

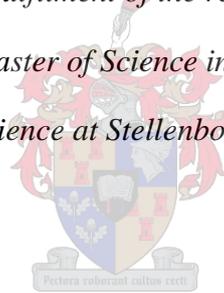
Cloning and expression of fungal alpha-amylase genes in *Saccharomyces cerevisiae* with integrated glucoamylase gene for raw starch conversion into bioethanol

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DECLARATION

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SUMMARY

Increasing population numbers and the rapid growth of technology and industry have resulted in an increase in energy demand. Biomass-based fuels (biofuels) have received considerable interest as an alternative transport fuel as biomass is abundant, cheap and renewable. Starch is a good feedstock for bioethanol production with a mature technology established in the USA. However, the current starch-to-ethanol conversion process requires a high energy input and high amyolytic enzyme loadings for liquefaction, resulting in economic challenges.

The yeast *Saccharomyces cerevisiae* is traditionally the preferred host for bioethanol production due to its high ethanol productivity, tolerance and high fermentation capacity, but is unable to utilise or ferment starch. Genetic engineering allows the construction of amyolytic *S. cerevisiae* strains that can convert starch to glucose and ferment the latter to ethanol. The application of raw starch hydrolysing enzymes could reduce the process time and cost of ethanol production, thus improving its economic feasibility. In this study, a literature and database search was conducted to obtain DNA sequences of genes encoding raw starch hydrolysing amylases. The *Aureobasidium pullulans* *ApuA*, *Aspergillus terreus* *AteA*, *Cryptococcus* sp. S-2 *CryA* and *Saccharomycopsis fibuligera* *SfiA* α -amylase encoding genes were synthesised and expressed on an episomal multicopy vector in a *S. cerevisiae* laboratory strain using the *Trichoderma reesei* *xyn2* secretion signal.

The *S. cerevisiae* Y294[*AteA*] and Y294[*ApuA*] strains displayed the highest levels of volumetric activity for the recombinant α -amylases (3.20 U.ml⁻¹ and 2.57 U.ml⁻¹, respectively) when grown in SC^{-URA} medium. The recombinant *AteA* and *ApuA* proteins were glycosylated and displayed pH optima between pH 4 and 5. Both enzymes were stable at 30°C and maintained up to 80% activity after 5 days. The *ApuA* and *AteA* genes were co-expressed with the *Aspergillus tubingensis* *GlaA* glucoamylase to generate the *S. cerevisiae* Y294[*ApuA-GlaA*] and Y294[*AteA-GlaA*] strains, respectively. When cultivated on 200 g.l⁻¹ raw starch, the Y294[*AteA-GlaA*] strain produced 43.81 g.l⁻¹ ethanol after 192 hours, which was significantly higher than the Y294[*AmyA-GlaA*] benchmark strain (41.02 g.l⁻¹) and the Y294[*ApuA-GlaA*] strain (32.83 g.l⁻¹).

The Y294[*AteA-GlaA*] strain displayed a maximum yield of 57 g.l⁻¹ ethanol in fermentations supplemented with STARGENTM 002 (commercial enzyme cocktail), indicating the margin of improvement possible in improving process efficiency. Assessment of the Y294[*AteA-GlaA*] strain using various optimisation strategies concluded that additional

glucoamylase would improve the fermentation rate and thus decrease the required fermentation time. High substrate loading reduced the fermentation efficiency, with up to a 50% improvement in starch conversion when the substrate loading was halved.

In this study, ethanol production was strain dependent (as only one parental strain was used), signifying that any further increase in enzyme production will not result in an increased ethanol yield, but will instead result in an improved fermentation rate. This study provides insights into the dynamics of hydrolysis of raw starch in a single-step Consolidated Bioprocessing (CBP) process. The importance of using appropriate enzyme ratios is highlighted as it ensures the improved efficiency and effectiveness of a CBP system. The knowledge obtained from this study is useful in the realisation of economic benefits of process integration in CBP for commercial starch-based biofuel production streams.

OPSOMMING

Toenemende bevolkingsgetalle en die vinnige groei in tegnologie en nywerheid het 'n toename in energiebehoefte tot gevolg. Biomassa-gebaseerde brandstof (biobrandstof) geniet groot belangstelling omdat biomassa volop, goedkoop en hernubaar is. Stysel is 'n goeie roumateriaal vir bio-etanolproduksie met volwasse proses tegnologie wat in die VSA gevestig is. Die huidige stysel-tot-etanol omskakelingsproses verg egter 'n hoë energie-inset en hoë amilolitiese ensiemladings vir vervloeiing, wat tot ekonomiese uitdagings lei.

Die gis *Saccharomyces cerevisiae* is tradisioneel die voorkeurgasheer vir bio-etanolproduksie weens sy hoë etanolproduktiwiteit, -verdraagsaamheid en hoë gistingskapasiteit, maar kan nie stysel benut of fermenteer nie. Genetiese manipulasie maak die konstruksie van amilolitiese *S. cerevisiae* stamme moontlik wat stysel na glukose kan omskakel en laasgenoemde tot etanol kan fermenteer. Die toepassing van rou stysel-hidroliserende ensieme kan die prosesseringtyd en koste van etanolproduksie verminder en gevolglik die ekonomiese lewensvatbaarheid daarvan verbeter. In hierdie studie is literatuur en databasisse deursoek vir DNS-volgordes van gene wat vir rou stysel hidroliserende amilases kodeer. Die geenvolgordes vir die *Aureobasidium pullulans* *ApuA*, *Aspergillus terreus* *AteA*, *Cryptococcus* sp. S-2 *CryA* en *Saccharomycopsis fibuligera* *SfiA* α -amilases is gesintetiseer en op 'n episomale multikopievektor in 'n *S. cerevisiae* laboratoriumstam uitgedruk deur die *Trichoderma reesei* *xyn2* sekresiesein te gebruik.

Die *S. cerevisiae* Y294 [*AteA*] en *S. cerevisiae* Y294 [*ApuA*] rasse het die hoogste vlakke van volumetriese aktiwiteit vir die rekombinante α -amilases (3.20 U.ml^{-1} en 2.57 U.ml^{-1} , onderskeidelik) tydens groei op $\text{SC}^{-\text{URA}}$ medium getoon. Die rekombinante *AteA* en *ApuA* proteïene was versuiker en het pH optima tussen pH 4 en 5 getoon. Beide ensieme was by 30°C stabiel en het tot 80% aktiwiteit na 5 dae behou. Die *ApuA* en *AteA* gene is saam met die *Aspergillus tubingensis* *GlaA* glukoamilase in *S. cerevisiae* uitgedruk om onderskeidelik die *S. cerevisiae* Y294 [*ApuA-GlaA*] en *S. cerevisiae* Y294 [*AteA-GlaA*] rasse te skep. Die *S. cerevisiae* Y294 [*AteA-GlaA*] ras het 43.81 g.l^{-1} etanol vanaf 200 g.l^{-1} rou stysel na 192 ure geproduseer, wat beduidend meer as die *S. cerevisiae* Y294[*AmyA-GlaA*] verwysingsras (41.02 g.l^{-1}) en die Y294[*ApuA-GlaA*] ras (32.83 g.l^{-1}) was.

Die *S. cerevisiae* Y294 [*AteA-GlaA*] ras het 'n maksimum opbrengs van 57 g.l^{-1} etanol getoon in fermentasies wat met STARGENTM 002 ('n kommersiële ensiemmengsel) aangevul is, wat die ruimte vir verbetering van die proses-effektiwiteit aandui. Assessering van die

S. cerevisiae Y294 [AteA-GlaA] ras met verskillende optimiseringstrategieë het aangedui dat bykomende glukoamilase die fermentasie koers kan verbeter en derhalwe die tydsduur van die fermentasie kan verkort. Hoë substraatladings verminder die fermentasie-effektiwiteit, met tot 'n 50% verbetering in styselomskakeling met die helfte van die substraatlading.

In hierdie studie was etanolproduksie ras-afhanklik (aangesien slegs een ouerras gebruik is), wat beklemtoon dat enige verdere toename in ensiemproduksie nie 'n toename in etanolproduksie teweeg sal bring nie, maar eerder 'n beter fermentasietempo. Hierdie studie bied insigte in die dinamika van rou stysel-hidrolise in 'n een-stap Gekonsolideerde Bioprosesserings (GBP) proses. Die belang van toepaslike ensiemverhoudings is beklemtoon siende dat dit verbeterde effektiwiteit van 'n GBP proses verseker. Die kennis wat uit hierdie studie voorspruit is nuttig om die ekonomiese voordele van prosesintegrasie in GBP vir kommersiële stysel-gebaseerde biobrandstofproduksie strome te realiseer.

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Table of Contents

DECLARATION	i
SUMMARY	ii
OPSOMMING	iv
ACKNOWLEDGEMENTS	vi
ABBREVIATIONS	x
 Chapter 1: Introduction and aims of the study	
1.1 Introduction.....	2
1.2 Aims of this study	3
1.3 References.....	4
 Chapter 2: Literature review	
2.1 Biofuels	8
2.2 Bioethanol.....	10
2.2.1 Global bioethanol production.....	12
2.2.2 Starch as bioethanol feedstock	14
2.3 Starch-hydrolysing enzymes.....	17
2.3.1 Enzyme synergy	19
2.3.2 Alpha-amylases	19
2.3.2.1 Structural and functional analysis	20
2.3.2.2 Mode of action	22
2.3.3 Glucoamylases	22
2.3.3.1 Structural and functional analysis	23
2.3.3.2 Mode of action	24
2.3.4 Industrial applications	25
2.4 Fermentation	27
2.4.1 Amylolytic yeast strains	27
2.4.2 Secretory systems.....	29
2.4.3 Fermentation configurations	30
2.5 Consolidated Bioprocessing of starch.....	30
2.6 This study.....	33
2.7 References.....	35

Chapter 3: Expression and evaluation of α -amylases required for the hydrolysis of raw starch

Abstract	55
3.1 Introduction.....	56
3.2 Materials and Methods.....	58
3.2.1 Media and strain cultivation.....	58
3.2.2 Strains and plasmids.....	58
3.2.3 Strain construction.....	60
3.2.4 Protein analysis	62
3.2.5 Amylase assays and enzyme characterisation.....	62
3.2.6 Fermentation studies	63
3.2.6.1 HPLC analysis.....	63
3.3 Results.....	64
3.3.1 Strain construction and evaluation	64
3.3.2 Enzyme characterisation	65
3.3.3 Fermentation of raw starch.....	67
3.4 Discussion.....	73
3.5 Competing interests	77
3.6 Acknowledgements.....	77
3.7 References.....	77
Chapter 4: General discussion and conclusion	
4.1 Introduction.....	84
4.2 Discussion and conclusions	85
4.3 Future work.....	87
4.4 References.....	89

ABBREVIATIONS

1G	First Generation
2G	Second Generation
3G	Third Generation
4G	Fourth Generation
CBP	Consolidated bioprocessing
DNS	Dinitrosalicylic acid
DNA	Deoxyribonucleic Acid
EC	Enzyme Commission
GRAS	Generally Regarded As Safe
HPLC	High Performance Liquid Chromatography
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
GHG	Green House Gas
GH	Glycosyl Hydrolases
CBM	Carbohydrate Binding Module
SBD	Starch Binding Domain
RSDE	Raw Starch Degrading Enzyme

Chapter 1

Introduction and aims of the study

1.1 Introduction

More than 80% of the world's energy requirements are met by fossil fuels (I.E.A., 2015). Fossil fuel combustion results in various gases, such as sulphur dioxide and carbon dioxide, being emitted into the atmosphere causing environmental problems, including acid rain and global warming (Yamada et al., 2009). Increasing concern regarding the lasting environmental impacts as well as the anticipated depletion of fossil fuel reserves has necessitated the search for sustainable alternative energy sources (Gupta and Verma, 2015) that are renewable and pose minimal to no risk to the environment.

