The expression of fungal enzymes in *Saccharomyces cerevisiae* for bio-ethanol production from raw cornstarch

by
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ABSTRACT

Reliable energy resources could be considered as one of the cornerstones of the prosperity of the human race. The growing human population is constantly exerting more pressure on the world's natural resources, which include natural fossil fuels that are non-renewable. There are concerns regarding the use of fossil fuels due to its growing scarcity and its negative impact on the environment. There is thus a growing need in the world for energy sources that are renewable, more or less carbon neutral and therefore with a minimum environmental impact. Renewable energy is currently being harnessed from the wind, water and sun, but to a limited extent. These forms of natural resources are very attractive for the production of renewable energy, but these technologies are difficult to apply in the current transportation sector. Biofuels provide an alternative to the current use of liquid fossil fuels and it could be able to sustain the current fleet of automobiles worldwide in the intermediate to long term with minimal adjustment to the engines of these vehicles.

Extensive research has been done on the production processes for biofuels. Previous processes included the use of high temperatures and acids that further increased the total production cost and thus making biofuels less attractive as an alternative energy source. Recent research has suggested a wide range of organic materials as substrate for the production of biofuels, which include lignin, hemi-cellulose, cellulose and starch. Processes based on hemi-cellulose, cellulose and lignin as substrate are still in its early research stages and commercial application of these processes will only occur over the medium- to long-term.

Starch is a very good alternative source for the production of biofuels, but there is a need for a microbial system for the conversion of starch to bio-ethanol in a single step, referred to as Consolidated Bioprocessing (CBP). This would reduce the overall production cost of bio-ethanol and thus making starch-based substrates more attractive as an alternative energy source. The cost saving will be mainly due to the elimination of the pre-treatment of raw starch at high temperatures and the addition of enzymes for the liquefaction and saccharification of starch to simple sugars. However, as there is no currently no known microbial organism known that can produce the required enzymes (i.e. amylases) as well as ferment the resulting sugars to ethanol, heterologous expression of these enzymes in a host strain able to ferment sugars could provide the best alternative system.

In the first part of this study, 36 fungal strains known for the production of amylases were screened and compared for the highest extracellular enzyme activity on raw corn starch. The best two candidates, i.e. *Aspergillus tubingensis* (T8.4) and *Mucor cincinelloides* (1180), were then further evaluated to determine which organism has the highest efficiency when combined with a *Saccharomyces cerevisiae* laboratory strain. In fermentation experiments, *A. tubingensis* (T8.4) in combination with *S. cerevisiae* Y102 yeast strain resulted in the highest yield of ethanol.

Literature on *A. tubingensis* is limited compared with other *Aspergillii* and it was previously accepted that *A. tubingensis* has the highest homology with *Aspergillus niger*. However, other reports – including the present study - found that *A. tubingensis* is closer related to other *Aspergillus* spp. with regard to its amylolytic enzymes. The α-amylase gene of *A. tubingenis* has a homology of 99.00% with that of *Aspergillus kawachii* whereas the glucoamylase gene has a homology of 99.26% with that of *Aspergillus shirousami*.

In the second part of this study, two recombinant *S. cerevisiae* strains were constructed to express the wild type *A. tubingensis* α-amylase (Atamy) and glucoamylase (Atglu), respectively. The combination of the two recombinant yeast strains was able to completely hydrolyse and also utilize raw corn starch for the production of bio-ethanol, with a yield of 11.04 g/l of ethanol, which translates to 98% of the theoretical yield from starch with a 52% conversion of the total raw starch. This rate of conversion is lower than other reports which indicated up to 82% and 96% of the theoretical yield of ethanol from raw and soluble starch, respectively, by α- and glucoamylase. Furthermore, the combined expressed of the two genes was much more effective than when only one of the two genes were expressed, with a yield of 0.32 g/l ethanol for only Atamy and 2.52 g/l ethanol for Atglu. This proved that the combination of the *A. tubingensis* genes were best suited for the production of biofuels from raw starch. This also proved that the concept of constructing an amylolytic yeast strain capable of raw starch hydrolysis and fermentation was indeed feasible.

OPSOMMING

Betroubare energiebronne kan as een van die boublokke vir die vooruitgang van die mensdom beskou word. Die groeiende menslike populasie is gedurig besig om meer druk op die wêreld se natuurlike hulpbronne te plaas, insluitende nie-hernubare fossielbrandstowwe. Daar is kommer rakende die gebruik van fossielbrandstowwe weens 'n afname in die beskikbaarheid en die negatiewe impak wat dit op die omgewing het. Daar is dus 'n groeiende behoefte in die wêreld vir 'n hernubare, min of meer koolstof-neutrale energiebron wat 'n minimale omgewingsimpak sal hê. Hernubare energie word tans tot 'n beperkte mate uit wind, water en die son verkry. Hierdie vorms van natuurlike energie hulpbronne is baie aanloklik vir die vervaardiging van hernubare energie, maar hierdie tegnologië is moeilik toepasbaar in die huidige vervoersektor. Biobrandstowwe voorsien 'n alternatief vir die huidige gebruik van fossielbrandstowwe en kan moontlik die huidige voertuigvloot wêreldwyd oor die medium- tot langtermyn onderhou met minimale enjin-aanpassings van hierdie voertuie.

Deeglike navorsing is alreeds op die vervaardigingsprosesse vir biobrandstowwe gedoen. Vorige prosesse het die gebruik van hoë temperature en sure ingesluit wat produksiekostes verder verhoog en gevolglik die gebruik van biobrandstowwe as 'n alternatiewe energiebron minder aantreklik gemaak het. Onlangse navorsing het die gebruik van organiese materiaal as substraat vir die produksie van biobrandstowwe voorgestel, wat lignien, hemi-sellulose, sellulose en stysel insluit. Prosesse met die gebruik van hemi-sellulose, sellulose en lignien as substraat is nog in die beginfase van ontwikkeling en kommersialisering van hierdie prosesse sal eers oor die medium- tot langtermyn plaasvind.

Stysel is 'n baie goeie alternatiewe bron vir die produksie van biobrandstowwe, maar 'n mikrobiese sisteem word vir die omskakeling van stysel in bio-etanol in 'n enkele stap benodig, bekend as gekonsolideerde bioprosessering (GBP). Dit sal die algemene produksiekoste van bio-etanol verlaag en dus styselsubstrate as 'n alternatiewe energiebron meer aantreklik maak. Die kostebesparing sal hoofsaaklik realiseer omdat die vooraf-behandeling van rou stysel byhoë temperature en die toevoeging van ensieme vir die vervloeiing en versuikering van stysel tot eenvoudige suikers, uitgeskakel word. Aangesien daar tans geen bekende mikrobe organisme is wat die nodige ensieme (nl. amilases) kan produseer en ook die suikers wat daardeur vrygestel is, na etanol kan fermenteer nie, kan die heteroloë uitdrukking van hierdie ensieme in 'n gasheer-ras wat die suikers kan fermenteer, moontlik die beste alternatief verskaf.

In die eerste deel van hierdie studie is 36 fungi rasse wat bekend is vir hul amilase produksie ge-evalueer en met mekaar vergelyk vir die hoogste ekstrasellulêre ensiemaktiwiteit op rou mieliestysel. Die beste twee kandidate, naamlik *Aspergillus tubingensis* en *Mucor cincinelloides,* is verder ge-evalueer om te bepaal watter organisme het die hoogste effektiwiteit in kombinasie met 'n *Saccharomyces cerevisiae* laboratorium gisras. In fermentasie-eksperimente het *A. tubingensis* in kombinasie met *S. cerevisiae* Y102 gisras die hoogste etanol opbrengs gelewer.

Inligting rakende *A. tubingensis* is beperk relatief tot ander *Aspergillii* en dit was voorheen aanvaar dat *A. tubingensis* die hoogste homologie met *Aspergillus niger* het. Ander verslae – insuitende die huidige studie - het egter gevind dat *A. tubingensis* nader verwant aan ander *Aspergillus* spp. in terme van amilolitiese ensieme is. Die α-amilase geen van *A. tubingensis* het 'n homologie van 99.00% met dié van *Aspergillus* kawachii en die glukoamilase 'n homologie van 99.26% met dié van *Aspergillus shirousami* getoon.

In die tweede gedeelte van hierdie studie is twee rekombinante *S. cerevisae* gisrasse gekonstrueer om onderskeidelik die α-amilase (Atamy) en glukoamilase (Atglu) van *A. tubingensis* uit te druk. Die kombinasie van die twee rekombinante gisrasse was in staat om die volledige hidrolise en benutting van rou mieliestysel vir die produksie van bio-etanol deur te voer met 'n opbrengs van 11.04 g/l wat gelykstaande is aan 98% van die teoretiese opbrengs vanaf stysel met 'n omskakeling van 52% van die totale rou stysel. Hierdie omskakelingskoers is laer as ander studies wat onderskeidelik 82% en 96% van die teoretiese opbrengs van rou en oplosbare stysel vir α- en glukoamilase getoon het. Verder was die kombinasie van die twee gene meer effektief as wanneer slegs een gebruik is, met 'n 0.32 g/l opbrengs vir Atamy en 2.52g/l vir Atglu. Hierdie het bewys dat die kombinasie van die *A. tubingensis* meergeskik vir die produksie van bio-etanol was. Dit het ook bewys dat die beginsel van 'n amilolitiese gisras wat in staat is om rou stysel te hidroliseer en te fermenteer, inderdaad moontlik is.

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TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION AND PROJECT AIMS 3

1. INTRODUCTION 4
2. AIMS OF THIS STUDY 5
3. REFERENCES 6

CHAPTER 2: LITERATURE REVIEW 7

1 INTRODUCTION 8
  1.1 The need for a new energy source 8
  1.2 The legacy of current fuels 8
2. ALTERNATIVE ENERGY SOURCES 9
  2.1 Biomass as a fuel source 11
    2.1.1 The history of biofuels 11
    2.1.2 Global biofuels production 12
    2.1.3 Biofuels on the African continent 13
    2.1.4 Biofuels in South Africa 14
  2.2 Starch crops as biomass for the production of bio-ethanol 16
    2.2.1 Current starch to bio-ethanol processes 17
3. ENZYMATIC HYDROLYSIS OF STARCH 19
  3.1 The origin and structure of starch 19
    3.1.1 Soluble starch 21
    3.1.2 Raw or native unmodified starch 22
  3.2 Enzymes required for starch hydrolysis 24
    3.2.1 Alpha-amylases 24
4. AMYLOLYTIC YEAST STRAINS 31
  4.1 Expression of amylases in yeast 31
  4.2 Simultaneous Saccharification vs. Consolidated Bioprocessing 33
  4.3 Consolidated Bioprocessing of Raw Starch – dream or reality? 34
5. REFERENCES 35

CHAPTER 3: BIOPROSPECTING FOR A FUNGUS EXPRESSING RAW STARCH DEGRADING ENZYMES 46

ABSTRACT 47

1 INTRODUCTION 47
2 MATERIALS AND METHODS 49
2.1 Microorganisms and growth conditions 49
2.2 Soluble/raw starch hydrolysis on agar plates 49
2.3 Liquid assays 50
2.4 Enzyme and yeast cocktails for ethanol production 51
3 RESULTS 51
3.1 Detection of hydrolysis of soluble and raw starch 51
3.2 Hydrolysis of soluble and raw starch (DNS assays) 52
3.3 α-Amylase and glucoamylase activity 53
3.4 Ethanol production 54
4 DISCUSSION 55
5 REFERENCES 57

CHAPTER 4: THE ISOLATION AND EXPRESSION OF THE *ASPERGILLUS TUBINGENSIS* A-AMYLASE AND GLUCOAMYLASE GENES IN *SACCHAROMYCES CEREVISIAE* 59

ABSTRACT 60
1 INTRODUCTION 60
2 MATERIALS AND METHODS 61
2.1 Strains and media 61
2.2 DNA manipulation 63
2.3 DNA sequencing 64
2.4 Yeast transformation 65
2.5 Amylase assays 65
2.6 Enzyme expression (SDS-PAGE) 66
2.7 Fermentation studies 66
3 RESULTS AND DISCUSSION 66
3.1 Cloning of *A. tubingensis* α-amylase 67
3.2 Cloning of *A. tubingensis* glucoamylase 69
3.3 SDS-PAGE 72
3.4 Amylase asssays 72
3.5 Fermentation studies 75
4 CONCLUSIONS 78
5 REFERENCES 79

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS 83

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION

An estimation by Royal Dutch Shell (a leading petroleum group) suggested that by the year 2050, 30% of the world chemical and fuel needs would be supplied by renewable resources with special reference to the agricultural sector (OECD, 1998). The ideal scenario would be to use agricultural residues as a resource as it could, together with other organic material, be transformed into fermentable sugars for the production of bio-ethanol. A number of biomass resources could be used for the production of bio-ethanol, including cellulose, hemicellulose, lignin and starch. Starch acts as a reserve energy storage compound in plants while cellulose, hemicelluloses and lignin form part of the cell walls of plants. Lignin is intertwined with cellulose and hemicellulose to form an impenetrable barrier, providing structural support and resistance to microbial attack. Starch and cellulose are homopolysaccharides composed of glucose units. Hemicelluloses are more complex and consists of combinations of glucose, mannose, xylose, galactose and arabinose sugars with the ratio of the sugars varying depending on the origin. Lignin is an aromatic polymer synthesized from phenylpropanoid precursors (Palmqvist and Hahn-Hägerdal, 2000).

Enzymes are being used in a number of industries, including agriculture, chemical production, cleaning products, energy production, food, pharmaceutical and the processing of raw materials. Technological developments are now rapidly stimulating the promotion of enzyme technology, based on health issues, energy, raw materials and environmental concerns (van Beilen and Li, 2002). The application of enzymes in industrial processes has led to a reduced need for chemicals (including organic solvents), the elimination of high process temperatures and extreme pH ranges, while producing a pure product with reduced environmental and financial impact. This is attributed to the ability of industrial enzymes to work at moderate temperatures and pH values, as well as its biodegradability (Cherry and Fidantsef, 2003).

The United States has proven that corn starch can be used for the large-scale production of bio-ethanol. Subsequently, the focus has shifted to optimising the cultivation of starch crops as well as starch production in cassava, maize and potatoes (Ulanov et al., 2003; McKibbin et al., 2006). In terms of the ethanol production process, it is economically more feasible to produce ethanol directly from raw starch than modified starch, because the latter requires heat pre-treatment. The use of raw starch, however, requires the use of raw starch degrading enzymes.

Several micro-organisms have the ability to grow on raw starch and therefore produce the enzymes of interest. Unfortunately, only a few of the genes coding for the raw starch degrading enzymes had been identified, but the DNA sequences have not been published. Some of the raw starch degrading enzymes had been purified and the amino acid sequence determined. However, once the DNA or

amino acid sequences are released, patents are already registered which restricts their application in biofuels, or any other industry.

The success of commercially feasible bio-ethanol depends on the development of strains that are efficient in utilizing the substrate (raw starch, in this case) while producing high levels of ethanol (Polagye et al., 2007). Strains of *Saccharomyces cerevisiae* are routinely used for the fermentation of glucose to ethanol since few other yeasts display similar levels of ethanol tolerance. Furthermore, *S. cerevisiae* has a long history with the fermentation industry (wine and beer) and has GRAS status (Generally Regarded as Safe) making it the ideal host for the production of bio-ethanol from starch (Dziezak, 1987). Unfortunately, *S. cerevisiae* is unable to convert starch to glucose as it lacks starch degrading enzymes. To enable *S. cerevisiae* to convert starch to bio-ethanol, it would require the genetic engineering of a suitable yeast strain capable of the successful production and secretion of raw starch degrading α-amylases and glucoamylases (Carlsen et al., 1996).

2. AIMS OF THIS STUDY

The aims of this study were to (1) isolate and identify a raw starch degrading organism, (2) to isolate the cDNA sequence of the α-amylase and glucoamylase genes, and (3) to express the amylases in a laboratory strain of *S. cerevisiae* to enable growth on raw starch.

The main objectives for this study were as follows:

- To evaluate fungal strains capable of hydrolyzing raw corn starch and identify the strain(s) with the highest extracellular α-amylase and glucoamylase activities.
- To determine the potential of these enzymes to produce bio-ethanol from starch in the presence of *S. cerevisiae* strains.
- To isolate, clone and sequence the α- and glucoamylase coding genes from the best amylase producer.
- To express the cDNA copies of the α-amylase and glucoamylase in *S. cerevisiae* and characterise the recombinant proteins expressed by the recombinant strains.
- To determine the feasibility of the recombinant strain(s) to hydrolyse raw starch and ferment the resulting glucose to bio-ethanol.

3. REFERENCES

Carlsen M, Nielsen J, and Villadsen J (1996) Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. *J Biotechnol* 45:81-93

Cherry JR and Fidantsef AL (2003) Directed evolution of industrial enzymes: an update. *Curr Opinion Biotechnol* 14:438-443

Dziezak JD (1987) Yeasts and yeast derivatives: Definitions, characteristics, and processing. *Food Technol* 44:104-121

McKibbin RS, Muttucumaru N, Paul MJ, Powers SJ, Burrell MM, Coates S, Purcell PC, Tiessen A, Geigenberger P, and Halford NG (2006) Production of high-starch, low-glucose potatoes through over-expression of the metabolic regulator SnRK1, *Plant Biotechnol J* 4:409–418

OECD (1998) Biotechnology for Clean Industrial Products and Processes. Paris, France

Palmqvist E, and Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technol* 74:25-33

Polagye BL, Hodgson KT, and Malte PC (2007) An economic analysis of bio-energy options using thinning from overstocked forests. *Biomass Bioener* 31:105-125

Ulanov A, Lygin A, Duncan D, Widholm J, and Lozovaya V (2003) Metabolic effects of glyphosate change the capacity of maize culture to regenerate plants. *J Plant Physiol* 166:978—987

van Beilen JB and Li Z (2002) Enzyme technology: an overview. *Curr Biotechnol* 13:338-344

CHAPTER 2

LITERATURE REVIEW

1. INTRODUCTION

1.1 *The need for a new energy source*

The depleting oil reserves and the climatic change caused by the combustion of fossil fuels is currently a big concern as it affects health, wealth and political stability. Fossil fuels are mainly responsible for the pollution of the atmosphere due to the release of SO_2, CO_2, NO_2 and NO associated with its combustion (Hoel and Kverndokk, 1996). Yet, fossil fuels remain the main source of energy despite the fact that these natural resources are not sustainable. At the present rate of global consumption, it is estimated that the current crude oil resources would be depleted in less than 50 years (Demirbas, 2006a). Although some studies have indicated that the crude oil and gas reserves will be sufficient for decades to come, the oil production might plateau in the near future due to other constrains such as a lack of investment in exploration. Efforts to address climate change may also influence demand in the long term (Kjarstad and Johnnson, 2009). Therefore, global energy security is currently viewed in the light of uncertainty emphasising the urgent need for alternative energy sources to meet the demand of an ever growing population (Kjarstad and Johnnson, 2009).

1.2 *The legacy of current fuels*

Coal, fuel oils, gasoline, diesel fuels, alcohol fuels, natural gas, liquefied petroleum gas and biodiesel are the major forms of fuels that have an impact on air quality and climate change. Coal is widely used for heating and the production of electricity (by means of boilers). The combustion of fossil fuels alone is responsible for 73% of the CO_2 production in the world (Wildenborg and Lokhorst, 2005). Under combustion conditions (above 300°C), non-condensable gasses such as CO_2, H_2O, SO_2, NO_2 and methane are released, while the organic compounds are released at temperatures below 300°C (Oros and Simoneit, 2000). A major by-product from coal combustion is fly ash, which contains potentially toxic components such as heavy metals and radionuclides (Dreher et al., 1996; Linak et al., 2000). Mercury is a toxic element that is currently a non-regulated air pollutant. Its emissions are expected to increase with an increased need for electricity and thus increased activity of coal by coal fired power plants (UNEP/WHO, 1992).

Fuel oils are placed into two groups, namely distillate oils and residual oils. Although these oils are not as widely used as coal, they play an important environmental role with the emissions of high levels of SO_2 with their use as a transportation fuel for large ocean transport vessels (Corbett and Fischbeck, 1997). More than half of the total sulphate aerosol column burden over the Mediterranean ocean is of cargo shipment origin (Marmer et al., 2007). Petroleum fuels are the most common culprit associated with a negative environmental impact. Lead has been used in petroleum petrol as an octane enhancer, but was banned for on-road vehicles due to its high levels

of toxicity. It is, however, still being used in aircrafts, farm equipment and marine engines. Another concern with petroleum is the production of carbon monoxide, volatile organic carbon and NO_x upon combustion. Standard vehicles (cars and trucks) are responsible for 10% of the global CO_2 emission (Gaffney and Marley, 2009). Diesel fuels have a far less impact on SO_2 emissions, but results in a five-fold emission of NO_x compared to petroleum (Kirchstetter et al., 1996).

Alcohol fuels are also referred to as biofuels. They represent an attractive alternative to fossil fuels due to their potential use in current transportation fuels. Methanol and petroleum blends have been developed to prevent possible corrosion and deterioration of motor vehicle engines. A convention has been established for the formulation of different blends, e.g. E85 for 85% ethanol and E50 for 50%. Compared to petroleum, E85 has a 31% lower carbon monoxide emission and a 84%, 93% and 70% reduction in benzene, 1,3-butadiene and acetaldehyde emission, respectively (Auto/oil Air Quality Improvement Research Program, 1992). Alcohols have broader flammability limits, high flame speeds and higher heats of vaporization, resulting in a shorter burn time compared to theoretical efficiency with petroleum fuels (MacLean and Lave, 2003). However, aldehydes are produced as a by-product during the combustion of biofuels, which can lead to the release of secondary atmospheric pollutants (Atkutsu et al., 1991; McNair et al., 1992). Compressed natural gas (CNG) and liquefied petroleum gas (LPG) have the lowest emission of carbon monoxide, benzene and aldehydes when compared to the combustion of petroleum, ethanol and methanol-based fuels (Blake and Rowland, 1995). Although compressed gas represents a cleaner fuel than petroleum-based fuels, a major drawback is the handling and transportation of large volumes of gas. Biodiesel is a plant oil methyl ester that produces formaldehyde during combustion, but it results in a lower emission of CO_2 (Turrio-Baldassari et al., 2004).

Clearly all the major energy sources (coal, fuel oils, petroleum, diesel fuels, alcohol fuels, natural gas and biodiesel) have advantages as well as drawbacks. While alternative energy sources are being investigated, it would in the meantime be advantageous to establish a means to control the emissions of harmful chemicals. Advancing technologies could help reduce the current energy consumption, increase efficiencies of energy conversion and utilization, monitor the carbon content of fuels and try to lower it, enhance CO_2 recycling and improve storing thereof (Demirbas, 2006b).

2. ALTERNATIVE ENERGY SOURCES

Alternative energy sources should have an insignificant environmental footprint, enable economic prosperity and represent a sustainable industry. This would include harvesting energy from wind (converted to electricity through turbines), solar energy, hydro energy and energy derived from

hydrogen with special reference to fuel cells (Figure 1). These alternatives are currently only used on small scale and their contribution as major role players in the future for the global energy supply is debatable (Adamson, 2004).

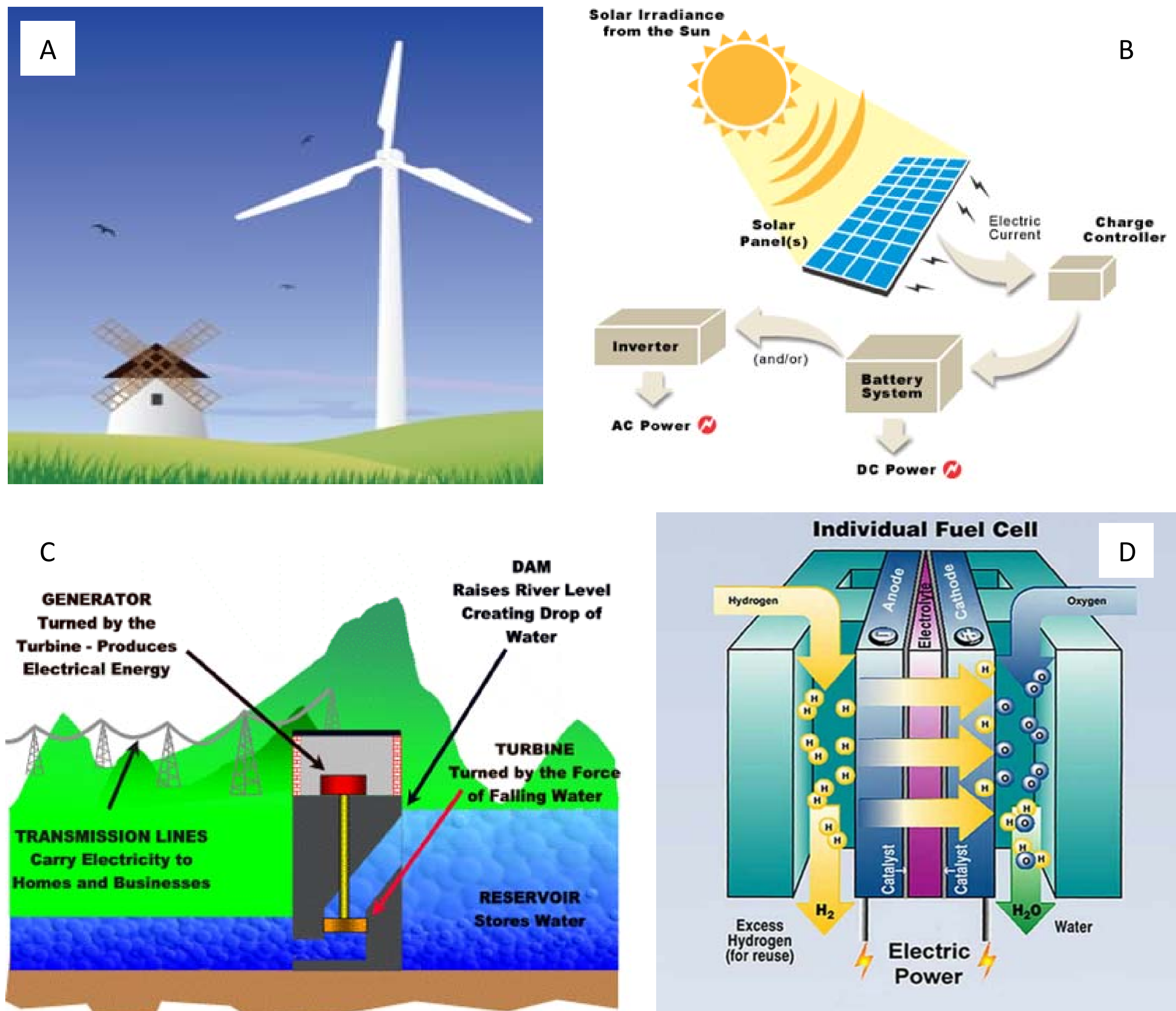

Figure 1: Alternative energy sources such as (A) energy from wind (http://www.scienceclassonline.com/), (B) solar energy (http://3.bp.blogspot.com/), (C) hydro energy (http://earthsci.org/mineral/energy/), and (D) energy derived from hydrogen are only used on small scale (http://climatelab.org/)

Biofuels is considered to be the most likely alternative energy source to address and help resolve the growing demand for transportation fuels. Alternative energy produced from biomass in the form of bio-ethanol seems to be feasible to play a major role in the long term. Brazil and the United States of America (USA) are currently the world leaders in bio-ethanol production with a combined production of about 65% of the bio-ethanol that is currently supplied globally (Demirbas and Balat, 2006). The Brazilian industry is based on sugarcane, whereas the USA, by far the largest producer of corn starch, uses maize as their main feedstock for the production of bio-ethanol (Figure 2).

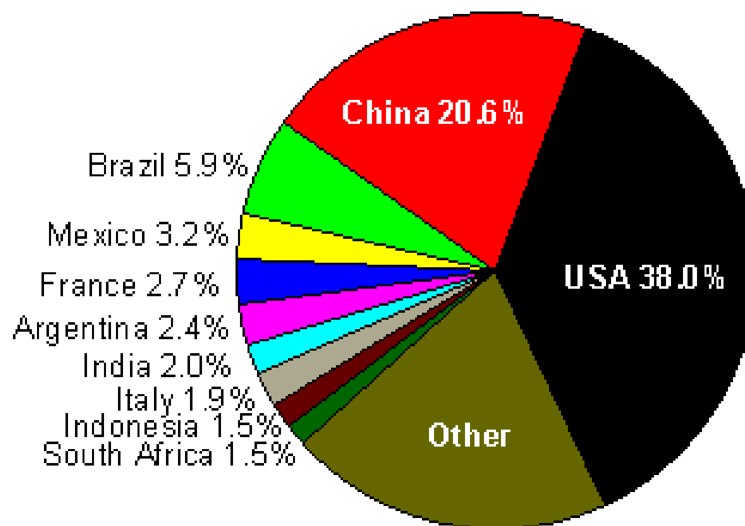

Figure 2: A representation of the world maize production for 2002 with the USA producing 229 million Metric tons (Mt) tons (http:www://orengonstate.edu/).

