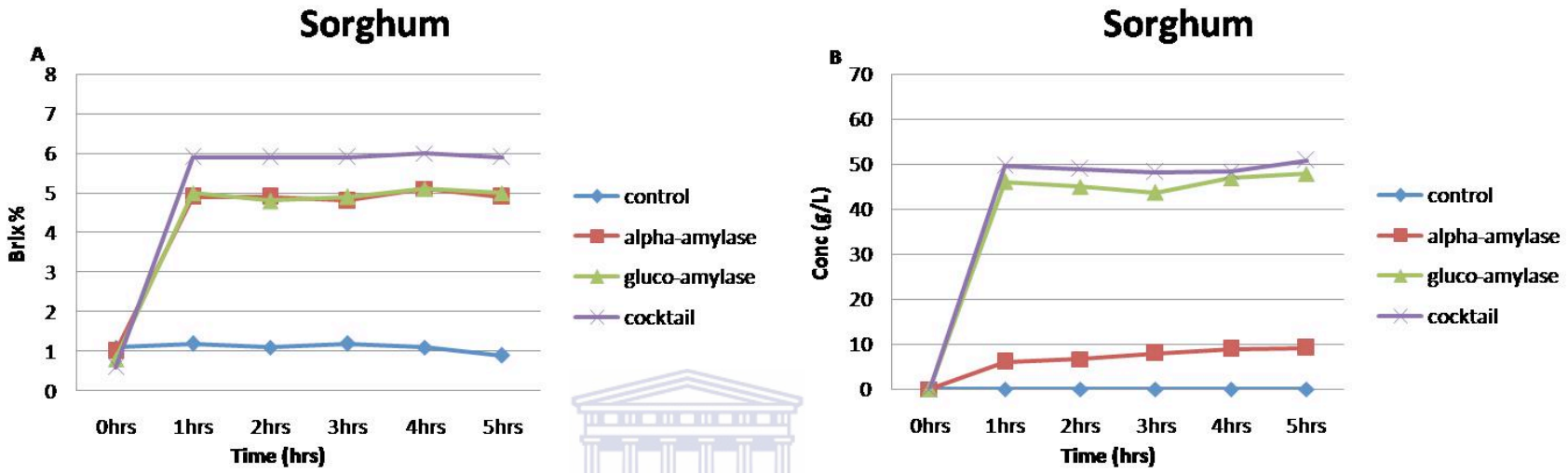

Figure 3. 7: This figure represents hydrolysis of sorghum starch at 70°C and different time-frame. *(A) Is the Brix % results which shows total soluble sugars in a solution and (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments Each time point is a mean of three technical replicates.*

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3.3.4 Screening and selection of ethanologens

Sorghum grain starch was extracted and hydrolysis was performed using the specified enzymes as stipulated in Section 2.5 and the measured glucose is portrayed in Figure 3.8.

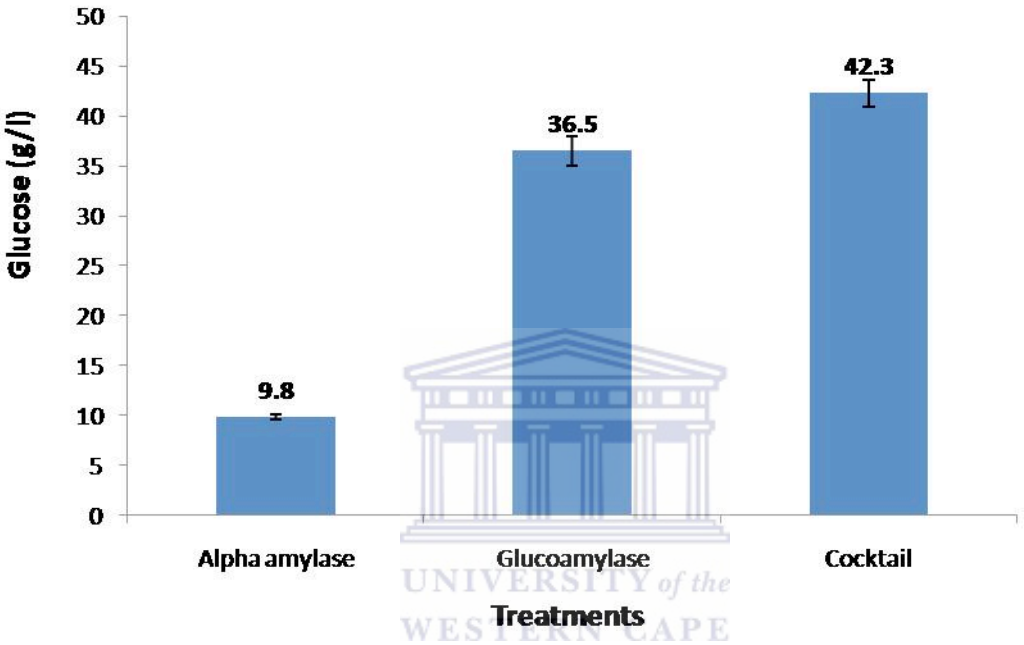

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Figure 3. 8: Glucose concentration after enzyme hydrolysis. *This figure shows the amount of liberated glucose units post α-amylase, gluco-amylase and cocktail hydrolysis in sorghum grains. The error bars represent two repetitions with a p-value of less than 0.05*

Previously (Section 3.3.2) we demonstrated that the enzymes have different functions in starch degradation and consequently results in liberation of different soluble sugars; α-amylase treatment resulted in multiple soluble sugars that also included glucose. As expected, gluco-amylase and the cocktail resulted to the sole production of glucose. Here (Figure 3.8), the amount of glucose each treatment produces from 60 g/L of grain material is shown. α-Amylase treatment produced 9.8 g/L glucose, gluco-amylase

produced 36.5 g/L and the cocktail produced an average of 42.3 g/L of glucose. Simultaneous liquefaction and saccharification (cocktail) treatment produced the highest amount of glucose (42.3 g/L) among the three treatments. Untreated solutions did not yield any glucose.

Hydrolysates were fermented at various temperatures that were conducive for the organisms. *Geobacillus thermoglucosidasius* fermentations were carried out at 60°C as recommended by Cripps *et al*., (2009) for 24 hours whereas *Saccharomyces cerevisiae* fermentations were carried out at 30°C for 48 hours. The figure below represents chromatograms displaying peak profiles of different hydrolysates.

These chromatograms display a typical production of ethanol and simultaneous consumption of glucose by *Saccharomyces* and *Geobacillus* strains. Figure 3.9 (A, B and C) represent the production of ethanol in all respective hydrolysates post fermentation with *Saccharomyces* strain. However chromatograms displaying *Saccharomyces* fermented hydrolysates also showed retention of a compound at approximately 13.4 minutes and we then traced back to the HPLC standard archives. The information gathered from the archives suggested that the retained compound was lactate.

A similar phenomenon was observed with hydrolysates fermented with *Geobacillus* (Figure 3.10; A, B and C). *Geobacillus* fermented glucose and produced ethanol, however, it yielded lactic acid as a major product and only a small fraction of ethanol was detected.

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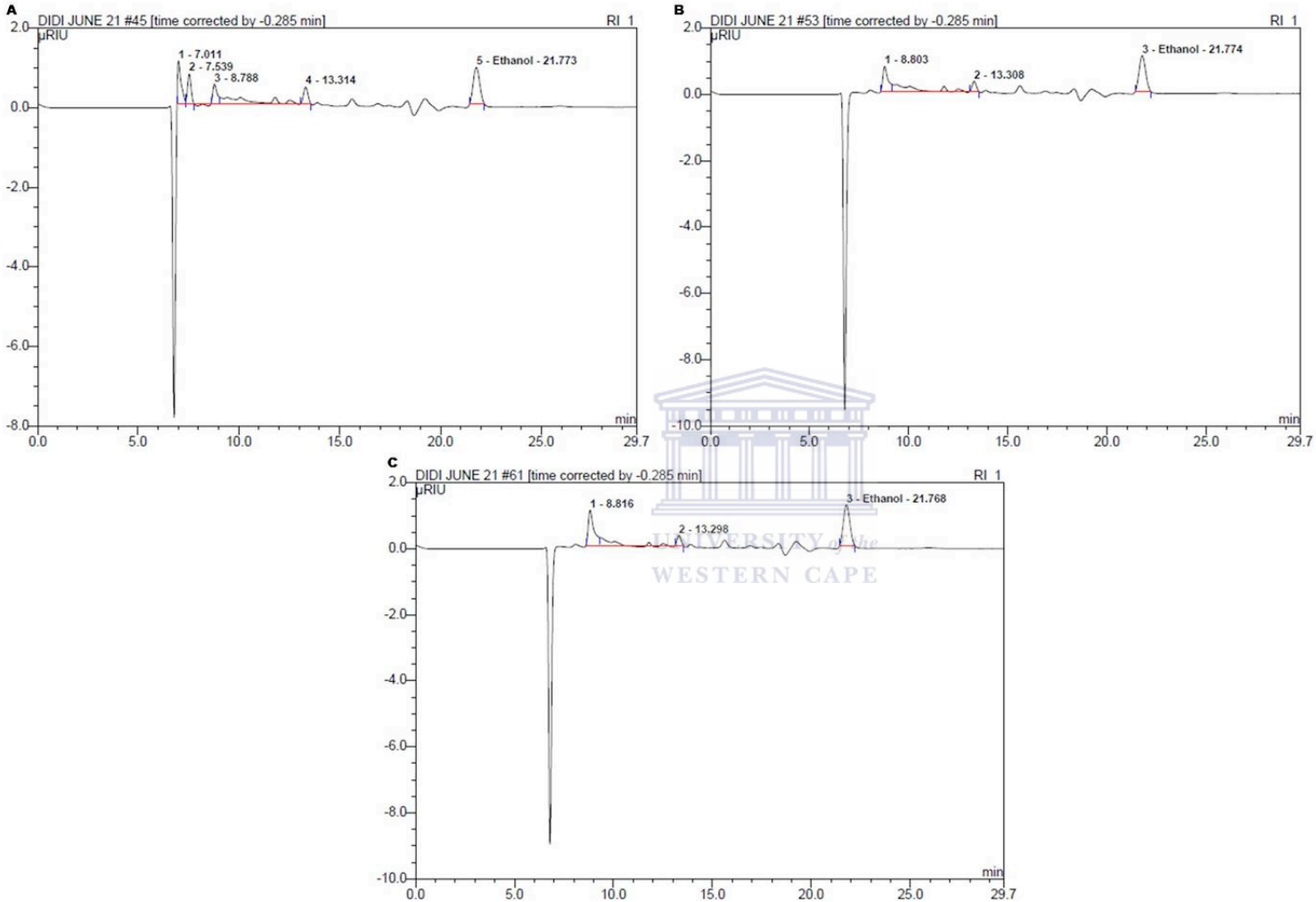

Figure 3. 9: Chromatograms displaying hydrolysates profiles post fermentation with Saccharomyces cerevisiae strain.

(A, B and C) Represent α-amylase, gluco-amylase and cocktail hydrolysates after fermentation respectively.

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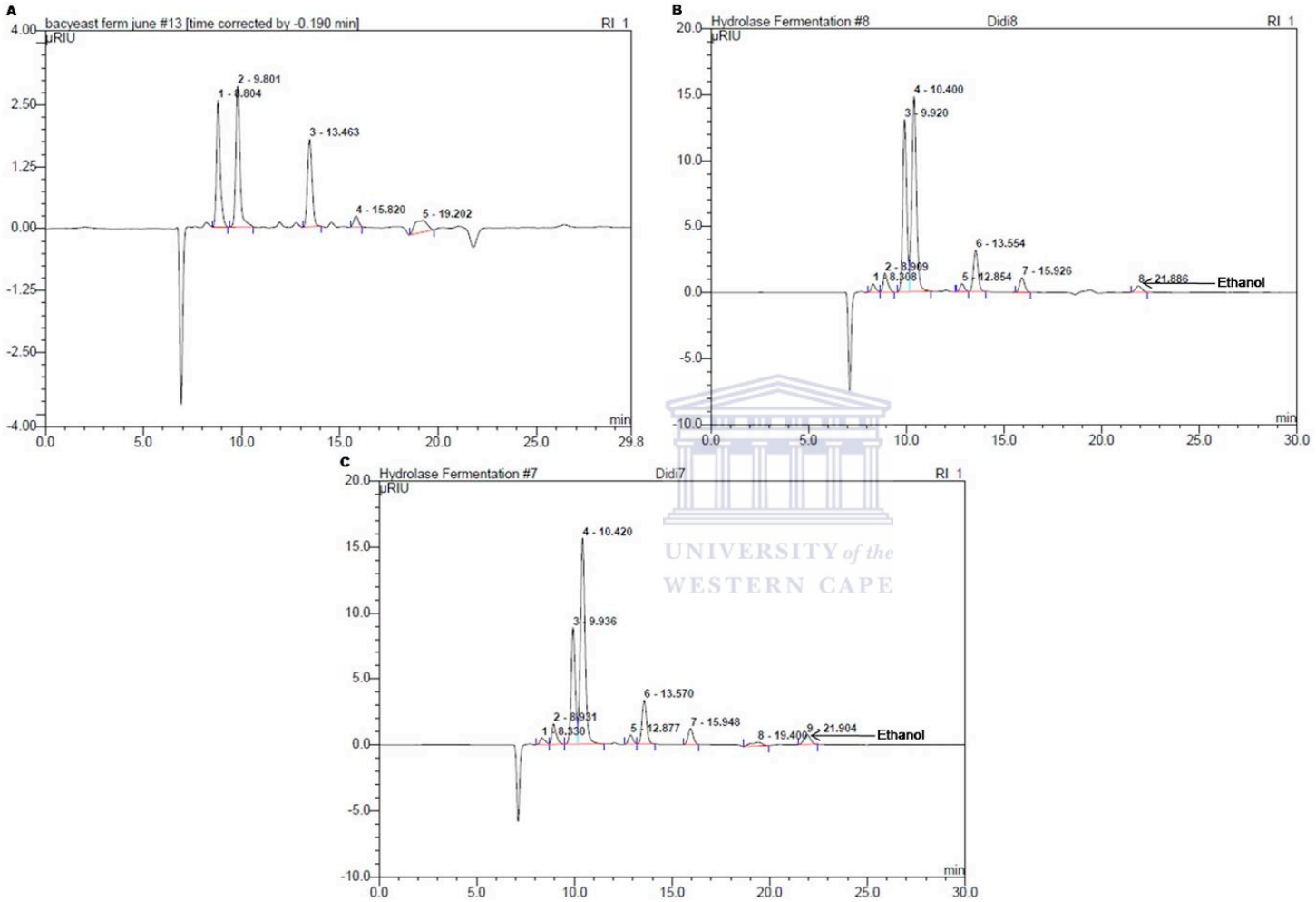