Various alternative energy sources are currently being exploited, namely wind, solar, biomass, geothermal and hydro energy. Of these, biomass is the only established renewable primary energy source that can provide alternative transportation fuels, such as bioethanol and biodiesel (Hamelinck et al., 2005; Alvira et al., 2010). The current (established) bioethanol production processes use starchy materials and sugars from crops and plant waste as feedstock. In 2015, the leading bioethanol producer was the USA using maize as feedstock, followed by Brazil that uses sugarcane (R.F.A., 2016). Lignocellulosic material is relatively abundant, renewable and does not compete with food crops (Tomás-Pejó et al., 2008; Alvira et al., 2010). Nevertheless, lignocellulose requires costly pre-treatment for efficient enzymatic hydrolysis and has a high enzyme requirement. In addition, the pre-treatment processes release inhibitors that negatively affect the fermentation process (den Haan et al., 2013). However, more research is required to develop technologies for the cost-effective commercial production of bioethanol from lignocellulosic plant material.

The cost of bioethanol production is higher than the cost associated with the utilisation of fossil fuels (Gupta and Verma, 2015), with biomass pre-treatment and enzyme addition representing the two major cost items in bioethanol production. Enzymes are applied as a substitute for acid hydrolysis of starch as the latter is linked to corrosion, higher energy requirements for heating and results in a high salt-ash content (<http://www1.lsbu.ac.uk/water/enztech/starch.html>). In starch hydrolysis, gelatinisation (heating of the feedstock) is essential as it increases the susceptibility of the starch to enzymatic attack. Ethanol production from starch involves three steps following gelatinisation: (1) liquefaction of starch by α -amylases, (2) saccharification by glucoamylases

and (3) fermentation of glucose to ethanol (Lee et al., 2012; van Zyl et al., 2012; Aydemir, 2014). Further optimisation of the current production processes is of paramount importance to improve cost and process efficiency. Although a higher enzyme load is required for raw starch hydrolysis, it is vital for cost reduction since the gelatinisation step (which requires heat) can be eliminated.

In industrial processes, the yeast *Saccharomyces cerevisiae* is commonly used for the conversion of sugars to ethanol; it has Generally Regarded As Safe (GRAS) status, is well-characterised and easy to manipulate (Görgens et al., 2014). However, this yeast is unable to hydrolyse starch, which has led to new technologies involving genetic manipulation to develop amyolytic yeast strains (Aydemir, 2014). These amyolytic yeast strains (expressing both α -amylases and glucoamylases) are able to hydrolyse starch and ferment the resulting sugars to ethanol in one step - a simultaneous saccharification and fermentation process. This process integration is referred to as consolidated bioprocessing (CBP) and has the potential to reduce biofuel production costs (Viktor et al., 2013). However, ethanol productivity has not been sufficiently improved and the production costs for starch-based ethanol remain high.

Although both α -amylases and glucoamylases are required for raw starch hydrolysis, some studies have indicated that α -amylases are the limiting factor in the starch-to-ethanol conversion process (Yamada et al., 2009). There is thus a need to identify more efficient raw starch hydrolysing α -amylases to enhance the efficiency of the process, thereby improving bioethanol production from starchy materials.

1.2 Aims of this study

The specific aims of this study were to:

- identify, clone and express fungal α -amylase encoding genes in *S. cerevisiae* and
- construct amyolytic *S. cerevisiae* strains to demonstrate raw starch conversion to ethanol.

Addressing these aims involved the following objectives:

- to perform a literature and database search to identify α -amylases with high activity on raw starch;
- to clone and express the candidate α -amylase genes in *S. cerevisiae*;
- to identify the recombinant *S. cerevisiae* strain(s) with the highest levels of α -amylase activity;
- to determine the optimum temperature and pH of the best-performing α -amylases;
- to sub-clone the *Aspergillus tubingensis* *GlaA* glucoamylase gene for co-expression with the best-performing α -amylase encoding gene(s) to construct amyolytic *S. cerevisiae* strains;
- to evaluate the amyolytic strains' ability to hydrolyse raw starch and ferment the resulting glucose to ethanol; and
- to investigate different strategies to optimise ethanol production by the amyolytic strains.

In this study, the secretion signal from the *Trichoderma reesei xyn2* was used to direct secretion of the amylases. The enzyme activity of the recombinant strains was compared to that of *S. cerevisiae* Y294[AmyA] expressing the *A. tubingensis* *AmyA* α -amylase, whilst for the fermentations, the amyolytic strains were compared to the *S. cerevisiae* Y294[AmyA-GlaA] strain expressing the *A. tubingensis* *GlaA* glucoamylase and *AmyA* α -amylase encoding genes. The *S. cerevisiae* Y294[AmyA-GlaA] strain displayed significant extracellular amyolytic activity in a related study (Viktor et al., 2013) and thus represented an excellent benchmark strain.

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Chapter 2

Literature review

2.1 Biofuels

Energy is an important global commodity with fossil fuels representing the major source. Fossil fuels include crude oil, coal, natural gas and their derivatives such as gasoline, diesel, kerosene and alcohol fuels (I.E.A., 2015). The transport sector is heavily dependent on fossil fuels as most transport fuels are petroleum based. Fossil fuel combustion results in the release of greenhouse gases into the atmosphere (Ali et al., 2016), which include SO₂, CO₂, CO, NO₂ and methane (I.E.A., 2015). These gases pollute the atmosphere, resulting in global warming and thus contributing towards climate change. With advancements in technology, industry and an ever-growing population, the demand for energy increases as well as the levels of air pollution.

By 2013, over 85% of the global energy consumption was derived from fossil fuels (coal, oil and natural gas), with other alternative sources of energy contributing a small fraction, thus highlighting the heavy dependence on non-renewable fossil fuels to meet the increasing global energy needs (Figure 2.1). An estimated 60% of global consumption of oil is by the transport industry and this is expected to increase as the global fleet of vehicles is projected to reach a peak of 2 billion by 2035 (I.E.A., 2008; I.E.A., 2016). As the demand from the transport sector continues to grow, so does the concern for the depletion of the global fossil fuel reserves (Cinelli et al., 2015). It is anticipated that the supply of crude oil will only last for another 45 years at the current consumption rate (Guo et al., 2015).

Biomass has been used for hundreds of years as an alternative energy source for cooking and heating. It has been widely accepted as a renewable resource for the production of a more sustainable energy. Although biomass is less effective than fossil fuels in terms of total energy output, it is the only alternative source that can competitively produce energy to supplement transportation fuel (van Zyl et al., 2011). This has resulted in the active development of the biofuels industry. Utilisation of surplus agricultural produce, volatile fuel prices (Avinash et al., 2014) as well as job creation have also been major factors in the drive for the development of a biofuels industry (Figiel and Hamulczuk, 2013; Pradhan and Mbohwa, 2014).

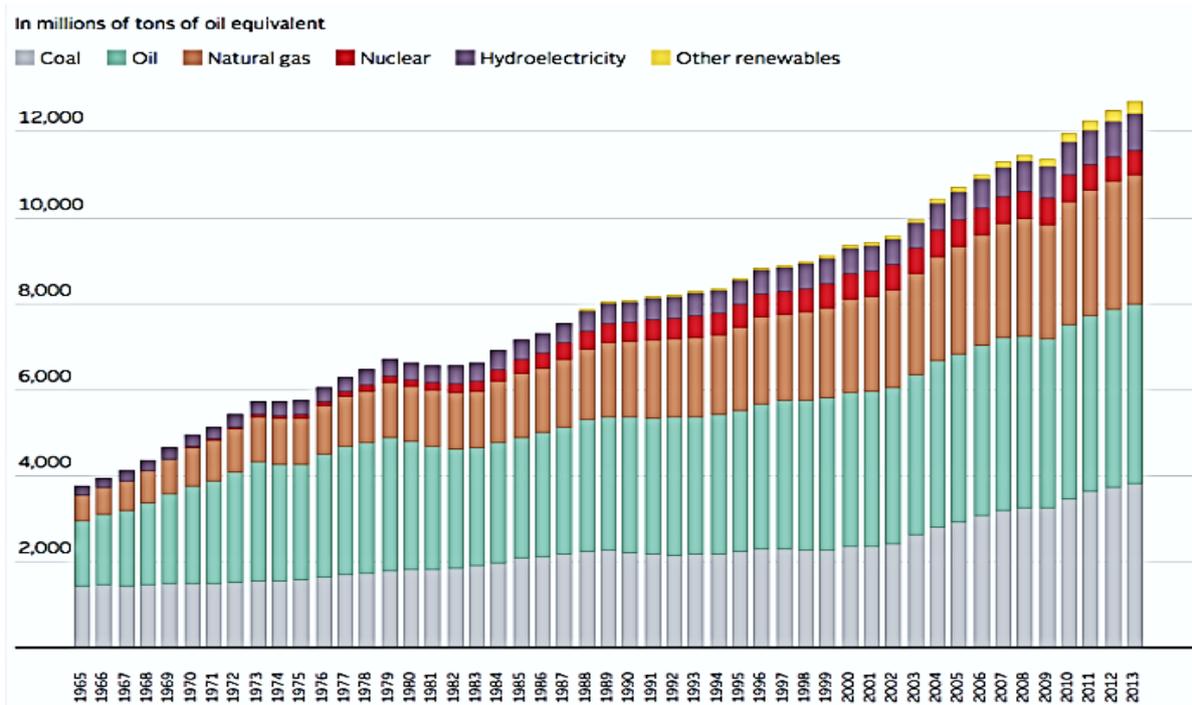


Figure 2.1: Average energy utilisation across the globe classified according to source or type used expressed in millions of tons of oil or equivalent (for coal, natural gas, nuclear, hydroelectricity and renewables). British Petroleum Statistical Review of World Energy (<http://www.bp.com/context/dam/bp/excel/energy-economics/statistical-reveiw-2016>).

Biofuels have been extensively researched and can be used in liquid, solid or gaseous forms. Gaseous biofuels extend to biogas and syngas; solid biofuels include wood chips, pellets and charcoal, whereas liquid biofuels include bioethanol, biodiesel, pyrolysis bio-oil and advanced hydrocarbon biofuels (Guo et al., 2015). Currently the two major biofuels produced globally are bioethanol and biodiesel, with bioethanol accounting for a greater proportion of production (Wang et al., 2012). Biomass-based fuels have many advantages over petroleum-based fuels, such as higher combustion efficiency, supply reliability, fuel sustainability and diversity, agricultural development and improved land and water use (Balat, 2011). Biofuels can be produced through biochemical means (Mohanty et al., 2009), pyrolysis (Balat et al., 2009a), gasification (de Kam et al., 2009; Balat et al., 2009b), liquefaction (Liu and Zhang, 2008) and supercritical fluid extraction (Sener et al., 2010).

Biofuels are classified according to the type of feedstocks used in production. Ethanol derived from food crops (such as sugar cane, wheat and potato waste) and vegetable oils (such as corn oil, sunflower oil and olive oil) are referred to as first generation (1G) biofuels

(den Haan et al., 2013; Azad et al., 2015). These are generally easier to convert than later generations of biofuels. The second generation (2G), third generation (3G) and fourth generation (4G) biofuels are known as advanced biofuels. The 2G biofuels are produced from non-food feedstocks such as lignocellulosic matter (including wood waste, wood and cotton seed), animal fat and municipal solid waste (Demirbas, 2009; Lee and Lavoie, 2013). Use of the 2G sources on a commercial scale is still under investigative development as the lignocellulosic matter is compact and complex, whereas the use of starchy feedstock (1G) has been well established and the focus is currently on process improvement.

Biofuels produced from microalgae are referred to as 3G biofuels. Algae are photosynthetic aquatic organisms that can be used as 3G feedstock owing to their being renewable, low cost substrates with high energy output (Tran et al., 2010). The biofuels are produced from a transesterification process and can be used as blends with aviation fuel, petrol or diesel. Advanced technologies such as geo-synthesis, electrochemical processing and petroleum-hydro-processing, capture carbon from the environment to produce 4G biofuels. This can be done by electrochemically capturing the carbon in CO₂ in water and converting it to ethanol, or by using geosequestration methods to store the CO₂ in old oil or gas fields for future use (referred to as new “fossil fuels”) (Azad et al., 2015). The 4G feedstocks are environmental CO₂, H₂O and heat energy (Glaser, 2009; Ganesh, 2014).