2.1 Biomass as a fuel source

2.1.1 The history of biofuels

The automotive industry has stayed virtually unchanged for the past few decades. Henry Ford and Nicholas Otto independently build engines in the late 1800's that used ethanol as fuel. The Model T (called the Quadricycle) was brought into production in 1908 with an adjustable carburettor that allowed it to use alcohol, petroleum or a blend named "gasohol" (Rosillo-Calle and Walter, 2006; Kovarik, 1998). Ethanol was primarily used as fuel in Europe (Germany, France and Italy) in the early 20th century, with ethanol production increasing by 60.5 million litres from 1887 to 1904 (Kovarik, 1998). The usage of alternative oils for fuel started around the same time as ethanol. Rudolf Diesel used peanut oil in engines as a form of liquid fuel in 1900 (Shay, 1993). Ethanol petroleum blends were widely used during the 1920's in all industrial countries excluding the USA. Europe produced ethanol from surplus food produce and paper mill waste, while Brazil and Australia made use of sugar cane (Kovarik, 1998).

The USA displayed a brief interest in ethanol during World War One due to the increasing scarcity of petroleum. After the war, the demand decreased due to petroleum abundance, which made petroleum supply less expensive compared to ethanol production (Kovarik, 1998). A combination of raised taxes and campaigning by petroleum producers led to a further loss of interest in ethanol

usage (Rothman et al., 1983). Ethanol production experienced a brief surge in the USA during the 1930's when the market value of maize dropped significantly. During the Second World War, ethanol production in especially Brazil and the USA again received significant interest due to the petroleum scarcity. After the end of the war, the petroleum production recovered and with its cheap availability, it kept the use of ethanol out of the market for almost 40 years (Hill, 2000). The constant discovery of new crude oil reserves also contributed to less interest in finding alternatives for a petroleum dependant world (Kovarik, 1998).

2.1.2 Global biofuels production

Organic material is viewed as the only sustainable resource for fuel production (Lynd et al., 1999). The major drawback of biomass as feedstock is that the availability is affected by seasonal changes and geographical locations. Plant biomass can be divided into sucrose containing feedstock, starchy substrates and lignocellulosic biomass (Balat et al., 2008). Sixty percent of the global bio-ethanol production is sourced from sugar cane with Brazil being the main producer (Lin and Tanaka, 2006), while the USA uses corn starch. Brazil and the USA together, produce more than 70% of the world's total bio-ethanol (Balat et al., 2008). Lignocellulose is the most abundant form of polysaccharides and has received much attention as a potential feedstock for bio-ethanol production (McAloon et al., 2000), but technologies for the conversion of cellulosic biomass to ethanol are still in its developmental stage and have not yet been demonstrated on a commercial scale (Balat et al., 2008).

The bio-ethanol production in the USA confirmed that a local biomass source should be used as substrate to overcome the limitations of supply and demand (Gray et al., 2006). A starch feedstock may also be supplemented by cellulosic agricultural waste in the future (Chen et al., 2007). The technology has been established to convert starch to bio-ethanol, but a reduction in production cost is required to produce bio-ethanol at a competitive price without the need for governmental subsidies. This reduction in cost can be accomplished by (1) using a less expensive feedstock (Table 1); (2) using a more efficient enzyme or a combination of enzymes for starch conversion (Gray et al., 2006); or (3) using a concept called Consolidated Bioprocessing (CBP). CBP is a single-step process where microorganisms hydrolyse the biomass to simple sugars while simultaneously fermenting the fermentable sugars to ethanol (Lynd et al., 1996).

Table 1: Different forms of biomass that could be used for the production of bio-ethanol and the expected theoretical yield (Linoj et al., 2006)

Feed stock	Bio-ethanol (l/ton)	Feed stock	Bio-ethanol (l/ton)
Barley	250	Sugar beet	110
Cassava	180	Sweet potato	125
Maize	360	Sweet sorghum	60
Potato	110	Wheat	340
Rice	430	Bagasse, cellulose	280
Sugar cane	70		

2.1.3 Biofuels on the African continent

Energy (and access thereto) has been a key factor assisting industrial growth and economic progress throughout history. It has been estimated that the energy supply in Africa and access thereto has to grow from 10% to at least 35% in the next 20 years to ensure positive growth on this continent (Singh and Sooch, 2004). Despite the continent's potential in harnessing renewable resources for the production of energy, a large number of countries are still importing energy (Table 2) (Marrison and Larson, 1996). Renewable energy exploitation in Africa would lead to foreign exchange savings for African countries that are currently dependant on foreign fossil fuels. It will also reduce the level of combustion emissions (preserve the air quality), reduce the environmental impact on the daily usage of traditional fuels, and stimulate the local agricultural sector. The extra revenue will also uplift the farming and rural community in the long term. The full impact of biofuels could only be fully understood when taking into consideration that more than one third of the African continent's population is starving and a large number of its residents has to survive on less than R20 a day.

The low level of modern energy usage on the African continent is a direct reflection on the overall poverty level and the usage of traditional energy (fire wood and coal). The African continent contains many land-locked countries that make the importation of foreign energy expensive to poverty stricken nations. The demand for African exports products are also declining, which is a major concern and setback to Africa's economy. Renewable energy could thus play a major role in filling this gap with self-sustainability that is confirmed by Brazil's success with biofuels produced from sugarcane (Biswas et al., 2001; Amigun et al., 2008).

Table 2: African countries that import and export energy (IEA, 2006)

Major energy exporters	Net energy exporters	Importers
Nigeria	Angola	Benin
Algeria	Cameroon	Eritrea
Libya	Congo	Ethiopia
South Africa	Democratic Republic of Congo	Ghana
Egypt	Cote d'Ivoire	Kenya
Gabon	Gabon	Morocco
Congo	Sudan	Mozambique
		Namibia
		Senegal
		Tanzania
		Togo
		Zambia
		Zimbabwe

First world countries and other developing countries have received significant support on legislative level for the production of bio-ethanol, whereas African countries have been generally left in the dark, despite its large potential (Marrison and Larson, 1996). Only countries on the southern tip of Africa (South Africa, Malawi, Swaziland, Mauritius and Zimbabwe) have ventured into bio-ethanol production. The lack of progress in Africa is mainly due to a lack of support on government. Most of the African countries are developing countries and lack social, economical and political stability which in turn also discourages foreign investment into sustainable energy projects. The instabilities on the African continent also results in the lack of a highly trained workforce required for self-sustainability (Amigun et al., 2008).

2.1.4 Biofuels in South Africa

Twenty percent of the gross domestic products (GDP) in South Africa are attributed to logistical expenses. Petroleum is largely used for personal and private use while diesel is primarily used for bulk produce transport. The limited crude oil resources has led the government to research other possibilities to substitute the country's fuel supply (Table 3). Liquid fuels produced from coal, supplying 37% of South Africa's current needs (Singh, 2006). The remaining 63% is dependent on the importation of crude oil from other countries. The introduction of light diesel engines to the personal transport market has led to diesel being viewed as a substitute for the petroleum market. Crude oil is one of SA's largest imports with a constantly varying price, therefore it makes a major contribution to the interest rate and thus to the general economy. This confirms the importance of

investing in an alternative fuel source. It would be beneficial to lower the current fossil fuel demand, but with the current transport fleet, it would be highly unlikely (Lynd et al., 2003).

Table 3: A summary of the current fuel situation in South Africa (Singh, 2006)

Option	Positives	Negatives	Current situation and future potential
Crude oil production	Lowest cost	Limited global reserves Non-renewable	±25 000 barrels a day offshore from the Southern Cape Limited reserves in future
Coal to liquid	Abundant for coal for ≥200 years	High capital expense. High levels of greenhouse gas emissions	± 150 000 barrels a day produced by Sasol Good future potential
Gas to liquids	Clean product	Limited gas reserves Non-renewable Limited by transport issues	±40 000 barrels a day produced by PetroSA SA reserves are limited. Can import by means of a pipeline from Mozambique
Biofuels	Cleanest fuel Reduction of GHG emissions High job creation Renewable and sustainable	Land intensive production Limited capacity	Requires governmental support

South Africa currently produces an excess of sugarcane and maize, which can act as feedstock for the production of bio-ethanol (Table 4). The excess maize and sugarcane is currently exported to other countries, thus its conversion to bio-ethanol would theoretically not affect the local food industry. Bio-ethanol costing should not therefore be based on the world food market, as only excess crops are being used. Therefore, the production costs of maize and sugarcane should be viable with the world price compensation. South Africa could therefore be a good producer of alternative fuel in view of the current climate and production surpluses. The current production of bio-ethanol is 400L/t of maize and 65L/t of sugar biomass, implying that South Africa can theoretically produce 800 million litres of ethanol per year (Lynd et al., 2003; Singh, 2006).

Table 4: The production and consumption of maize in South Africa (Singh, 2006)

Year	Production (1000 tons)	Consumption (1000 tons)	Surplus (1000 tons)
1997-1998	7203	6383	820
1998-1999	7461	6341	1120
1999-2000	11 001	6785	4216
2000-2001	7487	6924	563
2003-2004	8409	7751	658
2004-2005	9093	7956	1137
Average	**8605**	**6983**	**1623**

Current and future policies should be adjusted to create favourable frameworks (economical and legal) to benefit the biofuels market (Wiesenthal et al., 2009). European governments have already formed an organization to introduce and promote the usage of biofuels. Positive biofuel policies in Germany, France, Spain, Sweden, Italy, Austria, the UK, Portugal and Greece have contributed to an increase in biofuel consumption (Kondili and Kaldellis, 2007; van Dam et al., 2007), thus indirectly creating an alternative outlet for excess farm produce and the upliftment of rural areas. Sound governmental policies will also promote the reduction of greenhouse gas emissions and increase local energy supply security (reduction in oil imports) (Wiesenthal et al., 2009).

Initially government subsidies might be required to make the price of biofuels competitive with the current fossil fuel price. This will, however, lead to a loss in income for the government, but as the process becomes more cost-efficient, less subsidies will be required (e.g. Brazil). Fuel suppliers should be forced to introduce a fixed share of biofuels into their total fuel sales through governmental fuel policies. Past biofuel sales in Germany have shown that reduced taxation of biofuels has led to a vital promotion of sales (Wiesenthal et al., 2009).

2.2 Starch crops as biomass for the production of bio-ethanol

One of the major concerns regarding the production of biofuels is the use of a “universal” biomass such as starch. Unfortunately this raw material is also used as livestock feed and for human consumption, leading to competition with the feed/food markets and conflict with human rights groups in times of insufficient supply. The increase in feedstock demand could lead to an increase in food prices and also constrain the production of biofuels. Ideally more intensive agricultural practices would be required to increase the yields (more tons/ha) for these crops, which could ultimately lead to more pollution of natural resources due to the excessive use of fertilizers and pesticides to enhance crop production (von Blottnitz and Curran, 2007). Agricultural practices adopted during the cultivation of the biomass will therefore determine its environmental impact and long-term sustainability (Srinivasan, 2009).

The crops that are currently being used for the production of biofuels only account for approximately 4.2% of the total agricultural land globally (Rajagopal et al., 2007). The biofuel demand is expected to double in the next ten years, implying that its economic future is secured (Demirbas and Balat, 2006). A growing demand requires a growing production/supply, which would have an impact on global warming if deforestation is required to increase agricultural land (Srinivasan, 2009). Humans and livestock consume 48% and 35% respectively of the food grain currently produced; leaving approximately 17% for the production of biofuels and other needs. Agricultural, garden and municipal waste could also be used as feedstock, which would result in the

theoretical production of 491 billion litres of bio-ethanol each year, roughly 16 times more than the current output (Kim and Dale, 2004).

2.2.1 Current starch to bio-ethanol processes

A number of steps are required before bio-ethanol from biomass can be used as a fossil fuel supplement. These steps include the separation of the starch from the biomass, hydrolysis of the starch to simple sugars and the conversion of the sugars to ethanol. These processes are discussed below as generally applied in the USA (Kwiatkowski et al., 2006).

The production of bio-ethanol from corn starch makes use of two specific processes for the extraction of corn starch, i.e. the dry-mill and wet-mill processes (Figure 3) (Kim and Dale, 2008). During the dry-milling process, the maize kernels are hammered into coarse flour, which is cooked in water until the starch component becomes soluble. Amylase enzymes are introduced to hydrolyze the soluble starch, resulting in the release of glucose. The glucose-rich mash is then cooled and transferred to fermentation vessels where yeast is introduced to innitiate the fermentation process. After fermentation has taken place, the mash is distilled to obtain a high ethanol concentration end product. The alcohol-free mash is then further processed to obtain a protein-rich by-product that is used as animal feed. This by-product contains yeast, unfermented fibre and fat. About 1 kg by-product is produced for every kilogram of bio-ethanol produced. An increase in bio-ethanol production will therefore also lead to the increase of this low market value by-product (Belyea, et al 2004; Kelsall and Lyons, 2003; Schilling et al, 2004). Li *et* al. indicated that it would be possible to lower the overall by-product production and also increase the ethanol yield (Li et al., 2010) by using more effective starch hydrolyzing enzymes and including enzymes with an affinity for fibre structures.

During the wet-milling process, the maize kernels are added to water at 50°C, together with sulphur dioxide and lactic acid. A prolonged incubation period is necessary to soften the individual maize kernels. The maize kernels are then coarsely ground and separated into the germ and oils, fibrous plant material, starch and protein by means of a separator. The starch is hereafter subjected to a similar fermentation process as described with the dry-milling process (Ramirez et al., 2009).

The USA has proven that corn starch can be used with success for the production of bio-ethanol. The energy balance of producing ethanol from corn starch has, however, come under scrutiny by the industry. Some sections of the established processes could be altered to lower production cost and thus lower the overall cost of the final product, making it competitive with fossil fuel prices. Options for cost reduction would be (1) technological advances over a period of time, (2) genetic engineering of biomass used as substrate (higher sugar yield), and (3) optimizing the organism used for the fermentation (genetic engineering or adaptation) (Sticklen, 2006; Olempska-Beer et al., 2006).

Overall costing for the production of bio-ethanol is the lowest in Brazil, where the favourable climate, low cost of labour and a well-established infrastructure and governmental policies all contributed to the success (Yoosin and Sorapipatana, 2007).

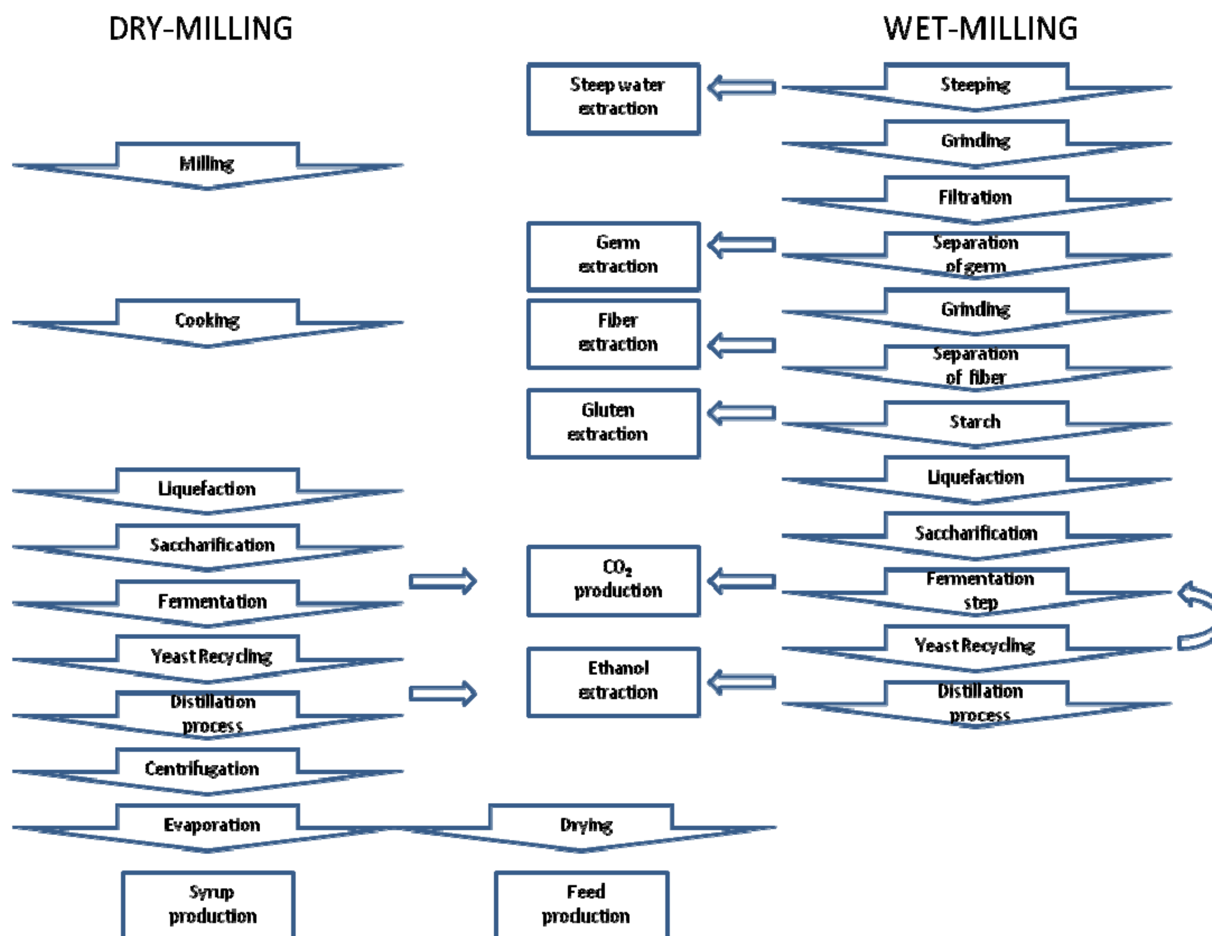

Figure 3: Bio-ethanol production through dry-mill and wet-mill processes. The USA produces more than 60% of the bio-ethanol through the dry milling process and nearly all new production plants are designed to use this method (Kim and Dale 2008).

The production costs of bio-ethanol are mainly affected by the price of the raw materials. Feed stocks account for more than 33% of the production costs of bio-ethanol, thus any means of lowering costs or maximizing yield is imperative (Balat et al., 2008). Ethanol production with the dry-milling process offers significant economic benefits with more money returned to the community than what is invested in the production of ethanol. The processes used in maize-based bio-ethanol production are directly influenced by the environmental production performance. Therefore, the amount of substrate (biomass and in this case maize) is directly influenced by the maize farming sites, which is based on the climatic variations, management of crop practices and soil properties

(Kim and Dale, 2008). The wet-milling process for maize is a conventional process for the extraction as well as the purification of starch and other co-products (e.g. germ, gluten, fibre and steep liquor). Shelled maize is the predominant substrate used in the wet-milling process and accounts for 75% of the overall production cost (Ramirez et al., 2009).

The hydrolysis of biomass and the fermentation of the resulting sugars to ethanol in a single step process would further lead to cost reductions using a single genetically engineered microorganism (Polagye et al., 2007). "Consolidated bioprocessing" will offer even further reductions in the production cost of bio-ethanol.

3. ENZYMATIC HYDROLYSIS OF STARCH

There has been a shift in the industrial conversion of starch to more simple structures through the usage of acid hydrolysis, which was discovered by Kirchoff in 1811 and de Saussure in 1815. It was, however, found that the yield of the desired end-product was low with large reversion reactions (James and Lee, 1997). A conversion has taken place over the past few decades where hydrolyzing enzymes have replaced the use of acid hydrolysis. Starch-converting enzymes comprise roughly 30% of global enzyme production, also including enzymes for other industrial applications including anti-staling agents for the baking industry and detergents for the laundry and porcelain industry (van der Maarel et al., 2002).

3.1 The origin and structure of starch

Plants have the unique characteristic of synthesizing biomass as a result of photosynthesis, a process during which light energy from the sun is converted into chemical energy. Starch is synthesized in the chloroplasts (Figure 4) of plants and accounts for a large fraction of the biomass. Starch is seen as a reserve energy storage compound that is metabolized through photorespiration during the absence of light. Plant organs such as tubers, seeds and roots are used for long-term storage of starch (Norouzian et al., 2005).

Starch is a homopolysaccharide consisting of D-glucose units linked primarily by α-1,4 bonds (Figure 5). Starch is present as small granules with its size being characteristic of its origin (Tester et al., 2004). Rice starch granules are 1-2 µm, whereas potato starch granules are approximately 100 µm in size. Starch contains two types of polysaccharides in varying concentrations: amylose (20-30%) and amylopectin (70–80%) (Peterson, 1998). Amylose forms a colloidal dispersion in hot water, whereas amylopectin is completely insoluble. Amylose is a flexible linear molecule consisting

of about a 1000 α-1,4-linked D-glucopyranose units with a molecular mass of $1x10^5$ to $1x10^6$ g/mole (Elmhurst, 2010; Tester et al., 2004). The amylose chain forms a spiral (resembling a coiled spring) due to the angle of the α-1,4 linkages.

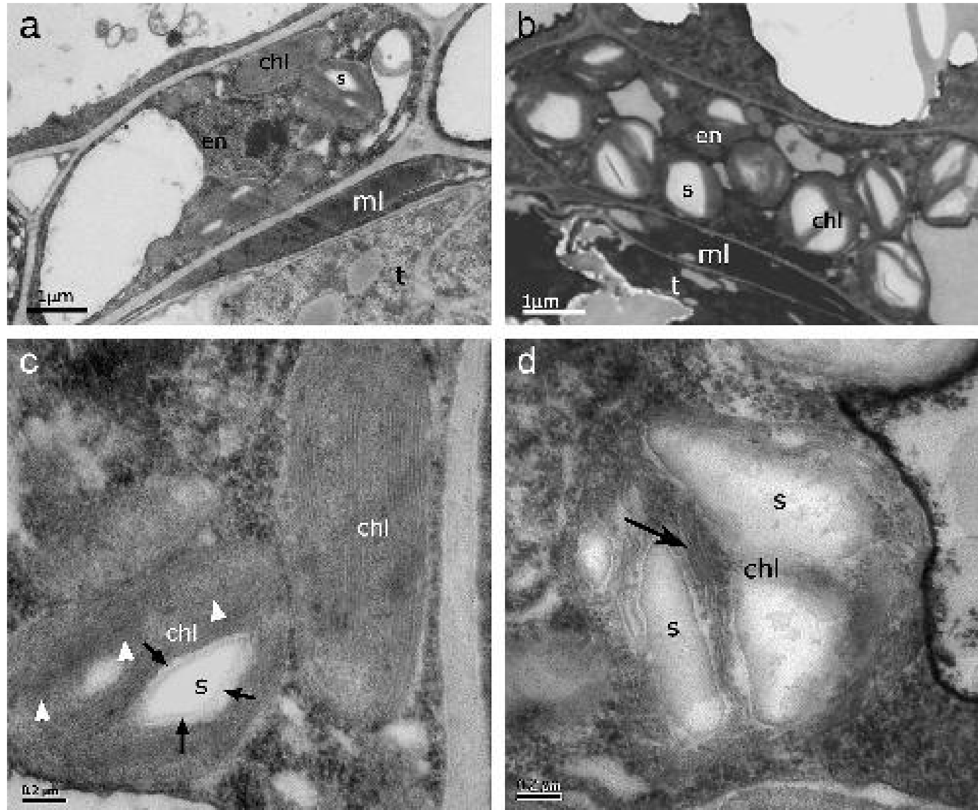

Figure 4: The starch molecules assemble in the chloroplast of plant cells: en, endothecium; chl, chloroplast; ml, middle layer; t, tapetum; and s, starch granule (http://www.cellbiolint.org/cbi/030/0583/cbi0300583f03.jpg).

Amylopectin consists of a α-1,4-D-glucopyranose chains with α-1,6 branching points found at 10 to 12 glucose unit intervals (Stevnebo et al., 2006) (Figure 5). It has a molecular mass of $1x10^7$ to $1x10^8$ g/mole (Elmhurst, 2010; Tester et al., 2004). Glycogen is similar to amylopectin, but the branching points are closer together, every 8 to 10 glucose units. Pullulan is another type of starch that consists of α-1,4 linked trisaccharides connected by an α-1,6 linkage.

Acids and enzymes can be used to hydrolyse starch into simpler carbohydrates called dextrins. The extend of the conversion is quantified as the dextrose equivalent (DE), which refers to the fraction of the glycosidic bonds that had been hydrolysed. Maltodextrin (DE 10-20) is an oligosaccharide used as a filler and thickener in the food industry, whereas maize syrups (DE30-70) are used as sweetners. Dextrose (DE100) is available as commercial glucose, prepared by the complete hydrolysis of starch (Daniel et al., 2000; Elmhurst, 2010).

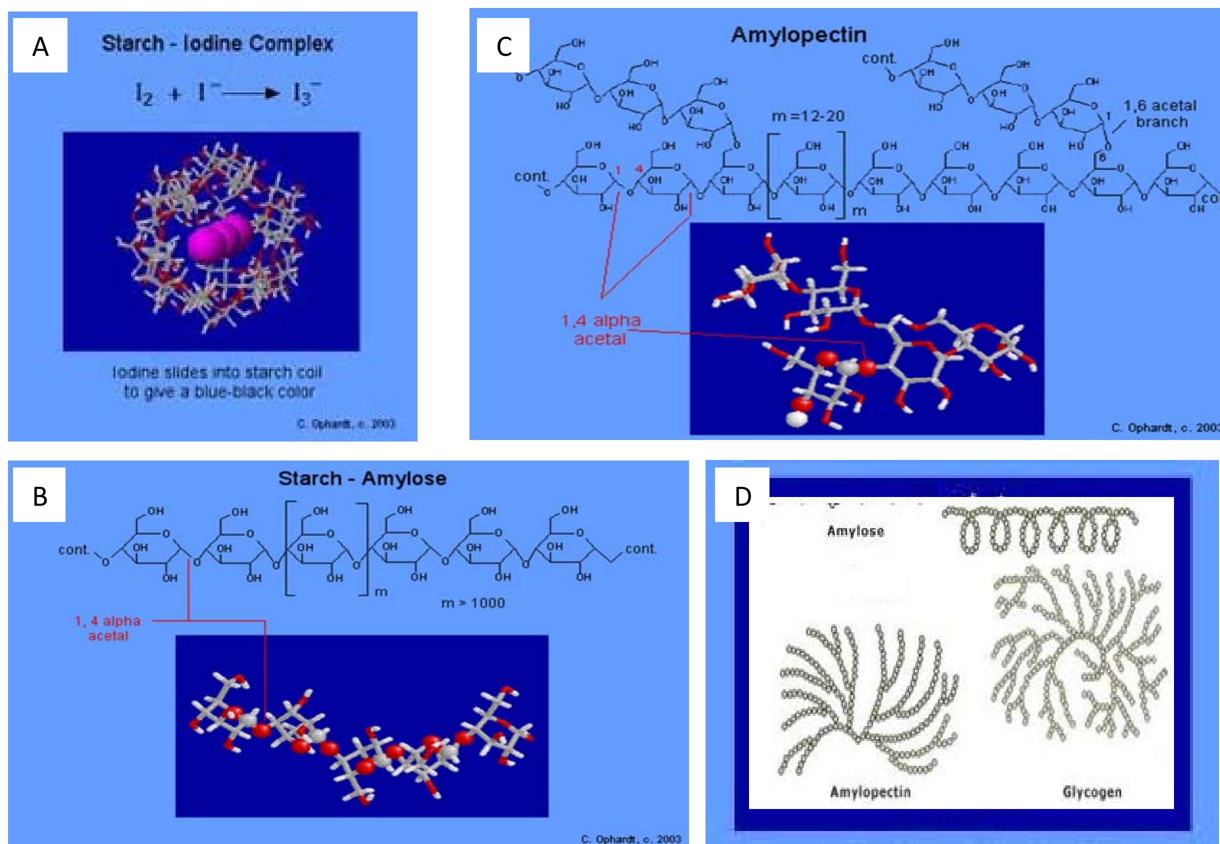

Figure 5: Amylose, amylopectin and glycogen are homopolysaccharides with different three-dimensional structures (http://www.elmhurst.edu/~chm/vchembook/547starch.html).

3.1.1 Soluble starch

The enzymes responsible for breaking down the starch structure are referred to as amylases. They can be sourced from animal, plant or microbial origin, with most sourced from microorganisms (Pandey et al., 2000). Isoamylases and α-amylases (EC 3.2.1.1) are endo-type enzymes (degrades the internal bonds of the starch) responsible for the liquefaction of starch (Figure 6). The isoamylases and pullulanases (EC 3.2.1.41) cleaves the α-1,6-bonds within the starch chain resulting in amylopectin being converted into amylose and oligosaccharides of various lengths (25-30 glucose-residues). The α-amylases hydrolyse the internal α-1,4-bonds of amylose and amylopectin at random, producing maltodextrins with a length of 10 to 20 glucose residues, as well as maltose and free glucose. The degradation of pullulan by pullulanases results in the formation of maltotriose as end-product (Gomes et al., 2003).

The β-amylases (EC 3.2.1.3) are exo-type enzymes, which hydrolyse the oligosaccharides from the non-reducing ends with the release of maltose residues. Maltases are responsible for hydrolyzing

the maltose into two D-glucose residues, whereas the glucoamylases (EC 3.2.1.3) release glucose from the non-reducing end of the dextrins (Miao et al., 2009; Saha et al., 1989; Kuriowa et al., 2005).