Figure 3. 10: Chromatograms displaying hydrolysates profiles post fermentation with Geobacillus thermoglucosidasius strain. *(A, B and C) Represent α-amylase, gluco-amylase and cocktail hydrolysates after fermentation respectively. The ethanol peaks (B and C) are clearly shown on the chromatograms.*

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Unlike *Saccharomyces*, the *Geobacillus* strains did not ferment most complex sugars such as short chains of glucose units that are present in α-amylase hydrolysate due to random hydrolysis of the enzyme (Figure 3.10 A). Ethanol produced from hydrolysate fermentations was quantified in order to select high fermenting strain(s) among the seven different strains. The obtained results are presented on the figure below (Figure 3.10).

The respective hydrolysates were fermented using various ethanologen strains to determine the amount of ethanol each strain is capable of producing. Fermentation of hydrolysates with *Saccharomyces* for a period of 48 hours at 30°C resulted in the formation of ethanol and the simultaneous disappearance of glucose. The same trend was observed with *Geobacillus* fermentations. Ethanol formation was observed after fermentation period of 24 hours at 60°C as well as the disappearance of glucose. However with *Geobacillus* only small traces of ethanol were identified and the dominating product that was identified was lactic acid.

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Geobacillus thermoglucosidasius M10 produced 4.4 g/L whereas the NCIMB11955 strain produced 5.1 g/L of ethanol. These were the organism maximum ethanol production when they fermented gluco-amylase treated sorghum starch. The 5.1 g/L produced by NCIMB strain was the highest between the two prokaryotic strains that were used and the main reason for this low ethanol content is mixed acid production.

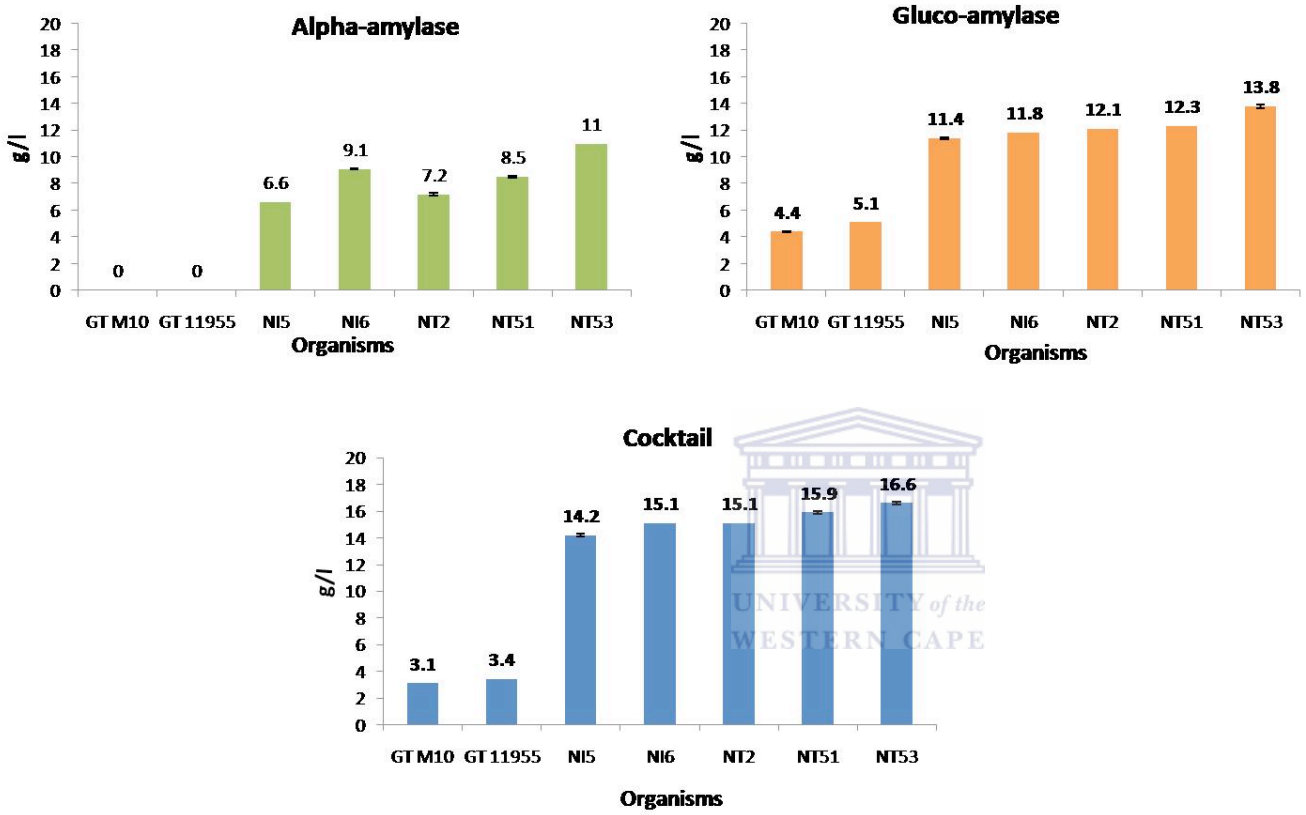

Figure 3. 11: The figure represents fermentation performances of the various organisms on different hydrolysate products. *(A) The amount of ethanol produced by the various organisms utilizing the dextrins in α-amylase treated solution. (B) Amount of ethanol produced by the organisms utilizing glucose in gluco-amylase treated solution as the sole carbon source. (C) Amount of ethanol produced by organisms utilizing glucose in cocktail treated solution as sole carbon source. The error bars represent two repetitions with a p-value of less than 0.05*

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Saccharomyces strains generally produced similar ethanol amounts throughout the treatments, but the NT 53 strain was the best performing strain among them. NT 53 produced an average of 16.6 g/L ethanol content, which translates to 0.39 g/g and 76.9% fermentation efficiency (Figure 3.11). The theoretical ethanol yield when converting a carbon source such as glucose into ethanol is 0.51 g/g (Nofemele *et al*., 2012).

3.4 Discussion

The aim of this chapter was to identify optimum conditions for starch hydrolysis using α-amylase and gluco-amylase and observe conditions that result in the production of high amounts of glucose. We also aimed to screen different ethanol-producing organisms and select the best ethanol producing strain(s) among them.

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We have shown that α-amylase and gluco-amylase enzymes result in different biochemical processes (Section 3.3.2) and thus as a consequence lead to different amounts of glucose being produced (Figure 3.8). α-Amylase from *Bacillus* is industrially important and has been studied extensively using both biochemical and protein engineering methods. *Bacillus licheniformis* (BLA), *Bacillus stearothermophilus* (BStA) and *Bacillus amyloquefaciens* (BAA) α-amylase have been investigated and utilized to identify some evident mechanisms of thermostability. In these three enzymes, BLA was found to be the most stable and effective enzyme (Klibanov, 1988). Although the α-amylase from *Bacillus licheniformis* is reported to be stable at various temperatures and pH, the α-amylase sourced from *Bacillus licheniformis* enzyme was used in this study and is reported to active and stable at a pH of 5-9. However, we tested all hydrolysis

processes between 45°C and 70°C using the grain's natural pH. At these temperatures, α-amylase managed to consume starch that was present in the solutions (Figure 3.1). Gluco-amylase was also subjected to the same conditions and starch was also well hydrolyzed. However, a different biochemical reaction was observed when gluco-amylase was applied, only glucose was produced, as expected since gluco-amylase is a specific cleaver (Figure 3.2). Belshaw and Williamson (1990) and Sigurskjold *et al*., (1994) isolated a starch binding domain structure (SBD) of gluco-amylase from *Aspergillus niger* that was reported to be unusually containing two polysaccharide-binding sites located on opposite sides of the SBD. The SBD have been implicated in several roles associated with facilitating the degradation of crystalline starch (Morrisa *et al*., 2005). Williamson *et al*. (1997) reported that the SBD could function as a recognition site, which locates the catalytic domain of the surface of the starch granules. However, Southall *et al*. (1999) reported that, alternatively, the SBD domain may in addition change the conformation of the substrate, thereby enhancing cleavage by the catalytic domain. This results in gluco-amylase having the capacity to degrade the amylose α-(1,4) bonds and the amylopectin α-(1,4) and α-(1,6) bonds, leading to the production of glucose as the sole product (Figure 3.2). α-Amylase, however is restricted to degrading amylopectin α-(1,4) and amylose α-(1,4) bonds only, thus resulting in the production of a mixture of dextrins together with glucose (Figure 3.1). Other published papers have adopted the method of saccharification with simultaneous fermentation (Nicolic *et al*., 2009). However, the disadvantage of employing this method is the fact that the saccharifaction and fermentation temperatures are usually not compatible to one another unless a thermo-tolerant organism is used. This method restricts the potential of both saccharifying enzyme and fermenting organism altogether, thus possibly lead to low

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amounts of glucose and subsequent ethanol production.

In this study a traditional method of independent hydrolysis and subsequent fermentation was applied. Fixed amounts of microorganism concentration were used in all fermentations processes (10%). Mojovic *et al*., (2006) reported that the difference in ethanologen amount does not affect the final product formation (ethanol). Chen *et al*., (2008) confirmed the findings of Mojovic *et al*., (2006) and suggested that different amounts of ethanologens only affect the duration of fermentation. *Geobacillus thermoglucosidasius* 11955 wild type isolate used in this study was reported by Cripps *et al*., (2009) to yield 0.1 g/g of ethanol. The fermentation ability of this strain was tested on pure glucose solutions. In the same study, Cripps *et al*., (2009) also reported a major production of lactate that was more than three times higher than the production of ethanol. Traces of other mixed products such as acetate and formate were also reported. We observed similar results in our study, where we detected a 0.14 g/g ethanol yield in gluco-amylase hydrolysates and 0.09 g/g in cocktail hydrolysates and large amounts of lactate were detected, although they were not quantified. All the various *Saccharomyces cerevisiae* strains used in this study managed to ferment dextrins that resulted from α-amylase treatment into ethanol. However, the ethanol amounts yielded from α-amylase hydrolysates were low compared to gluco-amylase and cocktail hydrolysates. Although NT 53 strain produced high amounts of ethanol, we observed no significant difference between ethanol amounts produced by the different yeast strains.

A phenomenon that was worth noting was that starch samples that were treated with α-amylase yielded very low amounts of glucose when compared to gluco-amylase and

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cocktail treatments, however, these samples ethanol amounts that were comparable to both gluco-amylase and cocktail treated fermentation products. This occurrence was exclusively seen in products that were fermented with yeast strains and these implied that the yeast strains possess amylolytic properties and were able to consume longer chains of glucose and convert them to ethanol. In a study by Yamakawa *et al*. (2012), a similar trend was observed where a diploid yeast strain displayed both α-amylase and gluco-amylase properties. The novel strain was reportedly to have naturally managed up to 46% fermentation efficiency using untreated starch as a substrate. This was compared to an engineered strain that was infused with α-amylase integrative plasmids that managed fermentation efficiencies of up to 76% from the theoretical yield (Yamakawa *et al*., 2012). In a separate study by Garcia *et al*. (2005), a yeast strain was modified by adding a starch binding domain with the capability to bind and hydrolyze insoluble starch. This resulted in elevated starch hydrolysis when compared to natural yeast strain.

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Both types of ethanologens (*Saccharomyces cerevisiae* and *Geobacillus thermoglucosidasius*) consumed all the available glucose within their respective prescribed fermentation periods, but they produced different major end products. *Geobacillus* species are predominantly producers of mixed organic acids such as lactate, acetate and small amounts of formate and ethanol. However, in a study by Cripps *et al*. (2009), an inefficient *Geobacillus* wild type isolate was engineered by up regulating the expression of pyruvate dehydrogenase (PDH) with simultaneous suppression of lactate dehydrogenase (LDH) and pyruvate formate lyase (PFL) by perturbing *ldh* and *pflB* genes. This transformation resulted in ethanol yields of up to 90% efficiency when compared to theoretical yield. Although *Saccharomyces* is a natural ethanol producer

and most of the time does not need modifications, *Geobacillus* has the potential to produce ethanol in the same scale as *Saccharomyces* when modified.