2.2 Bioethanol

Bioethanol is one of the most popular biofuels, particularly in the transport sector. Ethanol has applications in various industries including medical (antiseptic, antidote and as a solvent), recreational (alcoholic beverages), fuel (motor and rocket engines, household) and as feedstock to other processes (precursor for production of certain organic compounds) (McDonnell and Russel, 1999; Braeunig, 2006). One of the benefits of bioethanol use is that it produces lower CO₂ emissions than other liquid fuels (Celik, 2008; Sarris and Papanikolaou, 2016). Depending on the feedstock, emissions from bioethanol can be typically 20% (maize) to 90% (sugarcane) less than those from gasoline (Figure 2.2), emphasising their potential impact to reduce environmental pollution. The net energy balance for the conversion of biomass to bioethanol is an important parameter when evaluating the type of feedstock to use for production. Bioethanol yields can reach levels of up to nine times the amount of energy used in their manufacturing, which proves the capacity for sustainable

production (Philippidis, 2008). Ethanol is an ethyl alcohol that is biodegradable, water-soluble and has low toxicity, implying that less adverse environmental effects can be expected in the event of a spill compared to those associated with oil (McMillan, 1997).

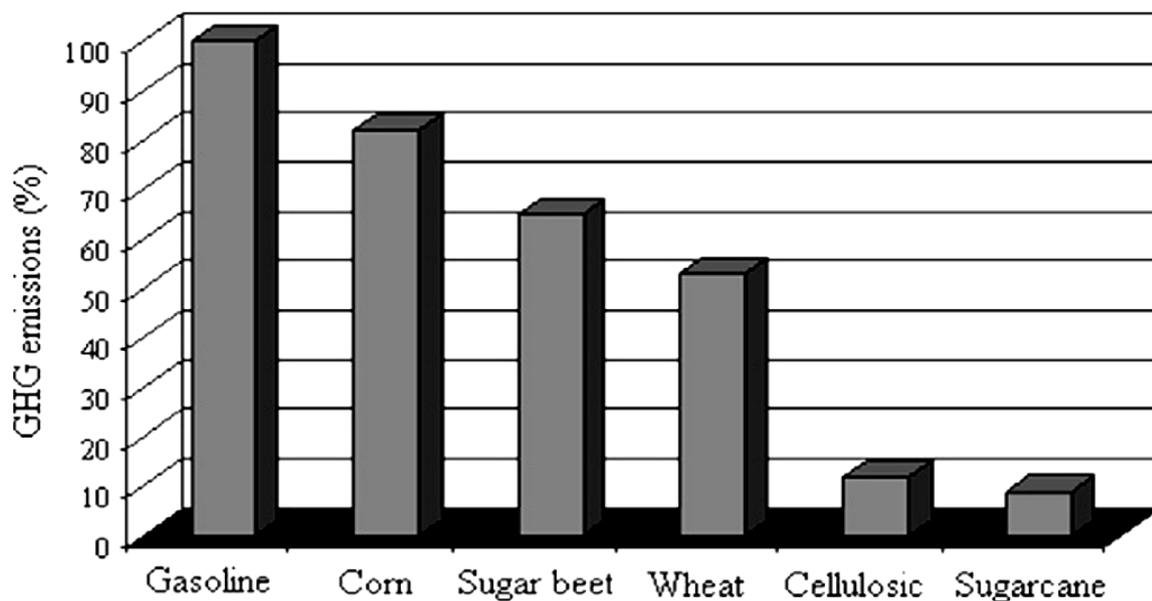


Figure 2.2: The estimated reduction in percentage Green House Gas (GHG) emissions released by bioethanol produced from a variety of feedstocks compared to that of gasoline (adapted from Philippidis, 2008).

The major feedstock for bioethanol production is starchy biomass, but it can also be produced from lignocellulosic matter such as herbaceous crops, forestry wastes, waste paper and agricultural wastes (Kim and Dale, 2004). Crops such as switch grass, grain sorghum, energy cane, napier grass, hybrid poplar cane and shrub willow are dedicated feedstock for ethanol production (Balat, 2011; Guo et al., 2015). In an effort to stimulate bioethanol production, government subsidies for biofuel crop production have been offered (Ali et al., 2016) and many countries have adopted mandatory biofuel policies and set targets for biofuel production. Incentives for blending biofuels with petroleum fuels are critical to support the continued growth of the biofuel industries and its commercial implementation (Pradhan and Mbohwa, 2014). Ethanol-gasoline blends can range from 5-85%, with the most popular blends being E85 (85% ethanol, 15% gasoline), E20 (20% ethanol, 80% gasoline) and E10 (10% ethanol, 90% gasoline) (Kim and Dale, 2006; Festel, 2008).

2.2.1 Global bioethanol production

The major ethanol producers, Brazil and the USA, account for up to 85.2% of the global ethanol production. A general increase in the global production of ethanol has been observed over the years (Figure 2.3), reaching 23.3 billion gallons in 2010 (Guo et al., 2015). In 2015, ethanol production was 25.6 billion gallons with 57.6%, 27.6%, 5.4%, 3.1% and 1.7% being produced in the USA, Brazil, Europe, China and Canada, respectively. The major feedstock used in the USA is maize, Brazil uses sugarcane while Canada and China use both maize and wheat (China also uses cassava). Wheat and sugar beet are primarily used as feedstock in Europe.

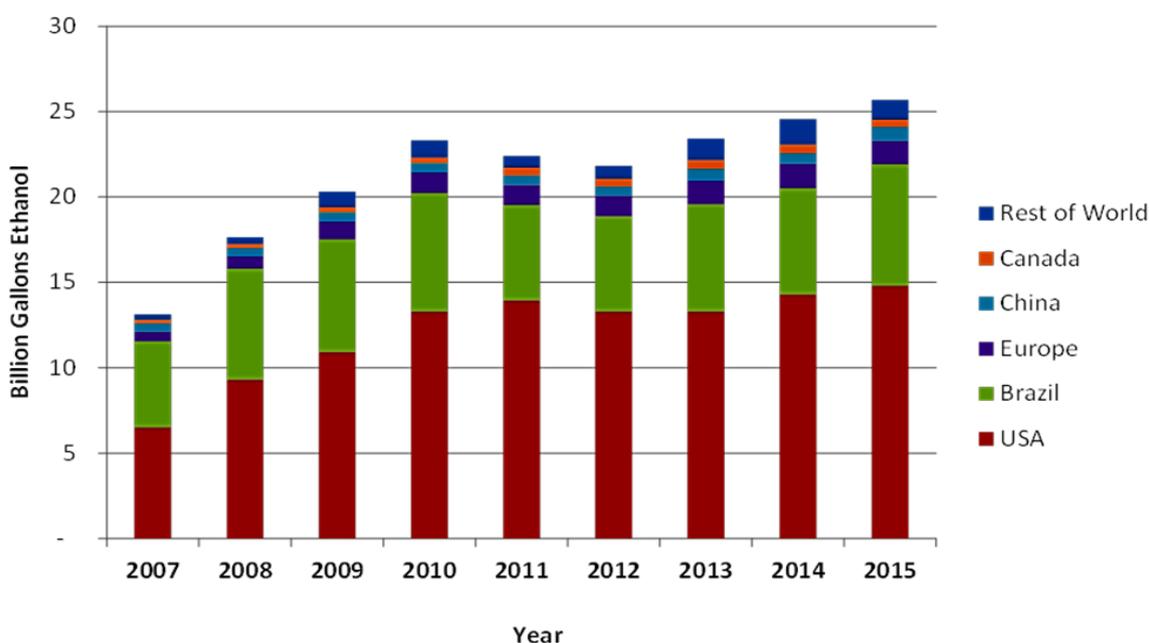


Figure 2.3: Total global bioethanol production over 9 years showing annual contribution in billions of gallons of ethanol of the main contributors by country and region (a combination of total ethanol from the producers from the rest of the world) (adapted from www.afdc.energy.gov/data).

Sub-Saharan African countries have potential for biofuel production (van Zyl et al., 2011) and various external initiatives have promoted the development of sustainable energy alternatives in countries such as Mozambique, South Africa, Malawi, Zimbabwe, Zambia, Tanzania and Kenya. The development of a biofuel industry in Africa has many advantages including reducing the reliance on the import of fossil fuels whilst increasing revenue, efficient use of current biofuel sources, energy supply diversification and improved

development of rural areas (Pradhan and Mbohwa, 2014). However, political and social instability as well as food shortages and droughts are factors hampering successful biofuel production in some African regions.

South Africa is both the largest consumer and producer of energy in Africa, but it still needs to import oil to meet the energy demands of the transportation sector (Pradhan and Mbohwa, 2014). Its global contribution to biofuel production was less than 0.01% in 2012. The South African Department of Energy (2012), stipulated mandatory regulations for blending of biofuels with fossil fuels to commence in late 2013, ensuring a 2 to 10% blend of bioethanol with petrol and a 5% blend for biodiesel (Gasparatos et al., 2015). Regardless of these policies and mandates set to promote the production and implementation of biofuels in South Africa, various challenges hinder their implementation, such as commodity prices, biodiversity, environmental degradation due to land use changes, as well as socio-economic concerns (Pradhan and Mbohwa, 2014).

A number of biofuel manufacturing plants have been established in South Africa, but production is still insufficient to meet the local demand. Current feedstocks include sorghum, soybean and sugarcane (Table 2.1). The capacity for biofuel production can be significantly improved since about 14% of arable land is under-utilised in South Africa. There is room to rehabilitate degraded land which can be dedicated to growing biofuel crops without contributing to food shortages or land use changes that may damage the ecosystem (Pradhan and Mbohwa, 2014).

Table 2.1: Biofuel plants in South Africa (adapted from South African Department of Energy, 2014, <http://www.energy.gov.za/files/policies/DraftpositionpaperontheSABiofuelsRegFrmwrk.pdf>)

Name	Capacity (10^6 .l.year ⁻¹), Type (feedstock)	Location
Arengo 316 (PTY) Ltd	90, Ethanol (sorghum and sugar beet)	Cradock, Eastern Cape
Mabele Fuels	158, Ethanol (sorghum)	Bothaville, Free State
Ubuhle Renewable Energy	50, Ethanol (sugarcane)	Jozinin, KwaZulu Natal
E10 Petroleum Africa CC	4.2, Ethanol (sugarcane)	Germiston, Gauteng
Rainbow Nation Renewable Fuels Ltd	288, Biodiesel (soya bean)	Port Elizabeth, Eastern Cape
Phyto Energy	≥ 500 , Biodiesel (canola)	Port Elizabeth, Eastern Cape
Exol oil refinery	12, Biodiesel(Waste vegetable oil)	Krugersdrop, Gauteng
Basfour 3528 (PTY) Ltd	170, Biodiesel (soya bean)	Berlin, Eastern Cape

2.2.2 Starch as bioethanol feedstock

Starch is a storage polysaccharide in the seeds, roots and tubers of higher plants that is produced in the green leaves of plants by means of photosynthesis. Carbon dioxide and water are transformed into glucose and oxygen in the presence of sunlight and chlorophyll. The polymerisation of these glucose monomers leads to starch granule formation. The size and shape of the granule and the extent of crystallinity are indicative of its botanical origins (Vengadaramana, 2013).

Starch consists of two types of D-glucose polysaccharides: amylose and amylopectin (Figure 2.4) (Marc et al., 2002), which are assembled to form a semi-crystalline starch granule (Burrell, 2003). Amylose comprises of linear chains of α -1,4 linked glucose units and is more crystalline compared to amylopectin. The chains can consist of up to 6 000 glucose units depending on the starch source. Marc et al. (2002) reported that the average amylose content in potato and maize starch amounts to 20 to 30% of the total starch content. Starch synthase is the enzyme responsible for amylose synthesis and it acts by elongating malto-oligosaccharides. Amylopectin constitutes about 70 to 80% of starch in maize and potato (Muralikrishna and Nirmala, 2005) and contains α -1,4 linked linear chains of 10 to 60 glucose units that are interlinked by occasional α -1,6 linkages of 15 to 45 glucose units (El-fallal et al., 2012).