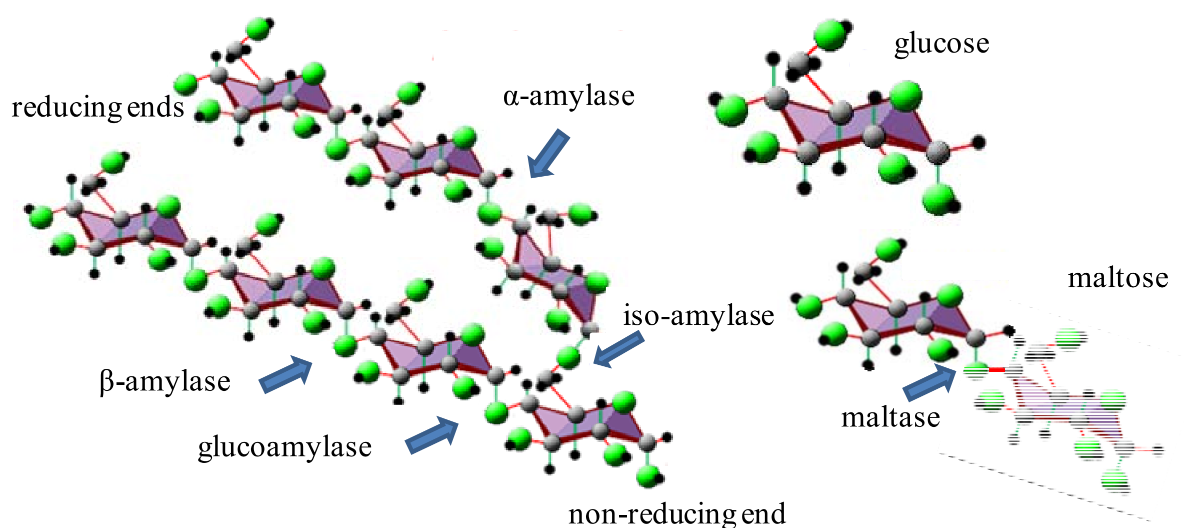

Figure 6: A schematic representation of a starch molecule with the different enzymes involved in the hydrolysis (http://www.elmhurst.edu.html). The β-amylase and glucoamylase can only act on an oligosaccharide once the non-reducing end is exposed by the action of the iso-amylase.

In some cases, α-amylases and glucoamylases would be sufficient for the complete degradation of amylases into D-glucose, depending on the composition and origin of the starch. The α-amylases could break down the starch into oligosaccharides, which forms the substrate for the glucoamylases, which in turn would then degrade the ends of the oligosaccharides resulting in the release of D-glucose. This implies that organisms producing these two enzymes would be able to hydrolyze amylose into D-glucose, but not necessarily utilize starch as a whole (Peterson, 1998).

3.1.2 Raw or native unmodified starch

Starch in its native state is referred to as raw or unmodified. The starch molecules are arranged in a dense polycrystalline state, which makes raw starch insoluble in cold water and results in the raw starch being resistant to enzymatic activity. Additional heating is therefore needed to gelatinize the raw starch in water (resulting in additional costs) prior to enzymatic degradation (Mitsuiki et al., 2005). Starch granules undergo gelatinization when it is heated above a specific temperature (an irreversible process). The breakage of the hydrogen bonds results in an uptake of water and overall swelling of the molecules, which lead to the disruption of the crystalline structure of the starch molecule. Leaching of the amylose molecules from the starch molecule takes place, resulting in the formation of soluble starch that is easy to hydrolyse (Daniel et al., 2000).

The temperature of gelatinization for most starches is between 60°C and 80°C, depending on the type of starch (Table 5). The swelling of the starch molecules and leaching of the starch have an effect on the viscosity of the paste solution. However, a portion of raw starch, commonly known as resistant starch, is not readily available for hydrolysis (Sharma et al., 2010) due to the physical structure rendering it inaccessible to the enzymes (Xie et al., 2006; Haralampu, 2000).

Table 5: Temperature of gelatinization for some starches (Zobel and Stephen, 2006)

	Temperature in °C		
Source	**Initiation**	**Midpoint**	**Completion**
Maize	62	67	72
Minot	61	66	71
Potato	50	60	68
Rice	66	72	78
Waxy maize	63	68	72
Wheat	52	58	64

Raw starch is difficult to degrade. In general, raw starch degrading organisms use only one raw starch degrading enzyme (RSDE) for hydrolysis, whereas a possible combination of RSDE would be more beneficial (Shigechi et al., 2002). The α-amylases hydrolyze the interior linkages of the starch molecule on the surface of the granules, supplying the glucoamylases with substrate (Figure 7). This hydrolysis results in the formation of small holes in the granular starch molecule, which allows the α-amylase entry into the interior of the starch molecule. Glucoamylases hydrolyse the shorter dextrins from the non-reducing ends of the starch molecule resulting in the exclusive formation of glucose (Figure 8). The combined action of the RSDE (α-amylases and glucoamylases) result in the complete degradation of raw starch into glucose units (Sun et al., 2010).

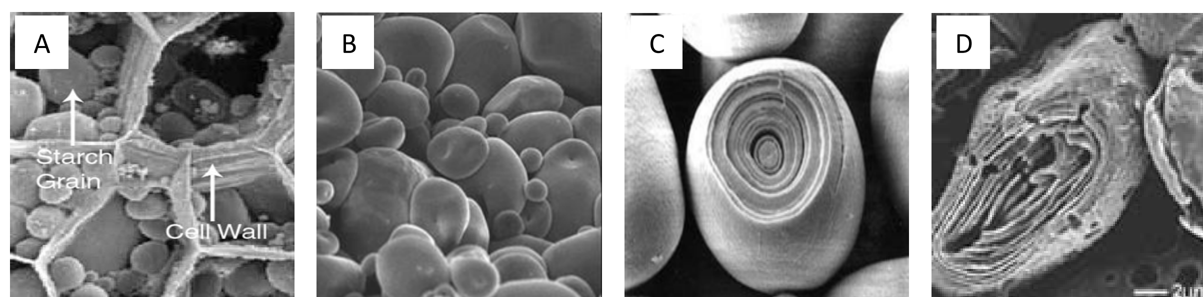

Figure 7: An image of granular corn starch visualized with electron microscopy (A) inside the plant cell, (B) intact granules, (C) the concentric layers inside the granule and (D) the granule after degradation by α-amylases (http://www.elmhurst.edu/~chm/vchembook/547starch.html).

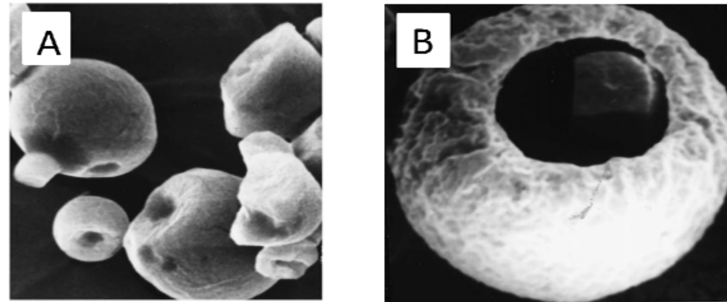

Figure 8: Scanning electron micrographs of (A) raw granular starch reacted upon by glucoamylase, (B) an enlarged single pitted granule (Dubey et al., 2007).

3.2 Enzymes required for starch hydrolysis

Efficient starch hydrolysis calls for the activities of both α-1,4 and α-1,6-debranching activities. Four groups of starch converting enzymes confer this activity and include endo-amylases, exo-amylases, debranching enzymes and transferases, of which enzymes from the first two groups are used in industries for starch hydrolysis. Endo-amylases display α-1,4-cleavage activity and include the α-amylases. Exo-amylases such as β-amylases only cleave α-1,4 glycosidic bonds, whereas glucoamylases and α-glucosidases display both α-1,4-cleavage and α-1,6-debranching activities (Sun et al., 2010).

3.2.1 Alpha-amylases

Alpha-amylases consists of hydrolases and transferases, which are multi-domain proteins with the similarity of each catalytic domain being in the form of a $(\beta/\alpha)_8$-barrel (eight parallel β-strands, surrounded by eight helices (Domain A in Figure 9). The active site of α-amylase is at the C-terminal end of the β-barrel strand (Farber and Petsko, 1990; MacGregor et al., 2001). Sequence similarities of the amino acid composition of the catalytic domain predicted that other hydrolyzing enzymes have a similar catalytic domain and thus fall in the same enzyme family (Katsuya et al., 1998).

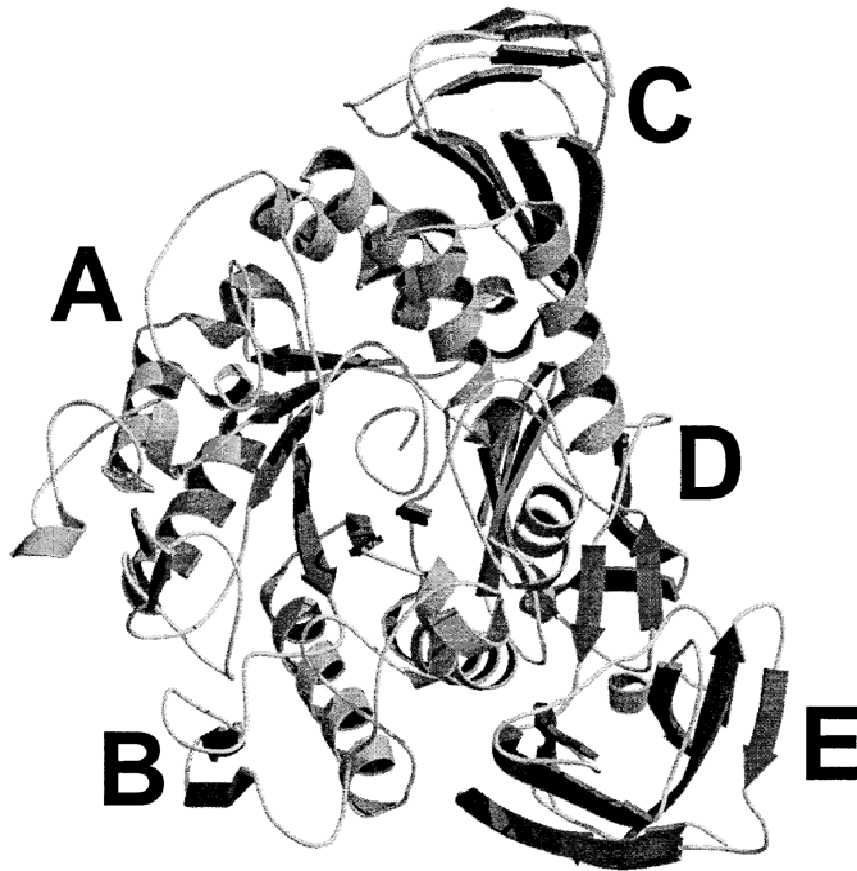

Figure 9: Ribbon model illustrating the individual domains in α-amylase (Lawson et al., 1994)

A large loop formed between the third β-strand and the third helix gives rise to Domain B. Domain A occurs at the N-terminal end of the protein in the majority of the α-amylases (Katsuya et al., 1998; Feese et al., 2000), whereas Domain C shields hydrophobic residues of Domain A from solvents, thus stabilizing it. This phenomenon might also assist with substrate binding (Dauter et al., 1999; Lawson et al., 1994). No role has yet been determined for Domain D, but Domain E is important for binding of granular starch. Domain E has also been found in other enzymes except α-amylase, for instance in glucoamylase (Hofmann et al., 1989; Jespersen et al., 1991). A number of enzymes were identified as part of the α-amylase family of enzymes (Table 6), with even more predicted to be part of the family.

Table 6: Different members of the α-amylase family (MacGregor et al., 2001)

Enzyme	EC Number
a) Enzymes with known three-dimensional structure	
α-Amylase	3.2.1.1
Oligo-1,6-glucosidase	3.2.1.10
Maltotetraohydrolase	3.2.1.60
Isoamylase	3.2.1.68
Maltogenic amylase	3.2.1.133
Neopullulanase	3.2.1.135
Malto-oligosyltrehalose trehalohydrolase	3.2.1.141
Amylosucrase	2.4.1.4
Cyclodextrin glucotransferase	2.4.1.19
Amylomaltase	2.4.1.25
b) Enzymes predicted to belong to the α-amylase family	
α-Glucosidases	3.2.1.10
Pullulanase (limit dextrinase)	3.2.1.41
Amylopullulanase	3.2.1.1/41
Cyclomaltodextrinase	3.2.1.54
Dextran glucosidase	3.2.1.70
Trehalose-6-phosphate hydrolase	3.2.1.93
Maltohexohydrolase	3.2.1.98
Maltotriohydrolase	3.2.1.116
Maltopentohydrolase	3.2.1.-
Branching enzyme	2.4.1.18
Glucan debranching enzyme	2.4.1.25/3.2.1.33
Maltosyl transferase	2.4.1.-
Dextran sucrase/Alternansucrase	2.4.1.5/2.4.1.140

During the catalytic process, α-amylase undergoes an anomeric configuration when the substrate is converted to product, i.e. the enzyme acts on α-linkages in glucans and yields α-linked products. The activity of α-amylase is believed to take place in a double displacement mechanism as shown in Figure 10.

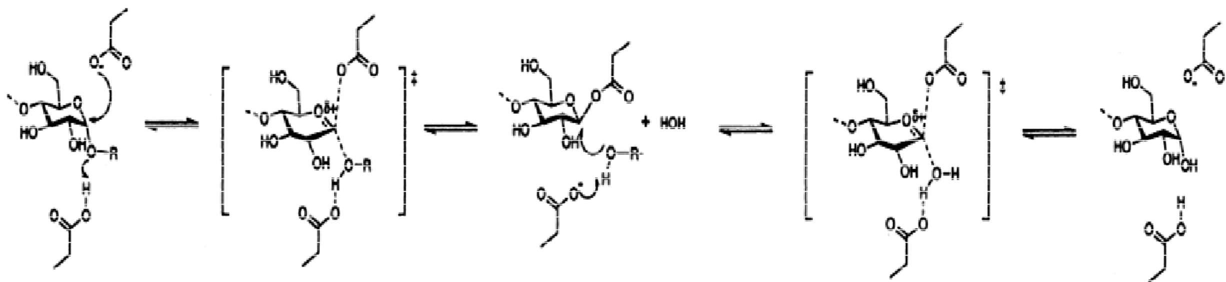

Figure 10: The catalytic steps used by α-amylases to hydrolyse glycosidic bonds (Macgregor et al., 2001)

An acid group on the enzyme protonates the glycosidic oxygen during the first displacement step, resulting in scission of the C1-O bond and thus the formation of an oxocarbenium ion-like transition state (Davies et al., 1998; Uitenhaag et al., 1999a). Next, the sugar anomeric centre is attacked by a nucleophilic acid group of amino acids, resulting in a β-glycosyl enzyme intermediate, while the aglycome of the substrate leaves the active site. This entire process is reversed during the second displacement when the anomeric centre is thereafter attacked by a water molecule that is activated by the carboxylate form of the former proton donor. Hereafter, an ion-like transition state takes place to produce an α-anomeric configuration and protonation of the original acid group. Transglycosylation can occur during the second displacement if the water is replaced by a free hydroxyl group of a sugar residue in the attacking group (Uitenhaag et al., 1999a; Uitenhaag et al., 1999b).

Extensive studies have been done on the DNA and amino acid sequences of α-amylases to assist in comparative studies of the different amylases that have been reported (Imanaka and Kuriki, 1999; Janacek, 1997), which display large variation in the length of the open reading frames (Kajiwara et al., 1997). DNA sequence analyses also assisted with functionality studies of its different domains (MacGregor et al., 2001), e.g. unravelling the evolutionary origin of the α/β-barrel of α-amylase (Farber and Petsko, 1990; Farber, 1993). As more α-amylase sequences are becoming known to scientists, it creates opportunities for further molecular studies as well as heterologous gene expression in other organisms (Janacek, 2002).

Amylases are one of the oldest enzymes to be used commercially, dating back to 1984 when used in the pharmaceutical industry for digestive disorders (Gupta et al., 2003). α-Amylases have been extensively applied in the bread and baking industry to produce higher volume, colour and softer texture (Hamer, 1995). Other enzymes, for example, lipases, pullulanases, cellullases and lipoxygenases, have been used in this industry for varied applications, but with none being able to compete with α-amylases (Si, 1999; Pintauro, 1979; Monfort, 1996).

Fungal α-amylases have already been used for over half a century in the baking industry in the USA and United Kingdom after confirmation of its GRAS status (Pritchard, 1992). α-Amylases have also been applied as an anti-staling agent in dough improvement (Gupta et al., 2003) and made a contribution to the textile industry for the desizing of textile fibres due to its ability to hydrolyse starch. It has also been used in the paper industry for low viscosity starch production for the coating of paper (Gupta et al., 2003) and in the detergent industry as a milder detergent relative to conventional detergents (van Ed et al., 1992). The physical properties of some α-amylases that have been reported are shown in Table 7.

Table 7: Properties of a selection of α-amylases (Robertson et al., 2006)

Organism	pH Optimum	Temperature Optimum (°C)	Raw starch Digestion
Aspergillus awamori	3.5	35	+
Aspergillus niger	3.5	35	+
Aspergillus fumigatus	5.5	35	+
Barley	4.5	40	+
Chalara paradoxa	5	30-40	+

+ = positive for raw starch digestion

3.3.2 Glucoamylase

Glucoamylase is known as an exo-acting enzyme that breaks down complex starch molecules from the outside towards the inside with the formation of β-D-glucose units. Glucoamylases have also been known as amyloglucosidase, glucoamylase or even I-amylase (Solomon, 1978). Glucoamylases could be found in a wide range of organisms including animals, microorganisms and plants. The glucoamylase enzyme normally consists of a catalytic domain, an O-glycosylated domain and a starch binding domain. (Ohnishi et al., 1992). The binding mechanism of glucoamylase originating from *A. awamori* was described by Goto *et al.* to cyclodextrins and raw starch (Goto et al., 1994). Glucoamylase forms an inclusion complex when β-cyclodextrin binds to the enzyme and the enzyme also recognizes the structure of the secondary OH-side, and not the primary OH-side (Goto et al., 1994). It has also been found that some *Aspergillus* spp. produce two forms of glucoamylase from the same structural gene.

It is evident that the gene size differs significantly within the glucoamylase family, and that some strains contain more than one glucoamylase (Table 8). It has thus been suggested that differential splicing of the mRNA results in two forms of the same enzyme in *A. niger* (Boel et al., 1984; Svensson et al., 1986). Other explanations for the different forms of glucoamylase include mRNA modification, the presence of a few structural genes, difference in carbohydrate content and limited proteolysis (Pretorius et al., 1991).

Table 8: Properties of some glucoamylases (James and Lee, 1997)

Organism	pH Optimum	Temperature Optimum (°C)	Molecular weight (d)	Raw Starch Digestion
Aspergillus awamori	4.5	60	83700-88000	+
Aspergillus niger I	4.5-5.0	60	99000	-
A. niger II	4.5-5.0	60	112000	-
A. niger	4.7	65	69810	-
var Teigham	3.5	60	89130	-
Aspergillus oryzae I	4.5	60	76000	+
A. oryzae II	4.5	50	38000	0
A. oryzae III	4.5	40	38000	0
Aspergillus saltoi	4.5	50	70000-90000	-
Clostridium thermohydrosulfuricum	4-6	75	-	-
Clostridium thermosaccharolyticum	5.0	70	75000	-
Flavobacterium sp.	5.5-6.5		-	-
Halobacterium sodomense RD-26	7.5	65	175000	-
Humicola lanuginosa K13/1	6.6	65-70	-	+
Rhizopus sp. I	4.5	40	74000	+
Rhizopus sp. II	4.5-5.0	-	58600	+
Rhizopus sp. III	4.5-5.0	-	61400	+
Rhizopus niveus	5.5	-	60000	+
Saccharomyces diastaticus I	-	-	68000	-
Saccharomyces diastaticus II	-	-	84000	-
Saccharomyces diastaticus III	-	-	79000	-
Lactobacillus amylovorus	6.0	60	47000	-

+ = positive for digestion; 0 = negative for digestion

Although glucoamylases are able to hydrolyse up to 100% of soluble starch, not all glucoamylases are capable of hydrolyzing raw starch (Table 8). Various studies have also been performed on the different binding domains of glucoamylases (Williamson and Belshaw, 1993) in an attempt to increase its substrate affinity (Juge et al., 2006). The same method has also been applied where the binding domain of *A. niger* has been linked to the glucoamylase of *Saccharomyces cerevisiae* var. *diastaticus* to increase its substrate affinity (Latorre-Garcia, 2005). Mutagenesis studies on the glucoamylase genes of *A. awamori* aimed to improve the thermostability of the enzyme and increase the overall glucose yield (Ford, 1999). Figure 11 indicates the binding of a glucoamylase to two starch molecules.

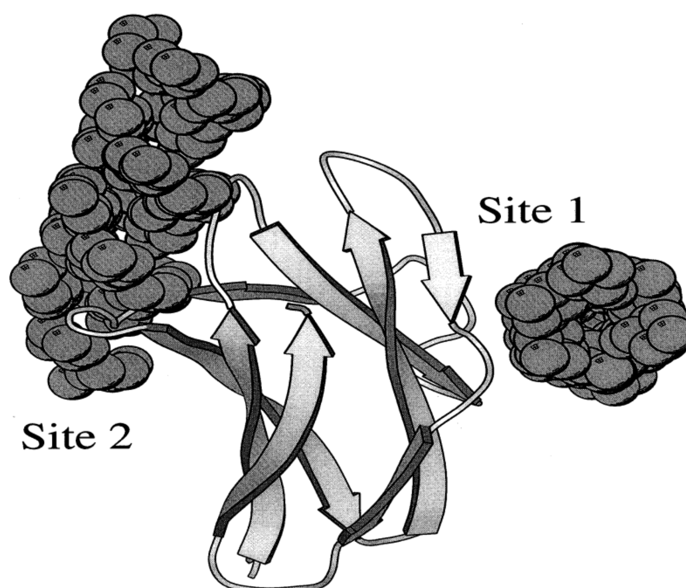

Figure 11: A ribbon model of the glucoamylase of *Aspergillus niger* starch binding domain and two starch molecules, indicating where the starch molecules would bind on its structure (Southall et al., 1999)

The catalytic reaction of glucoamylase takes place with the inversion of the configuration at the anomeric carbon. This process requires a pair of carboxylic acids at the active site of the enzyme that acts as a general acid with the anomeric carbon acting as a general base (McCarter and Withers, 1994). The hydrolytic mechanism of the glucoamylase consists out of three steps in which a proton is first transferred from the general acid catalyst to the glycosidic oxygen of the scissile bond. Hereafter the formation of an oxocarbenium ion take place and in the last step, a water-assisted nucleophilic attack is mediated by a general base catalyst (McCarter and Withers, 1994; Konstantindis and Sinnot, 1991; Tanaka et al., 1994). Glu179 was identified as the general acid and Glu400 as the general base in the catalytic processes of *A. awamori* and *A. niger* (Sierks et al., 1990; Svensson et al., 1990). Glucoamylase used in the industrial sector is predominantly isolated from *Aspergillus niger* and *Rhizopus* sp. for the production of glucose from malto-oligosaccharides (Pandey and Radhakrishnan, 1993), with *Aspergillus* being the most important industrial producer of products ranging from syrup production from starch to the production of secondary metabolites. Next to proteases, glucoamylases are the leading industrial enzyme with worldwide distribution and sales with a member of commercial production being developed (Table 9).

Table 9: Commercial glucoamylases (James and Lee, 1997)

Organism	Trade name	Company
Aspergillus niger	AMG 300 L	Novo Nordisk Biochem, N.A. Inc. Franklinton, N.C.
Aspergillus niger	Diazyme L-200	Solvay Enzymes, Elkhart, Indiana, U.S.A
Aspergillus niger	G-Zyme	Enzyme Biosystems Ltd., New York, N.Y., U.S.A
Aspergillus satoi	Molsin	Seishin Pharmaceutical Co., Japan
Endomethia bispora	Sure Curd	Phizer Inc., Milwaukee, Wisconsin, U.S.A
Rhizopus niveus	Glucozyme 12	Amano Enzymes U.S.A. Co. Ltd. Lombard, I.L., U.S.A
Rhizopus delemar	-	Miles Chemical Co., Elkhardt, Indiana, U.S.A.
Rhizopus delemar	-	Shin-Nihon Kagaku Kogyo Co. Japan
Rhizopus niveus	-	Seikagaku Kogyo Ltd., Japan

4. AMYLOLYTIC YEAST STRAINS

The aim of developing a raw starch-fermenting yeast is to reduce the processing costs of maize to ethanol. Having the yeast produce raw starch-degrading enzymes will reduce both the energetic and enzyme costs associated with raw starch conversion. Although some research groups have developed yeast strains that produce α-amylase and glucoamylase enzymes, none of the existing groups has developed yeast strains that produce sufficient quantities of raw starch-degrading enzymes to sustain growth and fermentation on this substrate for a commercially viable process.

Strains of *Saccharomyces cerevisiae* are currently being used for the conversion of glucose to bio-ethanol. Although some yeasts other than *Saccharomyces* have the capability of utilizing starch, they are not used in fermentation processes because of their low ethanol tolerance (McCann and Barnett, 1986) and repression of the catabolic systems (de Mot et al., 1984). Furthermore *S. cerevisiae* has a long history with the fermentation industry (wine and beer) and has GRAS status (Generally Regarded as Safe), making it the ideal host for the production of bio-ethanol from starch (Dziezak, 1987). *Saccharomyces cerevisiae* is unfortunately not capable of converting starch to glucose, but is able to utilize simple sugars as a carbon source in order to produce ethanol (Lin et al., 1998).

4.1 Expression of amylases in yeast

Previous studies with the expression of amylases in yeast have indicated that this molecular approach shows promise, but further research is required to optimise its expression and production in yeast (Randez-Gil et al., 1995). Table 10 indicates a selection of the different amylases that have

been successfully expressed in a yeast host. A number of successful expressions of amylases in *Saccharomyces* species have been reported, e.g. an α-amylase from *Aspergillus oryzae* in *Saccharomyces kluyveri* and *S. cerevisiae* (Moller et al., 2004). The two α-amylase genes of *Lipomyces kononkoae*, a *Saccharomycopsis fibuligera* glucoamylase (Knox et al., 2004), a wheat α-amylase (Rothstein et al., 1987), rice and germinating barley α-amylases were also successfully expressed in *S. cerevisiae* (Kumagai et al., 1990; Svensson and Sogaard, 1990; Juge et al., 1993), but were only able to hydrolyse soluble starch.

Cloning by PCR-based methods has resulted in the expression of some microbe's cDNA's in *S. cerevisiae* to yield amylolytic yeasts, i.e. yeast strains able to grow on starch as sole carbon source (Knox et al., 2004). The expression of the amylases in yeast is performed in one of two ways. In some instances the active enzymes are anchored to the cell wall of the yeast cell, resulting in a biocatalyst organism that can be constantly reused for further fermentations. The advantage of such a biocatalyst is that the risk of contamination is lowered during the fermentation process since the glucose that is produced by the activity of the enzymes, is readily utilised by the yeast. The disadvantage of the catalyst is that it is only able to hydrolyse the substrate in its immediate surroundings. In the alternative amylolytic yeast, enzymes are secreted and the amylolytic yeast therefore does not have to be in the immediate vicinity of the substrate to utilize the glucose produced by the free moving amylases. However, this is also to the amylolytic yeast's disadvantage as the glucose-enriched surroundings have a higher risk of contamination in comparison with the biocatalyst.

Table 10: Fungal amylases expressed in yeast

Source	Enzyme	Host	Reference
A. oryzae	α-amylase	*S. cerevisiae*	Randez-Gil et al., 1995
A. oryzae	α-amylase	*S. kluyveri*	Moller et al., 2004
A. oryzae	α-amylase	*S. cerevisiae*	Moller et al., 2004
Lipomyces kononenkoae	α-amylase	*S. cerevisiae*	Steyn et al., 1995
Rhizopus oryzae	glucoamylase	*S. cerevisiae*	Murai et al., 1998
Rhizopus oryzae	glucoamylase	*S. cerevisiae*	Shigechi et al., 2004
A. kawachii	α-amylase	*S. cerevisiae*	Kaneko et al., 1996

Amylases from different sources show differences in their modes of hydrolysis and thus different levels of ethanol yields could be expected with the expression of different amylases in a yeast cell (Planchot et al., 1995). For this reason, optimization studies are necessary to evaluate the expression levels of amylases (Leisola et al., 1980).

The yeast *S. cerevisiae* in particular is very good with the post-tanslational modification of heterologous gene expression, for instance the glycosylation of secreted proteins. Numerous other proteins have been expressed in *S. cerevisiae* for biological research, industrial and pharmaceutical purposes. These proteins have been secreted extracellularly, or exposed on the cell wall of the yeast itself or even intracellularly expressed for later isolation (Russo et al., 1995).

4.2 Simultaneous Saccharification vs. Consolidated Bioprocessing

A more energy-efficient process for the production of ethanol would require that hydrolysation of the biomass should result in a higher yield with lower processing costs (Gray et al., 2006). The hydrolysis temperature should thus be lowered for glucose production to that of the fermentation step, resulting in a simultaneous saccharification and fermentation step (SSF) as could be seen in Figure 12 (Devantier et al., 2005).