Chapter 4: Comparative analysis of sugars and ethanol production between sorghum and three other South African cereal grains

Abstract

Different regions worldwide use different feedstocks for bioethanol production. In Southern Africa there is still a debate as to which crop can be utilized efficiently and sustainably for bioethanol production, taking into account the unstable food security and environmental impacts. For this chapter we compared ethanol yields using grain crops (sorghum, maize, barley and maize) that are currently being explored as feedstocks for bioethanol production. Starch was extracted from grains and was degraded into soluble sugars by use of various enzymatic treatments. We then fermented the resultant sugars into ethanol and compared ethanol production from the various grain types. Our results showed similar glucose yields across the grain types with α-amylase, gluco-amylase and cocktail treatments averaging 11 g/L, 40.7 g/L 49.6 g/L respectively. Post fermentation these selected grains also showed similar ethanol yields with α-amylase, gluco-amylase and cocktail hydrolysates averaging 17.1 g/L, 19.2 g/L and 22.9 g/L respectively. When the ARC experimental hybrid strain (*Saccharomyces cerevisiae* NT 53) was compared with commercial baker's yeast (Anchor instant yeast), it produced comparable ethanol yields with a maximum of 0.5 g/g (98% fermentation efficiency) and 0.44 g/g (87% fermentation efficiency) respectively. The NT 53 strain displayed 11% higher fermentation efficiency compared to bakers yeast. Our conclusion is that these crops are all theoretically suitable feedstocks from which bio-ethanol can be sourced. The difference that sets sorghum apart as the most sustainable option is that bioethanol can be sourced from it's three different plant compartments (grain, stem and bagasse).

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4.1 Introduction

Starch is regarded as a high yield feedstock for bioethanol production but its hydrolysis to glucose is required to produce bioethanol by fermentation. Many crops contain starch and so these crops are looked upon as possible feedstocks for ethanol production, as well as foods. Surplus maize (often referred as corn) is close to exclusively used for the production of ethanol in the USA and it is milled for the extraction of starch, which is enzymatically treated to obtain glucose syrup. Although maize is the major starch-based feedstock in the US, research efforts are lately oriented towards the development of maize hybrids with higher extractable starch or higher fermentable starch content. Hybrid cereal crops that are commercially available and those under development have shown large variation in fermentation quality (Zhao *et al*., 2009). Laboratory-scale fermentation is considered as the most direct and reliable method of evaluating fermentation quality of grain (Ingledew *et al*., 1995; Zhan *et al*., 2003). Various crops are classified as potential ethanol feedstocks and are being aggressively reviewed with regard their extractable carbohydrate content and the economic implications their use might have. Although maize is considered conventional, in theory and practice crops such as sorghum, wheat/triticale, barley, rye and cassava could also be used as ethanol feedstocks (Wang *et al*., 1997; Zhan *et al*., 2003). The major distinguishing factor is the economic viability and food versus fuel ethics.

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First generation bioethanol feedstocks for commercial use vary from one region to another. For example, in France ethanol is mostly produced from sugarbeet molasses, however, it is also produced from wheat in a similar process to that of maize (Wang *et al*., 1997). In 2007, the South African Department of Energy published a national biofuels

strategy that was intended to develop the biofuels industry and achieve a market penetration of 2% in road transport fuels by 2013 (Department of energy, 2007). Two years later (2009), the Eastern Cape Department of Economic Development and Environmental Affairs also published a report of an ethanol plant in the Eastern Cape that will produce ethanol through the integration of sugarbeet and grain sorghum (DEDEA, 2009). Although all these strategies and policies were drafted and are available, by the year 2012 there is still not a single litre of bioethanol that is commercially produced in South Africa.

In this chapter we will be comparing glucose and subsequent ethanol producing abilities from the carbohydrate extracted from sorghum, maize, barley and wheat grains. These particular grains were chosen because they are amongst the 5 most important grains in South Africa and in the world. We will also take into consideration the production of these crops together with their consumption nationally.

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4.2 Materials and Methods

This section illustrates portions of methods that are specific for this chapter.

4.2.1 Determination of pH changes during the starch hydrolysis processes

In this section we investigated the effects of the various enzymes on the pH of the solutions. pH values were recorded after every enzyme treatment using Crison Basic 20 pH meter (Crison instruments Spain), and were compared to the untreated samples.

4.2.2 Performing fermentation process in small-scale volumes (500ml)

The alcohol-analyzing instrument (alcolyzer wine M) requires a minimum sample of 30ml for analysis. Substrate volumes were increased ten fold in order to accommodate the alcohol analysis instrument. We also increased the volumes to observe consistency in glucose and ethanol production from sorghum.

4.2.2.1 Preparation of grain material

Sorghum grains were selected for this section and they were milled to fine powder and processed further as stipulated in Section 2.1 of Chapter 2.

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4.2.2.2 Heat and enzyme treatment experiments

Thirty grams (30 g) of sorghum powder was added into 1 L Erlenmeyer flasks and 500 ml of dH_2O was added (6% w/v). Water submerged sorghum was autoclaved for 30 minutes. After the autoclaving step, the solutions were cooled down, enzymes were added (in the same manner as in Section 2.5 of Chapter 2) and incubated in a 70°C waterbath for 3 hours. Post incubation small part of the α-amylase, gluco-amylase and cocktail treated hydrolysates were transferred to falcon tubes for sugar analysis and the remainder was kept for further fermentation.

4.2.2.3 Fermentation

Overnight grown NT 53 broth culture (OD_{600} of approximately 1.7) was added directly into the hydrolysates (50 ml broth culture + 450 ml hydrolysate) and incubated at 30°C

with shaking at 170 rpm for 48 hours under anaerobic conditions. After the 48 hour fermentation period, the solutions were spun down and 50 ml supernatants were transferred to multiple 50 ml Falcon tubes for alcohol analysis.

4.3 Results

4.3.1 Measuring grain pH values subsequent to enzyme treatments

The pH values of each grain were measured after every enzyme treatment was completed and the obtained results are presented in Table 4.1.

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Table 4. 1: The table shows changes in the pH values of the four different grain starch solutions following hydrolysis treatments. *The pH measurements were taken after every enzyme treatment for all the grain solutions in order to investigate pH changes caused by various enzyme treatments in the respective solutions.*

Grain type	Control pH	α-Amylase pH	Gluco-amylase pH	Cocktail pH
Barley	5.48	5.14	4.69	4.72
Maize	5.84	5.7	5.33	5.22
Sorghum	6.15	6.09	5.65	5.53
Wheat	6.11	6.03	5.62	5.3

The above table represents pH values prior and throughout enzyme treatments. The general observation from the treated grains was that the enzymes led to slight change in the pH (acidification) of the solution, however, there were no radical changes observed

4.3.2 Determination of starch presence/degradation

The ability of iodine to bind amylose has been used to understand a variety of structural and functional aspects of starch in food systems. In starch granules, the linear amylose polymer binds a significantly higher proportion of iodine than does the branched amylopectin molecule (Morrison and Laignelet, 1983). The presence of starch in this study was determined by an iodine solution. Theoretically in this assay, when iodine is added to a starch solution, starch molecules force iodine atoms into a linear arrangement in the central groove of the amylose coil and this leads to a transfer of charge between starch and iodine. This causes the iodine/starch complex to have energy level spacings that absorb visible light and thus giving the complex its intense blue colour (Khera, n.d). A drop of the iodine solution was added onto the centrifuged slurry before and after hydrolysis. In this section, we wanted to show the visual differences between enzyme treated and untreated grains (Figure 4.1).

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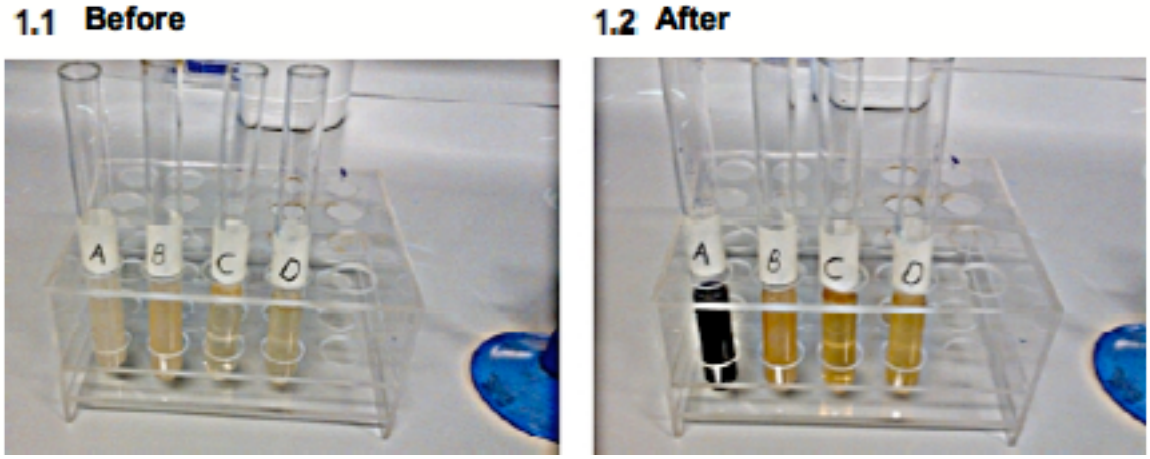

Figure 4.1: Demonstration of solutions colour transition post iodine addition. (1.1) represents solutions before (A) and after hydrolysis (B, C, and D) prior to iodine addition and (1.2) represents solutions before (A) and after hydrolysis (B, C, and D) post iodine addition.

The results obtained on Figure 4.1 (1.2) confirmed the presence and degradation of starch by the enzymes. Control (A) showed an intense blue/black colour when iodine solution was added, confirming the presence of starch. α-Amylase, gluco-amylase and cocktail treatments showed no colour change when iodine was added. The observed biochemical assay properties of the lack in colour change post hydrolysis by various treatments confirmed starch degradation by the enzymes [Figure 4.1 (1.2) B, C and D)], whereas the untreated control changed in colour, thus confirming that in the absence of the enzymes starch molecules were not degraded, as expected. This was observed in all the grains (barley, maize, sorghum and wheat).

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4.3.3 Measurement of total soluble sugars

Hydrolyzed samples were first analysed with a refractometer in an attempt to determine the presence of soluble sugar solids to water and approximate total soluble content across the hydrolyzed solutions. Brix % results are presented in Figure 4.2.

An elevation in overall soluble sugar content was observed across the grains after hydrolysis with the various enzymes. Untreated grain solutions (control) had an average of 1.1 Brix % with barley (1.3%) having slightly higher amount of soluble sugars compared to the rest. All three treatments (α-amylase, gluco-amylase and cocktail) relatively produced similar amounts of soluble sugars. α-Amylase produced an average of 5.5% with sorghum producing 5.8% when compared with other grains treated with α-amylase.

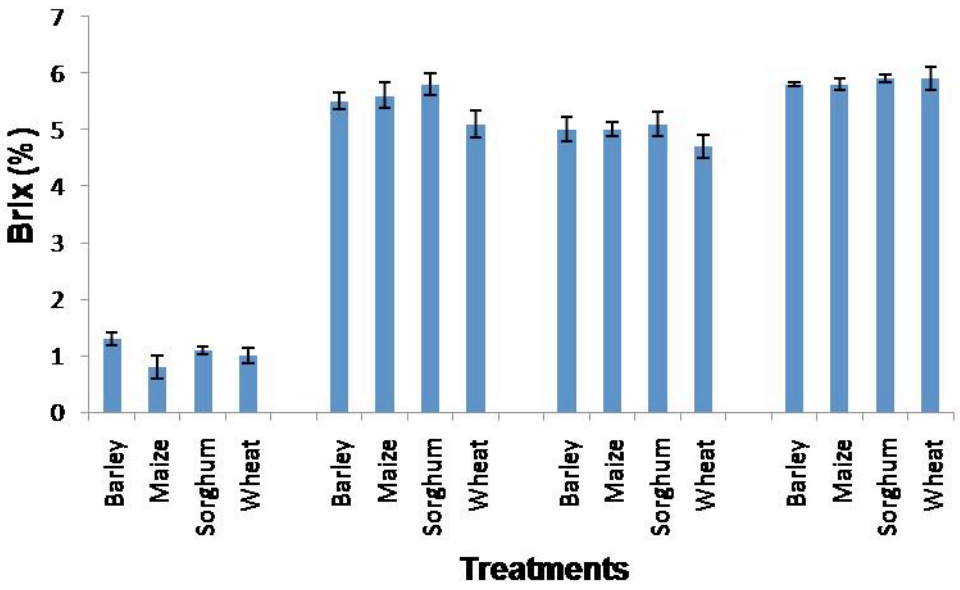

Figure 4.2: The figure presents amounts of total soluble sugars prior to and post hydrolysis. *Soluble sugars present in the solutions of the various grain types were measured with a refractometer and their brix % are displayed on the figure. The error bars represent three repetitions with a p-value of less than 0.05*

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Grains treated with gluco-amylase yielded similar results, with an average of 5% and sorghum yielding 5.1% soluble sugars whereas cocktail treated grains managed an average amount of 5.9% and all four other grains yielded similar amounts. The overall yield of soluble sugars was similar across the grain types when treated with the particular enzymes.