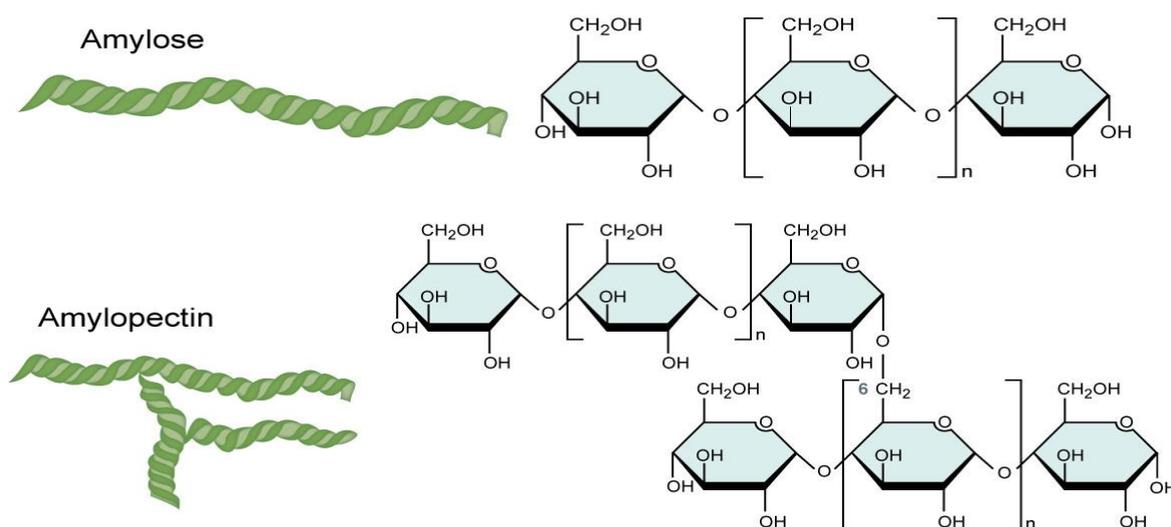


Figure 2.4: Partial structures of amylose (linear) and amylopectin (branched) polysaccharides present in starch molecules (<https://sites.google.com/a/aisr.org/mun-ib/home>).

Starch is one of the most accessible energy sources, with corn representing the largest industrial source; other common sources include wheat, tapioca and potato (Burrell, 2003). The steps involved in the conversion of starch to sugars are gelatinisation, liquefaction and saccharification. Gelatinisation involves expansion of the starch molecules in the presence of heat and water, resulting in the loss of crystallinity; while liquefaction and saccharification involves the breakdown of the molecule to sugars by amylolytic enzymes (Shigechi et al., 2002). Starch from agricultural crops and waste has found applications in the food industry (baking, processed foods, confectionary, beverages and animal feed) and the manufacturing of paper (and board), detergents, pharmaceuticals, cosmetics and bioethanol production (Table 2.2). However, there are limitations to starch applications due to its high tendency towards retrogradation, low shear resistance and thermal decomposition (van der Maarel et al., 2002; Goyal et al., 2005).

Table 2.2: Examples of the application of starch in different industries (adapted from Burrell, 2003)

Industry	Application
Food and beverage	Meat products, confectionary, bread, soft drinks, syrups
Agriculture	Animal feed and pellets, seed coating, fertiliser
Building	Mineral fibre, concrete, gypsum board
Textile	Fabrics, yarn, warp
Paper	Paper, cardboards
Pharmacy	Tablets, dusting powder
Energy	Bioethanol
Other	Biodegradable plastics, oil drilling, water treatment, glue manufacturing, laundry starch, explosives (nitro starch), cosmetics

The commercial importance of starch is mainly in its hydrolysis products, i.e. sugars such as maltose and glucose (Bai et al., 2012). In bioethanol production, starch is a raw material of interest as it is abundant, renewable, accessible in many parts of the world and is relatively inexpensive (Ulgen et al., 2002). However, raw or native starch is insoluble in cold water; heat is required to dissolve it into an almost transparent solution through gelatinisation. Effective gelatinisation can occur at temperatures of 54 to 85°C, with gelatinisation temperatures of 60°C and 65°C for potato and maize, respectively (Rahman, 1995). The appropriate temperature depends on a wide array of conditions, including the state and origin

of the starch. Nevertheless, this is an important step in the application of starch in various processes (Sundarram et al., 2014).

Wet milling and dry milling are the two basic methods for processing raw starch material. During dry milling, the feedstock is ground to flour or meal and consequently processed without separating its components (Figure 2.5). The meal is then slurried with water to form a mash, to which amylolytic enzymes are added for dextrose production via liquefaction and saccharification (Guo et al., 2015). Ammonia is added for pH control and as a nutrient for the yeast during fermentation. To reduce the bacterial population, the mash is processed in a high-temperature cooker before it is cooled and transferred to fermenters.

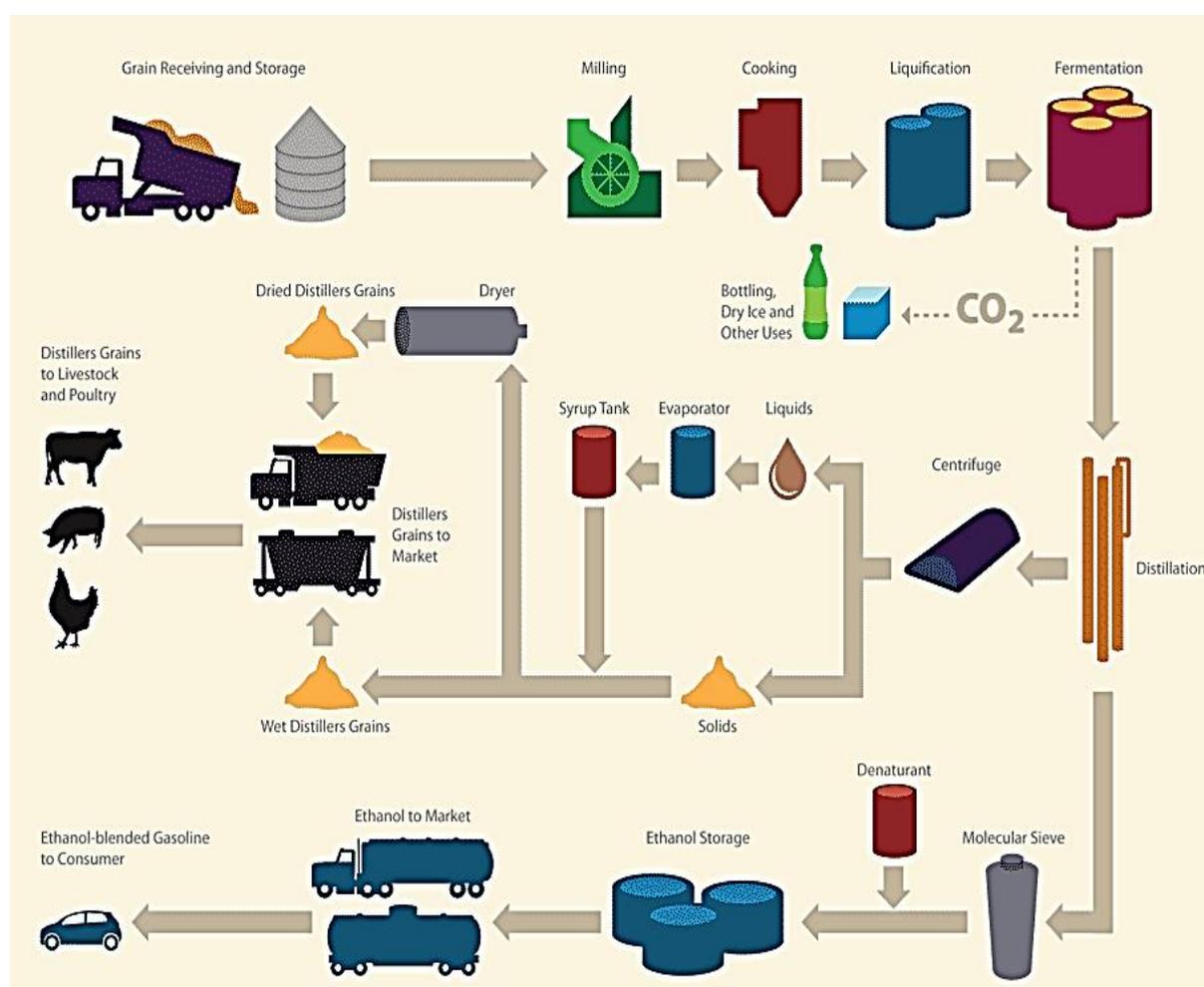


Figure 2.5: A flow chart of bioethanol production from maize using the dry milling process in the USA. By-products from the production process are used in the production of secondary products: carbon dioxide used in carbonated drinks and dry ice, and solid waste used as livestock and poultry feed. Renewable Fuels Association. (<http://ethanolrfa.org/how-ethanol-is-made/>).

Yeast is added to the slurry and fermentation (2 to 3 days) is performed at low temperatures with continuous agitation. The resulting ethanol is separated from the stillage and concentrated via distillation (Figure 2.5). Thereafter, it is concentrated with molecular sieves and denatured by adding two to five percent gasoline (Mosier and Ileleji, 2006; R.F.A., 2016). The stillage is processed to mainly solids that have varied uses, but mainly serve as livestock feeds. Commercial ethanol is currently produced using the dry milling process (Kim and Dale, 2004).

Wet milling is capital-intensive, but produces a variety of products that are used in the manufacture of volatile compounds. In this process, the grain is soaked in dilute aqueous sulphuric acid for 24 to 48 hrs. The maize slurry is passed through a series of grinders to separate the corn germ. The separated components are subjected to various processes and different products are collected at each processing step such as corn oil, corn syrup and gluten meal. The remaining starch at the end of the processes is either dried and sold, modified into value added products or fermented to ethanol (R.F.A., 2016).

2.3 Starch-hydrolysing enzymes

Enzymes are biological catalysts that accelerate biochemical reactions by lowering the activation energy of a reaction without being altered in the process (Emel et al., 2006). Enzymes involved in the hydrolysis of starch are collectively referred to as amylases. These are extracellular enzymes that randomly hydrolyse α -1,4 glycosidic linkages between adjacent glucose molecules (Singh et al., 2014). Amylases are produced by plants, animals and various organisms including yeast, fungi and bacteria (El-fallal et al., 2012) and vary in terms of activity, specificity and abundance (Pandey et al., 2000).

Enzymes that hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and any other compound are known as glycoside hydrolases (GH) (E.C 3.2.1.-). The classification into the GH families is based on the amino acid sequence homology of the enzymes (Henrissat, 1991). The majority of amylases belong to GH family 13 (largest GH family), whereas some amylases are grouped in GH families 14 and 15. Family 13 is also known as the amylase family and comprises a variety of enzymes with different specificities, including transferases and isomerases. The amylases are further grouped depending on their mode of action, that is, the manner in which the glycosidic bond

is attacked to result in either retaining or inversion of the product configuration (Mojsov, 2012).

Due the complexity of starch molecules, its complete hydrolysis requires the action of an assortment of enzymes, although prolonged incubation of starch with just one of these enzymes can lead to its almost complete hydrolysis (El-fallal et al., 2012). Three main groups of amylolytic enzymes, namely endo-amylases, exo-amylases and debranching enzymes (Figure 2.6) are involved in starch hydrolysis. Endo-amylases (e.g. α -amylases) are liquefying enzymes that cleave the internal α -1,4 bonds and produce α -anomeric compounds. Exo-amylases (e.g. glucoamylases and β -amylases) are saccharifying enzymes that cleave α -1,4 and α -1,6 bonds of the external glucose residues to yield glucose (β -amylases cleave the α -1,4 bonds of the second last external glucose residue yielding maltose and β -anomeric products) (Banks and Greenwood, 1977; Hill and McGregor, 1988; El-fallal et al., 2012). Debranching enzymes (e.g. pullulanase) exclusively degrade amylopectin by cleaving the α -1,6 bonds to produce linearised polysaccharide chains. Transferases cleave the α -1,4 bonds of the donor molecule and transfer the sugar to an acceptor through formation of a glycosidic bond, e.g. cyclomaltodextrin glycosyltransferases. Further discussions will focus on α -amylases and glucoamylases, which are the main enzymes of interest in this study.