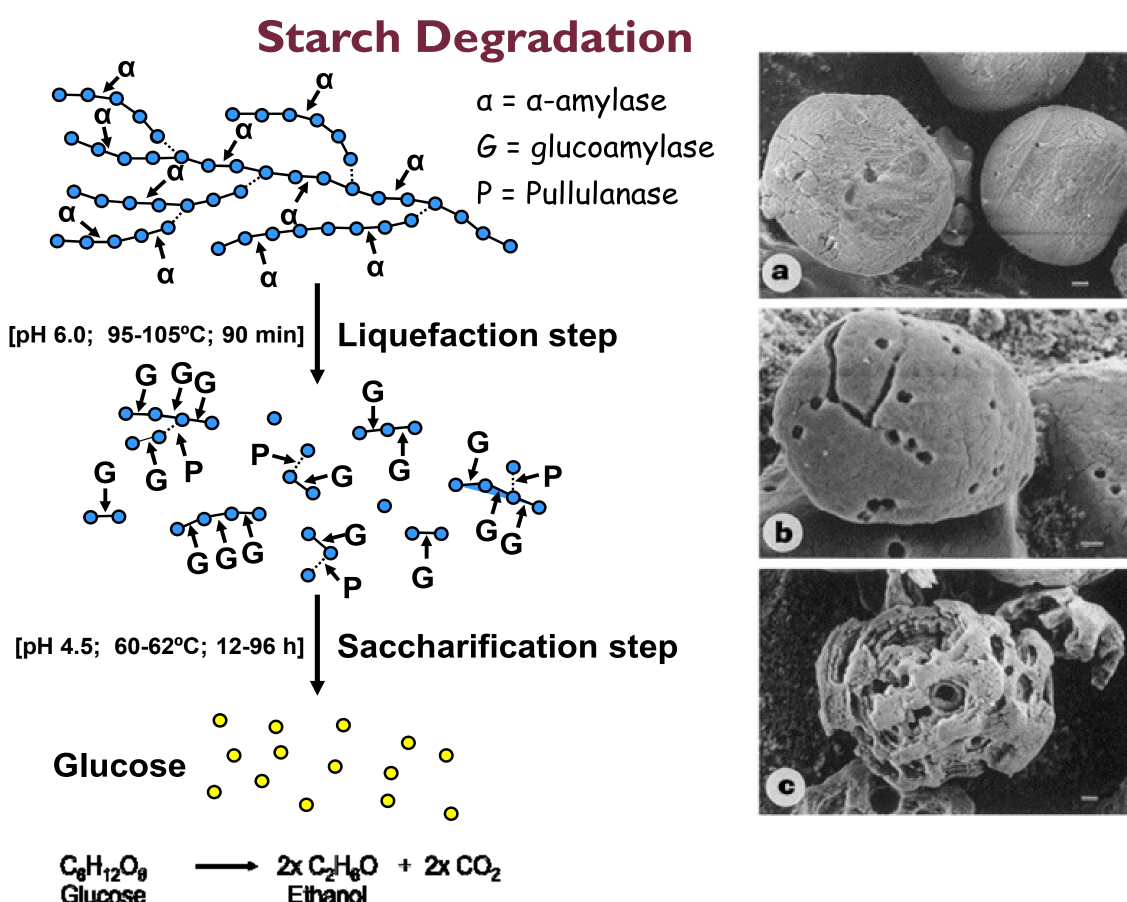

Figure 12: Basic steps involved in the liquefaction and saccharification of starch to glucose (WH van Zyl, Department of Microbiology, University of Stellenbosch).

Genecor has developed a raw starch hydrolysing cocktail, Stargen ™ 001, which converts starch into sugars during a SSF process (Figure 13). These sugars are then further fermented to yield ethanol. The drawback regarding such a process is the larger risk of contamination with lowered process temperatures (Shigechi et al., 2004). A genetically engineered organism which produces the nessesary amolytic enzymes would be able to hydrolyse and further convert the end product of the hydrolysis step into ethanol. Such an organism would be ideal for a Consolidated Bioprocessing (CBP) process (Lynd et al., 1999). It would thus be possible to convert biomass into ethanol in a single step in a biorefinery environment without the addition of commercial enzymes and also so reducing the risk of contamination.

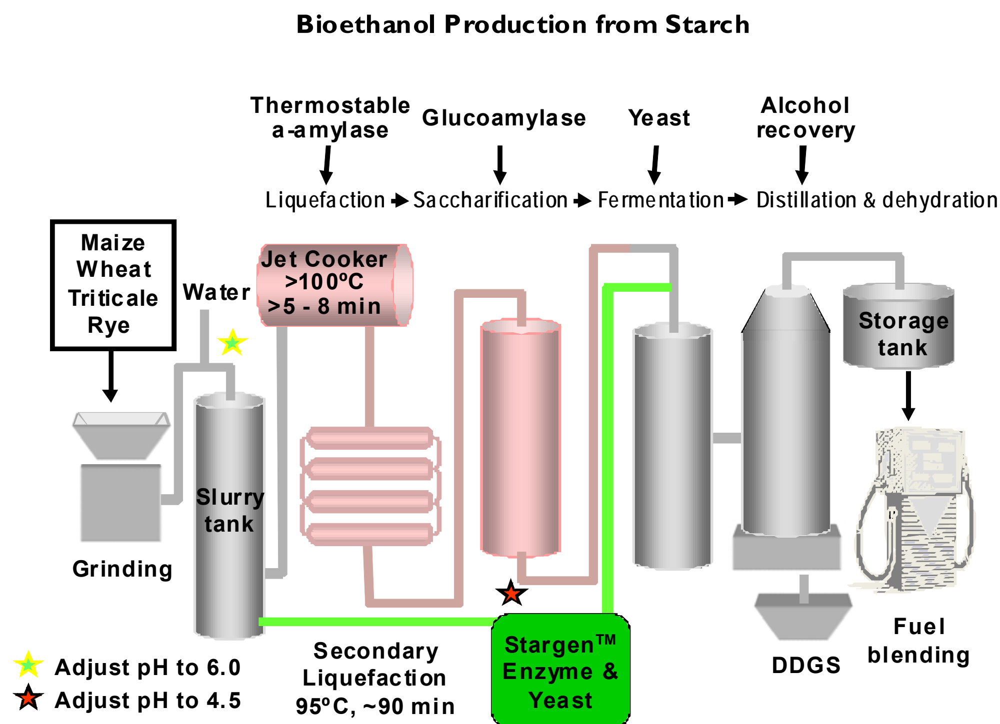

Figure 13: Traditional bioethanol production from starch involved liquefaction, saccharification and fermentation. The addition of amylases (such as Stargen™) omitted the need for cooking in Simultaneous Saccharification and Fermentation (WH van Zyl, Dept. Microbiology, University of Stellenbosch).

4.3 Consolidated Bioprocessing of Raw Starch – dream or reality?

The success of consolidated bioprocessing depends on the development of strains that are efficient in utilizing the substrate (raw starch, in this case) while producing high levels of ethanol. This would require genetic engineering of *S. cerevisiae*, which had already been successfully applied for the efficient utilization of other polysaccharides (Polagye et al., 2007). A recombinant yeast strain needs

to be constructed that can successfully produce and secrete raw starch degrading α-amylases (Carlsen et al., 1996) and glucoamylases. This strain would theoretically lead to higher ethanol production from starch without requiring pre-treatment of the substrate (Mukerjea et al., 2006; Barton et al., 1996). This would be beneficial if the different amylases originated from the same organism, thus complementing one another due to evolutionary development and compatibility.

Constructing an amylolytic yeast strain require the genetic engineering of an industrial yeast strain that can produce high levels of heterologous amylolytic activity. In the case of the raw starch-degrading yeast, glucoamylases and α-amylases capable of hydrolysing raw starch are required, but only a limited number of these are currently known. Bioprospecting for novel amylolytic enzymes is therefore essential to generate a sufficiently large collection of raw starch-degrading glucoamylases and α-amylases that can be screened for expression in yeast, The enzyme of choice should have high specific activity, high threshold and be available at low cost. In terms of biofuels, a combination of enzymes with these characteristics is required, which make the process even more expensive (Keller et al., 2001; Powell et al., 2001).

Limited successful results have been reported on the hydrolysis and utilization of raw starch by a single amylolytic transformed yeast strain. Different combinations of α- and glucoamylase genes have been expressed in yeast with the hope of a more refined hydrolysis system and thus higher ethanol yields. As previously discussed, numerous studies focused on the expression of different amylases in yeast, with some involving genetic modification before expression in yeast to optimize its expression and activity (Fang and Ford, 1998; Liu et al., 2000).

In this study, several fungal strains were screened for the presence of α- and/or glucoamylases. The α- and glucoamylases were also further investigated for their hydrolytic properties towards raw corn starch as substrate. Potential candidates were then used as a source of genes to be cloned for heterologous expression in yeast strains to create an amylolytic yeast strain capable of hydrolyzing and fermenting raw starch.

5. REFERENCES

Adamson KA (2004) Hydrogen from renewable resources – the hundred year commitment. *Ener Pol* 32:1231-1242

Amigun B, Sigamoney R, and von Blottnitz H (2008) Commercialisation of biofuel industry in Africa: A review. *Rev Sust Ener Rev* 12:690-711

Atkutsu Y, Toyoda F, Tomita KI, Yoshizawa F, Tamura M, and Yoshida T (1991) Effect of exhaust from alcohol fuel on ozone formation in the atmosphere. *Atmos Environ* 25A:1383–1389

Auto/Oil Air Quality Improvement Research Program (1992) Gasoline Reformulation and Vehicle Technology Effects on Exhaust Emissions. *Tech Bullet* 17:1–18

Balat M, Balat H, and Oz C (2008) Progress in bioethanol processing. *Prog Ener Combust Sci* 34:551-573

Barton S, Bullock C, and Weir D (1996) The effects of ultrasound on the activities of some glycosidase enzymes of industrial importance. *Enzyme Microb Technol* 18:190-194

Belyea RL, Rausch KD, and Tumbleson ME (2004) Composition of corn and distillers dried grains with solubles from dry grind ethanol processing. *Bioresource Technol* 94:293-298

Biswas WK, Bryce P, and Diesendorf M (2001) Model for empowering rural through renewable energy technologies in Bangladesh. *Environ Sci Policy* 4:333-344

Blake DR, and Rowland FS (1995) Urban leakage of liquefied petroleum gas and its impacts on air quality in Mexico City. *Sci* 269:953–956

Boel E, Hjort I, Svensson B, Norris F, Norris KE and Fill, N.P. (1984) Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs. *EMBO J* 3:1097-1102

Carlsen M, Nielsen J, and Villadsen J (1996) Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. *J Biotechnol* 45:81-93

Chen M, Xia L, and Xue P (2007) Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. *Int Biodeter Biodegr* 59:85-89

Corbett JJ, and Fischbeck PS (1997) Emissions from ships. *Sci* 278:823–824

Daniel JR, Whistler RL, and Röper H (2000) Starch. *Ullmann's Encyclopedia of Industrial Chemistry*, John Wiley & Sons, Inc., pp 1-25

Dauter Z, Dauter M, Brzozowski AM, Christensen S, Borchert TV, Beier L, Wilson KS, and Davies GJ (1999) X-ray structure of Novamyl, the five-domain 'maltogenic' α-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 Å resolution. *Biochem* 38:8385-8392

Davies G, Sinnott ML, and Withers SG (1998) Cycosyl transfer, in: M. Sinnott (Ed), Comprehensive Biological Catalysis, Academic Press, New York pp 119-208

de Mot R, Andries K, and Verachtert H (1984) Comparative study of starch degradation and amylase production by ascomycetous yeast species. *Syst Appl Microbiol* 5:106-118

Demirbas A (2006a) Progress and recent trends in biofuels. *Prog Energy Combust Sci* 33:1-18

Demirbas A (2006b) Hazardous emissions, global climate change and environmental precautions. *Energy Sources B* 1:75-84

Demirbas MF, and Balat M (2006) Recent advances on the production and utilization trends in bio-fuels: A global perspective. *Ener Convers Manage* 47:2371-2381

Devantier R, Pedersen S, and Olsson L (2005) Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. *Appl Microbiol Biotechnol* 68:622-629

Dreher K, Jaskot R, Richards JH, Lehmann JR, Winsett D, Hoffman A, and Costa D (1996) Acute pulmonary toxicity of size-fractionated ambient air particulate matter. *Amer J Resp Crit Care Medi* 153:A15

Dubey AK, Suresh C, Kavitha R, Karanth NG and Umesh-Kumar S (2007) Evidence that the glucoamylase and α-amylase secreted by *Aspergillus niger* are proteolytically processed products of a precursor enzyme. *FEBS Lett* 471:251-255

Dziezak JD (1987) Yeasts and yeast derivatives: Definitions, characteristics, and processing. *Food Technol* 44:104-121

Elmhurst (http://www.elmhurst.edu/~chm/vchembook/547starch.html) Cited 25 October 2010

Fang TY, and Ford C (1998) Protein engineering of *Aspergillus awamori* glucoamylase to increase its pH optimum. *Prot Eng* 11:383-388

Farber GK (1993) An α/β-barrel full of evolutionary trouble. *Curr Opinion Struct Biol* 3:409-412

Farber GK, and Petsko GA (1990) The evolution of α/β barrel enzymes. *Trends Biochem Sci* 15:228-234

Feese MD, Kato Y, Tamada T, Kato M, Komeda T, Miura Y, Hirose M, Hondo K, Kobayashi K, and Kuroki R (2000) Crystal structure of glycosyltrehalose trehalohydrolase from the hyperthermophilic archaeum *Sulfolobus solfataricus*. *J Mol Biol* 301:451-464

Ford C (1999) Improving operating performance of glucoamylase by mutagenesis. *Curr Opin Biotechnol* 10:353-357

Gaffney JS, and Marley NA (2009) The impacts of combustion emissions on air quality and climate – From coal to biofuels and beyond. *Atmos Environ* 43:23-36

Gomes I, Gomes J, and Steiner W (2003) Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: production and partial characterization. *Biores Technol* 90:207-214

Goto M, Tanigaw K, Kanlayakrit W, and Hayashida S (1994) The mechanism of binding of glucoamylase I from *Aspergillus awamori var. kawachi* to cyclodextrins and raw starch. *Biosci Biotech Biochem* 58:49-54

Gray KA, Zhao L, and Emptage M (2006) Bioethanol. *Curr Opin Chem Biol* 10:141-146

Gupta R, Gigras P, Mohapatra H, Goswami VK, and Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. *Process Biochem* 38:1599-1616

Hamer RJ (1995) Enzymes in the baking industry. In: Tucker GA, Woods LFJ, (eds). Enzymes in food processing. Galsgow: Blackie Academic and Professional pp 190-222

Haralampu SG (2000) Resistant starch: a review of the physical properties and biological impact of RS3. *Carbohydr Poly* 41:285-292

Hill K (2000) Fats and oils as oleochemical raw materials. *Pure Appl Chem* 72:1255-1264

Hoel M, and Kverndokk (1996) Depletion of fossil fuels and the impacts of global warming. *Resour Energy Econ* 18:115-136

Hofmann BE, Bender H, and Schulz GE (1989) Three-dimentional structure of cyclodextrin glucosyltransferase from *Bacillus circulans* at 3.4 Å resolution. *J Mol Biol* 209:793-800

IEA. Key world energy statistics 2005, IEA books, France, 2006

Imanaka T, and Kuriki T (1999) The concept of the α-amylase family: Structural similarity and common catalytic mechanism. *J Biosci Bioengineer* 87:557-565

James JA, and Lee BH (1997) Glucoamylases: Microbial sources, industrial applications and molecular biology – a review. *J Food Biochem* 21:1-52

Janecek S (1997) α-Amylase family: Molecular biology and evolution. *Prog Biophys molec Biol* 67:67-97

Janecek S (2002) How many conserved sequence regions are there in the α–amylase family? *Biologia, Bratislava* 57:29-41

Jespersen HM, MacGregor EA, Sierks MR, and Svensson B (1991) Comparison of the domain-level organization of starch hydrolases and related enzymes. *Biochem J* 280:51-55

Juge N, Nohr J, Le Gal-Coëffet MF, Kramhoft B, Furniss CSM, Planchot V, Archer DB, Williamson G, and Svensson B (2006) The activity of barley α-amylase on starch granules is enhanced by fusion of a starch binding domain from *Aspergillus niger* glucoamylase. *Biochem Biophys J* 1764:275-284

Juge N, Sogaard M, Chaix JC, Martin-Eauclaire MF, Svensson B, Marchis-Mouren G, and Guo XJ (1993) Comparison of barley malt α-amylase isozymes 1 and 2: construction of cDNA hybrids by in vivo recombination and their expression in yeast. *Gene* 130:159-166

Kajiwara Y, Takeshima N, Ohba H, Omori T, Shimoda M, and Wada H (1997) Production of acid-stable α-amylase by *Aspergillus kawachii* during barley Shochu-Koji production. *J Fermen Bioeng* 84:224-227

Kaneko A, Sudo S, Takayasu-Sakamoto Y, Tamura G, Ishikawa T, and Oba T (1996) Molecular cloning and determination of the nucleotide sequence of a gene encoding an acid-stable α-amylasefrom *Aspergillus kawachii*. *J Fermen Bioeng* 81:292-298

Katsuya Y, Mezaki Y, Kubota M, and Matsuura Y (1998) Three-dimentional structure of *Pseudomonas* isoamylase at 2.2Å resolution*. J Mol Biol* 281:885-897

Keller K, Friedman T, and Boxman A (2001) The bioseparation needs of tomorrow. *Trends Biotechnol* 19:438-441

Kelsall DR and Lyons TP (2003) Grain dry milling and cooking procedures: extracting sugars in preparation for fermentation. In: Jacques KA, Lyons TP, and Kelsall DR (eds) *The Alcohol Textbook*, 4th edition. Nottingham University Press, Nottingham, United Kingdom pp 9-21

Kim S, and Dale BE (2004) Global potential bioethanol production from wasted crops and crop residues. *Biomass Bioener* 26:361-375

Kim S, and Dale BE (2008) Life cycle assessment of fuel ethanol derived from corn grain via dry milling. *Biores Technol* 99:5250-5260

Kirchstetter TW, Singer BC, Harley RA, Kendall GR, and Chain W (1996) Impact of oxygenated gasoline use on California light-duty vehicle emissions. *Environ Sci Technol* 30: 661–670

Kjarstad J, and Johnsson F (2009) Resources and future supply of oil. *Ener Pol* 37:441-464

Knox AM, du Preez JC, and Kilian SG (2004) Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. *Enzyme Microb Technol* 34:453-460

Kondili EM, and Kaldellis JK (2007) Biofuel implementation in East Europe: current status and future prospects. *Renew Sust Ener Rev* 11:2137-2151

Konstantindis A and Sinnot ML (1991) The interaction of 1-fluoro-D-glucopyranosyl fluride with glucosidases. *Biochem J* 279:587-593

Kovarik B (1998) Henry Ford, Charles Kettering and the “fuel of the future”. *Auto Hist Rev* 32:7-27

Kumagai MH, Shah M, Terashima M, Vrkljan Z, Whitaker JR, and Rodriquez RL (1990) Expression and secretion of rice α-amylase by *Saccharomyces cerevisiae*. *Gene* 94:209-216

Kuriowa T, Shoda H, Ichikawa S, Sato S, and Mukataka S (2005) Immobilization and stabilization of pullulanase from *Klebsiella pneumonia* by a multipoint attachment method using activated agar gel supports. *Process Biochem* 40:2637-2642

Kwiatkowski JR, McAloon AJ, Taylor F, and Johnston DB (2006) Modelling the process and costs of fuel ethanol production by the corn dry-grind process. *Ind Crop Prod* 23:288-296

Latorre-Garcia L, Adam AC, Manzanares P, and Polaina J (2005) Improving the amylolytic activity of *Saccharomyces cerevisiae* glucoamylase by the addition of a starch binding domain. *J Biotechnol* 118:167-176

Lawson CL, van Montfort, Strokopytov B, Rozenboom HJ, Kalk KH, de Vries GE, Penninga D, Dijkhuizen L, and Dijkstra BW (1994) Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form. *J Mol Biol* 236:590-600

Leisola M, Ojamo H, and Kauppinen V (1980) Measurement of α-amylase and glucoamylase activities produced during fermentation. *Enzyme Microb Technol* 2:121-125

Li C, Rodríguez LF, Khanna M, Spaulding AD, Lin T, and Eckhoff SR (2010) An engineering and economic evaluation of quick germ–quick fiber process for dry-grind ethanol facilities: Model description and documentation. *Biores Technol* 101:5275-5281

Lin LL, Ma YJ, Chien HR, and Hsu WH (1998) Construction of an amylolytic yeast by multiple integration of the *Aspergillus awamori* glucoamylase gene into a *Sachharomyces cerevisiae* chromosome. *Enzyme Micro Technol* 23:360-365

Lin Y, and Tanaka S (2006) Ethanol fermentation from biomass resources: Current state and prospects. *Appl Microbiol Biotechnol* 69:627-642

Linak WP, Miller CA, and Wendt JOL, (2000) Comparison of particle size distributions and elemental partitioning from the combustion of pulverized coal and residual fuel oil. *J Air Waste Manage Ass* 50:1532–1544

Linoj Kumar NV, Dhavala P, Goswami A, and Maithel S (2006) Liquid biofuels in South Asia: resources and technologies. *Asian Biotechnol Develop Rev* 8:31-49

Liu HL, Doleyres Y, Coutinho PM, Ford C, and Reilly PJ (2000) Replacement and deletion mutations in the catalytic domain and belt of *Aspergillus awamori* glucoamylase to enhance thermostability. *Prot Eng* 13:655-659

Lynd LR, Elander RT, and Wyman CE (1996) Likely features and costs of mature biomass ethanol technology. *Appl Biochem Biotechnol* 57-58:741-761

Lynd LR, von Blottnitz H, Tait B, de Boer J, Pretorius IS, Rumbold K, and van Zyl WH (2003) Converting plant biomass to fuels and commodity chemicals in South Africa: a third chapter? *South African J Sci* 99:499-507

Lynd LR, Wyman CE, and Gerngross TU (1999) Biocommodity engineering. *Biotechnol Prog* 15:777-793

MacGregor EA, Janecek S, and Svensson B (2001) Relationship of sequence and structure to specificity in the α-amylase family of enzymes. *Biochem Biophysiol Acts* 1546:1-20

MacLean HL, and Lave LB (2003) Evaluating automobile fuel/propulsion system technologies. *Prog Ener Combust Sci* 29:1-69

Marmer E, Vignatti E, Langmann B, Dentener F, Hjorth J, Velchev K, Cavalli F, and van Aatdenne JA (2007) Assessment of impact of ship emissions over the summertime Mediterranean. EOS Transactions. American Geophysical Union. 88, Fall Meeting Supplement, Abstract A14D-08.

Marrison CI, and Larson ED (1996) A preliminary analysis of the biomass energy production potential in Africa in 2005 considering projected land needs for food production. *Biomass Bioener* 10:337-351

McAloon A, Taylor F, Yee W, Ibsen K, and Wooley R (2000) Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks. Report. http://www.doe.gov/bridge

McCann AK, and Barnett JA (1986) The utilization of starch by yeasts. *Yeasts* 2:109-115

McCarter JD, and Withers SG (1994) Mechanisms of enzymatic glycoside hydrolysis. *Curr Opin Struct Biol* 4:885-892

McNair L, Russell A, and Odman MT (1992) Airshed calculation of the sensitivity of pollutant formation to organic compound classes and oxygenates associated with alternative fuels. *J Air Waste Manage Ass* 42:174–178.

Miao M, Jiang B, and Zhang T (2009) Effect of pullulanase debranching and recrystallization on structure and digestibility of waxy maize starch. *Carbohydr Poly* 76:214-221

Mitsuiki S, Mukae K, Sakai M, Goto M, Hayashida S, Furukawa K (2005) Comparative characterization of raw starch hydrolyzing amylases from various *Bacillus* strains. *Enzyme Microb Techno*l 37:410-416

Moller K, Sharif MZ, and Olsson L (2004) Production of fungal α-amylase by *Saccharomyces kluyveri* in glucose-limited cultivations. *J Biotechnol* 111:311-318

Monfort A, Blasco A, Preito JA, and Sanz P (1996) Combined expression of *Aspergillus nidulans* endoxylanase X-24 and *Aspergillus oryzae* α-amylase in industrial baker's yeast and their use in bread baking. *Appl Environ Microbiol* 62:3712-3715

Mukerjea R, Slocum G, Mukerjea R, and Robyt JF (2006) Significant differences in the activities of α-amylases in the absence and presence of polyethylene glycol assayed on eight starches solubilised by two methods. *Carbohydr Res* 341:2049-2054

Murai T, Yoshino T, Ueda M, Haranoya I, Ashikari T, Yoshizumi H, and Tanaka A (1998) Evaluation of the function of arming yeast displaying glucoamylase on its cell surface by direct fermentation of corn to ethanol. *J Fermen Bioengineer* 6:569-572

Norouzian D, Akbarzadeh A, Scharer JM, and Young MM (2005) Fungal glucoamylases. *Biotech Adv* 24:80-85

Ohnishi H, Kitamura H, Minowa T, Sakai H, and Ohta T (1992) Molecular cloning of a glucoamylase gene from a thermophile *Clostridium* and kinetics of the cloned enzyme. *Eur J Biochem* 207:413-418

Olempska-Beer ZS, Merker RI, Ditto MD, and DiNovi MJ (2006) Food-processing enzymes from recombinant microorganisms – a review. *Reg Toxic Pharma* 45:144-158

Oros DR, and Simoneit BRT (2000). Identification and emission rates of molecular tracers in coal smoke particulate matter. *Fuel* 79:515–536

Pandey A, and Radhakrishnan S (1993) The production of glucoamylase by *Aspergillus niger* NCIM 1245. *Process Biochem* 28:305-309

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, and Mohan R (2000) Advances in microbial amylases. *Biotechnol Appl Biochem* 31:135-152

Peterson SH (1998) Genetic engineering of *Saccharomyces cerevisiae* for polysaccharide degradation. Thesis/Dissertation. University of Stellenbosch

Pintauro ND (1979) Bread and baked goods. In Pintauro ND (ed) Food processing enzymes – recent developments. Food technology review No. 52. Park Ridge, NJ, USA: Noyes Data Corporation

Planchot V, Colonna P, Gallant DJ, and Bouchet B (1995) Extensive degradation of native starch granules by alpha-amylase from *Aspergillus fumigates*. *J Cereal Sci* 21:163-171

Polagye BL, Hodgson KT, and Malte PC (2007) An economic analysis of bio-energy options using thinning from overstocked forests. *Biomass Bioener* 31:105-125

Powell KA, Ramer SW, del Cardeyre B, Stemmer WPC, Tobin MB, Longchamp PF, and Huisman GW (2001) Directed evolution and biocatalysis. *Angew Chem Int Ed* 40:3948-3959

Pretorius IS, Lambrechts MG, and Marmur J (1991) The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus*: an overview. *Biochem Mol Biol* 26:53-76

Pritchard PA (1992) Studies on the bread improving mechanisms of fungal α-amylase. *J Biol Educ* 26:12-18

Rajagopal D, Sexton SE, Roland-Holst D, and Zilberman D (2007) Challenge of biofuels: filling the tank without emptying the stomach? *Environ Research Lett* 2:1-9

Ramirez EC, Johnston DB, McAloon AJ, Yee W, and Singh Vijah (2009) Engineering process and cost model for a conventional corn wet milling facility. *Indust Crops Prod* 27:91-97

Randez-Gil F, Prieto JA, Murcia A, and Sanz P (1995) Construction of baker's yeast strains that secrete *Aspergillus oryze* alpha-amylase and their use in bread making. *J Cereal Sci* 21:185-193

Robertson GH, Wong DWS, Lee CC, Wagschal K, Smith MR, and Orts WJ (2006) Native or raw starch digestion: A key step in energy efficient biorefining of grain. *J Agric Food Chem* 54:353-365

Rosillo-Calle F, and Walter A (2006) Global market for bioethanol: historical trends and future prospects. *Ener Sustain Develop* 10:20-32

Rothman H, Greenshields R, and Rosillo-Calle F (1983) The Alcohol Economy: Fuel ethanol – the Brazilian Experience, Frances Pinter and Kentucky University Press, Lexington, USA

Rothstein SJ, Lahners KN, Lazarus CM, Baulcombe DC, and Gateby AA (1987) Synthesis and secretion of wheat α-amylase in *Saccharomyces cerevisiae*. *Gene* 55:353-356

Russo S, Siman-Tov RB, and Poli G (1995) Yeasts: from genetics to biotechnology. *J Environ Path Toxic Onco* 14:133-157

Saha BC, Shen GJ, Srivastava KC, LeCureux LW, and Zeikus JG (1989) New thermostable α-amylase-like pullulanase from thermophilic *Bacillus* sp. 3183. *Enzyme Microb Technol* 11:760-764

Schilling CH, Tomasik P, Karpovich DS, Hart B, Shepardson S, Garcha J, and Boettcher PT (2004) Preliminary studies on converting agricultural waste into biodegradable plastics, Part I: Corn distillers' dry grain. *J Polym Environ* 12:257-264

Sharma V, Rausch KD, Graeber JV, Schmidt SJ, Buriak P, Tumbleson ME, and Singh V (2010) Effect of resistant starch on hydrolysis and fermentation of corn starch for ethanol. *Appl Biochem Biotechnol* 160:800-811

Shay EG (1993) Diesel fuel from vegetable oils – status and opportunities. *Biomass Bioener* 4:227-242

Shigechi H, . Uyama K, Fujita Y, Matsumoto T, Ueda M, Tanaka A, Fukuda H, and Kondo A (2002) Efficient ethanol production from starch through development of novel flocculent yeast strains displaying glucoamylase and co-displaying or secreting α-amylase, *J Mol Catal Enzymatic* 17:179–187.