4.3.4 Glucose production by different grain types

In chapter 3 we have shown the biochemical action of the different enzymes towards the starch molecule. We have shown that gluco-amylase and the cocktail treatment resulted in the production of glucose as the sole sugar, whereas α-amylase resulted in the

production of multiple soluble sugars including glucose. Here we have measured and compared glucose production of the four different grain crops treated with α-amylase, gluco-amylase and the cocktail (Figure 4.3).

Different amounts of glucose were observed for the different treatments across the various grain types. However the variation was not significant for glucose amounts produced by grain types treated with the same enzyme. The untreated grain (barley, maize, sorghum and wheat) solutions did not yield any glucose amounts, hence there was no data presented as regards. α-Amylase treated grains produced average glucose amounts of 11 g/l, with barley producing high glucose amounts (11.9 g/l) among the grain types. A steep rise was observed for grains treated with gluco-amylase when they produced average glucose amounts of 40.7 g/l with sorghum producing high glucose amounts (43.8 g/l) when compared to other grain counterparts. A slight increase was also observed for grains treated with the cocktail with an average glucose production of 45.8 g/l with sorghum producing high glucose amounts (49.6 g/l) when compared to other grain types. In Chapter 3 it was shown that solutions treated with the enzyme cocktail produced glucose solely and the same trend was observed when we compared the grain types in this chapter. Sorghum grains treated with the cocktail produced high glucose amounts compared to other grains.

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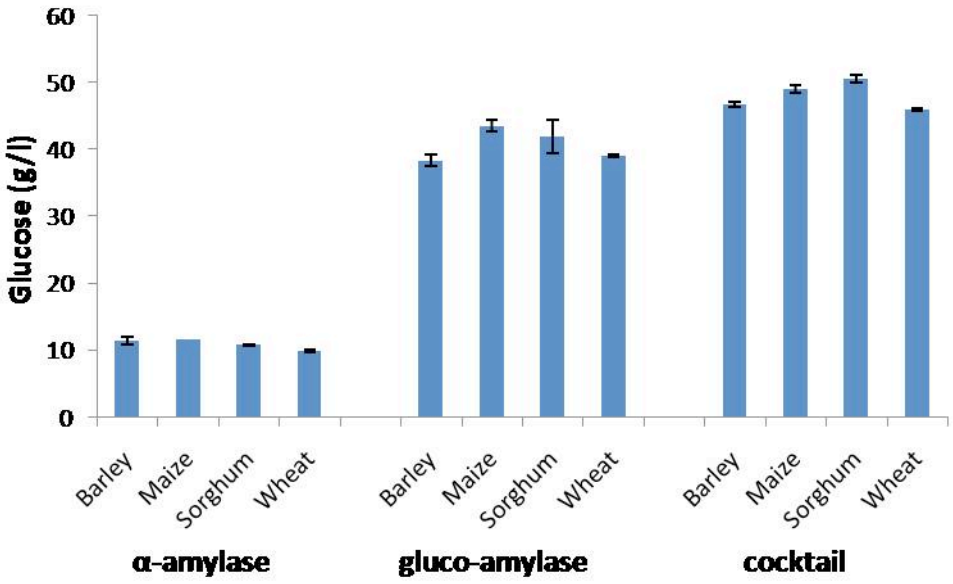

Figure 4.3: The figure represents glucose amounts produced by the different grain types. *Glucose produced by the different grain types (barley, maize, sorghum and wheat), as analysed by HPLC. The error bars represent two repetitions with a p-value of less than 0.05*

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4.3.5 Ethanol production by different hydrolysates fermented by *Saccharomyces cerevisiae* NT 53 strain

In Chapter 3 we compared the fermentation abilities of various *Geobacillus thermoglucosidasius* and *Saccharomyces cerevisiae* strains in an attempt to identify and select the best performing strain. *Saccharomyces cerevisiae* NT 53 strain was identified as the best fermenter among the other strains and was selected to further ferment hydrolysates obtained through hydrolysis of all other grain types (Figure 3.11).

The results suggest that the experimental strain yielded comparable ethanol amounts across all the grain hydrolysates (Figure 4.4). Cocktail treatment yielded high ethanol

amounts overall with maize cocktail hydrolysate yielding an average of 24.3 g/l (0.49 g/g) ethanol that translated to 97.2% fermentation efficiency when compared to the theoretical yield. The other grain types also yielded high ethanol amounts, with barley yielding an average of 22.8 g/l (0.49 g/g) that translated to 95.7% fermentation efficiency, wheat yielded 22 g/l (0,48 g/g) at 93.8% fermentation efficiency whereas sorghum yielded 22.5 g/l (0.44 g/g) and 87.4% fermentation efficiency.

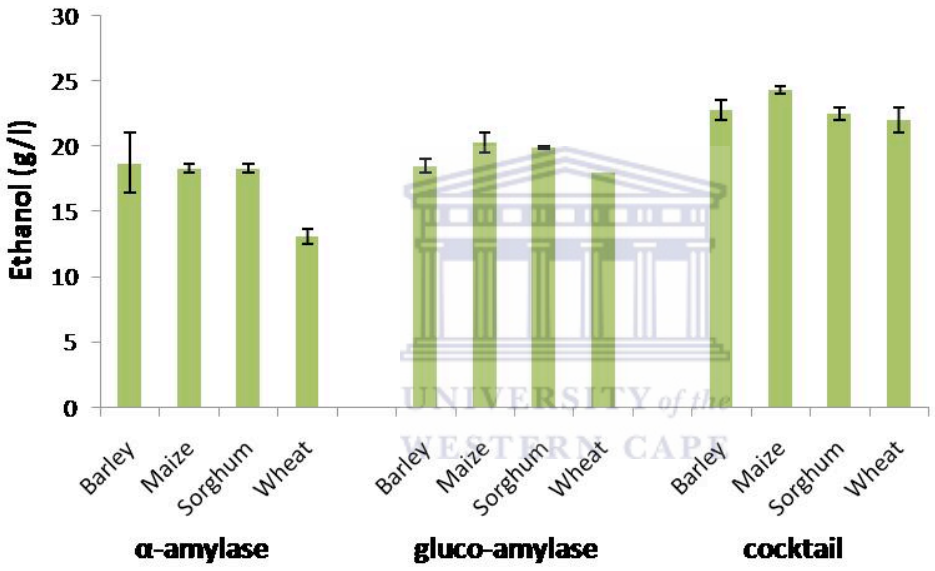

Figure 4.4: The figure represents ethanol amounts produced by the different hydrolysates. *Ethanol amounts produced by hydrolysates of different grain types (barley, maize, sorghum and wheat) was analysed by HPLC. The error bars represent two repetitions with a p-value of less than 0.05*

4.3.6 Comparing *Saccharomyces cerevisiae* NT 53 strain with commercial baker's yeast

The experimental strain (NT 53) produced a maximum average of 0.49 g/g ethanol amounts similar for gluco-amylase hydrolysates and 0.49 g/g for cocktail hydrolysates.

In this section we are comparing the fermentation and ethanol yield capability of the NT 53 strain with that of commercial baker's yeast (Figure 4.5).

The results indicate that both NT 53 strain and baker's yeast had an overall comparable performance, although at some instances NT 53 outperformed the baker's yeast. α-Amylase maize hydrolysates fermentation produced high ethanol amounts and resulted in both the NT 53 strain and baker's yeast yielding an average amount of 21.7 g/l. There was no significant difference in gluco-amylase hydrolysates fermentation products yielded by various grains. However, the NT 53 strain produced higher ethanol amounts when compared to baker's yeast during fermentation of sorghum hydrolysate.

Sorghum cocktail fermented with NT 53 produced an average ethanol amount of 0.5 g/g which translated to 98% fermentation efficiency and sorghum cocktail hydrolysate fermented with baker's yeast yielded an average amount of 0.44 g/g that translated to 87% fermentation efficiency. With the obtained overall results from soluble sugar measurements and ethanol yield using different grain crops, sorghum grains were selected for up scaling in larger vessels.

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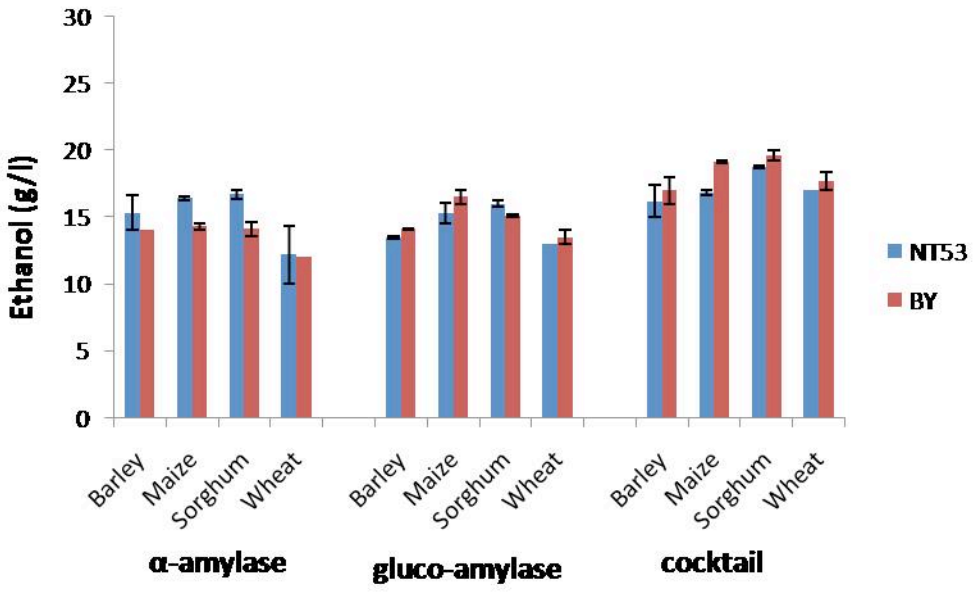

Figure 4.5: The figure represents ethanol amounts produced by the different grain types. *Ethanol amounts produced by the different grain type hydrolysates (barley, maize, sorghum and wheat) were fermented with NT 53 strain and commercial baker's yeast and then analysed by HPLC. The error bars represent two repetitions with a p-value of less than 0.05*

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4.3.7 Performing fermentation experiments using increased substrate volumes (small-scale)

The quantities of working solutions and substrates were raised ten times (50 ml to 500 ml) in an attempt to see whether at elevated quantities the consistency in soluble sugars and ethanol production from the grains would be retained. We also needed high volumes of fermentation products in order to measure the alcohol content using an alcolyzer that requires high volumes of samples for analysis. The alcolyzer is an instrument that measures alcohol content in a solution on a volume per volume (% v/v) percentage manner (Bastian *et al*., 2010). The initial step was analyzing soluble sugars from the different treatments with HPLC and the results are presented in Figure 4.6.

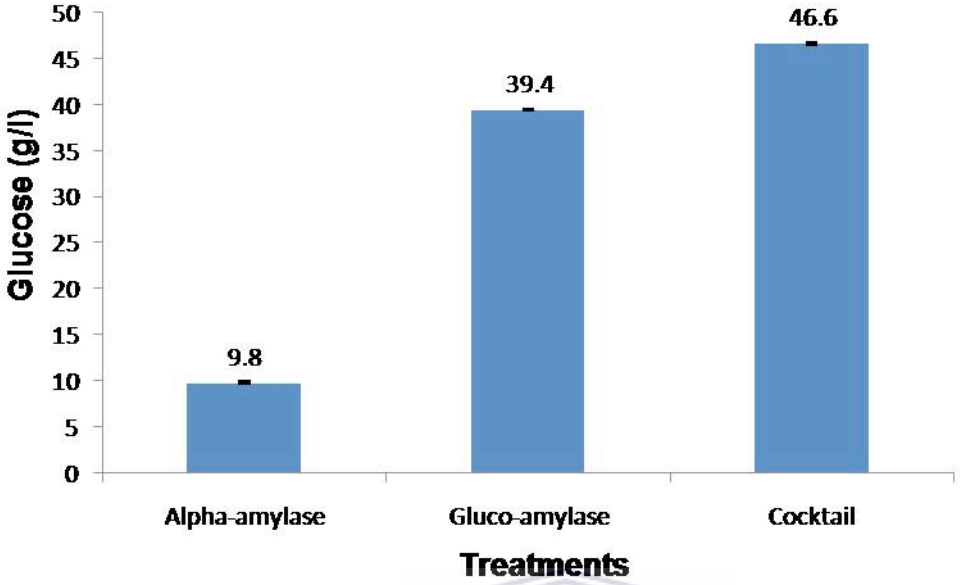

Figure 4. 6: Amounts of glucose produced by sorghum grains treated with different enzymes on small-scale conditions. *These hydrolysates resulting from α-amylase, gluco-amylase and cocktail treatments were analyzed with HPLC respectively. The error bars represent two repetitions with a p-value of less than 0.05*

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In Chapter 3, it was shown that at small-scale levels starch was completely hydrolyzed by the various enzyme treatments and cocktail treatments yielded high glucose concentration compared to other treatments. In this section (small-scale) we experienced a similar occurrence where all the treatments yielded similar glucose concentrations when compared to conventional lab-scale level. Untreated solutions did not yield any glucose amounts and cocktail treatment yielded high glucose concentration (46.6 g/l) followed by gluco-amylase with 39.4 g/l. The hydrolysates were fermented with the NT 53 *Saccharomyces cerevisiae* strain. Ethanol content post fermentation was analyzed with HPLC and the results are presented in Figure 4.7.