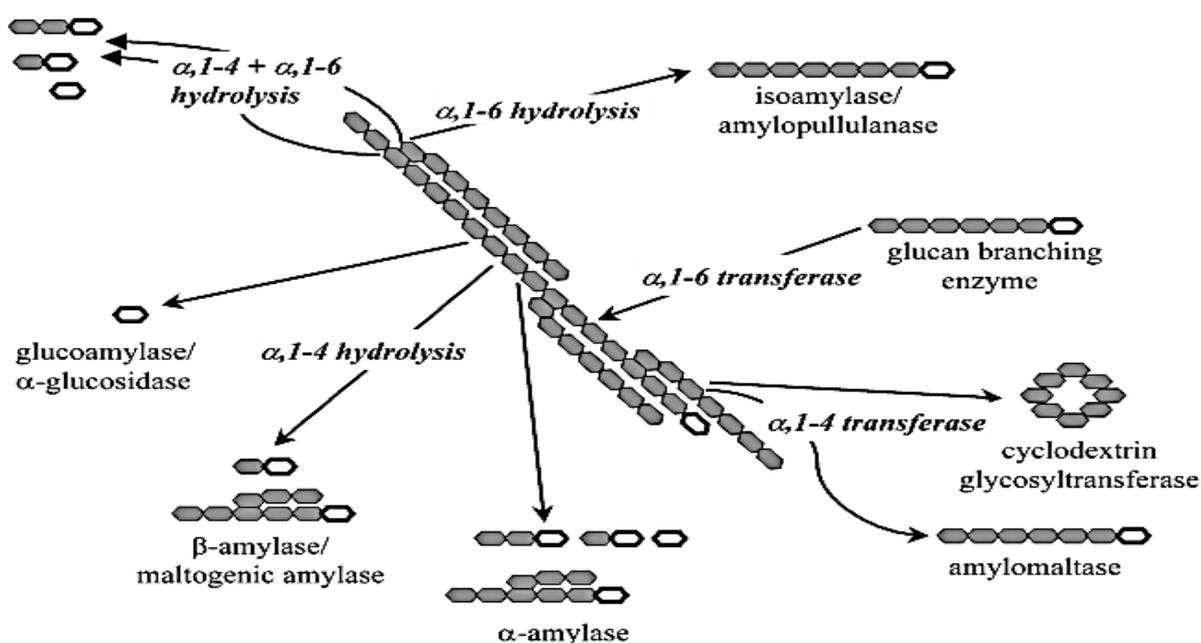


Figure 2.6: Enzymes involved in the hydrolysis of starch. The open hexagonal rings symbolise the reducing end of a poly-glucose molecule, the glucose molecules (van der Maarel et al., 2002).

2.3.1 Enzyme synergy

Enzyme synergy occurs when enzymes act together and consequently produce a higher or enhanced activity, as compared to the combined effect of the enzymes acting individually under the same process conditions (Wong et al., 2007; Kumar and Wyman, 2009). This concept is crucial in enzymology as enzymes act on different parts of the substrates, thereby breaking it into particles that are more accessible and which may possibly possess new sites for other enzymes to act on; also, synergy can result in hydrolysis of products that may be inhibitory to other enzymes. Enzyme cocktails are commonly used in starch hydrolysis. For efficient starch conversion, the synergy between α -amylases and glucoamylases is important, as enzymes contribute significantly to the overall cost of the conversion process. This synergy between these two enzymes is exploited as they are used in commercial enzyme cocktails for granular starch hydrolysis, such as STARGENTM 002, which contains *Aspergillus kawachii* α -amylases and glucoamylases from *Trichoderma reesei*. However, the level of synergy is affected by the type of substrate, intended use of the enzyme and the enzyme ratios (Presečki et al., 2013). Determination of enzyme ratio would aid in effective use of the enzymes by lowering enzyme loading and ultimately optimising starch hydrolysis (Gottschalk et al., 2010).

2.3.2 Alpha-amylases

Alpha-amylases catalyse the breakdown of α -1,4 linkages in starch to produce low molecular weight dextrans, oligosaccharides and small sugar molecules. In plants such as wheat, barley, rice, maize and peanuts, α -amylases are mainly associated with seed germination and maturation (Francis et al., 2003). In animals, its main sources are the salivary glands and pancreas where they are involved in the hydrolysis of carbohydrates. Alpha-amylases that hydrolyse starch to free sugar units are called saccharogenic or dextrinogenic enzymes, whilst starch-liquefying enzymes only liquefy starch and result in minimal to no production of free sugars. The α -amylases can also hydrolyse glycogen and other related polysaccharides (Ramachandran et al., 2004; Bai et al., 2012).

The α -amylases have largely replaced chemical hydrolysis in starch processing (Pandey et al., 2000). This is mainly attributed to the elimination of neutralisation steps, reaction specificity, product stability and the resultant lower energy requirement. Amylases

that are able to digest various raw granules are of particular interest as they provide access to new starch sources for direct starch hydrolysis (Bai et al., 2012). A limited number of these raw starch hydrolysing enzymes have been characterised, such as α -amylases from *Cryptococcus* sp. (Iefuji et al., 1996), *Bacillus* (Gupta et al., 2003; Demirkan et al., 2005; Sun et al., 2010; Bozic et al., 2011) and *Streptomyces bovis* (Yamada et al., 2009). Therefore, the α -amylases with desirable properties such as improved activity, thermal and pH stability as well as resistance to inhibitors, denaturing reagents and chemicals, are sought after for potential commercial applications.

2.3.2.1 Structural and functional analysis

Microbial α -amylases vary in their characteristics, but their molecular mass is usually in the 40 to 70 kDa range. The *Bacillus caldolyticus* α -amylase is the smallest α -amylase reported with a mass of 10 kDa (Gupta et al., 2003), whereas the *Chloroflexus aurantiacus* α -amylase is the largest with a mass of 210 kDa (Ratanakhonokchai et al., 1992). Glycosylation can contribute to the high molecular weight of some α -amylases. Typical glycosylation involves linkages to asparagine side chains or to threonine and serine residues, resulting in *N*-linked and *O*-linked glycosylation, respectively (Shental-Bechor and Levy, 2009). Increased molecular weight due to glycosylation has been reported for α -amylases from *Aspergillus oryzae* (Eriksen et al., 1998), *Bacillus subtilis* strains (Matsuzaki et al., 1974) and *Thymus vulgaris* (Abou Dohara et al., 2011). A variety of enzyme functions are affected by glycosylation such as folding, stability and secretion (Weerapana and Imperiali, 2006).

The amino acid sequences of α -amylases differ depending on the source, but α -amylases in general share about 30% amino acid identity and the catalytic action of the enzyme remains the same (Henrissat and Bairoch, 1993). The 3D structures of α -amylases published to date generally reveal a polypeptide chain folded into three domains (A, B and C). Domain A is the most conserved domain in the α -amylase family and is usually located at the N-terminal end and constitutes the core of the molecule with a $(\beta/\alpha)_8$ barrel. The active site (Figure 2.7) is located at the C-termini of the β -strands (Farber and Petsko, 1990).

Domain B is involved in Ca^{2+} binding and protrudes between β -sheet 3 and α -sheet 3 (Marc et al., 2002). This domain is less conserved in family GH 13 in relation to size and sequence, with the exception of the region around the Ca^{2+} binding site (Janecek, 1997). Domain B determines some functional and structural properties of the enzyme such as correct

folding, adaptation of the activity to pH and substrate specificity. Domain C is the C-terminal domain; its exact function has not yet been determined, but it is believed to be essential for catalysis and assisting in Ca^{2+} binding (Mojsov, 2012).

Most α -amylases contain a conserved Ca^{2+} binding site (Prakash and Jaiswal, 2010) between domains A and B, which is where the active site is located (Figure 2.7). The role of calcium ions is to protect the enzyme from denaturation and proteolytic attack. It helps in stabilising the tertiary structure of the enzyme to maintain correct configuration for activity (Agarwal and Henkin, 1987; Qian et al., 1993). Additional calcium ions and binding sites have been reported for the *Aspergillus niger* α -amylase (Boel et al., 1990). All animal and some bacterial amylases (such as *Bacillus thermooleovorans*) contain a chloride ion-binding site in its active site that enhances the catalytic action of the enzyme by acting as an allosteric activator (Levitzki and Steer, 1974; Malhotra et al., 2000; Prakash and Jaiswal, 2010). This binding site is generally located in the centre of domain A, but is not directly implicated in substrate binding.

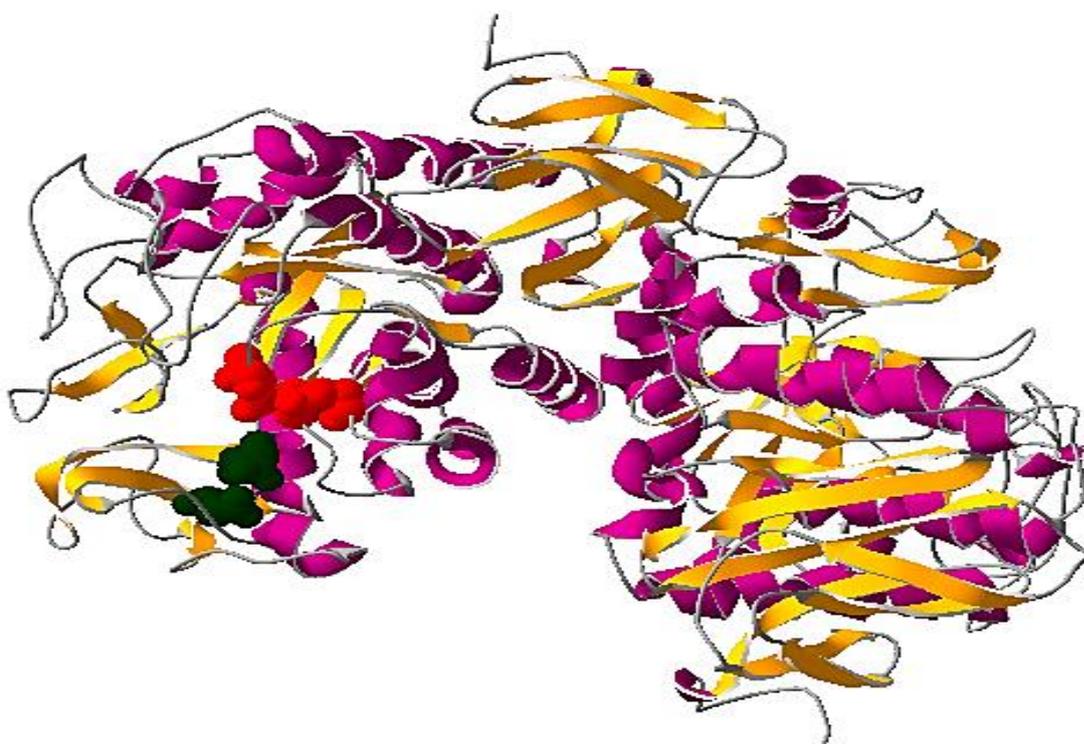


Figure 2.7: The crystal structure of an α -amylase from *Aspergillus niger* with the α -helical structures shown in purple and the β -sheets shown in orange. The calcium binding site is shown in green which comprises of residues Asp121 and Asn175. The active site is shown in red and comprises of residues Asp195, Glu223 and Asp300 (Vujicic-Zagar and Dijkstra, 2006).

The carbohydrate-binding module (CBM) is a conserved region of approximately 90 to 130 amino acids responsible for binding of the enzyme to starch. It is found mainly at the C-terminal of the catalytic domain. The starch strands attached to the CBM are stretched thereby increasing the surface area for attack by the hydrolytic enzymes (Mojsov, 2012). The CBMs are classified as part of 74 families based on sequence homology (www.cazy.org).