Shigechi H, Fujita Y, Koh J, Ueda M, Fukuda H, and Kondo A (2004) Energy-saving direct ethanol production from low-tempearture-cooked corn starch using a cell-surface engineered yeast strain co-displaying glucoamylase and α-amylase. *Biochem Engineer J* 18:149-153

Si JQ. (1999) Enzymes, baking bread making. In: Flickinger MC, Drew SW, editors. Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol.2 Wiley, 947- 58

Sierks MR, Ford C, Reilly PJ, and Svensson B (1990) Catalytic mechanism of fungal glucoamylase as defined by mutagenesis of Asp176, Glu179 and Glu180 in the enzyme from *Aspergillus awamori*. *Protein Eng* 3:193-198

Singh KJ, and Sooch SS (2004) Comparative study of economics of different models of family size biogass plants for state of Punjab, India. *Ener Convers Manage* 45:1329-1341

Singh M (2006) Economics of biofuels for the transport sector in South Africa. *Ener Sust Develop* 10:40-47

Solomon B (1978) Starch hydrolysis by immobilized enzymes: Industrial Applications. *Adv Biochem Engineer* 10:131-177

Southall SM, Simpson PJ, Gilbert HJ, Williamson G, and Williamson MP (1999) The starch-binding domain from glucoamylase disrupts the structure of starch. *FEBS Lett* 447:58-60

Srinivasan S (2009) The food v. fuel debate: A nuanced view of incentive structures. *Renew Ener* 34:950-954

Stevnebo A, Sahlstrom S, and Svihus B (2006) Starch structure and degree of starch hydrolysis of small and large strarch granules from barley varieties with varying amylase content. *Anim Feed Sci Technol* 130:23-38

Steyn AJC, Marmur J, and Pretorius IS (1995) Cloning, sequence analysis and expression in yeasts of a cDNA containing *a Lipomyces kononenkoae* s-amylase-encoding gene. *Gene* 166:65-71

Sticklen M (2006) Plant genetic engineering to improve biomass characteristics for biofuels. *Curr Opinion Biotechnol* 17:315-319

Sun H, Zhao P, Ge X, Xia Y, Hao Z, Liu J, Peng M (2010) Recent advances in microbial raw starch degrading enzymes. *Appl Biochem Biotechnol* 160:988-1003

Svensson B, and Sogaard M (1990) Expression of cDNAs encoding barley α-amylase 1 and 2 in yeast and characterization of the secreted proteins. *Gene* 94:173-179

Svensson B, Clarke AJ, Svendsen I, and Moller H (1990) Identification of carboxylic acid residues in glucoamylase G2 from *Aspergillus niger* that participate in catalysis and substrate binding. *Eur J Biochem* 188:29-38

Svensson B, Larsen K and Gunnarsson A (1986) Characterization of a glucoamylase G2 from *Aspergillus niger*. *Eur J Biochem* 154:497-502

Tanaka Y, Tao W, Blanchard JS, and Hehre (1994) Transition-state structures for the hydrolysis of alpha-d-glucopyranosyl fluoride by retaining and inverting reactions of glycosylases. *J Biol Chem* 269:32306-32312

Tester RF, Karkalas J, and Qi X (2004) Starch – composition, fine structure and architecture. *J Cereal Sci* 39:151-165

Turrio-Baldassarri L, Battistelli CL, Conti L, Crebelli R, De Berardis B, Iamiceli AL, Gambino M, and Iannaccone S (2004) Emission comparison of urban bus engine fueled with diesel oil and 'biodiesel' blend. *Sci Environ* 327:147–162

Uitenhaag JCM, Mosi R, Kalk KH, van der Veen BA, Dijkhuizen L, Withers SG, and Dijkstra BW (1999a) X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the α-amylase family. *Nat Struct Biol* 6:432-436

Uitenhaag JCM, Kalk KH, van der Veen BA, Dijkhuizen L, and Dijkstra BW (1999b) The cyclization mechanism of cyclodextrin glycosyltransferase (CGTase) as revealed by a γ-cyclodextrin-CGTase complex at 1.8 Å resolution. *J Biol Chem* 274:34868-34876

UNEP/WHO (United Nations Environmental Program/World Health Organization) (1992) Urban Air Pollution in Megacities of the World. Blackwell, Oxford

van Dam J, Faaij APC, Lewandowski I, and Fischer G (2007) Biomass production potentials in Central and Eastern Europe under different scenarios. *Biomass Bioener* 31:345-366

van der Maarel MJ, van der Veen B, Uitenhaag JC, Leemhuis H, and Dijkhuizen L (2002) Porperties and applications of starch-converting enzymes of the alpha-amylase family. *J Biotechnol* 94:137-155

van Ed JH, van Rijswijk WC, and Bollier M (1992) Enzymatic automated dishwash detergents. *Chim Oggi* 10:21-24

von Blottnitz H, and Curran MA (2007) A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective. *J Cleaner Prod* 15:607-619

Wiesenthal T, Leduc G, Christidis P, Schade B, Pelkmans L, Govaerts L, and Georogopoulos P (2009) Biofuel support policies in Europe: Lesssons learnt for the long way ahead. *Renew Sust Ener Rev* 13:789-800.

Wildenborg T, and Lokhorst A (2005) Introduction of CO_2 Geological storage-classification of storage options. *Oil Gas Sci Technol Rev* 60:513-515

Williamson G, and Belshaw NJ (1993) Properties of the binding domain of glucoamylase. *Carbohydr Poly* 21:147-149

Xie X, Liu Q, and Cui SW (2006) Studies of granular structure of resistant starches (type4) from normal, high amylase and waxy corn starch citrates. *Food Research Int* 39:332-341

Yoosin S, and Sorapipatana C (2007) A study of ethanol production cost for gasoline substitution in Thailand and its competitiveness. *Int J Sci Technol* 12:69-80

Zobel HF, and Stephen AM (2006) Starch: structure, analysis, and application. In: Stephen AM, Philips GO, and Williams PA (eds) Food polysaccharides and their applications, 2nd edition. CRC/Taylor and Francis, Boca Raton, Florida, United States of America pp 32-39

CHAPTER 3

Bioprospecting for a fungus expressing raw starch degrading enzymes

Bioprospecting for a fungus expressing raw starch degrading enzymes

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Abstract

The United States has proven that corn starch can be used for the large scale production of bio-ethanol. However, it is economically more feasible to produce ethanol directly from raw starch than modified starch that requires heat pretreatment. The objective of this study was to screen wild type fungi for the ability to metabolise raw starch. After selecting suitable wild type strains capable of raw starch hydrolysis, the fungi were further screened on plates for the production of α- and glucoamylases. The best amylase-producing strains were further evaluated in liquid media with starch as sole carbon source. The strain *Aspergillus tubingensis* (T8.8) displayed the highest levels of both α-amylase and glucoamylase activity. When the supernatant of *A. tubingensis,* grown in 2% raw starch, was inoculated with either *S. cerevisiae* N96 or Y102, 1 g/l and 1.6 g/l ethanol was produced respectively from 6 g/l raw starch under fermentative conditions.

Keywords: starch hydrolysis • bio-ethanol • *Aspergillus tubingensis*

1. INTRODUCTION

Biofuels produced from wood is an attractive alternative to fossil fuels, but the technology development is still in its early stages (Polaye et al., 2007; Hahn-Hagerdal et al., 2007), whereas the production of bio-ethanol from starch is already established. However, further studies to optimize the production of bio-ethanol from starch could improve current processes to make it more cost-effective and sustainable as an alternative energy source. The current biorefining steps use up to the equivalent of 20% of the total energy content of ethanol in the production of this fuel source (Robertson et al., 2006). The hydrolysis of biomass and the fermentation of the resulting sugars to ethanol in a single step process would result in significant cost reductions (Polaye et al., 2007). "Consolidated Bioprocessing" or CBP, where one organism can both hydrolyse the starch and ferment the resulting sugars will offer even further reductions in the production cost of bio-ethanol. CBP will thus replace high temperature processes and thus overall minimize energy usage and so raise the net yield of energy of bio-ethanol production (Robertson et al., 2006).

One aspect of starch hydrolysis that needs further development to render it more economically feasible, is to produce ethanol from raw starch as is found in corn kernels. The starch molecules are in a dense polycrystalline state, which makes raw starch (natural and untreated), insoluble in cold water and resistant to enzymatic hydrolysis. Additional heating is therefore needed to gelatinize the raw starch in water, which significantly increases the production costs (Mitsuiki et al., 2005).

Filamentous fungi are frequently applied in industrial processes of a biotechnological nature. The ability of a filamentous fungus in its high efficiency in substrate decomposition makes it attractive as a source of the isolation of novel degrading enzymes (Maeda et al., 2005). Fungi are exceptional organisms due to their ability to secrete enzymes into the substrate on which they grow. Hydrolysis takes place in the substrate itself, making it semi-soluble and thus allowing the fungi to absorb the necessary nutrients. A number of fungal species produce α-amylases (Yoon et al., 2007; Mukerjea et al., 2006) and glucoamylases (Jin et al., 1999) that enable these fungi to hydrolyse raw starch into D-glucose. Some strains of *Aspergillus* and *Rhizopus* spp. are currently being exploited for the commercial production of glucoamylases for application in the food industry (Jin et al., 1999; Koutinas et al., 2003). It has also been reported that *Mucor*, *Rhizopus*, *Rhizomucor*, *Lipomycetes*, *Penicillium*, *Aspergillus* and *Fusarium* species express α-amylases or glucoamylases, or both in most cases (Mukerjea et al., 2006; Yoon et al., 2007).

Although fungi in general secrete a vast array of enzymes into the substrate on which they grow, few have been identified with the ability to grow well on raw starch. Bioprospecting could lead to the isolation of potentially important organisms producing raw starch degrading enzymes. The first step in bioprospecting requires a large scale screening of diverse microbes for the enzymes required for the selected industrial process (Burton et al., 2002). The protein needs to be isolated and characterized since its working temperature and pH range will determine the industrial application. The three-dimensional structure and amino acid sequence may be required to design synthetic genes for expression in large quantities in a more favourable microorganism, such as *S. cerevisiae* (Beilen and Li, 2002).

Saccharomyces cerevisiae is a heterotrophic, non-motile, unicellular organism, which relies on its immediate environment for the ingestion of nutrients to sustain biomass and energy production (Gagiano et al., 2002). The positive attributes of *S. cerevisiae* is its high fermentation, ethanol yield and ethanol tolerance capabilities (Ibragimova et al., 1995), as well as its GRAS status (Generally Regarded As Safe) (Nakamura et al., 1997; Knox et al., 2004). This unique organism is however not capable of hydrolyzing starch (Tubb, 1986). The exception will be *S. cerevisiae var diastaticus* with its production of extracellular glucoamylase. The latter however does not have any α-amylase activity and thus no real activity in hydrolyzing α-1,6 branch points (McCann and Barnett, 1986; Sills and Stewart, 1982). The combination and finally the expression of amylase genes in *S. cerevisiae* giving

rise to an amylolytic yeast strain will be of great advantage to the hydrolysis and fermentation of starchy substrates in a single step. This will be of great commercial benefit to the industries which produces potable alcohol.

In this study, we investigated the ability of 36 wild-type fungal strains to hydrolyse soluble and raw starch, followed by the characterization of the α-amylase and glucoamylase activity of the best hydrolyzing strains. Finally, the potential of the best candidate – used together with fermenting yeast strains – for the production of bio-ethanol was determined.

2. MATERIALS AND METHODS

2.1 Microorganisms and growth conditions

Fungal strains used in the study are listed in Table 1. All 36 isolates were cultivated on Malt Extract Agar (MEA, Biolab Diagnostics, South Africa) and incubated at 26ºC, with the exception of *Rhizomucor spp.* that were grown at 40ºC. The *A. tubingensis* strain was cultured in 250 ml Erlenmeyer flasks containing 50 ml of YPD medium with shaking at 100rpm for 3 days. The supernatant was concentrated by freeze-drying and used inoculum for the enzyme activity assays. The optimal enzyme activity was determined using a modified version of the DNS method (Miller, 1959), using 0.1% soluble corn starch in citrate-phosphate buffer at different pH values and temperatures. This method is a quantitative measurement of the amount of glucose released by the enzymes with relative activity expressed in percentage, with the highest activity taken as 100%.

2.2 Soluble/raw starch hydrolysis on agar plates

Starch Agar comprised (per litre) of 6.7 g yeast nitrogen base (Difco Laboratories, Germany), 5 g $(NH_4)_2SO_4$, 20 g corn starch (Sigma, Germany) and 12 g agar. Raw Starch Agar comprised of corn starch that was sterilised with gamma radiation and added to the cooled medium after autoclaving. Phadebas Agar comprised (per liter) 6.7 g yeast nitrogen base (Difco Laboratories, Germany), 5 g $(NH_4)_2SO_4$, 1 g corn starch (Sigma, Germany), 20 Phadebas tablets (Kabi Pharmacia Diagnostics, Sweden) and 12 g agar (Sigma, Germany).

Cultures were incubated at 26°C or 40°C for *Rhizopus* spp. until colonies in 2cm diameter formed. The formation of clearing zones around the colonies indicated starch hydrolysis, whereas clearing zones on Phadebas agar plates confirmed the extracellular production of α-amylases.

Table 1. Fungal strains used in this study

Genus	Species	Soluble[1]	Raw[2]	Phadebas[3]	Strain reference[4]
Aspergillus	*flavus*	+	+	+	1220
	fumigatus	+	+	+	T4.1
	fumigatus	+	+	+	T4.4
	fumigatus	+	+	+	T10.4
	niger	+	0	0	578
	tubingensis	+	+	+	T8.2
	tubingensis	+	+	+	T8.4
Fusarium	*cerealis*	0	0	0	1371
	boothii	0	0	0	29020
	pseudograminearum	0	0	0	28062
	mesoamericanum	0	0	0	29184
	acaciae-mearnsii	0	0	0	34207
	brasilicum	+	0	0	31281
	cortaderiae	0	0	0	29297
	meridionale	0	0	0	28436
	culmorum	+	0	0	3288
	graminearum	0	0	0	28334
	austroamericaum	0	0	0	28585
	asiaticum	0	0	0	13818
Lipomycetes	*kononenkoae*	+	0	0	1004
Penicillium	*antrovenetum*	+	0	+	C11
	citrinum	+	+	0	C58
	griseofulvin	+	+	+	C33
	janczewskii	+	+	+	C26
	janczewskii	+	+	+	C111
	roseopurpureum	+	+		C59
Mucor	*circinelloides*	0	0	0	1180
	hiemales	+	0	+	1211
	luna	0	0	0	1119
Rhizomucor	*pusillus*	0	0	0	T3.2
	pusillus	0	0	0	T3.3
	pusillus	0	0	0	T3.4
Rhizopus	*digosporus*	0	0	0	1219
	oryzae	0	0	0	1220
	stolonifer	+	0	0	1221
Stachybotrys	*atra*	+	0	0	369

[1] produced a halo on soluble starch plates
[2] produced a halo on raw starch plates
[3] produced a halo on Phadebas plates
[4] Dept. Microbiology culture collection, University of Stellenbosch

2.3 Liquid assays

Liquid fungal cultures were prepared in Synthetic Complete medium (per liter): 6.7 g yeast nitrogen base (Difco Laboratories, Germany), 5 g $(NH_4)_2SO_4$ and 20 g soluble starch (Sigma, Germany) for all the fungal strains. The medium was inoculated at a concentration of $2x10^4$ spores/ml unless stated otherwise and incubated at 26°C (or 40°C for *Rhizopus* spp.) and on a rotary shaker at 100 rpm. The

spores were inoculated in 250ml Erlenmeyer flasks. Small volumes of supernatant were sampled at regular intervals. Reducing sugars (DNS) assays were performed (Miller, 1959) using soluble starch (pH 6) as substrate and glucose as standard at 60°C for 5 min. Activity was expressed as nkat/ml with one unit of enzyme defined as the amount of enzyme activity needed to produce 1 nmol of glucose equivalents per second under the given assay conditions.

The Red-Starch kit (Megazyme, Ireland) was used to quantify the α-amylase activity of *Aspergillus tubingensis* (T8.4), *Mucor* sp. (1180) and *Penicillium janczewskii* (C111) inoculated in triplicate at a concentration of $2x10^4$ spores/ml in 50 ml liquid SC-complete medium with 0.6% (w/v) raw starch in 250 ml Erlenmeyer flasks. Cultures were incubated at 26°C on a rotary shaker at 100 rpm for 3 days (negative control had no inoculum). A D-Glucose kit (Roche, Germany) was used to quantify the amount of glucose produced by glucoamylases expressed in the cultures.

2.4 Enzyme and yeast cocktails for ethanol production

A. tubingensis (T8.4) and *Mucor* sp. (1180) were inoculated in triplicate in SC liquid cultures at a concentration of $1x10^6$ spores/ml. The strains were monitored over a period of 3 days after which the supernatant was harvested by means of filtration through a 0.22 µm AcetatePlus filter paper (Lasec, South Africa). The supernatant was then transferred to sterile serum bottles to which of 0.6% (w/v) of sterile raw starch was hereafter added. Two yeast industrial strains, *S. cerevisiae* N96 and *S. cerevisiae* Y102, were inoculated into the supernatant of each fungal strain to a final concentration of $1x10^6$ cells/ml and cultivated on a rotary shaker (100rpm)at 30°C and 100 rpm in air-restricted serum bottles. The respective fungal supernatant and yeast combinations were monitored for 7 days for the production of ethanol, which was quantified with an Ethanol kit (Roche, Germany).

3. RESULTS

3.1 Detection of hydrolysis of soluble and raw starch

All 36 fungal strains had the ability to grow on the soluble starch media, but only 17 of the strains produced halos around the colonies (Table 1, Figure 1). Strains producing zones of hydrolysis were transferred to raw starch agar plates and incubated for 48 hours. All the strains were able to grow on the plates, but mainly the *Aspergillus* and *Penicillium* spp. grew better than the rest with the distinct formation of halos on the raw and Phadebas plates.

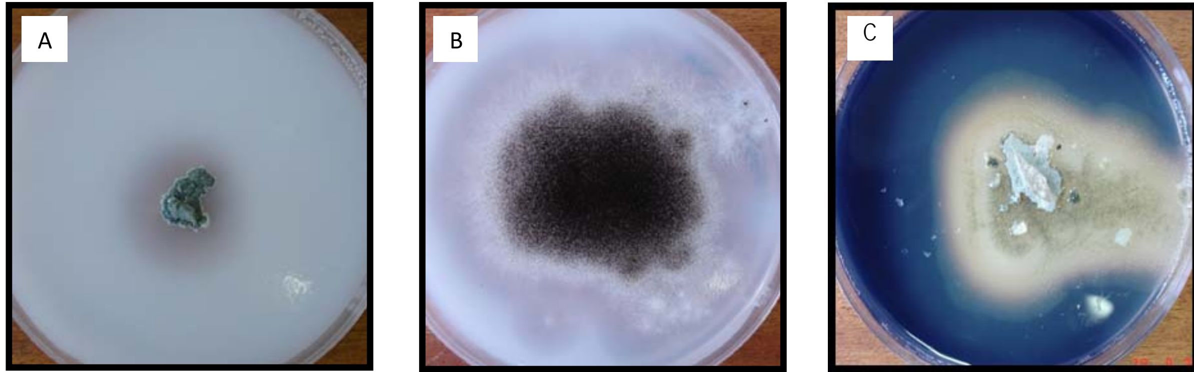

Figure 1: Halo formation by a fungal strains (A) *Penicillium jaczewskii* grown on soluble starch, (B) *Aspergillus tubingensis* grown on raw starch, and (C) *Aspergillus flavus* grown on Phadebas agar plates.

3.2 Hydrolysis of soluble and raw starch (DNS assays)

The fungal strains were further evaluated for their ability to reduce complex sugars into simple sugars (glucose). All 36 strains were grown in liquid SC-media that contained 2% soluble starch at 26ºC for 7 days at 100 rpm. DNS assays were performed on the supernatant using soluble starch as substrate. The absorbance values were compared to give a rough indication of which fungi optimally hydrolyzed soluble starch in this qualitative study. All fungal strains showed activity in contrast with results of activity on solid media (Table 1), but large differences were observed in the levels of activity, with *A. tubingensis* displaying the highest activity of 31.00 nkat/ml and *A. fumigatus* the lowest with 14.76 nkat/ml.

The ten fungal strains displaying the highest activity on soluble starch were further evaluated with 0.6% raw starch as sole carbon source in liquid cultures. These fungal strains included *Mucor sp* (1180), *Mucor* sp (1211), *Penicillium citrinum* (C58), *Penicillium roseopurpureum* (C59), *Penicillium griseofulvin* (C33), *Penicillium janczewskii* (C111), *Penicillium atrovenetum* (11), *Fusarium acaciae-mearnsii* (34207), *Aspergillus fumigatus* (T2.2) and *Aspergillus tubingensis* (T8.4). The results of the five best amylase producers are shown in Figure 2.

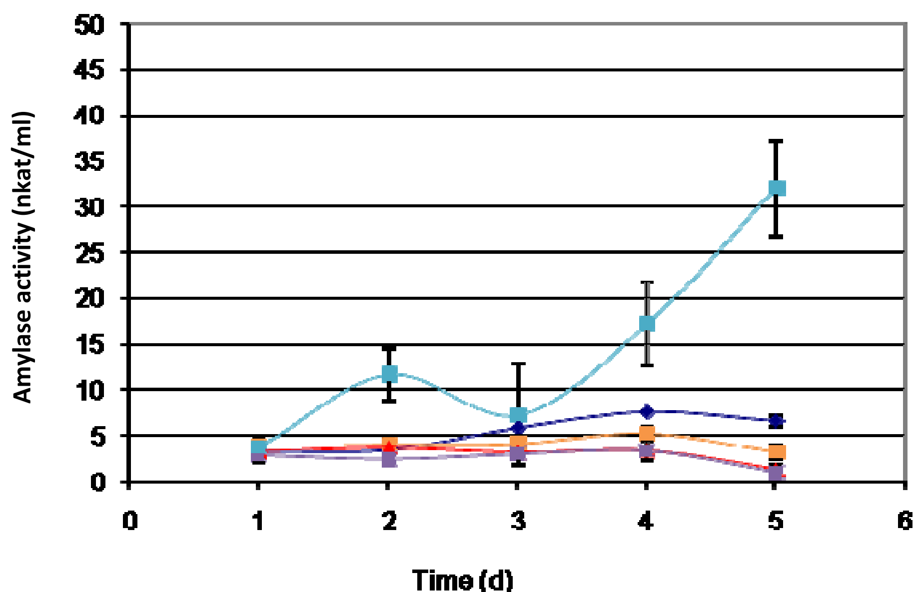

Figure 2: Enzymatic activity of the five fungal strains which produced the highest levels of reducing sugars upon cultivation in raw starch. (◆) *Penicillium janczewskii* (C111); (■) *Penicillium atrovenetum* (C11); (▲) *Fusarium* (34207); (X) *Aspergillus fumigatus* (T2.2); and (■) *Aspergillus tubingensis* (T8.4).

3.3 α-Amylase and glucoamylase activity

The *Mucor sp*. (1180), *A. tubingensis* (T8.4) and *P. janczewskii* (C111) strains showed varying levels of α-amylase activity, with that of A. *tubingensis* significantly higher than for *Mucor sp.* and *P. janczewskii* (Figure 3). Similarly, the *A. tubingensis* (T8.4) strain produced the highest level of extracellular glucoamylase activity.

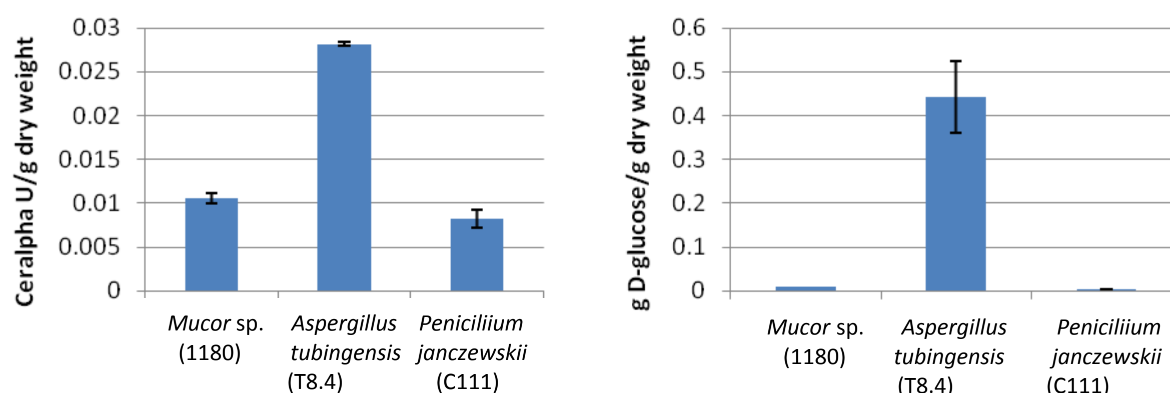

Figure 3: α-Amylase and β-glucoamylase activities displayed by the fungal strains upon cultivation on raw starch for 72 hours.

The pH optimum of the *A. tubingensis* amylase activity was determined to be pH 4.0, with significant activity observed at pH 3 – 5 (Figure 4). This compared well with that reported for other *Aspergillus* α-amylases and glucoamylases (James and Lee, 1997; Robertson et al., 2006) and is also in agreement with the optimal growth pH for the yeast *Saccharomyces cerevisiae*, which is ideal for the production of bio-ethanol in a single-step fermentation process (Moller et al., 2004; Murai et al., 1998).

An optimum temperature of 50°C was observed for the amylase activity of *A. tubingensis* (Figure 4). Previous studies showed that the optimum temperature of *Aspergillus* α-amylases and glucoamylases are generally in the range of 40 - 50°C and 40 - 60°C, respectively (Gupta et al., 2003; James and Lee, 1997; Robertson et al., 2006). High levels of activity at a lower temperature would be advantageous to ensure more cost-effective starch hydrolysis in industrial processes (Kim and Dale, 2008; Ramirez et al., 2008).

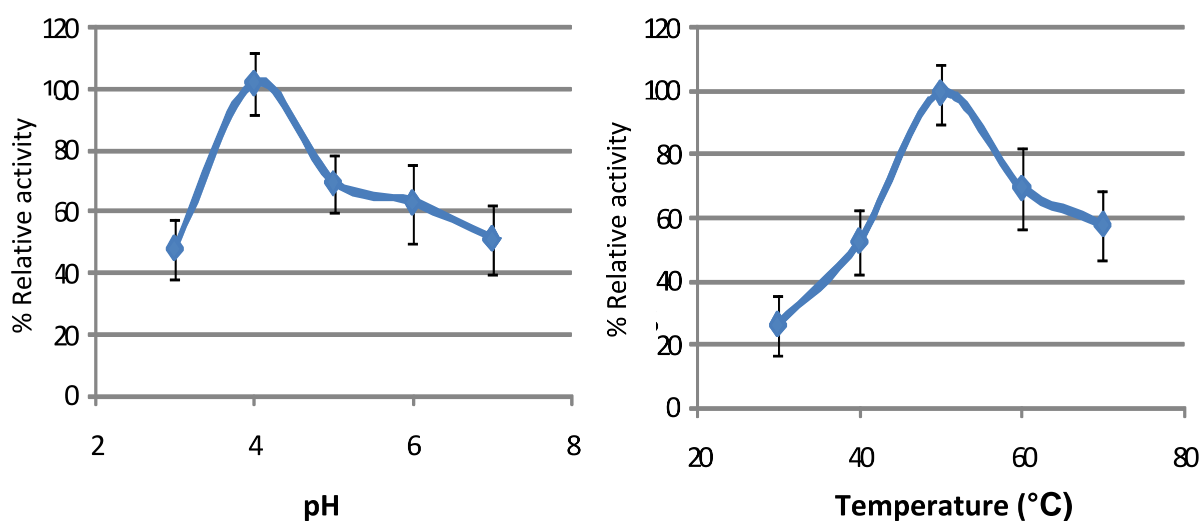

Figure 4: The optimal (A) pH and (B) temperature for the *A. tubingensis* extracellular amylase activity.

3.4 Ethanol production

When co-inoculated with *S. cerevisiae* strains, the *A. tubingensis* (T8.4) strain was able to produce enough extracellular amylase activity to support growth of both *S. cerevisiae* strains on 0.6% (w/v) raw starch. Furthermore, the combination with strains Y102 and N96 yielded 1.6 g/l and 1.0 g/l ethanol after 7 and 6 days, respectively (Figure 5).