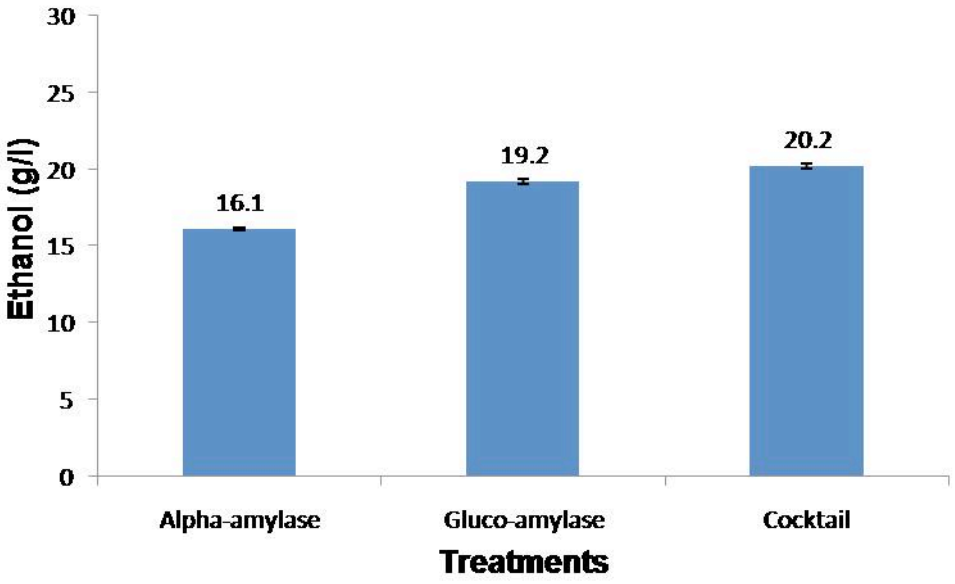

Figure 4.7: Amounts of ethanol yielded by sorghum hydrolysates fermented with Saccharomyces cerevisiae NT 53 strain on small-scale conditions. *Fermentation products resulting from the fermentation of α-amylase, gluco-amylase and cocktail treatments were analyzed with HPLC respectively. The error bars represent two repetitions with a p-value of less than 0.05*

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Ethanol formation was observed after the fermentation period of 48 hours at 30°C and the simultaneous consumption of glucose. Cocktail treatments yielded more ethanol concentration (20.2 g/l) when compared to other two treatments; however gluco-amylase treatments yielded more ethanol in terms of g/g conversions. Gluco-amylase yielded 0.48 g/g that translated to 95% fermentation efficiency, whereas cocktail treatments yielded 0.43 g/g that translated to 85% fermentation efficiency. To determine alcohol (% v/v) in the solution using we used an Anton Paar wine analysis system and the results are presented in Figure 4.8

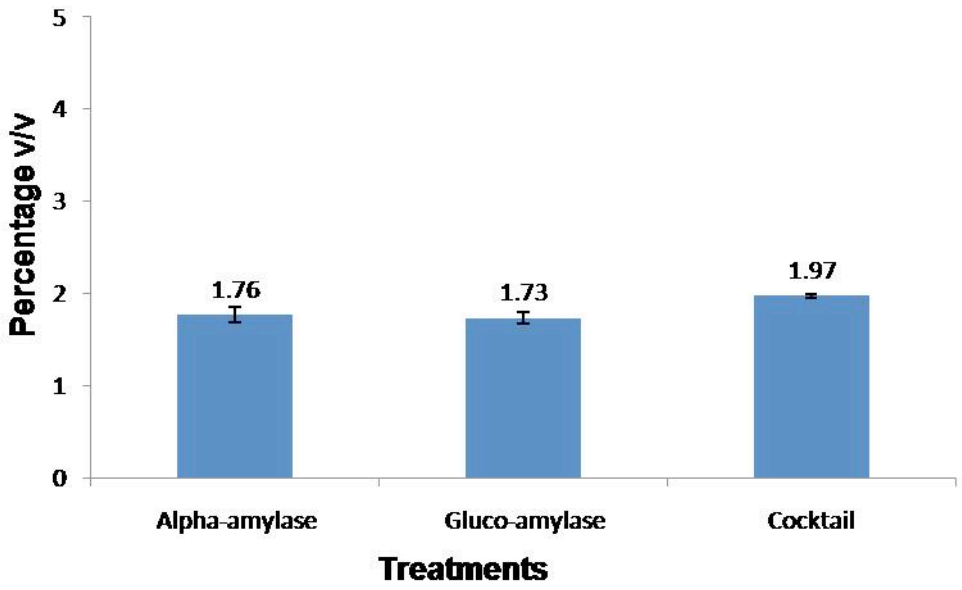

Figure 4.8: Amount of alcohol % v/v present in various fermented solutions. *Sorghum grain starch treated with various enzymes yielded different alcohol volumes when measured with the alcolyzer after fermentation. The error bars represent three experimental repetitions with a p-value of less than 0.05*

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The amount of alcohol/ethanol within the fermented products was determined in terms of volume per volume percentage. The fermentation products that were obtained from different treatments had similar alcohol contents. α-Amylase samples produced 1.76% of alcohol after fermenting with experimental NT 53 strain. The second largest alcohol producer (in terms of % v/v) was gluco-amylase with an average of 1.73%, and as expected, cocktail treatment dominated the production with almost 2% alcohol. We must take into consideration that the approximate concentration of 2% was before distillation and further dehydration of excess water in the fermentation products.

4.4 Discussion

The aim of this chapter was to compare glucose production and subsequent ethanol yield among four most important South African grain crops, namely sorghum, maize, barley and wheat. Preparation of grains and starch extraction were prerequisites for hydrolysis experiments and soluble sugar measurement was a prerequisite for the subsequent fermentation and ethanol quantification experiments. Presence of starch was tested using the fundamental biochemical iodine test (Figure 4.1). The test allows pentaiodide (I_5) ions of iodine to attach themselves in the coils of beta amylose molecules when starch is present. After starch degradation by the enzymes the intense blue colour did not form when iodine was added. The deficiency in colour formation was attributed by the enzymes degrading amylose bonds, leaving I_5 ions with no molecules to attach themselves to and hence no blue colour formation.

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Soluble sugars present before and after starch hydrolysis were measured with a refractometer. A study by Audilakshumi *et al*. (2010) measured soluble sugar content in sorghum stems and they explained that high stem sugar content translated to higher brix % amounts and low sugar content translated to low brix % amounts. In this study, post starch degradation brix amounts were elevated when compared to untreated starch. This was a result of the enzymes breaking down the complex insoluble starch polymer into soluble sugars and thus increasing the sugar content of grain solution. The analysis gave a rough indication of the amount of soluble sugars that are present in the hydrolysates. There was no considerable difference between the amounts produced by various grain crops. A further analysis was done to determine the amount of glucose that was present in the different grain crops.

Cereal grain starch is structured the same way irrespective of the type of feedstock and as a result they all require the same biochemical action to break down amylose bonds into soluble sugars. However grain feedstocks differ in the amounts of starch polymers they contain and the different amounts of starch polymers dictate the amount of glucose produced by a particular grain feedstock and subsequently the amount of ethanol yielded through fermentation. In a study conducted by Shingechi *et al*., (2004), 50 g/L of maize starch was extracted through high temperatures (120°C for 20 minutes) and low temperatures (80°C for 5 minutes) respectively. The different starch solutions were hydrolyzed and fermented with a yeast strain displaying amylolytic degrading properties. The maximum amount of ethanol recorded post fermentation was 0.5 g/g for both and they translated to 97.2 and 97.8% fermentation efficiencies respectively. Duvernay *et al*. (2013) compared soluble sugar production facilitated by various enzymes and ethanol yield post-fermentation between dried (flour) and freshly prepared sweet potatoes. Maximum average ethanol amounts of 34.9 g/L for the freshly prepared solution and 33.6 g/L for the dried flour were yielded from initial glucose amounts of 61.2 g/L and 62.6 g/L respectively. These translated to 0.57 g/g and 0.54 g/g, and fermentation efficiencies that were well above 100%, given that theoretical ethanol yield is 0.51 g/g. However, these unusual amounts were justified by the non-quantified fructose and maltose that were detected in the hydrolysates.

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Alcohol content that is observed after a fermentation process depends on many factors including the type of material that is being fermented, the starting concentration of soluble sugars and the organism that is used for fermentation. A study by Martín *et al*. (2010) investigated the effects that sugar reduction in grape must have on the alcohol

content. Grape must was reduced through filtration in a two-step filtration process and after every filtration step the sugar concentration was reduced and the same trend was observed after fermentation and distillation. Unfiltered grape must managed 209 g/L sugar concentration and post fermentation and distillation the must produced 12% v/v alcohol content. The filtered must in the two-step process had 133 and 95 g/L, and managed to produce 7.3 and 5% v/v alcohol content respectively. In our study we obtained approximately 2% v/v (Figure 4.8) alcohol content post fermentation (without distillation) from total soluble sugar content of 46.6 g/L (Figure 4.6).

As mentioned before, a wide range of grain crops are explored world wide as feedstocks for bioethanol production. The exploitation of crops in most parts of the world is mostly due to the crops of interest being produced in excess amounts and processing facilities that are in place. In Sub-Saharan Africa (SSA), edible crops are valuable and are consumed by human beings as staple food sources. In South Africa different crops were considered and were investigated as possible feedstocks for bioethanol production. We conclude here that sorghum is the grain crop with the highest potential for ethanol production. In a document released by the South African Grain Information Service (SAGIS), production of grain sorghum for the 2011/12 financial year was 163.700 tons and it is projected that for 2012/13 the production will decline to 137.200 tons (sagis.org.za, 2012). The document also reported that in the production year of 2009/10 South Africa imported 4 000 tons of grain sorghum and exported 52 000 tons, whereas in 2011/12, 57.800 tons were imported and 25.200 tons were exported. Consumption of sorghum in the country is mainly shared between malting, meal and grits, and a small portion is used for animal feed. A separate document presented by the Sorghum Section

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7 committee (2007) reported that the production of grain sorghum in 2003/04 was 373 000 tons and in 2004/05 was 260 000 tons. Sorghum production has declined over the years and projections have continued to indicate a continued decline in sorghum production in South Africa. The decline in sorghum production is caused by the decrease in traditional local market demand for sorghum (energy.gov.za). This makes sorghum an attractive feedstock for other non-traditional uses, such as bioethanol production.

Sugarbeet, sugarcane and sorghum are currently the leading contenders for commercial ethanol production (energy.gov.za). In this chapter we have shown that sorghum grain starch yields comparable sugars and ethanol when compared with other crops. Additionally, sorghum also contains readily fermentable stem soluble sugars that can yield up to 220 g/L of sugars (Makaula BSc Hons Thesis, 2010). This gives sorghum a considerable advantage and more importantly sorghum requires less water and other farming input costs when compared to other crops.

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Various enzymes treatments were used for starch breakdown and all of them lead to similar ethanol production after fermentation. When taking into consideration the prices and efficiencies of the enzymes in starch degradation, gluco-amylase is recommended for starch degradation treatment.

Chapter 5: General discussion and concluding remarks

5.1 General discussion

Human activities are amongst the primary causes for climate change and fossil energy depletion. These activities lead to higher energy price inflation and thus cripple the global economic development. This is a situation that will inevitably lead to rise in demand and therefore a rise in limited resources, cost and affordability. As mentioned before, various studies are undertaken worldwide and they range from solar energy, wind power, biomass to biofuels (including bioethanol) and in some cases these have already been commercialized. Our study focused on first generation ethanol production in the South African context using a selection of starchy grain crops as feedstocks. Crops such as maize and wheat are explored in some parts of the world as primary feedstocks for ethanol production. In South Africa, as with most developing countries, the luxury of exploiting these crops is limited due to the fact that most of the crops are considered as vital sources for staple food. Although most grain crops are cultivated for food purposes, the national market demand has increasingly showed less interest in sorghum and therefore this presents an opportunity for sorghum as a suitable crop for bioethanol production. However, we still had to prove that grain sorghum is capable of yielding a high/similar ethanol content compared with other starch crops.

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The main aim of this thesis was to perform a comparative analysis of soluble sugars and ethanol yield between sorghum and other three grain crops that are grown in South Africa. This was done with the hope of identifying the type of grain with the ability to yield high soluble sugars and subsequently high ethanol yields. Out of the various

enzyme pre-treatments, all starch molecules were successfully degraded into soluble sugars. The soluble sugars were also successfully converted into ethanol across all the grain feedstocks.

In Chapter 3 it was shown that the enzyme treatments degraded starch and formed soluble sugars and subsequently it was also demonstrated convincingly in chapter 4 that at scaled-up (small-scale) levels, the enzymes maintained their starch degradation efficiencies and produced similar chromatogram profiles and glucose amounts at small scale and lab-scale levels. Two different enzymes were used separately and also in a cocktail form. Traditionally, the enzymes (α-amylase and gluco-amylase) are used as a collective when degrading starch. The process that is currently employed for industrial scale ethanol production from starchy material involves the initial hydrolysis step of adding α-amylase and subjecting it to high temperatures, followed by addition of gluco-amylase (Shigechi *et al*., 2004). This method was efficient in starch degradation and converted all available starch to glucose monomers. In this study the same was observed when both enzymes were used, however the sole application of gluco-amylase saw the production of glucose amounts that were similar to the cocktail treatments. Thus, if costs implications are taken into consideration for sorghum starch degradation, sole application of gluco-amylase is recommended.