2.3.2.2 Mode of action

Alpha-amylases are responsible for the endo-hydrolysis of α -1,4 glycosidic linkages in polysaccharides containing three or more α -1,4 linked D-glucose units. Their action results in a decrease in the viscosity of a starch solution. The saccharide composition of products from starch hydrolysis is dependent on various factors such as temperature and pH, conditions of hydrolysis and the origin of the enzyme (Parka and Son, 2007). However, the products are always oligosaccharides that vary in length, but all have an α -configuration at the C₁-carbon of the reducing glucose units (Cantarel et al., 2009; Jin et al., 1999). The term alpha thus refers to the anomeric configuration of the free sugar group that is released. Hydrolysis of amylose yields glucose, maltose and maltotriose, whereas amylopectin hydrolysis also yields α -limit dextrans with α -1,6 linked glucose residues.

The catalytic residues in α -amylases are the Asp195, Glu223 and Asp300 amino acids, which are located on the C-terminal side of the β -strands of domain A. Their side chains are oriented towards the catalytic cleft found between domains A and B. The role of the Asp300 is not exactly determined; but might be linked to the stabilisation of the oxocarbenium intermediate state during hydrolysis (Uitdehaag et al., 1999). The Asp195 acts as a nucleophile, whilst the Glu223 acts as an acid base catalyst.

2.3.3 Glucoamylases

Glucoamylase (E.C. 3.2.1.3) is a hydrolysing enzyme belonging to GH family 15 that saccharifies native starch as well as other starchy substrates such as glycogen (Kumar and Satyanarayana, 2009). Though it hydrolyses both types of bonds in starch, the activity of the glucoamylases towards α -1,6 linkages is greatly reduced relative to its activity towards α -1,4 linkages (Sauer et al., 2000); this debranching activity is nevertheless essential in processes requiring the complete hydrolysis of starch.

2.3.3.1 Structural and functional analysis

Many forms of glucoamylase have been isolated, with some variations of the enzyme within the same organism. Some microbes, mostly fungi, produce up to six different forms of glucoamylases. Pretorius et al. (1991) reported that these intra-species differences may be due to factors such as the presence of many structural genes, limited proteolysis, mRNA modifications as well as varied carbohydrate content. The commercially used *Aspergillus awamori* and *A. niger* each possess two glucoamylases, GAI and GAII, which may vary in molecular mass, glycosylation levels, activity on certain substrates or ultimately in amino acid composition (Jensen and Olsen, 1999; Kumar and Satyanarayana, 2009). In this case, only the GAI has raw starch digesting ability, whilst both GAI and GAII are able to hydrolyse soluble starch.

The glucoamylase carbohydrate binding module consists of eight β -strands arranged in an antiparallel fashion, forming an open-sided barrel organised into two sheets held together by hydrophobic and electrostatic interactions (Jacks et al., 1995; Juge et al., 2002; Liu et al., 2007). The CBM has two binding sites (Figure 2.8) on opposite sides of its domain. A 40-amino acid residue serves as a linker backbone to maintain the distance between the CBM and the catalytic domain (Williamson et al., 1992; Sauer et al., 2000). This linker region has been associated with roles in stability, secretion and hydrolysis of raw starch (Figure 2.8) (Christensen et al., 1999).

The catalytic domain contains 13 α -helices, of which 12 form a $(\alpha/\alpha)_6$ barrel with the polypeptides arranged in a fold with an inner core consisting of six mutually parallel α -helices connected through a set of six peripheral α -helices (Aleshin et al., 1992; Sauer et al., 2000; Kumar and Satyanarayana, 2009). Based on studies on the glucoamylase from *A. niger*, the two catalytic residues act respectively as an acid and a base (James and Lee, 1997). Tryptophan residues that interact by means of hydrogen bonds to the catalytic Glu residue on the active site are involved in substrate interactions (Sauer et al., 2000).

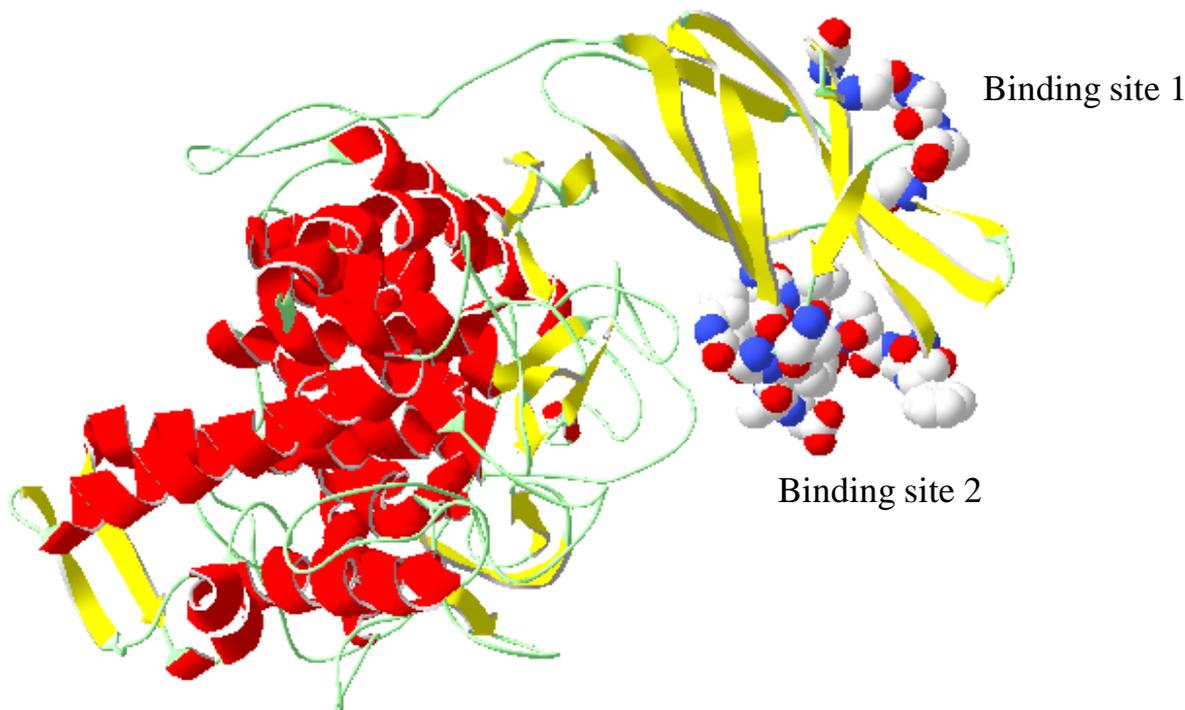


Figure 2.8: The crystal structure of *Hypocrea Jecorina* (*Trichoderma reesei*) glucoamylase showing two binding sites (1 and 2) characteristic of this enzyme. The red shows α -helices and the yellow represents the β -sheets in the protein structure. The green shows the polypeptide coils joining the structure. The two binding sites are separated by a polylinker region seen between the binding sites (Bott et al., 2008).

2.3.3.2 Mode of action

Glucoamylases catalyse the sequential cleavage of α -1,6 and α -1,4 glycosidic bonds from the non-reducing end of starch and related polysaccharides, releasing glucose as the end product. Catalysis occurs with an inversion of the anomeric carbon configuration. The CBM enhances amyolytic activity by binding to the substrate and disrupting the α -glucan interchain (Sauer et al., 2000), thereby facilitating hydrolysis. The hydrolysis mechanism involves proton transfer to the glycosidic oxygen of the scissile bond from the acid catalyst; formation of an oxocarbenium ion is followed by a nucleophilic attack of water assisted by a base catalyst (Figure 2.9) (Sinnot, 1990; Tanaka et al., 1994; McCarter and Withers, 1994).

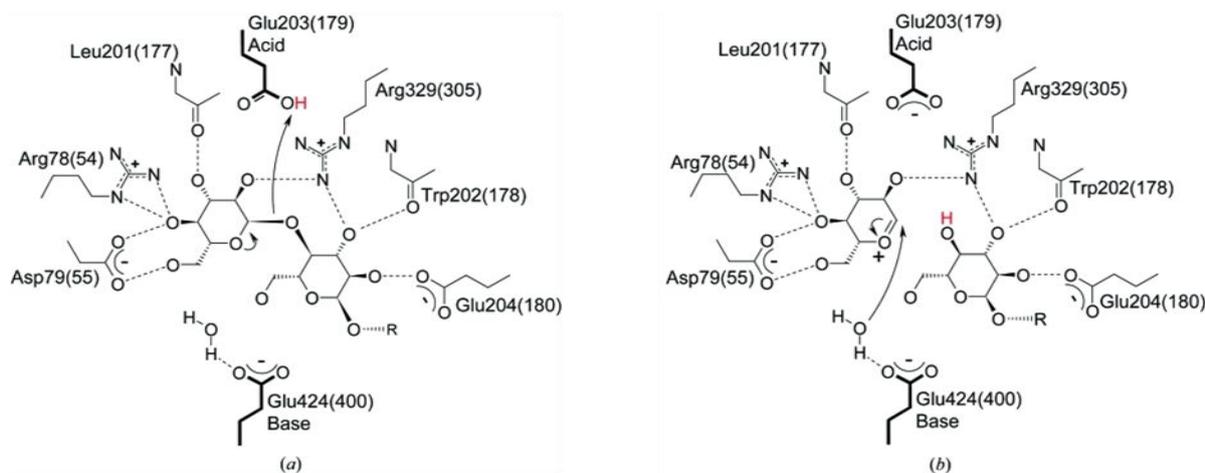


Figure 2.9: The *Aspergillus niger* glucoamylase active-site residues and their interactions with (a) the α -1,4 linked glucose substrate and (b) the oxocarbenium-ion transition state during glycosidic bond hydrolysis (Lee and Paetzel, 2011)

2.3.4 Industrial applications

Microbes are used to produce commercial quantities of amylases as plant and animal sources are associated with low enzyme production levels and poor stability (Tanyildizi et al., 2005). Microbial production of amylases is advantageous as the microbes are easily manipulated to obtain specific enzymes and they are able to bulk produce these enzymes at a lower cost. Enzyme specificity, thermostability and pH response are critical factors that facilitate their industrial use (Kandra, 2003). Fungi and bacteria are the dominant sources of microbial amylases, although some yeasts and actinomycetes have also been known to produce these enzymes (Horváthová et al., 2000; El-fallal et al., 2012).

For efficient industrial-scale amylase production, the enzyme should be produced extracellularly and the producing microbe grown on inexpensive substrates (Sajitha et al., 2010; Ahmadi, 2012; Mohammadabadi and Chaji, 2012). The microbial strain should be stable and not produce toxic substances. Characteristics of many different microbes have been and are currently studied to evaluate and in some cases improve their application in industry. The *Aspergillus* and *Rhizopus* spp. are ubiquitous and widely studied for the production of amylases since they have relaxed nutritional requirements (Bilal and Figen, 2007; Afifi et al., 2008; Pascoal et al., 2010).

Alpha-amylases are very popular and are among the most important industrial enzymes, constituting about 30% of the world's enzyme production. Alpha-amylases have been

isolated from *Rhizopus*, *Aspergillus*, *Bacillus*, *Pseudomonas* and *Clostridium* species. However, *Bacillus* species such as *Bacillus subtilis* (Rajput et al., 2013) and *Bacillus licheniformis* are preferred as industrial strains as they produce significant amounts of the enzyme (Kokab et al., 2007; Naizi et al., 2010). When thermal stability is of paramount importance in the industrial application, microbes producing thermostable α -amylases are selected, such as extreme thermophilic bacteria like *Rhodothermus marinus*, as well as hyperthermophilic and thermophilic archaea such as *Pyrococcus furiosus*, *Thermococcus hydrothermalis* and *Sulfolobus acidocaldarius* (Goyal et al., 2005; Arikan, 2008). Different species of *Aspergillus* are currently being used to produce α -amylases on an industrial scale, including *A. niger*, *A. oryzae*, *Aspergillus flavus*, *Aspergillus fumigatus* and *A. kawachii* (Hussein and Janabi, 2006; Rasooli et al., 2008; Hunter et al., 2011).

Glucoamylases have found many applications, including the production of glucose (for bioethanol production), high glucose and fructose syrups (Saha and Zeikus, 1989; Sauer et al., 2000), pharmaceuticals and baking products (Selvakumar et al., 1996; Pandey et al., 2000). They are the key enzymes in the production of sake, soy sauce and beer (Pavezzi et al., 2008). Most industrial saccharification processes require thermostable glucoamylases, but most of the characterised glucoamylases are unstable at high temperatures. Glycosylation has also been shown to increase thermostability of some enzymes. Protein engineering techniques such as the deletion of some amino acids (Chen et al., 1996) or addition of disulphide bonds (Allen et al., 1998; Li et al., 1998) were able to improve the performance of these enzymes at the desired temperature, thus making them more suitable for industrial use.