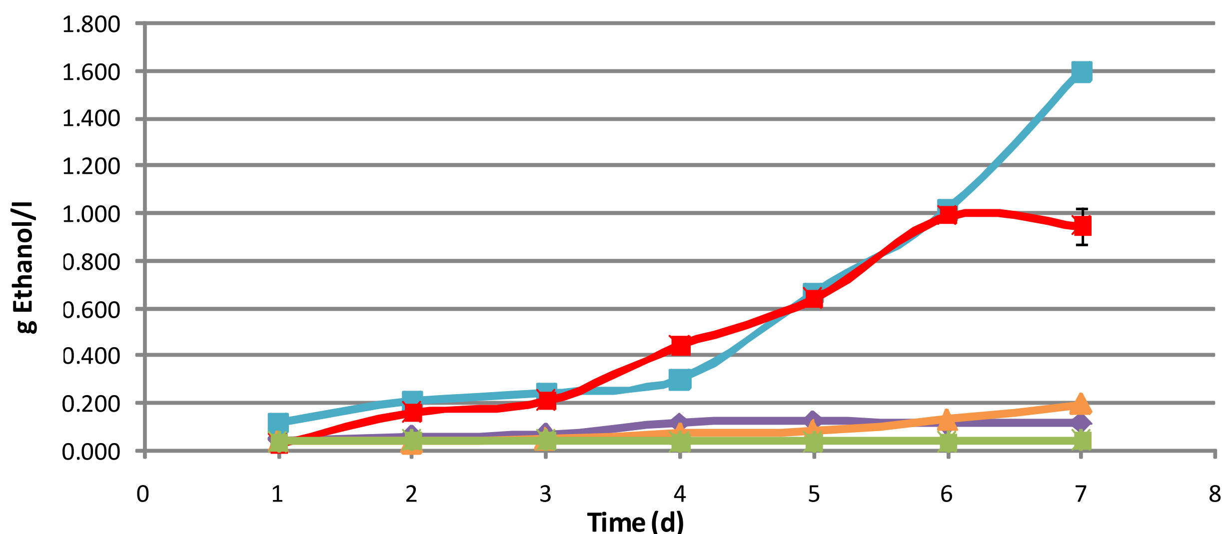

Figure 5: Ethanol production by (■) *A. tubingensis* T8.4 + *S. cerevisiae* Y102; (X) *A. tubingensis* T8.4 + *S. cerevisiae* N96; (◆) *Mucor circinelloides* 1180 + *S. cerevisiae* Y102; (▲) *Mucor circinelloides* 1180 + *S. cerevisiae* N96; relative to the reference strains (●) *S. cerevisiae* Y102 + *S. cerevisiae* N96.

4. DISCUSSION

The 36 wild type fungal strains were screened for their ability to metabolise starch, were all able to grow on soluble starch agar plates, but not all the fungi produced halos (hydrolysis zones). This was also the case for strains grown on raw starch agar plates. Halos are a clear indication of the hydrolysis of the starch substrate surrounding the colony, whereas growth does not imply starch utilization, since some fungal strains are oligotrophic and able to grow on SC medium when starch is omitted (Russo et al., 1995). The formation of halos was therefore used as an indicator of extracellular activity and strains that did not produce halos were not further evaluated.

Halo size can be influenced by a number of factors, including the agar concentration, size of the protein, level of glycosylation of the enzyme, number of branching points of the fungus, etc. (Cornesa et al., 2001). Therefore, the size of the halos was regarded as a qualitative test and not quantitative measurement. The presence of α-amylase activity, as indicated by a halo on Phadebas agar plates, were found in half of the strains (Table 1). The reducing sugar assays indicated the total extracellular amylase activity, which includes α-amylase, β-amylase and glucoamylase activities (Fleet, 2003). In the initial screening, using soluble starch as substrate, all the fungal strains confirmed amylase activity after 7 days, with *Mucor sp.* (1180), *A. tubingensis* (T8.4) and *P. janczewskii* (C1 11) identified as the strains with the best total extracellular amylase activity. However, *A. tubingensis* (T8.4) clearly outperformed the other strains with both the highest α-

amylase and glucoamylase activity, as was also evident when the substrate in the samples became less cloudy as the starch particles were hydrolysed.

It has also been found in this study that the optimum pH and temperature of *Aspergillus tubingensis* is at pH 4 and 50°C. It should, however, be taken into consideration that this is the optimum temperature of activity of the total extracellular enzymes excreted by this organism. It could therefore be possible that the optima for the individual extracellular enzymes of *A. tubingensis* may differ when expressed in a different host. This is of importance for the hydrolysis of raw starch at lower temperatures to reduce production costs.

The supernatant of *Mucor sp*. (1180) and *A. tubingensis* (T8.4) was evaluated in liquid cultures together with selected strains of *S. cerevisiae* at 30ºC to determine which fungi and yeast is most compatible. The *S. cerevisiae* N96 strain is an industrial wild type yeast used in the production of wine, whereas strain Y102 was used in the 1980's for bio-ethanol production from molasses (Saayman, 2005). Both *S. cerevisiae* strains were able to produce ethanol when cultivated on 0.6% (w/v) raw starch with the addition of the *A. tubingensis* (T8.4) supernatant. The supernatant, which contained the amylase enzymes, had the ability to hydrolyse the raw starch substrate to simple sugars that could be used as substrate by the yeast strains for the production of ethanol under anaerobic conditions.

Although fungi and yeast can grow together under certain conditions, co-cultivation has little potential since the low levels of ethanol tolerance of filamentous fungi will always be problematic. Yet, *A. tubingensis* can still contribute to bio-ethanol production if the genes encoding for glucoamylase and α-amylase could be isolated and expressed in *S. cerevisiae*. Clearly, *A. tubingensis* possesses unique raw starch degrading enzymes that can hydrolyse raw starch into glucose. To our knowledge, this is the first report of a study conducted on the starch hydrolyzing abilities of *A. tubingensis* and its potential for the production of bio-ethanol from starch in general. Characterisation of this strain has taken place with molecular techniques, but few genes have been sequenced (Giraud et al., 2007). This would render *A. tubingensis* an untapped source of interesting enzymes waiting to be discovered (Zhan et al., 2007; Achal et al., 2007).

5. REFERENCES

Achal V, Savant VV, and Reddy MS (2007) Phosphate solubilisation by a wild type strain and UV-induced mutants of *Aspergillus tubingensis*. *Soil Biol Biochem* 39:695-699

Beilen JB, and Li Z (2002) Enzyme technology: an overview. *Curr Opinion in Biotechnol* 13:338-344

Burton SG, Cowan DA, and Woodley JM (2002) The search for the ideal biocatalyst. *Nat Biotechnol* 20:37-45

Cornesa A, Punt PJ, van Luijk N, and van den Hondel CAMJJ (2001) The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Gene Biol* 33:155-171

Fleet GH (2003) Yeast interactions and wine flavour. *Int J Food Microb* 86:11-22

Gagiano M, Bauer FF, and Pretorius IS (2002) The sensing of nutritional status and relationship to filamentous growth in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2:433-470

Giraud F, Dupont J, Haon M, Bouzid O, Alibeu O, Navarro D, Sage L, Seigle-Murandi F, Asther M, and Lesage-Meessen L (2007) Phylogenetic analysis of the *Aspergillus niger* aggregate in relation to feruloyl esterase activity. *Res Microbiol* 158:413-419

Gupta R, Gigras P, Mohapatra H, Goswami VK, and Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. *Process Biochem* 38:1599-1616

Hahn-Hagerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, and Zacchi G (2007) Bio-ethanol – the fuel of tomorrow from the residues of today. *TRENDS Biotechnol* 24:549-556

Ibragimova SI, Kozlov DG, Kartasheva NN, Suntsov NI, Efremov BD, and Benevolensky SV (1995) A strategy for construction of industrial strains of distiller's yeast. *Biotechnol Bioeng* 46:285-290

James JA, and Lee BH (1997) Glucoamylases: microbial sources, industrial applications and molecular biology – a review. *J Food Biochem* 21:1-52

Jin B, van Leeuwen HJ, Patel B, Doelle HW, and Yu Q (1999) Production of fungal protein and glucoamylase by *Rhizopus oligosporus* from starch processing wastewater. *Process Biochem* 34:59-65

Kim S, and Dale BE (2008) Life cycle assessment of fuel ethanol derived from corn grain via dry milling. *Bioresource Technol* 99:5250-5260

Knox AM, du Preez JC, and Kilian SG (2004) Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipmyces kononenkoae* and *Saccharomyces fibuligera*. *Enzyme Microb Technol* 34:453-460

Koutinas AA, Wang R, Kookos IK, and Webb C (2003) Kinetic parameters of *Aspergillus awamori* in submerged cultivations on whole wheat flour under oxygen limiting conditions. *Biochem Eng J* 16:23-34

Maeda H, Yamagata Y, Abe K, Hasegawa F, Machida M, Ishioka R, Gomi K, and Nakajima T (2005) Purification and characterization of a biodegradable plastic-degrading enzyme from *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 67:778-788

Murai T, Yosino T, Ueda M, Haranoya I, Ashikari T, Yoshizumi H, and Takana A (1998) Evaluation of the function of arming yeast displaying glucoamylase on its cell surface by direct fermentation of corn to ethanol. *J Fermen Bioeng* 86:569-572

McCann AK, and Barnett JA (1986) The utilization of starch by yeast. *Yeast* 2:109-115

Miller GL (1959) Use of dinitrosalicyclic acid reagent for determination of reducing sugars. *Anal Chem* 31:426-428

Mitsuiki S, Mukae K, Sakai M, Goto M, Hayashida S, and Furukawa K (2005) Comparitive characterization of raw starch hydrolyzing – amylases from various *Bacillus* strains. *Enzyme Microb Technol* 37:410-416

Moller K, Sharif MZ, Ollson L (2004) Production of fungal α-amylase by *Saccharomyces kluyveri* in glucose-limited cultivations. *J Biotechnol* 111:311-318

Mukerjea R, Slocum G, Mukerjea R, and Robyt JF (2006) Significant differences in the activities of α-amylases in the absence and presence of polyethylene glycol assayed on eight starches solubilised by two methods. *Carbohydr Res* 341:2049-2054

Nakamura Y, Kobayashi F, Ohnaga M, and Sawada T (1997) Alcohol fermentation of starch by a genetic recombinant yeast having glucoamylase activity. *Biotechnol Bioeng* 53:21-25

Polagye BL, Hodgson KT, and Malte PC (2007) An economic anaylysis of bio-energy options using thinning from overstocked forests. *Biomass Bioener* 31:105-125

Ramirez EC, Johnston DB, McAloon AJ, Yee W, and Singh V (2008) Engineering process and cost model for a conventional corn wet milling facility. *Indust Crops Prod* 27:91-97

Robertson GH, Wong DWS, Lee CC, Wagschal K, Smith MR, and Orts WJ (2006) Native or raw starch digestion: A key step in energy efficient biorefining of grain. *J Agric Food Chem* 54:353-365

Russo S, Siman-Tov RB, and Poli G (1995) Yeasts: from genetics to biotechnology. *J Environ Path Toxic Onco* 14:133-157

Saayman M (2005) Characterisation of the malate transporter and malic enzyme from *Candida utilis*. PhD/Dissertation. University of Stellenbosch

Sills AM, and Stewart GG (1982) Production of amylolytic enzymes by several yeast species. *J Inst Brew* 88:313-316

Tubb RS (1986) Amylolytic yeasts for commercial applications. *TIBTECH* 4:98-104

Yoon S, Fulton DB, and Robyt JF (2007) Formation of covalent β-linked carbohydrate-enzyme intermediates during the reactions catalyzed by α-amylases. *Carbohydr Res* 342:55-64

Zhan J, Gunaherath GMKB, Wijerathe EMK, and Gunatilaka AAL (2007) Asperpyrone D and other metabolites of the plant-associated fungal strain *Aspergillus tubingensis*. *Phytochem* 68:368-372

CHAPTER 4

The isolation and expression of the *Aspergillus tubingensis* α-amylase and glucoamylase genes in *Saccharomyces cerevisiae*

The isolation and expression of the *Aspergillus* tubingensis α-amylase and glucoamylase genes in *Saccharomyces cerevisiae*

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Abstract

Starch is one of the most abundant polysaccharides produced in nature and is being used especially in the USA for the production of bio-ethanol as a fossil fuel supplement. A number of steps are involved in the utilisation of starch as a feedstock, including the separation of the starch from the biomass, hydrolysis of the starch to simple sugars and the conversion of the sugars to ethanol. The combined hydrolysis of biomass and fermentation of the resulting sugars in a single step process (simultaneous saccharification and fermentation, SSF) would result in cost reductions, whilst using a single genetically engineered microorganism that could perform both the starch hydrolysis and fermentation (consolidated bioprocessing, CBP) will offer even further reductions in the production cost of bio-ethanol (Polaye et al., 2007). In this study, the α-amylase and glucoamylase genes were isolated from a *Aspergillus tubingensis* strain and expressed separately on episomal plasmids in a laboratory strain of *Saccharomyces cerevisiae*. The resulting strain was able to perform CBP in liquid media where raw starch was used as the sole carbon source for the production of ethanol. The combination of the yeast strains produced ethanol at a final concentration of 11.04 g/l, which is comparable with ethanol yields reported in previous studies with CBP yeast strains (Khaw et al., 2006; Shigechi et al., 2004).

Keywords: raw starch • genetic engineering • amylases • *Aspergillus tubingensis*

1. INTRODUCTION

Starch is one of the most abundant polysaccharides produced in nature and is being used in the USA for bio-ethanol production as a fossil fuel supplement. A number of steps are involved in the utilisation of starch as a feedstock, including the separation of the starch from the biomass, hydrolysis of the starch to simple sugars and the conversion of the sugars to ethanol. The combined hydrolysis of biomass and fermentation of the resulting sugars in a single step process (simultaneous saccharification and fermentation, SSF) would result in cost reductions, whilst using a single genetically engineered microorganism (Polaye et al., 2007) that could perform both the starch hydrolysis and fermentation (consolidated bioprocessing, CBP) will offer even further reductions in the production cost of bio-ethanol. *Saccharomyces cerevisiae* is currently used for the production of

bio-ethanol due to its high ethanol tolerance. Unfortunately the yeast lacks starch degrading enzymes which prevents the efficient utilization of starch (Lin et al., 1998). Several other yeast species are able to degrade starch, but their low ethanol tolerance makes co-fermentation with *S. cerevisiae* problematic.

Starch consists of α-1,4 linked glucose units with α-1,6 branching points (Tester et al., 2004). The α-amylases (EC 3.2.1.1) degrade the starch chain at random, releasing oligosaccharides (dextrins) of varying lengths. Glucoamylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3) is an exo-acting enzyme that release β-D-glucose from starch through the hydrolysis of α-1,4 and α-1,6 glucosidic linkages from the non-reducing ends of starch molecules and other related poly- and oligosaccharides (Sauer et al., 2000). To date, several amylase encoding genes have been identified, cloned and sequenced (Randez-Gil et al., 1995; Moller et al., 2004; Steyn et al., 1995), but few of these enzymes display the ability to degrade raw starch. In terms of bio-ethanol production from starch, it would be economically more feasible to use raw starch degrading enzymes, as the need for heat pretreatment is circumvented. A number of fungal species produce α-amylases (Yoon et al., 2006; Mukerjea et al., 2006) and glucoamylases (Jin et al., 1999) with strains of *Aspergillus* and *Rhizopus* spp. exploited for the commercial production of glucoamylase for the food industry (Jin et al., 1999; Koutinas et al., 2003). An *Aspergillus tubingensis* strain had previously been identified with both α-amylase and glucoamylase activity in a study that - to our knowledge, constitutes the first report on the starch hydrolysing capabilities of *A. tubingensis* (Viktor et al., 2011). This fungus is known for the production of ochratoxin A in grapes and is usually associated with grape contamination (Medina et al., 2008). It also has other unique abilities such as a strong cytotoxic activity against some cancer cells (Zhan et al., 2007) and good bio-solubilisation of soluble rock phosphates (Achal et al., 2007; Reddy et al., 2002).

In this study, the α-amylase and glucoamylase genes were isolated from the wild type *A. tubingensis* strain and expressed in a laboratory strain of *S. cerevisiae*. This yielded an amylolytic yeast strain with the capability of consolidated bioprocessing in liquid media where raw starch was used as the sole carbon source for the production of ethanol.

2. MATERIALS AND METHODS

2.1 Strains and media

All strains and plasmids used in the study are listed in Table 1. All the chemicals, media components and supplements used in this study were of analytical grade standard. The recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α, which was cultivated at 37°C in Terrific Broth containing 100 μg/ml ampicillin or on Luria Bertani agar on a rotating wheel (Sambrook et al. 1989).

Table 1: Microbial strains and plasmids used in this study

Strains or plasmids	Genotype	Source/Reference
Strains:		
E. coli DH5α	*supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook *et al*. (1989)
A. tubingensis (T8.4)	Wild type	Dept. Microbiology, US
S. cerevisiae Y294	*α leu2-3,112 ura3-52 his3 trp1-289*	ATCC 201160
S. cerevisiae Y294[yBBH]	*URA3* $ENO1_P$-$ENO1_T$	This study
S. cerevisiae Y294[Atamy]	*URA3* $ENO1_P$-*Atamy*-$ENO1_T$	This study
S. cerevisiae Y294[Atglu]	*URA3* $ENO1_P$-*Atglu*-$ENO1_T$	This study
Plasmids		
pTZ57R/T	*bla*	Fermentas
pTZ-Atamy	*bla Atamy*	This study
pTZ-Atglu	*bla Atglu*	This study
yBBH1	*bla URA3* $ENO1_P$-$ENO1_T$	Dept. Microbiology, US
yBBH1-Atamy	*bla URA3* $ENO1_P$-*Atamy*-$ENO1_T$	This study
yBBH1-Atglu	*bla URA3* $ENO1_P$-*Atglu*-$ENO1_T$	This study

A. tubingensis was cultivated at 30°C on a rotary shaker at 100 rpm in maltodextrin medium (5% maltodextrin, 0.6% $NaNO_3$, 0.15%KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl and trace elements. *A. tubingensis* was maintained on MEA agar plates (12.75% maltose, 2.75% dextrin, 2.35% glycerol, 0.78% peptone, 15% agar) at 30°C. To induce the amylase genes prior to mRNA isolation, *A. tubingensis* was cultivated in liquid SC medium (1.7 g/l yeast nitrogen base without amino acids (Difco Laboratories), 5 g/l $(NH_4)_2SO_4$, 2% raw corn starch) for 3 days at 30°C on a rotary shaker at 100 rpm.

The *S. cerevisiae* Y294 parental strain was cultivated in YPD medium (1% yeast extract, 2% peptone and 2% glucose) and the transformants in synthetic complete (SC^{-URA}) medium (1.7 g/l yeast nitrogen base without amino acids (Difco Laboratories), 20 g/l glucose, 5 g/l $(NH_4)_2SO_4$ and supplemented with additional amino acids, without uracil). Cultivation took place aerobically on a rotary shaker at 250 rpm at 26°C in 250 ml Erlenmeyer flasks containing 50 ml of SC^{-URA} medium. Soluble starch (Sigma) was added prior to autoclaving at a concentration of 2% (w/v) when required. For the fermentation studies, recombinant strains were cultivated in SC^{-URA} media with 2% raw corn starch (Sigma) as sole carbon source. The raw corn starch was sterilized by the addition of absolute ethanol to a previously weighed amount of starch powder and allowing the ethanol to evaporate before adding to the autoclaved SC^{-URA} medium.

2.2 DNA manipulation

Standard protocols were followed for DNA manipulation (Sambrook *et al*., 1989). Enzymes used for restriction digests and ligations were sourced from Roche Diagnostics (Randburg, South Africa) or Fermentas Inc. (Maryland, USA). Where applicable, DNA was eluted from agarose gels with the Zymoclean™ Gel Recovery Kit (Zymo Research).

The *A. tubingensis* strain used in this study was verified with PCR amplification of the ITS region from its genomic DNA using the universal ITS1 and ITS4 primers (White et al., 1990). The sequence of the amplified region was compared to sequences in the NCBI database. Restriction fragment length polymorphism (RFLP) analysis was used to distinguish the *A. tubingensis* strain from *Aspergillus niger.*

For the α-amylase, the Asptalf-L + Asptalf-R and Asamy-L + Asamy-R primer sets were designed based on the genomic and cDNA sequences of the *Aspergillus kawachii* amylase gene, respectively (Genbank Accession nr. AB008370). For the glucoamylase, the Atglu-L + Atglu-R primer set was designed based on the genomic and cDNA sequences of the *A. shirousami* glucoamylase gene (Genbank Accession nr. D10460 and E02366, respectively). PCR was used for the amplification of the open reading frames of the genes using a Perkin Elmer Gene Amp® PCR System 2400 and TaKaRa Taq™ (Takara Bio Inc, Shiya, Japan) according to the manufacturer's recommendations. Table 2 lists the primers (Whitehead Scientific) used for PCR amplification.

Table 2: PCR primers used in the study with the relevant restriction sites underlined

Primer name	Sequence (5'-3')	Accession nr.
genomic Atamy		
Asptalf-L	<u>ATGAATTC</u>GAATTCATCTCGCTTC	AB008370
Asptalf-R	<u>ATCTCGAG</u>TACCTCCACGTATCAA	
cDNA Atamy		
Asamy-L	<u>AAGAATTC</u>CGCTTCGCCAAG	AB008370
Asamy-L	<u>CTCTCGAG</u>ATCAACCACCGTC	
cDNA Atglu		
Atglu-L	<u>CAGAATTC</u>CACCGCAATGTCGTTC	E02366
Atglu-R	<u>AGCTCGAG</u>AATAGTCTACCGCCAGGT	
Identification		
ITS1	TCCGTAGGTGAACCTTGCGG	
ITS4	TCCTCCGCTTATTGATATGC	

For cDNA preparation, total nucleic acid was isolated from *A. tubingensis* using liquid nitrogen (La Grange et al., 1996) and mRNA was retrieved using the FastTrack 2.0 mRNA isolation kit (Invitrogen, Corporation, Carlsbad, CA, USA). First strand cDNA was amplified from the mRNA with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Inqaba). The complete cDNA copies of the α-amylase and glucoamylase genes were amplified from the cDNA with the Atamy-L + Atamy-R and Atglu-L + Atglu-R primer sets, respectively.

The Atglu and Atamy genes were cloned into the *Eco*RI and *Xho*I sites of yBBH1, yielding yBBH1-Atamy and yBBH1-Atglu, respectively (Fig 1). Plasmid yBBH1 is similar to yENO1 previously described (Den Haan *et al.* 2007), but contains an additional 150 bp spacer region (non-coding) flanking the *ENO1* cassette designed to enable the consecutive cloning of additional cassettes into its *Bam*HI and *Bgl*II sites (Sadie et al., 2011).

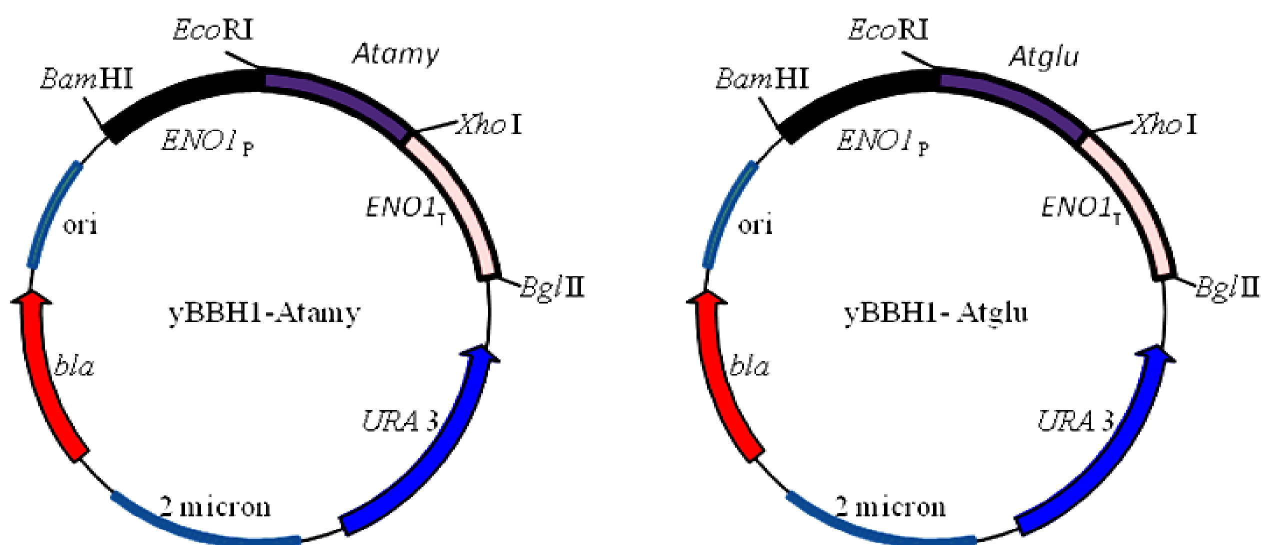

Figure 1: Plasmid maps of vectors used in this study: plasmid yBBH1 containing the (A) *Atamy* and (B) *Atglu* genes cloned in the *Eco*RI and *Xho*I sites of yBBH1.

2.3 DNA sequencing

The *Atamy* and *Atglu* cDNA fragments were ligated into the pTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas), now designated pTZ-Atamy and pTZ-Atglu, for sequencing on the ABI PRISM™ 3100 Genetic Analyser. The sequence data was analysed with the BLAST program (www.ncbi/nih.gov/BLAST) and restriction sites identified with DNAMAN (version 4.1) (Lynnon Biosoft).

2.4 Yeast transformation

The *S. cerevisiae* Y294 host strain was transformed with the respective recombinant plasmids using the dimethyl sulphoxide-lithium acetate method (Hill et al., 1991). The presence of amylase genes in the transformed yeast strain was verified with PCR using gene-specific primers (Table 1). Transformants were screened on SC^{-URA} and further selection was done on SC-agar containing either 2% soluble or 2% raw corn starch.

2.5 Amylase assays

The *S. cerevisiae* Y294 transformants were streaked out on SC^{-URA} plates containing 2% soluble corn starch or 2% raw corn starch to determine the hydrolyzing effect of the extracellular enzymes produced. Residual starch in the plates were stained with an iodine solution (3% KI, 0.3% iodine) resulting in a clear zone around the colonies producing the amylase enzymes. SC plates were prepared containing 0.1% Ostrazin Brilliant Red coupled (OBR) starch (Sigma) which also resulted in a clear halo around the colonies producing extracellular amylases.

Transformants were cultured in 250 ml Erlenmeyer flasks at 100 rpm containing 50 ml of YPD medium for 3 days at 30°C. The supernatant containing the extracellular α-amylase or glucoamylase were concentrated by freeze-drying and served as amylolytic source in the liquid enzyme activity assays. The optimal enzyme activity was determined using a modified version of the DNS method (Miller, 1959), using 0.1% soluble corn starch in citrate-phosphate buffer at different pH values and temperatures after 48 hours. This method is a quantitative measurement of the amount of glucose released by the enzymes with relative activity expressed in percentage, with the highest activity taken as 100%.

A growth curve was used to evaluate growth of the transformants relative to the reference strain *S. cerevisiae* Y294[yBBH1]. The yeast strains were inoculated at $1X10^5$ cells/ml and cultured at 30°C and 100 rpm in 250 ml Erlenmeyer flasks containing 50 ml of $2XSC^{-URA}$, until the different cultures reached stationary phase. Samples were taken periodically and absorbance readings were done at 600 nm. These readings were converted to dry weight using a standard curve compiled with *S. cerevisiae* Y294. The α-amylase activity was determined with the Red Starch assay (Megazyme, Ireland) at 30°C and pH 5.4 with activity expressed as Celalpha Units per gram dry weight. Glucoamylase activity was determined at 30°C for 10 minutes with modification (100 µl of the enzyme solution was added to 900 µl of a 0.1% raw starch substrate solution, pH 5.4) of with the D-glucose assay (Megazyem, Ireland). Glucoamylase activity was expressed as enzyme units (U) per gram of dry weight.

2.6 Enzyme expression (SDS-PAGE)

Characterization of the recombinant enzymes was performed by separating the protein fractions on 10% SDS-polyacrylamide gel using a 5% stacking gel and Tris-glycine buffer (Sambrook et al., 1989). Concentrated α-amylase and glucoamylase samples were obtained with freeze-drying as previously mentioned. The proteins were denatured by boiling for 3 minutes and electrophoresis was carried out at 100 V for ± 90 minutes at ambient temperature. Protein bands were visualized with the silver staining method (Laemmli, 1970).

2.7 Fermentation studies

The different recombinant yeast strains were cultivated in SC^{-URA} media, containing 2% raw corn starch (Sigma) as sole carbon source. Pre-cultures were cultivated in SC^{-URA} media with 2% corn starch and inoculated ($1x10^5$ cells/ml) into 120 ml glass serum bottles (in quadruplicate) containing 100 ml SC^{-URA} media with 2% corn starch. Mixing and anaerobic incubation was done on a magnetic multi-stirrer at 30°C until stationary phase. Sampling was done at 12 hour time intervals using a syringe needle pierced through the rubber plug.

Ethanol concentrations were determined with high performance liquid chromatography (HPLC), using a Waters 717 injector (Milford, MA, USA) and Agilent 1100 pump (PaloAlto). The compounds were separated on a Phenomenex RHM Monosaccharide 7.8 x 300 mm column (00H0132-K0, Bio-Rad Inc., CA, USA) at 60°C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min and detected with a Waters 410 refractive index detector.

3. RESULTS AND DISCUSSION

Rapid developments in the enzyme technology over the past four decades have revolutionized modern biotechnology (Kirk et al., 2002). These advantages also made a significant contribution to the development of new technologies for microbial raw starch digestion that could also be applied for more efficient ethanol production (Robertson et al., 2006; Sun et al., 2010). The construction of a recombinant *S. cerevisiae* yeast strain is of particular interest to the biofuel industry due to its high ethanol production levels, GRAS status and characterised genome sequence (Russo et al., 1995).