In Chapter 3 it was also shown that all the tested ARC *Saccharomyces* and *Geobacillus* strains consumed all the available glucose and the production of ethanol was observed and measured. As expected, in this study yeast strains produced high alcohol yields but the *Geobacillus* wild isolate strains yielded lactate as a major product. Another

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observation was that the yeast strains consumed oligoglycans (short glucose chains) that were liberated during α-amylase treatment. The NT 53 strain from the ARC culture collection was selected for further use and, in Chapter 4 the strain fermented sugars into ethanol in all tested grain types. The NT 53 experimental strain was then compared to commercial baker's yeast and it was shown that NT 53 generally produced higher ethanol content. Although *Saccharomyces* strains performed very well, *Geobacillus* have many advantages such as withstanding high temperatures and tolerance to high ethanol content, however wild type strains have poor ethanol production. With the engineered strains available and tested at lab scale levels to produce ethanol amounts that are comparable to yeast, *Geobacillus* could provide a good alternative or complementation to yeast in the future.

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5.2 Concluding remarks

This study does not provide a wholesome solution to the already suppressed energy supply; however, it provides a baseline for South African bioethanol prospects. In this thesis it was proved that the grain crops produced similar amounts of glucose and ethanol, and it was also shown that the use of gluco-amylase alone was sufficient to degrade starch and produce glucose amounts that were comparable to cocktail treatments. This cuts down the costs associated with using both enzymes. Although all the crops yielded similar amounts of ethanol, due to the controversy linked with using food crops that are in demand such maize and wheat, crops such as sorghum provide an advantageous, controversy-free alternative feedstock for the national bioethanol industry. Sorghum has agronomical and bio-energy source advantages compared to maize, wheat

and barley because it produces similar amounts of grain-based ethanol and sorghum also has an added advantage of having stem juices that are high in sugar content, thus intensifying the relevance of sorghum as a credible bioethanol feedstock. The overall objective of this thesis was to justify the potential application of sorghum as the most suitable and sustainable grain crop for the bioethanol industry in South Africa.

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Appendix 1

Brix percentages of different sugars

Sugars (80 mM)	Brix %
Fructose	1.5 ± 0.1
Glucose	1.6 ± 0.2
Sucrose	3 ± 0.1

The Table shows Brix % of various pure 80 mM sugars, the Brix % of fructose and glucose is different (less) to that of sucrose even though they have the same concentration, proving the brixometer to be biased towards sucrose.

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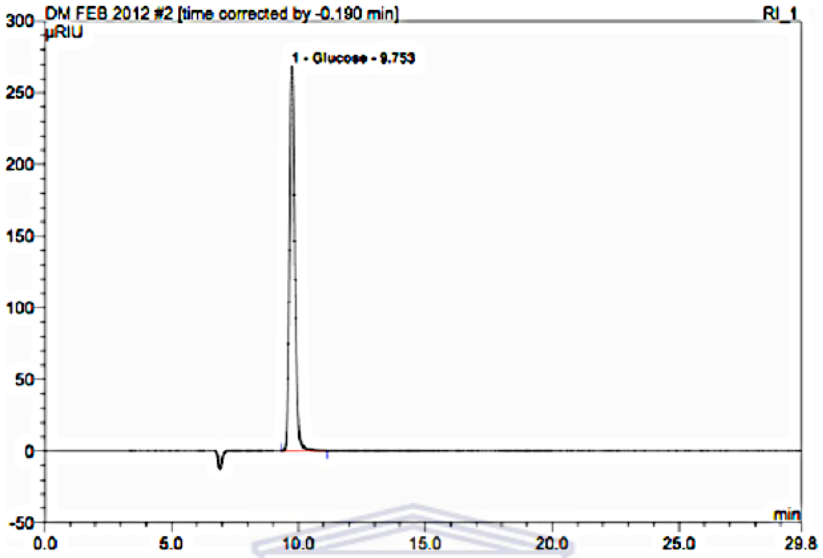
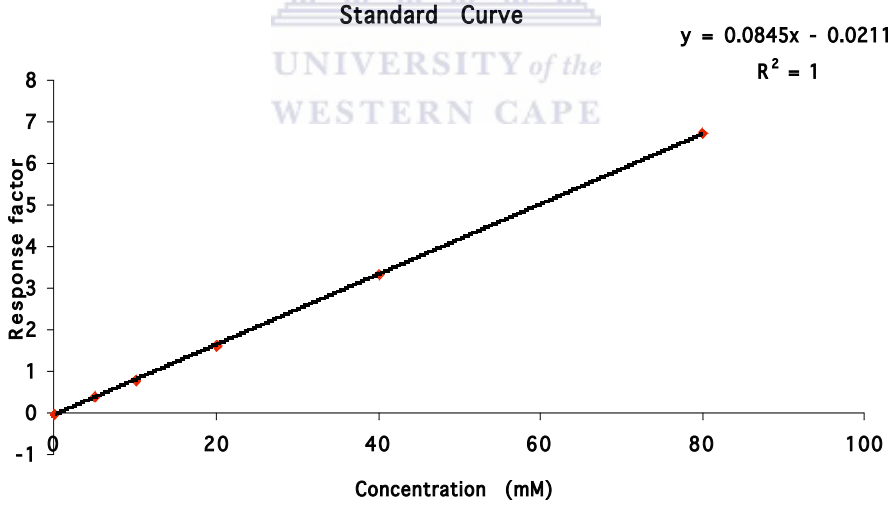
Appendix 2

Presentation of chromatograms and standard curves of glucose pure samples

(A)



(B)



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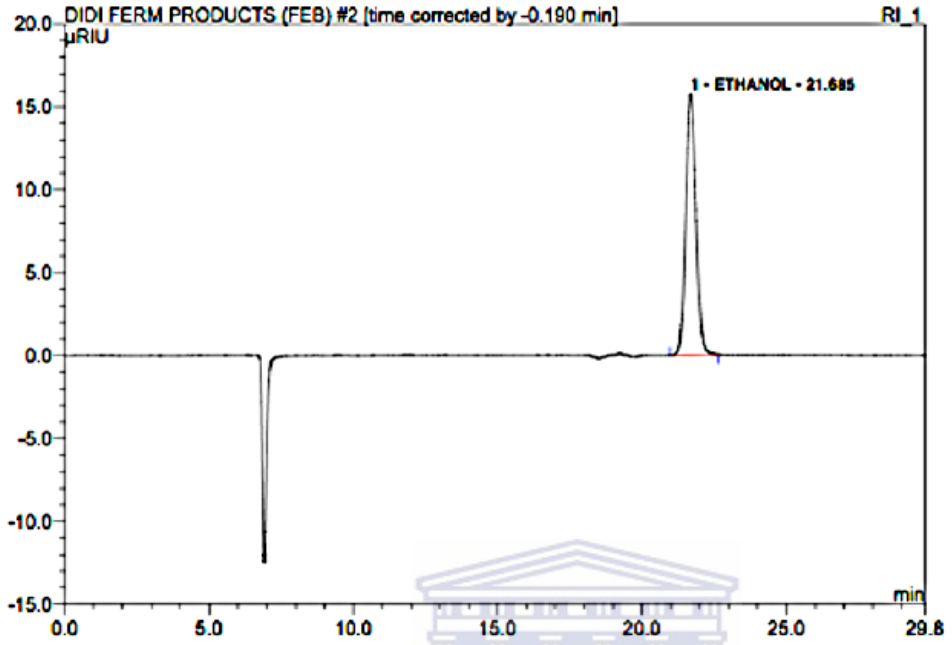
(A) Chromatogram depicting 80 mM of pure glucose used in preparing a standard curve. (B) Standard curve obtained by HPLC after injection of different amounts of pure glucose. X - axis represents different concentrations of glucose sugars and Y - axis represents response factor.

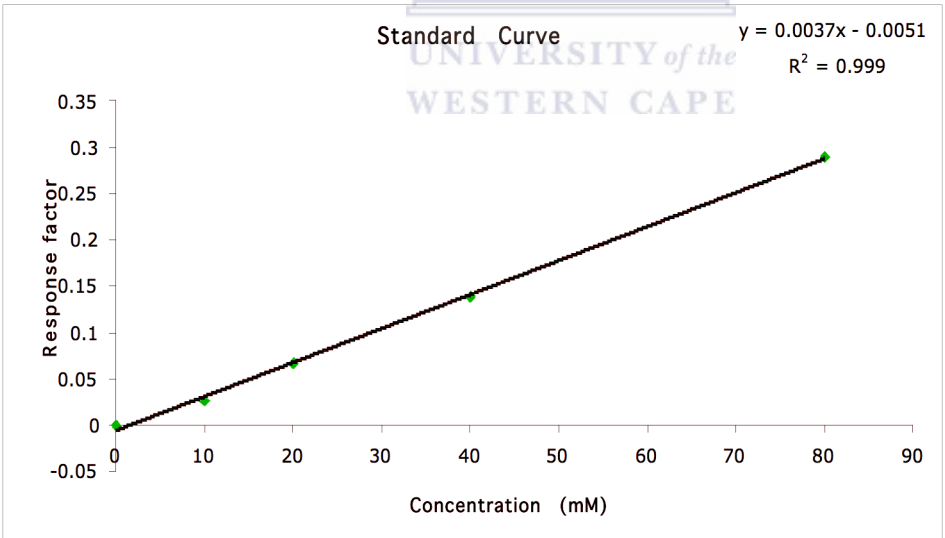
Appendix 3

Presentation of chromatograms and standard curves of ethanol pure samples

(A)



(B)



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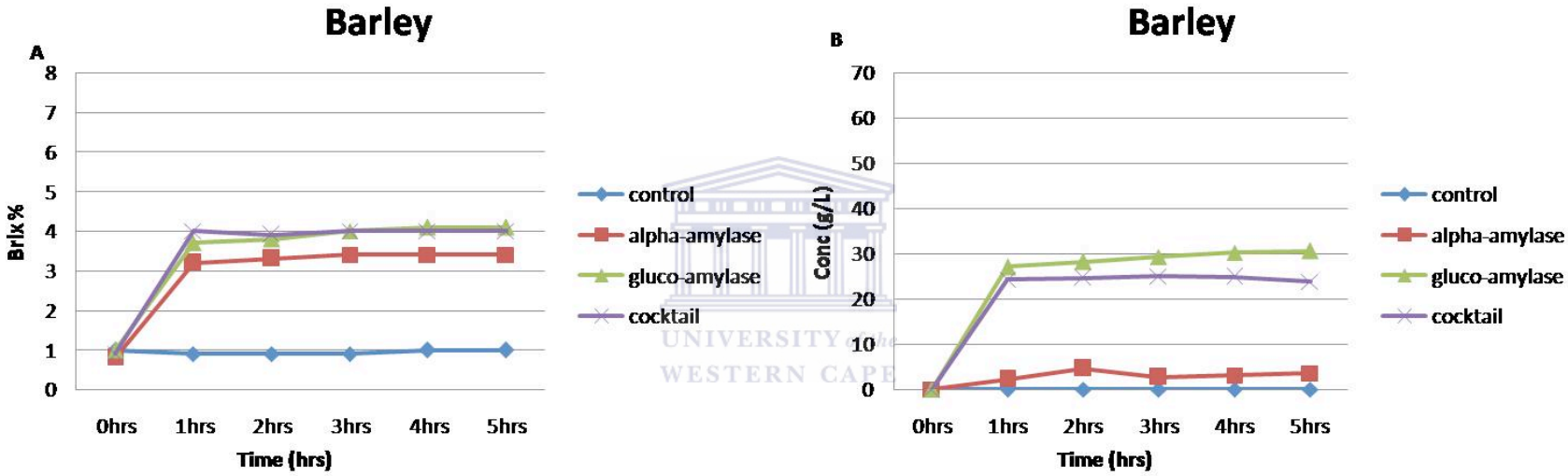
(A) Chromatogram depicting 80 mM of absolute ethanol used in preparing a standard curve. (B) Standard curve obtained by HPLC after injection of different amounts of absolute ethanol. X - axis represents different concentrations of ethanol and Y - axis represents response factor.