Fungi are the most proficient microbes that produce glucoamylases, with *A. niger*, *A. awamori* and *Rhizopus oryzae* being the most important producers for industrial use (Pandey, 1995; Coutinho and Reilly, 1997; Mikai et al., 2015). Studies on improving the action of glucoamylases for application in bioethanol production are of particular importance. A number of raw starch hydrolysing enzymes have been successfully cloned and expressed in yeast, including glucoamylases from *A. tubingensis* (Viktor et al., 2013), *A. awamori* (Favaro et al., 2012), *R. oryzae* (Yamada et al., 2009) and *Saccharomycopsis fibuligera* (Eksteen et al., 2003). These have potential in starch-based bioethanol production as they contribute to a more economical process.

2.4 Fermentation

Fermentation is a process whereby sugars (glucose, fructose, sucrose) are converted into ethanol, CO₂ and energy. The main metabolic pathway used by yeast in production of ethanol is the glycolytic pathway (Embden-Meyerhof-Parnas pathway). Under anaerobic conditions, fermentation occurs and glucose is metabolised to two molecules of pyruvate, which is further reduced to two molecules of ethanol (Figure 2.10). Production of ethanol occurs concurrently with the continued cell growth as energy produced in glycolytic pathway is used to drive the cells biosynthetic processes (Bai et al., 2008).

A number of secondary products are produced during fermentation. Glycerol is the main by-product, formed as a result of high pH and increased osmotic pressure whilst organic acids and higher alcohols are produced at much lower levels (Bai et al., 2008). The by-product formation is a sign of cell stress as some intermediates from the glycolytic pathway are diverted for their production, and this inevitably results in a decrease in ethanol yield. Stress factors that affect yeast cell growth and fermentation capacity include environmental stresses: high temperature, pH instabilities, nutrient deficiency, contamination and accumulation of secondary products of the yeast metabolism (Ingledeew, 1999; Bai et al., 2008).

2.4.1 Amylolytic yeast strains

Heterologous gene expression in yeast has received considerable interest over the years as the yeast have been used in production of foreign proteins for industrial use, as well as for use in proteomics research. The yeast *Saccharomyces cerevisiae* is well characterised as a model organism for heterologous protein expression (Liu et al., 2012). It is commonly used in industry because it is Generally Regarded As Safe (GRAS), exhibits natural robustness, has a high ethanol production rate, tolerance to high ethanol concentration, high sugar and high osmotic pressure (Kuyper et al., 2005; den Haan et al., 2007; Palmqvist and Hahn-Hägerdal, 2000; den Haan et al., 2013). The *S. cerevisiae* strains are easily manipulated and can perform post-transcriptional modification of secreted proteins. However, the yeast is unable to utilise starch materials as it lacks amylolytic enzymes necessary for starch hydrolysis (van Zyl et al., 2012; Favaro et al., 2012).

Successful foreign gene expression involves cloning of genes into an expression vector, transformation of gene cassettes into the host and synthesis of the protein by the host under suitable conditions (Cereghino and Cregg, 2000). Since liquefaction and saccharification processes are essential for *S. cerevisiae* to utilise raw starch (Shigechi et al., 2002), several studies have focused on engineering yeasts to enable them to produce and secrete amyolytic enzymes for these processes (Aydemir, 2014). Some raw starch hydrolysing α -amylase encoding genes from different sources including bacteria (Tawil et al., 2012; Roy et al., 2013), yeasts (Viktor et al., 2013; Sundarram et al., 2014), mould, barley (Liao et al., 2010) and rice (Sundarram et al., 2014) have been cloned and expressed in *S. cerevisiae*. The source of the enzyme is important as its activity and stability should be compatible with fermentation conditions (Aydemir, 2014).

Glucoamylase is paramount for the complete hydrolysis of starch. Glucoamylase encoding genes from various fungal strains have been cloned and expressed in *S. cerevisiae* strains (Sauer et al., 2000; Mikai et al., 2015). Glucoamylase from *R. oryzae* produced up to 5% ethanol and 2400 U.l⁻¹ activity, which is one of the highest activity levels obtained in flask fermentation experiments to date (Yang et al., 2011). Studies have shown that it is possible to increase the ethanol production rate by increasing exogenous glucoamylase enzyme concentrations (Liao et al., 2012; Aydemir, 2014). However, this is not applicable in industry as pure enzymes are costly, which increases the overall cost of ethanol production.

2.4.2 Secretory systems

Use of enzymes in industry requires efficient expression systems with high productivity. Bacteria are able to produce large quantities of proteins, but are unable to perform eukaryotic post-translational processing. Eukaryotic yeasts are able to secrete proteins in their native and biologically functional forms hence they are preferred for heterologous protein production (Idris et al., 2010). The secretion of some heterologous proteins into culture supernatant however remains low, as a result of a host of factors including vector systems used, host strain, codon usage, post-translational processing and folding, signal sequences, promoter choice and glycosylation (Li et al., 2002; Neibaur and Robinson, 2005; Kim and Lee, 2006). Differences in amino acid demands and folding patterns of peptides affects the overall level of secretion of the protein (Liu et al., 2012).

In recombinant protein production, promoters that initiate strong and constitutive expression are used to ensure high levels of desired enzyme (Liu et al., 2012). Constitutive promoters are advantageous in that they ensure expression of genes without induction and they are active at high glucose concentrations. Although constitutive *T. reesei* promoters from the pyruvate kinase (*PDC*) and enolase genes (*ENO1*) resulted in high expression levels of recombinant proteins in *Saccharomyces* spp. (Li et al., 2012), the trend is to use the promoters that are native to the host. One widely preferred promoter used in recombinant protein production in *S. cerevisiae* is the translation elongation factor 1 (*TEF1*) promoter which results in high expression levels in both oxygen rich and deprived conditions (Partow et al., 2010).

2.4.3 Fermentation configurations

Several strategies have been employed in an effort to optimise bioethanol production from biomass; namely separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bioprocessing (CBP). In SHF the feedstock undergoes enzymatic hydrolysis, followed by the addition of the fermenting microbes for ethanol production (Shigechi et al., 2004). This process has the advantage that the processes occur separately hence optimum conditions for both enzymes and microbes can be applied resulting in a higher conversion rate. The major setback of this approach is end-product inhibition of the enzymes by the glucose during the hydrolysis stage.

In SSF, the hydrolysis of the feedstock and fermentation processes are combined in one bioreactor. It reduces the risk of contamination, duration of fermentation and amount of enzyme needed for the hydrolysis (Sarris and Papanikolaou, 2016). The end-product inhibition is reduced because the sugars are used by the fermenting microbe as they are produced and converted to ethanol. Disadvantages include ethanol inhibition of yeast and different temperature optima of the two processes reducing process efficiency (Aggelis, 2007). A more promising alternative configuration is CBP.

2.5 Consolidated Bioprocessing of starch

CBP involves the combination of amylolytic enzyme production, hydrolysis of biomass to simple sugars and fermentation of the sugars to ethanol by one organism in a single vessel (den Haan et al., 2013; Ali et al., 2016). An ideal CBP organism will completely hydrolyse

raw starch and ferment the resultant sugars to produce ethanol without requiring any pre-treatment of the substrate. The CBP process has many advantages over the independent hydrolysis and fermentation performed separately. The independent processes can lead to enzyme inhibition by the products (sugars and acetates), whereas CBP efficiency circumvents this problem as it prevents glucose accumulation (Olofsson et al., 2008; Ali et al., 2016), resulting in increased efficiency of ethanol production owed to the synergistic action of both the α -amylase and glucoamylase enzymes. Another advantage of the CBP is that it offers cost reduction as the processes are combined in a single bio-reactor. However, the combined processes of CBP (microbial growth, enzymatic hydrolysis and fermentation) have different optimal conditions, as such, optimal performance of the individual processes is impossible to achieve (Ali et al., 2016).

Studies in search for suitable microbial agents for CBP have focused on engineering yeasts that express amyolytic enzymes. These amyolytic CBP yeasts have been constructed to utilise starch feedstock (Table 2.3) and their application should reduce operation costs by eliminating the need for exogenous commercial enzyme (Lynd et al., 2015). One of the strategies is cell-surface engineering, which involves anchoring the enzymes onto the cell walls of the yeast. This is suitable for repeated large-scale fermentations requiring long-term stability of enzymes (Aydemir, 2014). Yamakawa et al. (2012) constructed a recombinant amyolytic *S. cerevisiae* strain with the glucoamylase and α -amylase anchored onto its cell surface that maintained its activity and produced ethanol in 23 continuous fermentation cycles. However, this method has a major disadvantage in that the yeast and ultimately the enzymes can only interact with substrate in its immediate vicinity.

Co-cultures of two different strains can also be used to ferment starch to ethanol in one step (Verma et al., 2000; Manikandan and Viruthagiri, 2009). It involves growing the amyolytic microbes together with the fermenting yeast. Despite the saving in enzyme cost, ethanol yields were low as starch utilisation mainly supported the growth of the amyolytic microbes (Aydemir, 2014). Direct starch-fermenting recombinant *S. cerevisiae* strains have since been developed that co-express both α -amylase and glucoamylase encoding genes enabling the yeast to hydrolyse and ferment starch in a single CBP step (Yamakawa et al., 2012; Favaro et al., 2015; Ali et al., 2016), reducing the energy requirement and cost of the overall process (van Zyl et al., 2007).

Table 2.3: Ethanol production from various CBP systems (adapted from Jouzani and Taherzadeh, 2015)

Biomass	Microorganism	Technology	Ethanol yield	References
Starch	Flocculent yeast YF207	Recombinant: cell surface engineering expressing system, glucoamylase/ α -agglutinin fusion protein	Fed-batch: 50 g.l ⁻¹ ethanol after 120 h	Shigechi et al., 2002
Starch (40 g.l ⁻¹) supplemented with 4 g.l ⁻¹ glucose	<i>S. cerevisiae</i> strain YPG/AB	Recombinant: expressing <i>B. subtilis</i> α -amylase and <i>Aspergillus awamori</i> glucoamylase as secreted polypeptides	Batch: 15.6 g.l ⁻¹ ethanol Fed-batch: 47.5 g.l ⁻¹ ethanol	Ulgen et al., 2002
Starch	<i>S. cerevisiae</i>	Recombinant: expressing <i>Lipomyces kononenkoae</i> α -amylase genes (<i>LKA1</i> and <i>LKA2</i>), or <i>S. fibuligera</i> α -amylase (<i>SFA1</i>) and glucoamylase (<i>SFG1</i>) genes	61 g.l ⁻¹ of ethanol after 6 days	Eksteen et al., 2003
Raw corn starch	<i>S. cerevisiae</i>	Recombinant: cell surface engineering system, codisplaying <i>Rhizopus oryzae</i> glucoamylase and <i>Streptococcus bovis</i> α -amylase	61.8 g.l ⁻¹ (86.5% of theoretical yield and 0.31 g ethanol.g ⁻¹ starch)	Shigechi et al. 2004
Raw corn starch	Non-flocculent <i>S. cerevisiae</i> (YF237)	Recombinant: cell surface engineering system, displaying glucoamylase and secreting α -amylase	Batch: 0.18 g.g cell.h ⁻¹	Khaw et al., 2006
Starch (150 g.l ⁻¹)	Polyploid <i>S. cerevisiae</i>	Combining δ -integration and polyploidisation: polyploidy yeasts expressing <i>S. bovis</i> α -amylase and <i>R. oryzae</i> glucoamylase/ α -agglutinin fusion protein genes	75 g.l ⁻¹ ethanol after 72 h	Yamada et al., 2010a,b
Raw corn starch (20% (w/v))	Industrial strain <i>S. cerevisiae</i>	Expressing <i>A. awamori</i> glucoamylase gene (<i>GAI</i>), and <i>D. occidentalis</i> α -amylase gene (<i>AMY</i>)	10.3% (v/v) ethanol (80.9 g.l ⁻¹) after 6 days	Kim et al., 2010, 2011
Raw starch	<i>S. cerevisiae</i> (<i>RaGA</i>)	Recombinant: expressing <i>R. arrhizus</i> glucoamylase gene in <i>S. cerevisiae</i>	50 g.l ⁻¹ ethanol	Yang et al., 2011
Raw corn starch (100 g.l ⁻¹)	Diploid <i>S. cerevisiae</i>	Recombinant: cell surface engineering system, co-displaying glucoamylase and modified α -amylase	Productivity of 1.61 g.l.h ⁻¹ , 76.6% of the theoretical yield	Yamakawa et al., 2012