The fungus *A. tubingensis* was identified in a previous study (see Chapter 3) to have the ability of secreting enzymes that are capable of hydrolysing raw corn starch. Although hydrolytic enzymes of *A. tubingensis* have been reported before (Reddy et al., 2002), there has not yet been an extensive study on its ability to hydrolyse raw starch (Martinez-Culebras and Ramon, 2007; van Diepeningen et al., 2004; Kimura et al., 2000; Biely et al., 2000). Some reports indicated that *A. tubingensis* is closely related to *A. niger* (Giraud et al., 2007; Reddy et al., 2002), although others suggested that it is more

closely related to *Aspergillus kawachii* and *Aspergillus shirousami* based on ITS sequence homology (Manger-Jacob et al., 2005).

3.1 Cloning of *A. tubingensis* α-amylase

Amplification of the genomic DNA of *A. tubingensis* with primer set Asptalf-L + Asptalf-R yielded a fragment of approximately 2 800 base pairs (bp) displaying 99.00% homology with the α-amylase sequence of *A. kawachii*. Based on this result, the Asamy-L + Asamy-R primerset was designed for amplification of the cDNA of *A. tubingensis* which yielded a fragment of approximately 1923 bp with a 99.77% homology with the corresponding *A. kawachii* α-amylase sequence. The cDNA sequence of the *A. tubingensis* α-amylase gene (*Atamy)* and predicted amino acid sequence are shown in Figure 2.

```
1     ATGAGAGTGTCGACTTCAAGTATTGCCCTTGCTGTGTCCCTTTTTGGGAAGCTGGCCCTT
1      M  R  V  S  T  S  S  I  A  L  A  V  S  L  F  G  K  L  A  L

61    GGGCTGTCAGCTGCAGAATGGCGCACTCAATCCATCTACTTCCTTTTGACGGATCGGTTC
21     G  L  S  A  A  E  W  R  T  Q  S  I  Y  F  L  L  T  D  R  F

121   GGTAGGACGGACAATTCGACTACAGCTACGTGCAATACGGGTGACCAAATCTACTGTGGT
41     G  R  T  D  N  S  T  T  A  T  C  N  T  G  D  Q  I  Y  C  G

181   GGAAGTTGGCAAGGAATTATCAACCATCTGGACTATATCCAGGGCATGGGATTCACAGCT
61     G  S  W  Q  G  I  I  N  H  L  D  Y  I  Q  G  M  G  F  T  A

241   ATCTGGATCTCGCCTATCACTGAGCAGCTACCCCAGGATACTTCGGATGGTGAAGCCTAC
81     I  W  I  S  P  I  T  E  Q  L  P  Q  D  T  S  D  G  E  A  Y

301   CATGGATACTGGCAGCAGAAGATATACTATGTGAACTCCAACTTCGGCACGGCAGATGAT
101    H  G  Y  W  Q  Q  K  I  Y  Y  V  N  S  N  F  G  T  A  D  D

361   CTGAAGTCCCTCTCCGATGCTCTTCACGCCCGCGGAATGTACCTCATGGTCGACGTCGTC
121    L  K  S  L  S  D  A  L  H  A  R  G  M  Y  L  M  V  D  V  V

421   CCTAACCACATGGGCTACGCAGGTAACGGCAACGATGTGGATTACAGCGTCTTCGACCCC
141    P  N  H  M  G  Y  A  G  N  G  N  D  V  D  Y  S  V  F  D  P

481   TTCGACTCCTCCTCCTACTTCCATCCATACTGCCTCATCACAGATTGGGACAACTTGACC
161    F  D  S  S  S  Y  F  H  P  Y  C  L  I  T  D  W  D  N  L  T

541   ATGGTCCAAGACTGTTGGGAGGGTGACACCATCGTGTCTCTGCCAGATCTGAACACCACG
181    M  V  Q  D  C  W  E  G  D  T  I  V  S  L  P  D  L  N  T  T

601   GAAACCGCCGTGAGAACCATTTGGTACGATTGGGTAGCCGACCTGGTATCCAACTACTCA
201    E  T  A  V  R  T  I  W  Y  D  W  V  A  D  L  V  S  N  Y  S

661   GTCGACGGCCTCCGTATCGACAGTGTCGAAGAAGTCGAACCCGACTTCTTCCCGGGCTAC
221    V  D  G  L  R  I  D  S  V  E  E  V  E  P  D  F  F  P  G  Y

721   CAAGAAGCAGCAGGAGTCTACTGCGTCGGTGAAGTCGACAACGGCAACCCTGCTCTCGAC
241    Q  E  A  A  G  V  Y  C  V  G  E  V  D  N  G  N  P  A  L  D

781   TGCCCATACCAAAAATATCTAGATGGTGTTCTCAACTATCCCATCTACTGGCAACTCCTC
261    C  P  Y  Q  K  Y  L  D  G  V  L  N  Y  P  I  Y  W  Q  L  L
```

```
841     TACGCCTTTGAATCCTCCAGCGGCAGCATCAGCAACCTCTACAACATGATCAAATCCGTC
281      Y  A  F  E  S  S  S  G  S  I  S  N  L  Y  N  M  I  K  S  V

901     GCCAGCGACTGCTCCGATCCGACCCTCCTGGGCAACTTTATCGAAAACCACGACAACCCC
301      A  S  D  C  S  D  P  T  L  L  G  N  F  I  E  N  H  D  N  P

961     CGCTTCGCCTCCTACACATCCGACTACTCCCAAGCCAAAAACGTCCTCAGCTACATCTTC
321      R  F  A  S  Y  T  S  D  Y  S  Q  A  K  N  V  L  S  Y  I  F

1021    CTCTCCGACGGCATCCCCATCGTCTACGCCGGCGAAGAACAGCACTACTCCGGCGGCGAC
341      L  S  D  G  I  P  I  V  Y  A  G  E  E  Q  H  Y  S  G  G  D

1081    GTGCCCTACAACCGCGAAGCTACCTGGCTATCAGGCTACGACACCTCCGCGGAGCTCTAC
361      V  P  Y  N  R  E  A  T  W  L  S  G  Y  D  T  S  A  E  L  Y

1141    ACCTGGATAGCCACCACAAACGCGATCCGGAAACTAGCTATCTCAGCAGACTCGGACTAC
381      T  W  I  A  T  T  N  A  I  R  K  L  A  I  S  A  D  S  D  Y

1201    ATTACTTACAAGAACGACCCAATCTACACAGACAGCAACACCATCGCGATGCGCAAAGGC
401      I  T  Y  K  N  D  P  I  Y  T  D  S  N  T  I  A  M  R  K  G

1261    ACCTCCGGCTCCCAAATCATCACCGTCCTCTCCAACAAAGGCTCCTCCGGAAGCAGCTAC
421      T  S  G  S  Q  I  I  T  V  L  S  N  K  G  S  S  G  S  S  Y

1321    ACCCTCACCCTCAGCGGAAGCGGCTACACGTCCGGCACGAAGCTCATCGAAGCGTACACC
441      T  L  T  L  S  G  S  G  Y  T  S  G  T  K  L  I  E  A  Y  T

1381    TGCACGTCCGTGACGGTGGACTCGAACGGGGATATCCCTGTGCCGATGGCTTCGGGATTA
461      C  T  S  V  T  V  D  S  N  G  D  I  P  V  P  M  A  S  G  L

1441    CCTAGAGTTCTCCTCCCTGCTTCGGTGGTTGATAGTTCTTCGCTTTGTGGGGGGAGTGGT
481      P  R  V  L  L  P  A  S  V  V  D  S  S  S  L  C  G  G  S  G

1501    AACACAACCACGACCACAACTGCTGCTACCTCCACATCCAAAGCCACCACCTCCTCTTCT
501      N  T  T  T  T  T  T  A  A  T  S  T  S  K  A  T  T  S  S  S

1561    TCTTCTTCTGCTGCTGCTACTACTTCTTCATCATGCACCGCAACAAGCACCACCCTCCCC
521      S  S  S  A  A  A  T  T  S  S  S  C  T  A  T  S  T  T  L  P

1621    ATCACCTTCGAAGAACTCGTCACCACTACCTACGGGGAAGAAGTCTACCTCAGCGGATCT
541      I  T  F  E  E  L  V  T  T  T  Y  G  E  E  V  Y  L  S  G  S

1681    ATCTCCCAGCTCGGAGAGTGGCATACGAGTGACGCGGTGAAGTTGTCCGCGGATGATTAT
561      I  S  Q  L  G  E  W  H  T  S  D  A  V  K  L  S  A  D  D  Y

1741    ACCTCGAGTAACCCCGAGTGGTCTGTTACTGTGTCGTTGCCGGTGGGGACGACCTTCGAG
581      T  S  S  N  P  E  W  S  V  T  V  S  L  P  V  G  T  T  F  E

1801    TATAAGTTTATTAAGGTCGATGAGGGTGGAAGTGTGACTTGGGAAAGTGATCCGAATAGG
601      Y  K  F  I  K  V  D  E  G  G  S  V  T  W  E  S  D  P  N  R

1861    GAGTATACTGTGCCTGAATGTGGGAGTGGGAGTGGGGAGACGGTGGTTGATACGTGGAGG
621      E  Y  T  V  P  E  C  G  S  G  S  G  E  T  V  V  D  T  W  R

1921    TAG
641      *
```

Figure 2: cDNA sequence and predicted amino acid sequence of the α-amylase gene of *A. tubingensis* (*Atamy*).

3.2 Cloning of *A. tubingensis* glucoamylase

The Atglu-L + Atglu-R primer set was designed based on the genomic and cDNA sequences of the *A. shirousami* glucoamylase gene. PCR amplification of the genomic DNA of *A. tubingensis* yielded a fragment of approximately 2 600 bp with 98.42% homology with the genomic sequence of *A. shirousami* (D10460). Amplification of the *A. tubingensis* cDNA yielded a fragment of approximately 1 920 bp with 99.26% homology with the cDNA sequence of *A. shirousami*. The cDNA sequence and predicted amino acid sequence for *Atglu* are shown in Figure 3.

```
1       ATGTCGTTCCGATCTCTTCTCGCCCTGAGCGGCCTTGTCTGCTCGGGGTTGGCAAGTGTG
1        M  S  F  R  S  L  L  A  L  S  G  L  V  C  S  G  L  A  S  V

61      ATTTCCAAGCGCGCGACCTTGGATTCGTGGTTGAGCAACGAAGCGACCGTGGCTCGTACT
21       I  S  K  R  A  T  L  D  S  W  L  S  N  E  A  T  V  A  R  T

121     GCCATCCTGAATAACATCGGGGCGGACGGTGCTTGGGTGTCGGGCGCGGACTCTGGCATT
41       A  I  L  N  N  I  G  A  D  G  A  W  V  S  G  A  D  S  G  I

181     GTCGTTGCCAGTCCCAGCACCGATAACCCGGACTACTTCTACACCTGGACTCGCGACTCT
61       V  V  A  S  P  S  T  D  N  P  D  Y  F  Y  T  W  T  R  D  S

241     GGTATCGTCCTCAAGACCCTCGTCGACCTCTTCCGCAATGGAGATACTGATCTCCTTTCC
81       G  I  V  L  K  T  L  V  D  L  F  R  N  G  D  T  D  L  L  S

301     ACCATTGAGCACTACATCTCCTCTCAGGCAATTATTCAGGGTGTCAGTAACCCCTCTGGT
101      T  I  E  H  Y  I  S  S  Q  A  I  I  Q  G  V  S  N  P  S  G

361     GATCTGTCCAGCGGTGGTCTTGGTGAGCCCAAGTTCAATGTCGATGAGACTGCCTACGCC
121      D  L  S  S  G  G  L  G  E  P  K  F  N  V  D  E  T  A  Y  A

421     GGTTCTTGGGGACGGCCGCAGCGTGATGGTCCTGCCCTGAGAGCAACTGCTATGATCGGC
141      G  S  W  G  R  P  Q  R  D  G  P  A  L  R  A  T  A  M  I  G

481     TTTGGGCAGTGGCTGCTTGACAATGGCTACACCAGCGCTGCAACAGAGATTGTTTGGCCC
161      F  G  Q  W  L  L  D  N  G  Y  T  S  A  A  T  E  I  V  W  P

541     CTCGTTAGGAACGACCTGTCGTATGTGGCTCAGTACTGGAACCAGACGGGATATGATCTC
181      L  V  R  N  D  L  S  Y  V  A  Q  Y  W  N  Q  T  G  Y  D  L

601     TGGGAAGAAGTTAATGGCTCGTCCTTCTTCACTATTGCCGTGCAACACCGCGCCCTCGTC
201      W  E  E  V  N  G  S  S  F  F  T  I  A  V  Q  H  R  A  L  V

661     GAAGGTAGTGCCTTCGCGACGGCCGTCGGCTCGTCCTGCTCCTGGTGTGATTCGCAGGCA
221      E  G  S  A  F  A  T  A  V  G  S  S  C  S  W  C  D  S  Q  A

721     CCTCAGATTCTCTGTTACTTGCAGTCCTTCTGGACCGGCAGCTACATCCTGGCCAACTTT
241      P  Q  I  L  C  Y  L  Q  S  F  W  T  G  S  Y  I  L  A  N  F

781     GACAGCAGCCGTTCCGGCAAGGACACAAACACCCTCCTGGGAAGCATCCACACCTTTGAT
261      D  S  S  R  S  G  K  D  T  N  T  L  L  G  S  I  H  T  F  D

841     CCTGAGGCTGGATGCGACGACTCCACCTTCCAGCCCTGCTCCCCGCGTGCGCTCGCCAAC
281      P  E  A  G  C  D  D  S  T  F  Q  P  C  S  P  R  A  L  A  N

901     CATAAGGAGGTTGTAGACTCTTTCCGCTCGATCTATACTCTCAACGATGGTCTCAGTGAC
301      H  K  E  V  V  D  S  F  R  S  I  Y  T  L  N  D  G  L  S  D
```

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961     AGTGAGGCGGTTGCGGTCGGTCGGTACCCTGAGGATAGCTACTACAACGGCAACCCGTGG
321      S  E  A  V  A  V  G  R  Y  P  E  D  S  Y  Y  N  G  N  P  W

1021    TTCCTGTGCACCTTGGCTGCCGCGGAACAGCTGTACGATGCTCTGTACCAGTGGGACAAG
341      F  L  C  T  L  A  A  A  E  Q  L  Y  D  A  L  Y  Q  W  D  K

1081    CAGGGGTCGTTGGAGATCACAGACGTGTCACTTGACTTCTTCAAGGCTCTGTACAGTGGT
361      Q  G  S  L  E  I  T  D  V  S  L  D  F  F  K  A  L  Y  S  G

1141    GCTGCCACCGGCACGTACTCTTCGTCCAGCTCGACCTATAGCAGCATTGTGAGTGCCGTC
381      A  A  T  G  T  Y  S  S  S  S  S  T  Y  S  S  I  V  S  A  V

1201    AAGACTTTCGCTGATGGTTTTGTTTCTATTGTGGAAACTCACGCCGCAAGCAACGGCTCT
401      K  T  F  A  D  G  F  V  S  I  V  E  T  H  A  A  S  N  G  S

1261    CTGTCTGAGCAATTCGACAAGTCTGATGGCGACGAGCTTTCTGCTCGCGATCTGACCTGG
421      L  S  E  Q  F  D  K  S  D  G  D  E  L  S  A  R  D  L  T  W

1321    TCTTACGCTGCTCTGCTGACCGCCAACAACCGTCGTAATTCTGTCGTGCCCCCGTCTTGG
441      S  Y  A  A  L  L  T  A  N  N  R  R  N  S  V  V  P  P  S  W

1381    GGTGAGACCTCTGCCAGCAGCGTGCCCGGCACCTGTGCGGCTACCTCTGCCTCTGGTACC
461      G  E  T  S  A  S  S  V  P  G  T  C  A  A  T  S  A  S  G  T

1441    TACAGCAGTGTGACCGTCACCTCGTGGCCGAGCATCGTGGCTACTGGTGGCACCACTACG
481      Y  S  S  V  T  V  T  S  W  P  S  I  V  A  T  G  G  T  T  T

1501    ACGGCTACCACCACTGGATCGGGCGGCGTGACCTCGACCAGCAAGACCACCACAACTGCT
501      T  A  T  T  T  G  S  G  G  V  T  S  T  S  K  T  T  T  T  A

1561    AGTAAGACCAGCACCACTACGTCCTCGACCTCCTGCACCACCCCCACTGCCGTAGCTGTG
521      S  K  T  S  T  T  T  S  S  T  S  C  T  T  P  T  A  V  A  V

1621    ACCTTTGATCTGACGGCGACCACCACCTACGGCGAGAACATCTACCTGGTCGGGTCGATC
541      T  F  D  L  T  A  T  T  T  Y  G  E  N  I  Y  L  V  G  S  I

1681    TCTCAGCTCGGTGACTGGGAGACCAGCGATGGCATAGCTCTGAGCGCTGACAAGTACACT
561      S  Q  L  G  D  W  E  T  S  D  G  I  A  L  S  A  D  K  Y  T

1741    TCCAGTAACCCGCCTTGGTATGTAACTGTGACTCTGCCGGCTGGTGAGTCATTTGAGTAC
581      S  S  N  P  P  W  Y  V  T  V  T  L  P  A  G  E  S  F  E  Y

1801    AAGTTCATCCGCGTTGAGAGCGATGACTCCGTGGAGTGGGAGAGCGACCCGAACCGGGAA
601      K  F  I  R  V  E  S  D  D  S  V  E  W  E  S  D  P  N  R  E