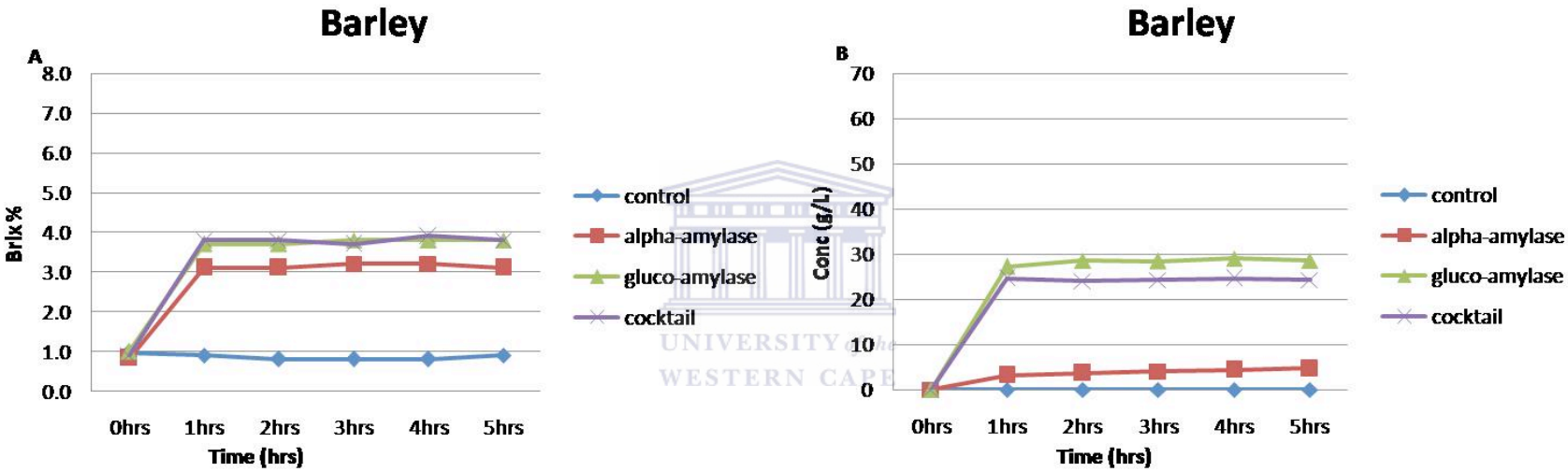
Appendix 4

Appendix 4 I



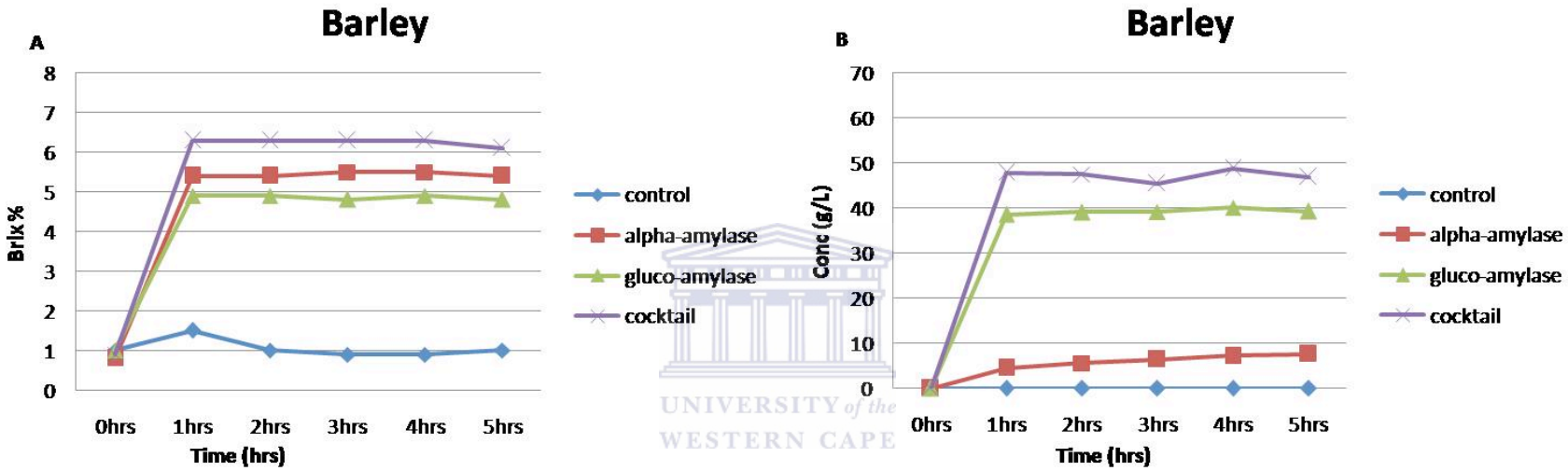
Appendix 4 I: represents hydrolysis of barley starch at 45 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 II



Appendix 4 II: represents hydrolysis of barley starch at 50 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 III



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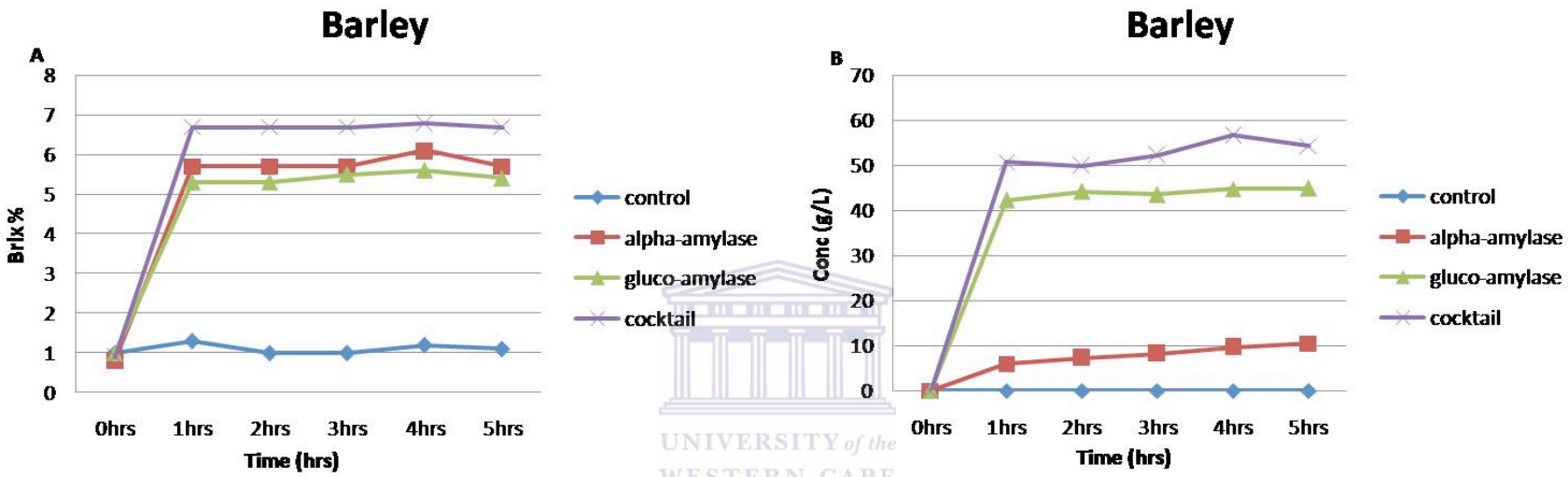
Appendix 4 III: represents hydrolysis of barley starch at 60 °C and different time-frame. (A) Is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 IV



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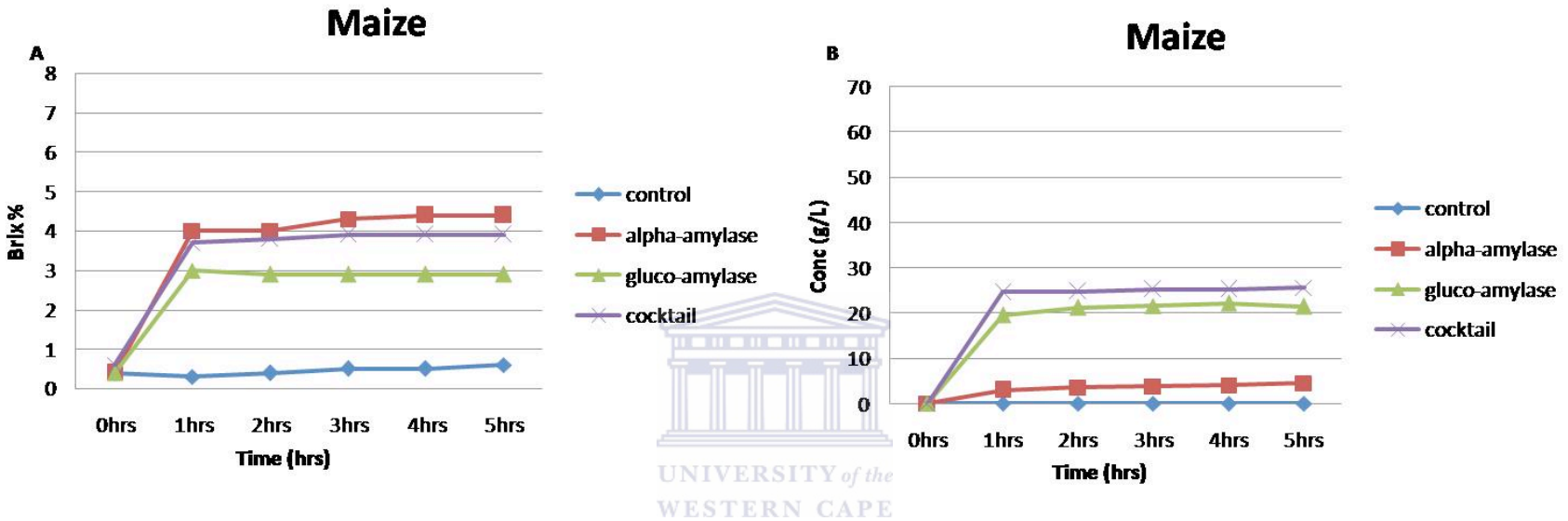
Appendix 4 IV: represents hydrolysis of barley starch at 70 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 V



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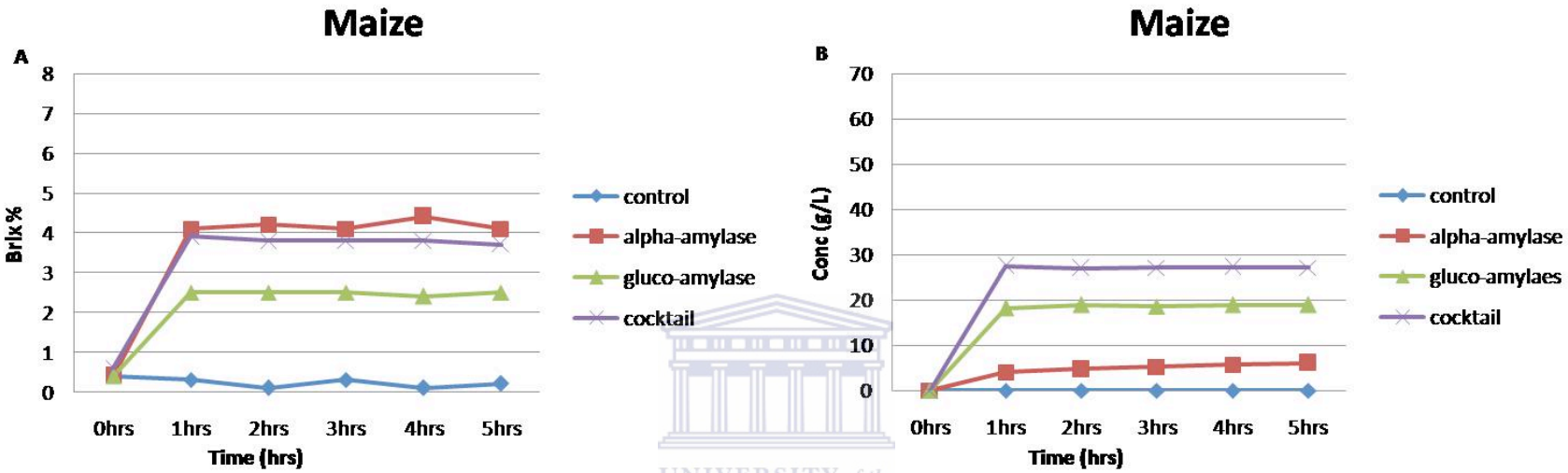
Appendix 4 V: represents hydrolysis of maize starch at 45 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 VI



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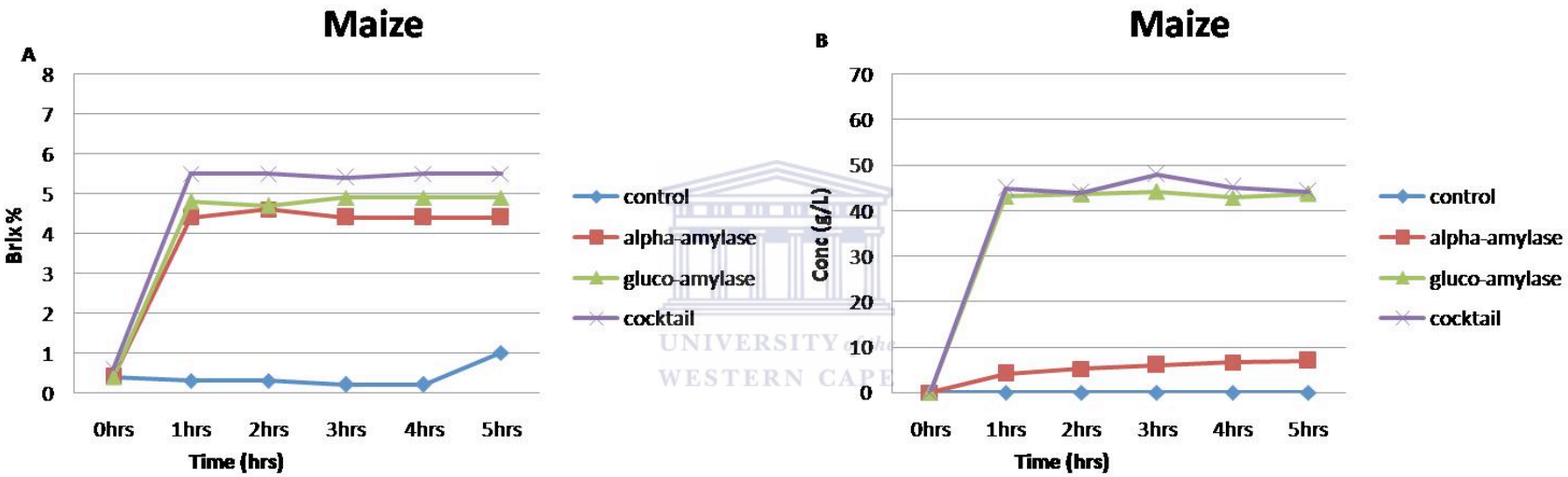
Appendix 4VI: represents hydrolysis of maize starch at 50 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 VII



Appendix 4 VII: represents hydrolysis of maize starch at 60 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

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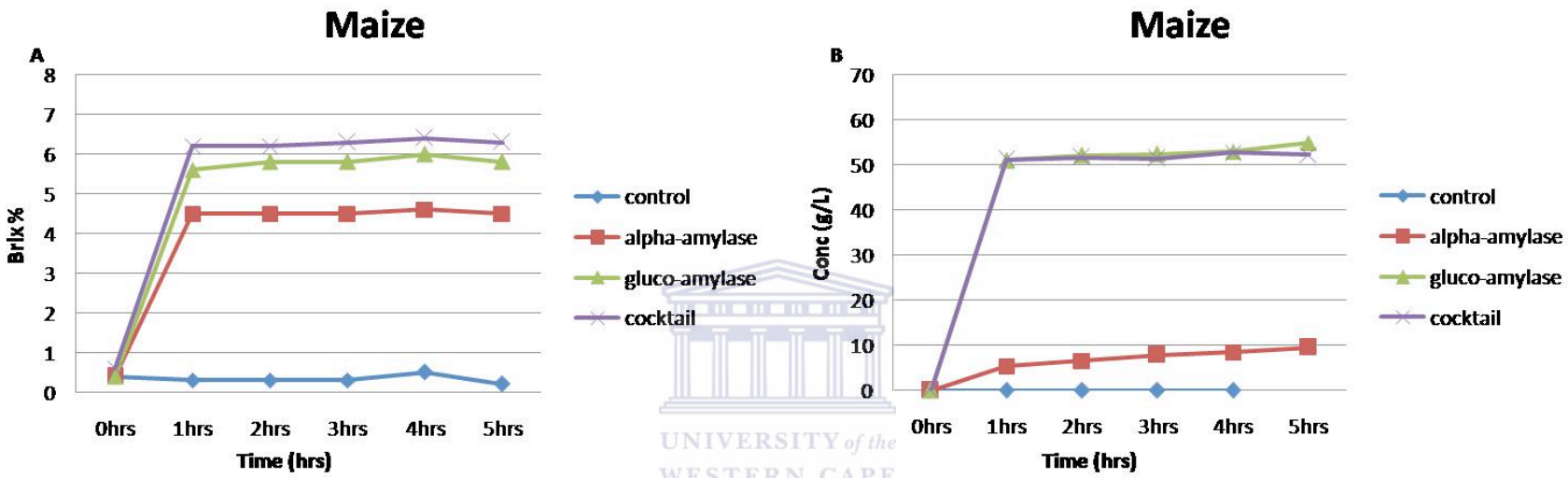
Appendix 4 VIII



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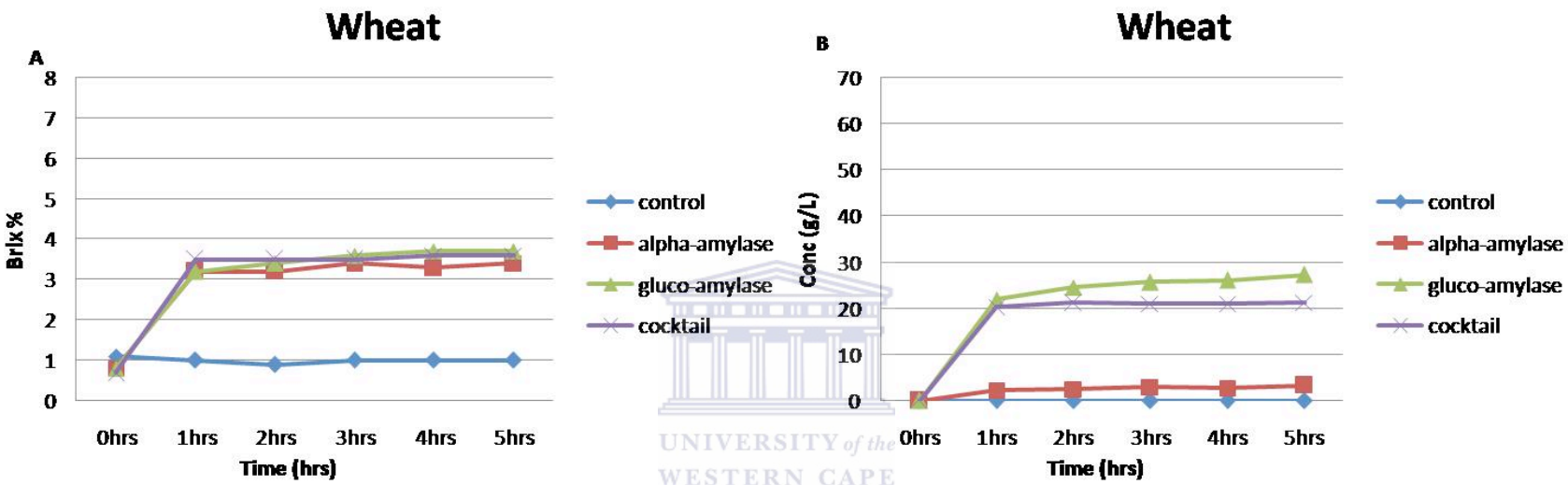
Appendix 4VIII: represents hydrolysis of maize starch at 70 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 IX



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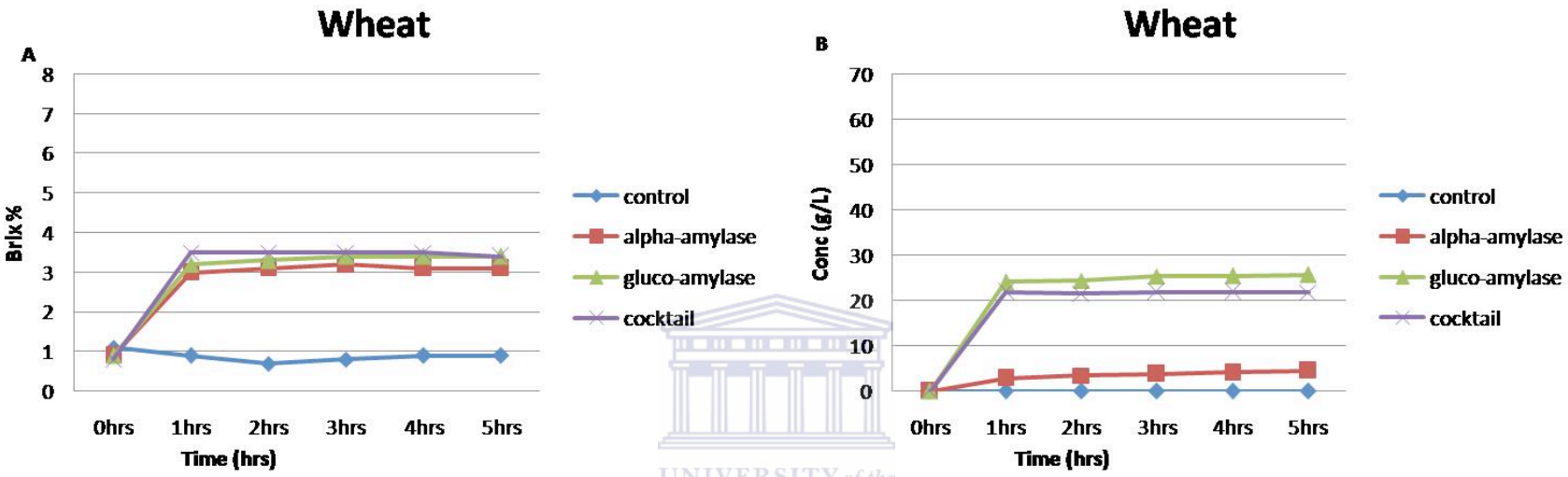
Appendix 4 IX: represents hydrolysis of wheat starch at 45 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 X



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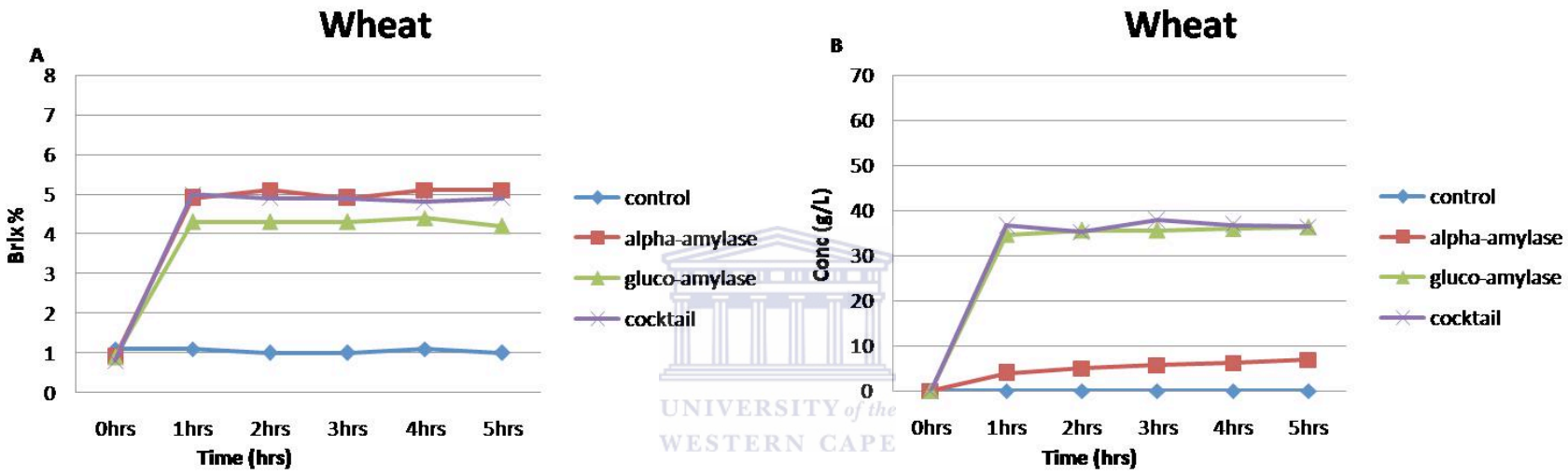
Appendix 4 X: represents hydrolysis of barley starch at 50 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 XI



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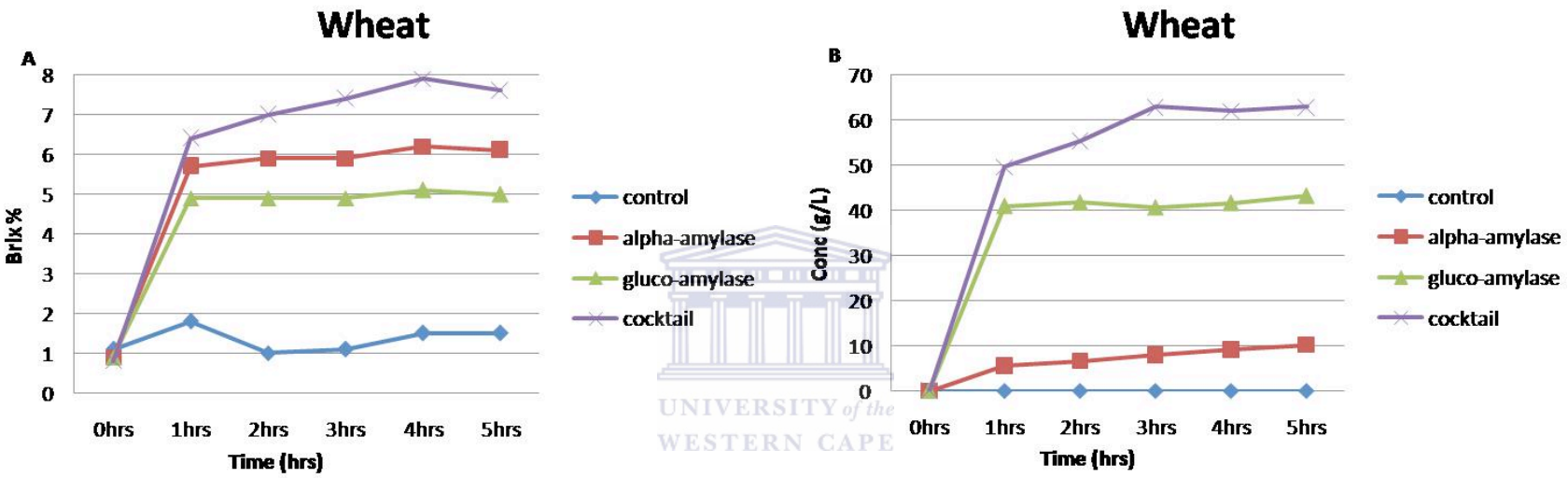
Appendix 4 XI: represents hydrolysis of wheat starch at 60 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

Appendix 4 XII



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Appendix 4 XII: represents hydrolysis of wheat starch at 70 °C and different time-frame. (A) is the Brix % results which shows total soluble sugars in the hydrolysate, (B) shows the amounts of glucose present in hydrolysates after various enzyme treatments. Each time point is a mean of three technical replicates.

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