1861    TACACCGTTCCTCAGGCGTGCGGCGAGTCGACCGCGACGGTGACCGACACCTGGCGGTAG
621      Y  T  V  P  Q  A  C  G  E  S  T  A  T  V  T  D  T  W  R  *
```

Figure 3: cDNA and predicted amino acid sequence of the glucoamylase gene of *A. tubingensis* (Atglu)

A number of studies have been conducted on the hydrolytic effect of *A. awamori* (Nunberg et al., 1984; Goto et al., 1994), whose gene sequences also served as the reference for cloning of the α-amylase and glucoamylase genes of *A. tubingensis* in the present study (Kaneko et al., 1996; Manger-Jacob et al., 2005). Functional α-amylase and glucoamylase genes were successfully isolated from *A. tubingensis* with DNA sequences nearly identical to that of *A. kawachii*. The *A. tubingensis*

genes were cloned on the yBBH1 plasmid and were individually expressed in *S. cerevisiae* Y294. The recombinant *S. cerevisiae* Y294[Atamy] strain, expressing the α-amylase, showed zones of hydrolysis (halo's) when grown on 2% starch solid media (Figure 4A). The exo-type glucoamylase usually doesn't produce halo's on solid medium and OBR-starch plates were used to visualise starch hydrolysis by the recombinant *S. cerevisiae* Y294[Atglu] strain, expressing the glucoamylase (Figure 4B). This was a clear indication that the recombinant fungal enzymes were secreted into the media and enabled the yeast strain to hydrolyse the complex starch structure.

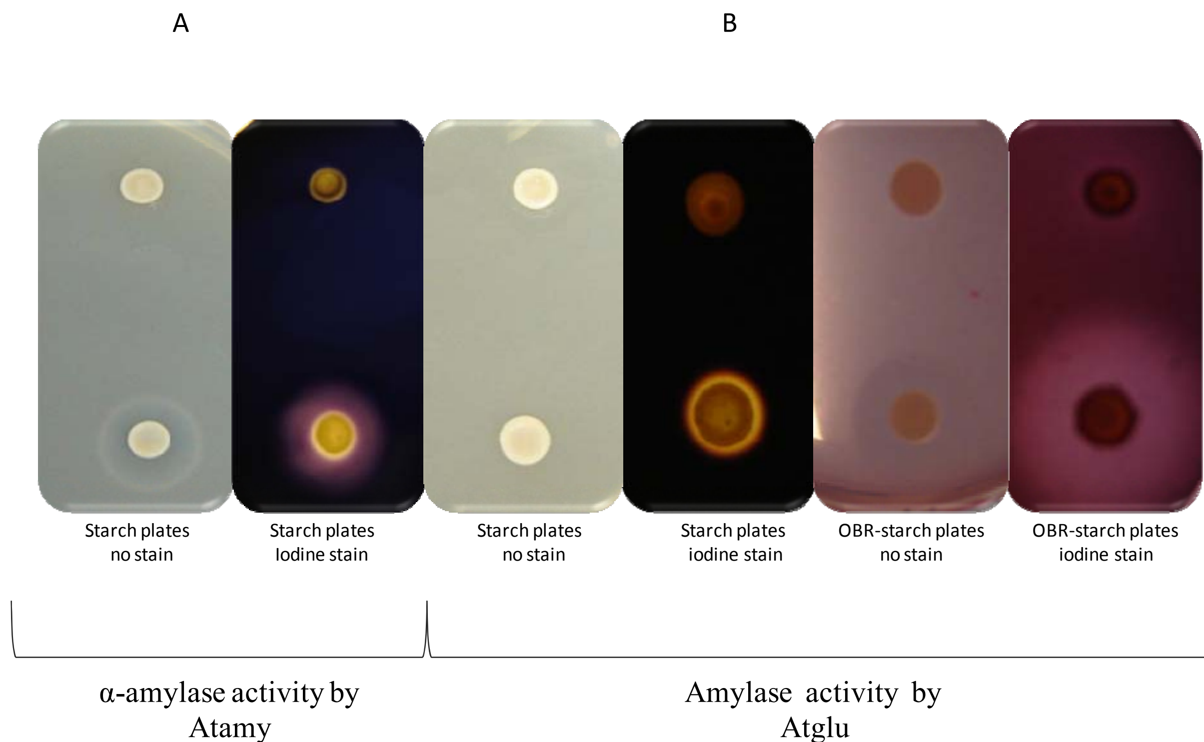

Figure 4: Hydrolysis zones formed by the (A) *S. cerevisiae* Y294[Atamy] and (B) *S. cerevisiae* Y294[Atglu] strains, relative to the control *S. cerevisiae* Y294[yBBH1] strain shown at the top of each photograph**.**

3.3 SDS-PAGE

SDS-PAGE analysis of the supernatant indicated the presence of proteins that correspond to the estimated size of the respective α-amylase and glucoamylase. The molecular weight of the transformed fungal α-amylases is expected to be 60 - 85 kDa based on sequence homology with *A. kawachii* (Kaneko et al., 1996). Previous studies indicated that a molecular weight of approximately 85 kDa could be expected for the native *A. tubingensis* α-amylase (66 kDa without carbohydrates) (Gupta et al., 2003; Kaneko et al., 1996). There was no single protein species visible in the *S. cerevisiae* Y294[Atamy] supernatant (Figure 5, Lane 3) at the expected size of 66 – 85 kDa, but a large smear was observed at 130 kDa, which could indicate a differentially glycosylated protein. The putative recombinant glucoamylase was observed at approximately 90 kDa, which is within the range expected for fungal glucoamylases (Suresh et al., 1999). The predicted molecular mass of the glucoamylase based on previous sequence studies has suggested a protein which is 68 kDa in size (Manger-Jacob et al., 2005). The predicted molecular weight of the α- and glucoamylase based on the amino acid sequences was 69.6 kDa and 68 kDa, respectively.

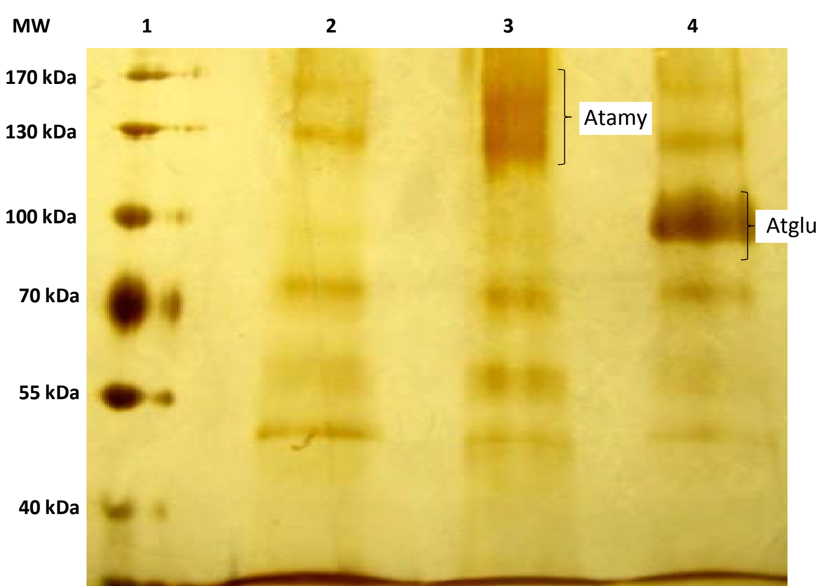

Figure 5: SDS-PAGE of supernatant isolated from yeast strains. Lane (1) Molecular Weight Marker; (2) *S. cerevisae* Y294[BBH1] as reference strain; (3) *S. cerevisiae* Y294[Atamy]; and (4) *S. cerevisiae* Y294[Atglu]. The brackets in lane 3 and 4 indicate the positions of the irrespective proteins.

3.4 Amylase asssays

The pH optima of the recombinant α-amylase and glucoamylase were confirmed to be pH 4.0 and pH 4.5, respectively, with significant activity observed for both enzymes at pH 3 – 5 (Figure 6A). The

optima for both enzymes compared well with that reported for other *Aspergillus* α-amylase and glucoamylases (James and Lee, 1997; Robertson et al., 2006). The optimal enzyme activities are also in agreement with the optimal pH for the growth of the host organism, which is ideal for the production of bio-ethanol in a single-step fermentation process (Moller et al., 2004; Murai et al., 1998).

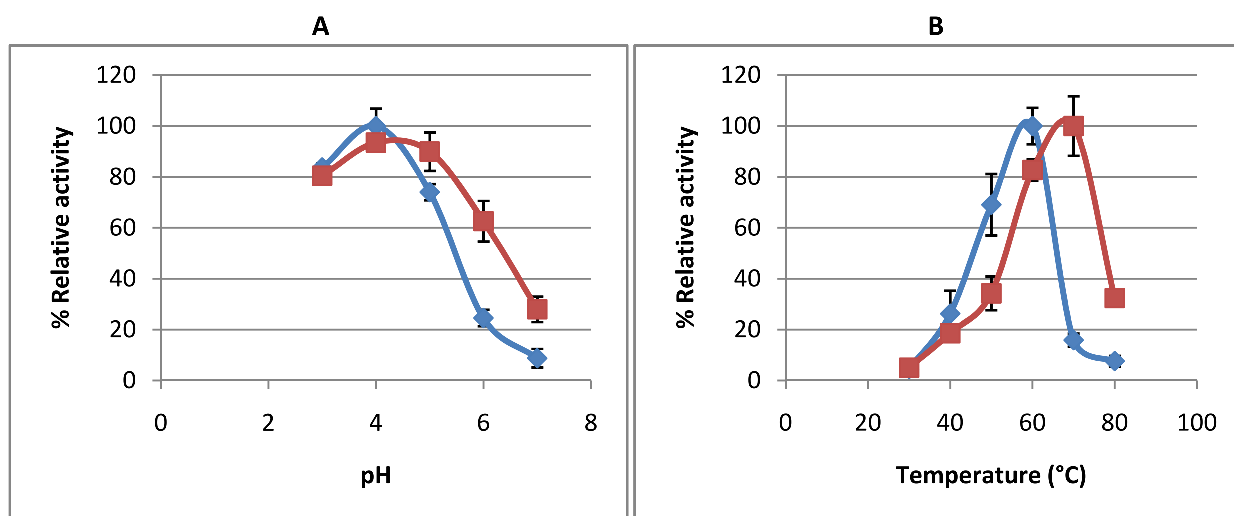

Figure 6: The optimal (A) pH and (B) temperature for the extracellular amylases produced by (◆) *S. cerevisiae* Y294[Atamy] and (■) *S. cerevisiae* Y294[Atglu].

Optimum temperatures of 60°C and 70°C were observed for the recombinant α-amylase and glucoamylase, respectively (Figure 6B). Previous studies showed that the optimum temperature of *Aspergillus* α-amylases and glucoamylases are generally in the range of 40 - 50°C and 40 - 60°C, respectively (Gupta et al., 2003; James and Lee, 1997; Robertson et al., 2006). High levels of activity at a lower temperature would be advantageous to ensure more cost-effective starch hydrolysis in industrial processes (Robertson et al., 2006).

Figure 7 shows the growth rate of the different strains over a period of 72 hrs on double strength SC^{-ura} liquid media, but with the normal strength of glucose at 20 g/L, with exponential growth slowing down after 24 hrs. The negative control *S. cerevisiae* Y294[yBBH1] (containing only the episomal yBBH1 plasmid) grew at a similar rate than the *S. cerevisiae* Y294[Atglu] strain, with a dry weight of 4.3 mg/ml after 72 hrs. The *S. cerevisiae* Y294[Atamy] strain grew much slower, reaching only 2.25 mg/ml after 72 hrs, indicating a possible metabolic burden for the expression of the α-amylase in the yeast strain.

The glucoamylase strain appeared to grow without any constraints, but the slower growth rate of the α-amylase strain may render the combination of the *A. tubingensis* α-amylase and glucoamylase less effective in a consolidated process. The α-amylases are endo-type enzymes, whereas glucoamylases are exo-type enzymes, which implies that the level and activity of the glucoamylase is

in general the limiting factor in the hydrolysis of starch, and therefore also in the bioconversion of raw starch to bio-ethanol (Robertson et al., 2006). Co-expression of both genes in the same yeast strain may thus impede the growth rate (and therefore expression of the recombinant glucoamylase) in the host strain. The enzyme activity of the recombinant α-amylase and glucoamylase (Figure 7) correlated with the growth of the respective strains, with a steep increase over the first 24 hrs. Hereafter, the α-amylase showed a constant rate of activity up to 72 hrs. The glucoamylase activity reached its peak at 18 hrs, with a 33% decline after 24 hrs and thereafter remaining constant up to 72 hrs.

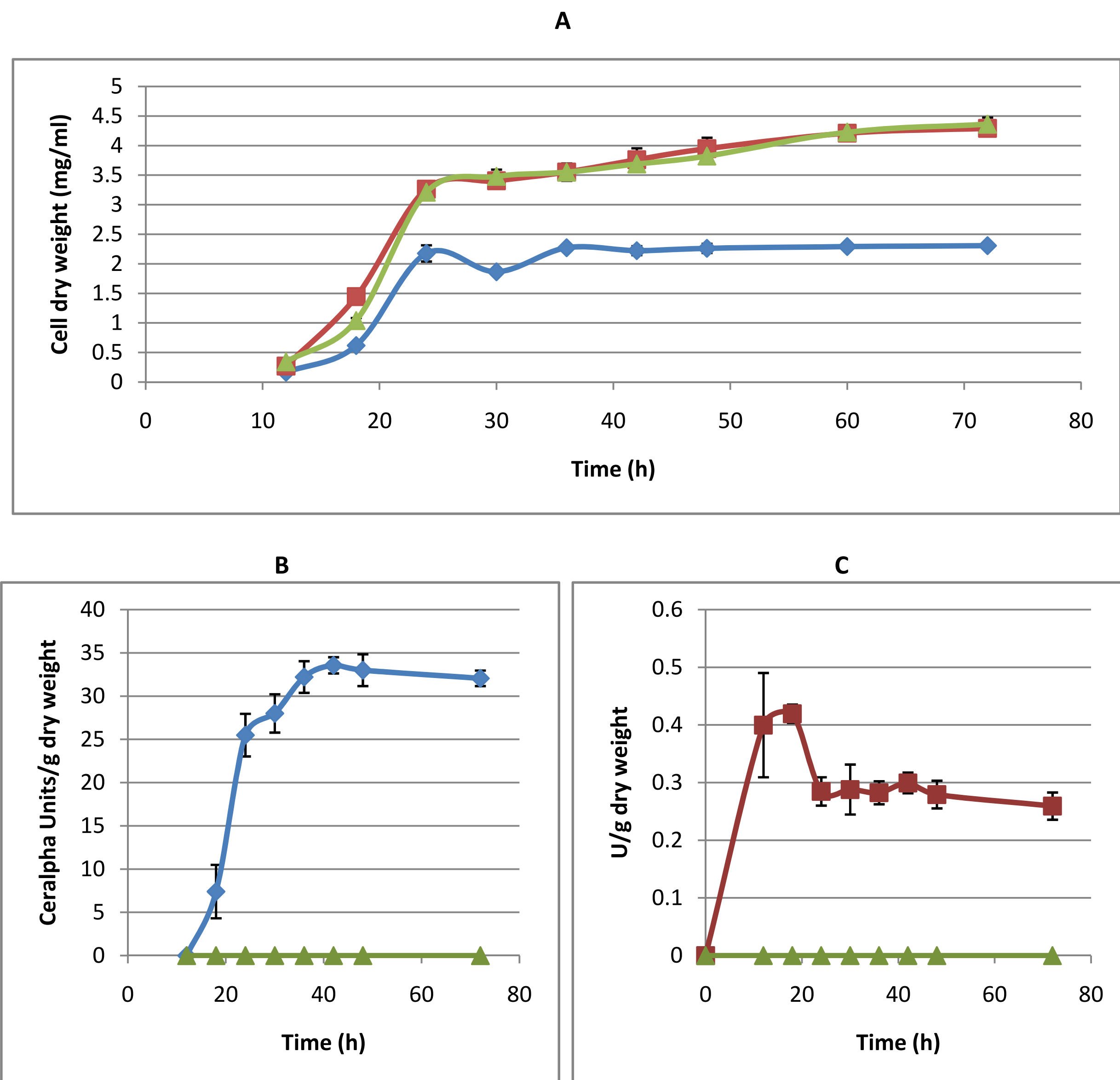

Figure 7: (A) Growth rate of strains (▲) *S. cerevisiae* Y294[yBBH1], (◆) *S. cerevisiae* Y294[Atamy] and (■) *S. cerevisiae* Y294[Atglu] and (B) Extracellular α-amylase activity of *S. cerevisiae* Y294[Atamy] and (C) glucoamylase activity of *S. cerevisiae* Y294[Atglu] relative to the reference strain *S. cerevisiae* Y294[yBBH1], cultured in double strength SC-URA medium with 20 g/L glucose.

3.5 Fermentation studies

Figure 8 indicates the differential hydrolytic abilities of the different transformants and combinations thereof. For the reference strain, *S. cerevisiae* Y294[yBBH1], a large amount of starch precipitated to the bottom of the bottle and the clarity of the supernatant indicates that the starch was not hydrolysed as shown in serum bottle A (Figure 8). The recombinant *S. cerevisiae* Y294[Atamy] strain producing the α-amylase of *A. tubingensis*, showed less accumulation of non-degraded starch, suggesting that the presence of the α-amylase improved the hydrolytic ability of the yeast strain, as shown in serum bottle B (Figure 8). The yeast strain producing the *A. tubingensis* glucoamylase resulted in even less starch precipitation than for the *S. cerevisiae* Y294[Atamy], suggesting a higher efficacy on raw starch, as shown in serum bottle C (Figure 8). The supernatant also appeared to be more turbid relative to that of *S. cerevisiae* Y294[Atamy] and the reference strain, which is a further confirmation of the hydrolytic effect of the extracellular glucoamylase. The co-fermentation of the recombinant strains producing the α-amylase and glucoamylase, respectively, resulted in the absence of a raw starch precipitate with all the remaining starch in suspension as shown in serum bottle D (Figure 8). This confirms that the combination of the two different enzymes yielded the best level of hydrolysis of the raw corn starch.

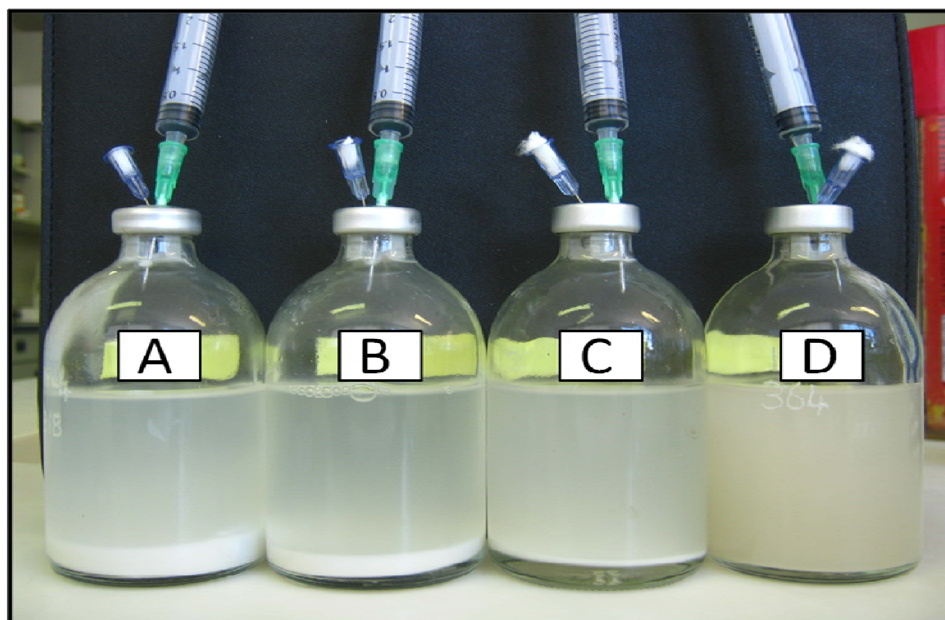

Figure 8: The effect of (A) *S. cerevisiae* Y294[yBBH1], (B) *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[yBBH1], (C) *S. cerevisiae* Y294[Atglu] + *S. cerevisiae* Y294[yBBH1], and (D) a combination of both *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu] when grown anaerobically in serum bottles containing in double strength SC^{-URA} media with 2% corn starch as sole carbon source after 15 days of cultivation.

During fermentation under anaerobic conditions, a weight loss due to CO_2 production is a rough indication of the ability of the yeast cells to metabolise starch through its conversion to simple sugars. The combination of the α-amylase and glucoamylases in *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu] resulted in the highest weight loss (representing CO_2 production) after 16 days, with a total weight loss of approximately 1 g (Figure 9). The *S. cerevisiae* Y294[Atglu] + Y294[yBBH1] strains expressing only the glucoamylase had the second highest weight loss of 0.4 g after 16 days, followed closely by *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[yBBH1], expressing only the α-amylase, with a weight loss of 0.38 g.

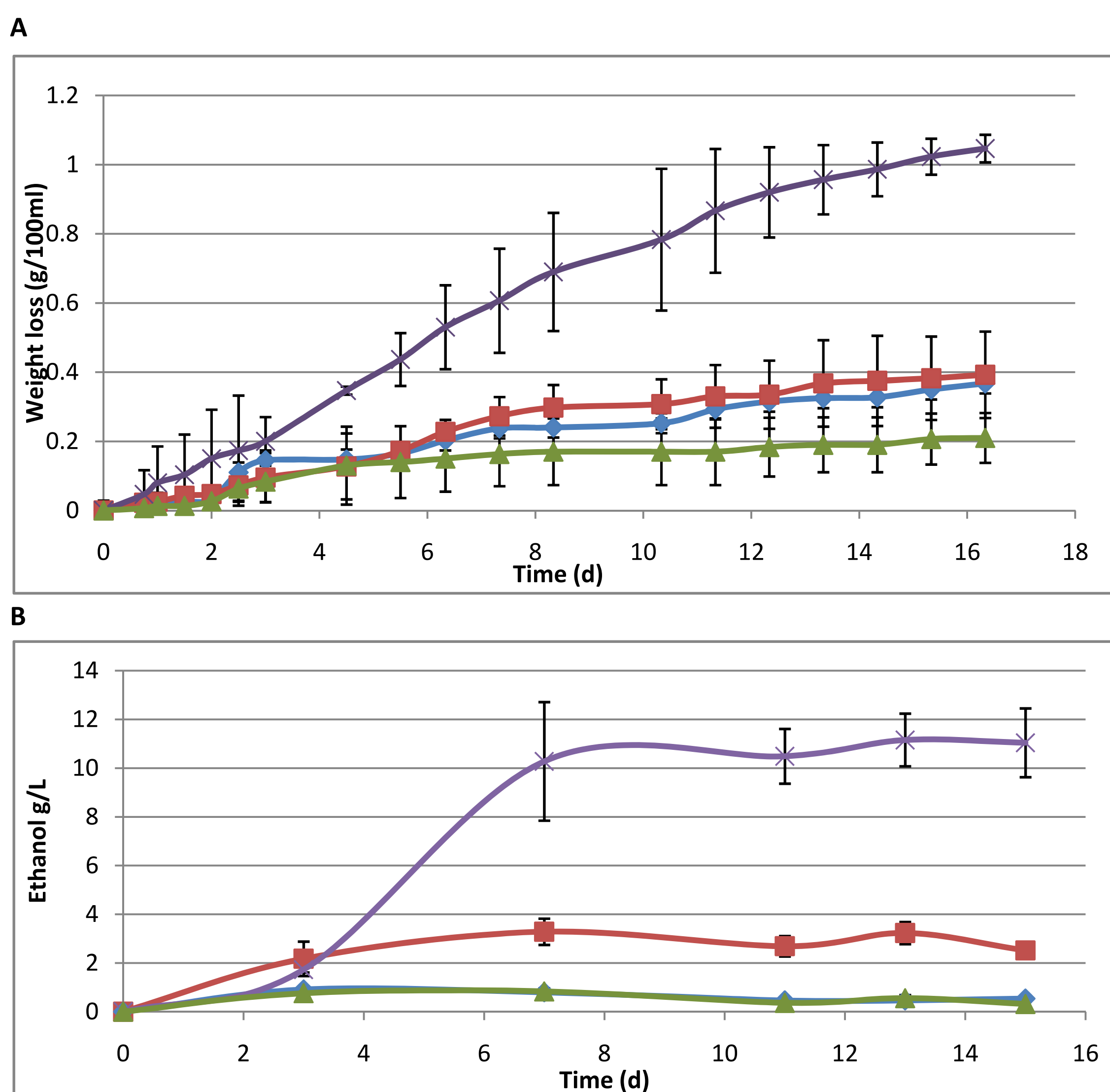

Figure 9: Weight loss incurred (A) and ethanol production (B) by (◆) *S. cerevisiae* Y294[Atamy] + Y294[yBBH1], (■) *S. cerevisiae* Y294[Atglu] + Y294[yBBH1] and (X) *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu], relative to the reference strain (▲) *S. cerevisiae* Y294[yBBH1], cultured in double strength SC^{-URA} media with 2% corn starch as sole carbon *source* under anaerobic conditions (note that data points for (◆) and (▲) overlap).

The negative control had a weight loss of 0.2 g after 16 days, indicating a 5-fold improvement for the *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu] combination relative to the host strain, whereas production of either the α-amylase or glucoamylase could improve the fermentation rate only by 2-fold. This confirmed that the combination of the exo- and endo- type enzymes is superior for fermentation of the raw corn starch, relative to the expression of only one of the enzymes.

HPLC analysis confirmed that the *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu] combination resulted in the highest ethanol yield, i.e. 11.04 g/l after 15 days under fermentative conditions (Figure 9B). This was followed by *S. cerevisiae* Y294[Atglu] + *S. cerevisiae* Y294[yBBH1] with 2.5 g/l, whereas *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[yBBH1] yielded only 0.53 g/l, very similar to the 0.32 g/l of the reference strain. These results confirmed that the combination of the α-amylase and glucoamylase of *A. tubingensis* improved the production of ethanol from raw corn starch under fermentative conditions by 37-fold. Cell counts confirmed that the *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu] combination was able to grow on raw starch, with the number of cells increasing from 1×10^5 cells/ml (0.5×10^5 cells/ml per strain) to 2.65×10^6 (combined) at the end of fermentation.

During the fermentation experiments, the raw starch was hydrolysed to reducing sugars (glucose) that could be fermented to produce ethanol and CO_2, or could be utilised for biomass production (i.e. cell growth). Residual sugars present in the culture indicated a varying degree of consumption by the yeast cells (Table 3). Once again, the *S. cerevisiae* Y294[Atamy] + *S. cerevisiae* Y294[Atglu] combination was superior with 62% less reducible sugars than the reference strain. The *S. cerevisiae* Y294[Atglu] strain showed a higher sugar consumption than the reference strain, the *S. cerevisiae* Y294[Atamy] was less effective, resulting in higher reducible sugars relative to the reference strain. This phenomenon might be due to the fact that the α-amylase expressed by the yeast is an endo-acting enzyme, releasing glucose from starch quicker than it could be utilised by the yeast strain, resulting in a net increase in free reducible sugars.

Table 3: Residual total reducible sugars in different recombinant fermentations

Recombinant yeast strain combination	Day 3	Day 15
	µmol/ml	
Y294[Atamy]+Y294[BBH]	49.18	47.82
Y294[Atglu]+Y294[BBH]	51.28	38.22
Y294[Atamy]+Y294[Atglu]	45.88	15.29
Y294[BBH]	45.44	40.11

4. CONCLUSIONS

In this study, the α-amylase and glucoamylase genes from *A. tubingensis* were isolated and individually expressed in *S. cerevisiae*. When used in combination, it allowed the yeast strains to grow on raw starch as the sole carbon source and utilize the substrate for the production of ethanol in liquid cultures. When taking into consideration that the medium contained 20 g/l raw starch, a yield of 11.04 g/l ethanol translates to 98% of the theoretical yield from starch with a 54% conversion of the total raw starch (as per www1.eere.energy.gov/biomass/ ethanol_yield_calculator.html).

The rate of conversion was however lower than in comparison with other reports. These other studies reported ethanol yields ranging from 20 to 61.8 g/l after 2.5 to 3 days with different recombinant strains with the conversion of raw starch (Khaw et al., 2006; Shigechi et al., 2004). However, these genes were integrated into the genomic DNA of the host *S. cerevisiae* strain and fermentation processes were optimised for ethanol production (de Villiers, 2008). To our knowledge, this is the first combined expression of two different amylases from the same organism in a yeast host for the production of bio-ethanol from raw corn starch. It is thus evident that the co-expression of amylolytic enzymes from the same organism might complement one another for higher ethanol yields in comparison with the expression of amylolytic enzymes from different organisms in a yeast host.

In the present study, the genes were expressed on episomal plasmids to demonstrate the ability of the *A. tubingensis* genes to convey amylolytic activity to a laboratory strain of *S. cerevisiae*. Further research will include testing different copy numbers of the α-amylase and glucoamylase genes in different industrial strains of *S. cerevisiae*. It would also be advantageous to integrate the α-amylase and glucoamylase genes of this study in different industrial strains of *S. cerevisiae*, which in turn might lead to higher ethanol yields. It might also be the case that by just transforming the yeast strain with episomal genes will lead to a higher metabolic burden and thus placing extra strain on the transformed yeast strain.

Since the cost of biomass used in the production of bio-ethanol amounts to nearly one third of the total price of production, it is imperative that the yield of bio-ethanol from a specific carbon source should be optimised (Balat et al., 2008). This study indicated that higher yields of bio-ethanol production from raw starch are indeed possible without any pre-treatment. This single advancement will result in significant reductions in the production cost of bio-ethanol.

The production of bio-ethanol from starch has received a lot of criticism in past as starch is also an important source of human food, but this has not stopped continuous efforts to optimise its production processes and/or find alternative technologies to improve current processes (Balat et al.,

2008; Bungay, 2004). It should also be kept in mind that bio-ethanol is not the only alternative and sustainable fuel source available, but it could play a significant role towards a better future (Viktor, 2011).

5. REFERENCES

Achal V, Savant VV, and Reddy MS (2007) Phosphate solubilisation by a wild type strain and UV-induced mutants of *Aspergillus tubingensis*. *Soil Biol Biochem* 39:695-699

Balat M, Balat H, and Oz C (2008) Progress in bioethanol processing. *Prog Ener Combust Sci* 34:551-573

Biely P, de Vries RP, Vrsanska M, Visser J (2000) Inverting character of α-glucuronidase A from *Aspergillus tubingensis*. *Biochem Biophys J* 1474:360-364

Bungay HR (2004) Confessions of a bioenergy advocate. *TRENDS Biotechnol* 22:67-71

de Villiers T (2008) Fungal enzymes and microbial systems for industrial processing. Thesis/Dissertation. University of Stellenbosch

den Haan R, McBride JE, La Grange DC, Lynd LR, and van Zyl WH (2007) Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. *Enzyme Microb Technol* 40:1291-1299

Giraud F, Dupont J, Haon M, Bouzid O, Alibeu O, Navarro D, Sage L, Seigle-Murandi F, Asther M, and Lesage-Meessen L (2007) Phylogenetic analysis of the *Aspergillus niger* aggregate in relation to feruloyl esterase activity. *Res Microbiol* 158:413-419

Goto M, Semimaru T, Furukawa K, and Hayashida S (1994) Analysis of the raw starch-binding domain by mutation of a glucoamylase from *Aspergillus awamori* var. *kawachi* expressed in S*accharomyces cerevisiae*. *Appl Environ Microbiol* 60:3926-3930

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. *Process Biochem* 38:1599-1616

Hill J, Donald KA, and Griffiths DE (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res* 19:5791

James JA, and Lee BH (1997) Glucoamylases: microbial sources, industrial applications and molecular biology – a review. *J Food Biochem* 21:1-52

Jin B, van Leeuwen HJ, Patel B, Doelle HW, and Yu Q (1999) Production of fungal protein and glucoamylase by Rhizopus oligosporus from starch processing wastewater. *Process Biochem* 34:59-65

Kaneko A, Sudo S, Takayasu-Sakamoto Y, Tamura G, Ishikawa T, and Oba T (1996) Molecular cloning and determination of the nucleotide sequence of a gene encoding an acid-stable α-amylase from *Aspergillus kawachii*. *J Fermen Bioeng* 81:292-298

Khaw TS, Katakura Y, Koh J, Kondo A, Ueda M, and Shioya S (2006) Evaluation of performance of different surface-engineered yeast strains for direct ethanol production from raw starch. *Appl Microbiol Biotechnol* 70:573-579

Kimura I, Yoshioka N, Kimura Y, and Tajima S (2000) Cloning, sequencing and expression of an α-L-arabionfuranosidase from *Aspergillus sojae*. *J Biosci Bioeng* 89:262-266

Kirk O, Borchert TV, and Fuglsang CC (2002) Industrial enzyme applications. *Curr Opinion Biotechnol* 13:345-351

Koutinas AA, Wang R, Kookos IK, and Webb C (2003) Kinetic parameters of *Aspergillus awamori* in submerged cultivations on whole wheat flour under oxygen limiting conditions. *Biochem Eng J* 16:23-34

La Grange DC, Pretorius IS, Van Zyl WH (1996) Expression of a *Trichoderma reesei* beta-xylanase gene (*XYN2*) in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 62:1036-1044

Laemmli UK, Beguin F, and Gujer-Kellenberger (1970) A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J Mol Biol* 47:69-85

Lin LL, Ma YJ, Chien HR, and Hsu WH (1998) Construction of an amylolytic yeast by multiple integration of the *Aspergillus awamori* glucoamylase gene into a *Sachharomyces cerevisiae* chromosome. *Enzyme Micro Technol* 23:360-365

Manger-Jacob F, Muller T, Janssen M, Hofer M, and Holker U (2005) Isolation and sequencing of a new glucoamylase gene from an *Aspergillus niger* aggregate strain (DSM 823) molecularly classified as *Aspergillus tubingensis*. *Ant Leeuwen* 88:267-275

Martinez-Culebras PV, and Ramon D (2007) An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. *Int J Food Microb* 113:147-153

Medina A, Mateo EM, Valle-Algarra FM, Mateo F, Mateo R, and Jimenez M (2008) Influence of nitrogen and carbon sources on the production of ochratoxin A by ochratoxigenic strains of *Aspergillus* spp. Isolated from grapes. *Int J Food Microb* 122:93-99

Miller GL (1959) Use of dinitrosalicyclic acid reagent for determination of reducing sugars. *Anal Chem* 31:426-428

Moller K, Sharif MZ, and Olsson L (2004) Production of fungal α-amylase by *Saccharomyces kluyveri* in glucose-limited cultivations. *J Biotechnol* 111:311-318

Mukerjea R, Slocum G, Mukerjea R, and Robyt JF (2006) Significant differences in the activities of α-amylases in the absence and presence of polyethylene glycol assayed on eight starches solubilised by two methods. *Carbohydr Res* 341:2049-2054

Murai T, Yoshino T, Ueda M, Haranoya I, Ashikari T, Yoshizumi H, and Tanaka A (1998) Evaluation of the function of arming yeast displaying glucoamylase on its cell surface by direct fermentation of corn to ethanol. *J Fermen Bioengineer* 6:569-572

Nunberg JH, Maede JH, Cole G, Lawyer FC, McCabe P, Schweickart V, Tal R, Wittman VP, Flatgaard JE, and Innis MA (1984) Molecular cloning and characterization of the glucoamylase gene of *Aspergillus awamori*. *Mol Cell Biol* 4:2306-2315

Polaye BL, Hodgson KT, and Malte PC (2007) An economic analysis of bio-energy options using thinning from overstocked forests. *Biomass Bioener* 31:105-125

Randez-Gil F, Prieto JA, Murcia A, and Sanz P (1995) Construction of baker's yeast strains that secrete Aspergillus oryzae alpha-amylase and their use in bread making. *J Cereal Sci* 21:185-193

Reddy MS, Kumar S, Babita K, and Reddy MS (2002) Biosolubilization of poorly soluble rock phosphates by *Aspergillus tubingensis* and *Aspergillus niger*. *Bioresour Technol* 84:187-189

Robertson GH, Wong DWS, Lee CC, Wagschal K, Smith MR, and Orts WJ (2006) Native or raw starch digestion: A key step in energy efficient biorefining of grain. *J Agric Food Chem* 54:353-365

Russo S, Siman-Tov RB, and Poli G (1995) Yeasts: from genetics to biotechnology. *J Environ Path Toxic Onco* 14:133-157

Sadie CJ, Rose SH, den Haan R, and van Zyl WH (2011) Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by *Saccharomyces cerevisiae. Appl Microbiol Biotechnol* submitted for publication

Sambrook J, Fritsch EF, and Maniatis T (1989) Molecular cloning: A laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sauer J, Sigurskjold BW, Christensen U, Frandsen TP, Mirgorodskaye E, Harrison M, Roepstorff P, and Svensson B (2000) Glucoamylase: structure/function relationship, and protein engineering. *Biochem Biophys J* 1543:275-293

Shigechi H, Koh J, Fujita Y, Matsumoto T, Bito Y, Ueda M, Satoh E, Fukuda H, and Kondo A (2004) Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and alpha-amylase. *Appl Environ Microbiol* 70:5037-5040

Steyn AJC, Marmur J, and Pretorius IS (1995) Cloning, sequence analysis and expression in yeasts of a cDNA containing *a Lipomyces kononenkoae* s-amylase-encoding gene. *Gene* 166:65-71

Sun H, Zhao P, Ge X, Xia Y, Hao Z, Liu J, Peng M (2010) Recent advances in microbial raw starch degrading enzymes. *Appl Biochem Biotechnol* 160:998-1003

Suresh C, Dubey AK, Srikanta S, and Kumar US (1999) Characterization of starch hydrolyzing enzyme of *Aspergillus niger*. *Appl Microbiol Biotechnol* 51:673-675

Tester RF, Karkalas J, and Qi X (2004) Starch – composition, fine structure and architecture. *J Cereal Sci* 39:151-165

van Diepeningen AD, Debets AJM, Varga J, van der Gaag M, Swart K, and Koekstra RF (2004) Efficient degradation of tannic acid by black *Aspergillus* species. *Mycol Res* 108:919-925

White TJ, Bruns T, Lee S, and Taylor J (1990) in: A. Innis, Gelfand D. H. and J. J. Sninsky (eds.), PCR Protocols, Academic Press, San Diego, USA, pp. 315-322

Yoon S, Fulton DB, and Robyt JF (2006) Formation of covalent β-linked carbohydrate-enzyme intermediates during the reactions catalyzed by α-amylases. Carbohydr Res 342:55-64

Zhan J, Gunaherath GMKB, Wijerathe EMK, and Gunatilaka AAL (2007) Asperpyrone D and other metabolites of the plant-associated fungal strain *Aspergillus tubingensis*. *Phytochem* 68:368-372

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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It is clear that biofuels alone will not be able to meet the growing energy demand of the world, but it could play a significant role in achieving this goal. The current cost of producing bio-ethanol is still very high and it is thus important that the overall cost should be reduced to ensure the cost-effective production of biofuels in the future. The development of CBP technology and thus the engineering of a microbial system for the conversion of raw starch to ethanol would be able to result in significant cost reductions as the need of for expensive pre-treatment processes and the addition of industrial enzymes for the liquefaction and saccharification of starch is eliminated. Not only will such a microbial organism be capable of converting the simple sugars to ethanol, but it will also be able to produce its own enzymes for the hydrolysis of the substrate.

With this study it was possible to further emphasize the significant contribution that natural enzymes could make towards the industrial sector with their rate of activity and high specificity towards a specific substrate, in this case raw corn starch. Bioprospecting empowers us to utilize organisms from nature in assisting us to develop better industrial applications. From the 36 strains evaluated, *Aspergillus tubingensis* was the best organism with the highest specificity towards raw corn starch and also the highest compatibility to the laboratory strains of *S. cerevisiae*. Although *A. tubingensis* has been studied in the past by other groups, there has been no report on the expression of the α-amylase (Atamy) and the glucoamylase (Atglu) genes from this organism in *S. cerevisiae* for the hydrolysis of raw corn starch.

The expression of Atamy and Atglu in *S. cerevisae* Y294 indicated that the glucoamylase is the limiting factor in the complete hydrolysis of raw corn starch. Furthermore, the combination of the two recombinant yeast strains that respectively expressed Atamy and Atglu was able to completely hydrolyse and utilize raw corn starch without any additional treatment of the substrate. Fermentation studies revealed that the highest yield of ethanol from the combination of the recombinant strains was 11.04 g/l, which implies 98% of the theoretical yield of ethanol from starch and a 52% conversion of the total starch content. Although this rate of conversion is lower as that achieved with other studies, none of the fermentation steps have been optimised. We are also convinced that if the Atamy and Atglu genes are co-expressed and integrated within the genome of a single *S. cerevisiae* cell, it could result in an amylolytic yeast strain with a higher rate of activity.

Future research could investigate different sets of α-amylase and glucoamylase genes originating from different organisms to find the best combination. Different *S. cerevisiae* strains could be evaluated, with the ultimate aim of expressing the best set of genes in an industrial strain capable of producing very high levels of ethanol. Future research would also focus on the optimisation of the fermentation process itself, i.e. temperatures, pH, which will also include raw starch substrates from different biomass sources.