To date, starch-CBP yeasts have been unable to completely saccharify the feedstock as a result of many different factors, including end-product inhibition, low ethanol tolerance and low enzyme titres (den Haan et al., 2013). This has necessitated the development of more effective and proficient CBP microorganisms (Ali et al., 2016). Increased ploidy of the host strain had been explored and indicated increased enzyme levels in culture medium and consequently improved starch conversion (Yamada et al., 2009; Yamakawa et al., 2010). Strategies to improve gene expression for application in CBP includes genetic manipulation of target genes to improve secretion, codon optimisation of genes (Eksteen et al., 2003; Shigechi et al., 2004; Yamada et al., 2009; Favaro et al., 2010) as well as the expression of the relevant genes in industrial strains that are robust and have high tolerance to extracellular metabolites (den Haan et al., 2013; Aydemir, 2014)

Expression of amylolytic genes in industrial yeast strains is essential, as industrial strains possess valuable properties such as high tolerance to ethanol, sugar and acid concentration as well as exhibit high ethanol productivity and thermostability (Favaro et al., 2014). Viktor et al. (2013) compared ethanol production in a *S. cerevisiae* Y294 laboratory strain and a semi-industrial strain, *S. cerevisiae* Mnu α 1 [AmyA-GlaA], both expressing the α -amylase and glucoamylase from *A. tubingensis*. After 10 days of cultivation on 20 g.l⁻¹ raw corn starch, the semi-industrial strain performed better than the laboratory strain with 9.03 and 6.67 g.l⁻¹ ethanol produced, respectively.

2.6 This study

Currently, most of the bioethanol produced worldwide is starch-based, which has led to increased interest in amylolytic enzymes. An improvement in biomass conversion technologies requires a switch from separate hydrolysis and fermentation to simultaneous hydrolysis and fermentation (den Haan et al., 2013). Cold starch hydrolysis (also known as raw or native starch hydrolysis) is proposed whereby the hydrolysis occurs without the costly gelatinisation step (Robertson et al., 2006). This significantly reduces the total energy input, thus reducing production cost. These low temperature fermentation systems have been applied on industrial scale and have reached the same fermentation efficiency (when using high enzyme concentrations) as the conventional high temperature cooking system (Shigechi et al., 2004; de Souza and de Magalhães, 2010).

The development of raw starch CBP yeast strains is a welcomed low-cost alternative for biomass processing (van Zyl et al., 2011; den Haan et al., 2013). Combinations of α -amylases and glucoamylases from different microbial sources have been expressed in yeast strains in an effort to improve productivity as well as ethanol yield by finding the best enzyme combination (Yamada et al., 2011; Yamakawa et al., 2012). Alpha-amylases act prior to glucoamylases by reducing the chain length of dextrans, thus facilitating the saccharification and fermentation processes. When the α -amylase converts the starch biomass effectively, it maximises the required action of the glucoamylase, thereby leading to an efficient fermentation process. This highlights the importance of the α -amylases, hence the focus on these enzymes in this study.

The ideal microorganism for biofuel production requires high substrate utilisation and processing capacities, fast sugar transport, good tolerance to inhibitors and end products, high metabolic fluxes and produce a single fermentation product (Robertson et al., 2006; van Zyl et al., 2012; Aydemir, 2014). It is unlikely that a naturally occurring organism would display all the required traits. Fortunately, current progress in metabolic engineering and synthetic biology will enable the construction of a desirable strain that produces raw starch hydrolysing amylases efficient at the required fermentation conditions. Bioprospecting is a technique that can be used to obtain novel amylases that can be applied for efficient and cost effective ethanol production (Puspasari et al., 2011; van Zyl et al., 2012; Aydemir, 2014). Some fungi have GRAS status, are ubiquitous, have good tolerance to low water activity, tolerance to high osmotic pressure and have non-fastidious nutritional requirements (Raimbault, 1998; Singh and Singh, 2014). They are competitive microbes for the bioconversion of solid substrates, hence their increasing attention for use in industrial applications, but they do not produce high levels of ethanol.

Several studies have indicated that raw starch hydrolysis by α -amylases is considered the rate limiting step in starch conversion (Verma et al., 2000; Khaw et al., 2006; Yamada et al., 2009). In addition, the amylases exhibit reduced activity at low temperatures, therefore a high enzyme loading is required for raw starch hydrolysis (van Zyl et al., 2012; Presečki et al., 2013). Improved conversion of raw starch to ethanol thus requires α -amylases that are more efficient in hydrolysing raw starch. Raw starch-hydrolysing activity has been confirmed for the α -amylases from *A. tubingensis* (Viktor et al., 2013), *A. terreus* (Sethi et al., 2016), *Cryptococcus* sp. S-2 (Iefuji et al., 1996), *Saccharomycopsis fibuligera*

(Hostinová et al., 2010; Sun et al., 2010) and *Aureobasidium pullulans* (Li et al., 2011). These fungal α -amylases were screened in this study for their ability to hydrolyse raw starch. The α -amylase encoding gene sequences were obtained from the NCBI database and used to construct synthetic genes. The genes were cloned onto an episomal plasmid for heterologous expression in *S. cerevisiae* Y294 (to ensure high copy numbers) under regulation of the enolase 1 (*ENO1*) constitutive promoter and terminator sequences.

A major shortcoming of current recombinant *S. cerevisiae* strains is insufficient extracellular enzyme activity due to limited secretion of the recombinant enzymes (Eksteen et al., 2003; Favaro et al., 2012). Therefore, the XYNSEC secretion signal from the *Trichoderma reesei* xynB (Tsuchiya et al., 2003; Favaro et al., 2012; Njokweni et al., 2012) was selected for use in this study to facilitate recombinant protein secretion. The performance of the recombinant strains was compared to the *S. cerevisiae* Y294[AmyA] and the best α -amylase encoding genes were co-expressed with the *A. tubingensis* *GlaA* gene in the *S. cerevisiae* Y294 laboratory strain. The recombinant amyolytic *S. cerevisiae* Y294 strains were then evaluated for their efficiency in the hydrolysis and fermentation of raw corn starch for bioethanol production using the *S. cerevisiae* Y294[AmyA-GlaA] strain from Viktor et al. (2013) as benchmark strain.

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Chapter 3

Expression and evaluation of α -amylases required for the hydrolysis of raw starch

Expression and evaluation of α -amylases required for the hydrolysis of raw starch

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Abstract

Starch-based bioethanol has emerged as a sustainable and renewable alternative energy source, but exogenous enzymes and heat is required for the conventional liquefaction process. The cost-effective utilisation of raw starch requires consolidated bioprocessing (CBP), which entails starch hydrolysis and glucose fermentation by a single organism. This requires the co-expression of recombinant α -amylases and glucoamylases in a strain of *Saccharomyces cerevisiae*. The *Aureobasidium pullulans* *ApuA*, *Aspergillus terreus* *AteA*, *Cryptococcus* sp. S-2 *CryA* and *Saccharomycopsis fibuligera* *SfiA* α -amylase encoding genes were cloned and expressed in the *S. cerevisiae* Y294 laboratory strain under the transcriptional control of the enolase 1 promoter [*ENO1_P*] and terminator [*ENO1_T*] sequences. The *S. cerevisiae* Y294[*ApuA*] and Y294[*AteA*] strains were superior to the other strains, producing extracellular α -amylase activities of 2.57 U.ml⁻¹ and 3.20 U.ml⁻¹, respectively. When co-expressed with the *Aspergillus tubingensis* glucoamylase gene *GlaA*, the amyolytic *S. cerevisiae* Y294[*ApuA*-*GlaA*] and Y294[*AteA*-*GlaA*] strains were able to produce 32.83 and 43.81 g.l⁻¹ ethanol, respectively, from 200 g.l⁻¹ raw starch after 8 days of fermentation. Optimisation strategies concluded that the *S. cerevisiae* Y294[*AteA*-*GlaA*] strain will benefit from additional glucoamylase to increase both the fermentation rate and ethanol yield.

Keywords

raw starch • amyolytic yeast • biofuels • consolidated bioprocessing • amylase

3.1 Introduction

The growing population and global economy depend heavily on fossil fuels as the major energy source. The impending depletion of fossil fuels coupled with the negative environmental impacts associated with their use has necessitated the development of alternative sources of energy. Ideally, the alternative fuel must be derived from readily available and low-cost sources, which should be easy to manipulate and pose minimal threat to the environment (Meher et al., 2006). Biomass-derived fuels, known as biofuels, have received considerable interest as a renewable alternative source of energy; these include biodiesel, bioethanol, biomethanol, biogas, biosyngas, bio-oil and biohydrogen (Balat, 2011). Bioethanol has potential as an alternative fuel as it compares well with current fossil fuel sources in terms of total energy production, with the added advantage of a reduced negative impact on the environment (Pradhan and Mbohwa, 2014; Sarris and Papanikolaou, 2016).

Of the possible biomass sources, starch and lignocellulose have been the focus for the production of bioethanol (van Zyl et al., 2012). Lignocellulose is relatively abundant and inexpensive, but expensive pre-treatment processes are required for its conversion and different microbial species are required to ferment the various sugars released during its hydrolysis (Balat, 2011). Nevertheless, much research still needs to be done to optimise its use for bioethanol production on a commercial scale. Starch is an attractive low-cost feedstock and is currently used for large-scale bioethanol production in the USA (Wang et al., 2012; den Haan et al., 2013). It is one of the most abundant polysaccharides in plants with corn being the largest industrial starch source (Burrell, 2003). Starch consists of 20-30% amylose and 70-80% amylopectin (Yamada et al., 2009), both comprising of mainly α -1,4 linked glucose units. Amylose contains occasional α -1,6 branching points, while amylopectin is highly branched (Burrell, 2003; Stevnebøa et al., 2006). Apart from bioethanol production, starch hydrolysis products have various uses in the textile, detergent, baking and confectionary industries.

Starch hydrolysis involves gelatinisation, liquefaction and saccharification steps. Gelatinisation involves heating of the starch, which increases the susceptibility of the molecules to enzymatic hydrolysis. This is an energy-intensive process and accounts for substantial production cost. The synergistic action of α -amylases (EC 3.2.1.1) and glucoamylases (1,4- α -D-glucan glucohydrolase; EC 3.2.1.3) results in the conversion of

amylose and amylopectin into short oligosaccharide chains (dextrins of 10 to 20 glucose units in length), glucose and maltose units (van der Maarel et al., 2002; Robertson et al., 2006; Puspasari et al., 2012). The α -amylases contribute to the liquefaction of starch, whilst glucoamylases predominantly result in the saccharification of the resulting oligosaccharides (Robertson et al., 2006). The use of raw starch hydrolysing amylases have additional economic value due to the elimination of the heating process required for gelatinisation (Kaneko et al., 2005; Omemu et al., 2005; Nurachman et al., 2010; Puspasari et al., 2011).

The cost-effective bioethanol production would ideally require consolidated bioprocessing (CBP) whereby liquefaction, saccharification and fermentation of raw starch occur in a single step by a single organism (van Zyl et al., 2012). The yeast *Saccharomyces cerevisiae* remains the ideal host organism for bioethanol production due to its high ethanol tolerance, ability to perform post translational modification, robustness and status as being generally regarded as safe (GRAS) (Görgens et al., 2014). As the yeast is unable to produce the required enzymes, amyolytic yeast strains have been developed by means of recombinant DNA technology to enable strains to hydrolyse raw starch (Shigechi et al., 2002; Kim et al., 2010; Kim et al., 2011; Viktor et al., 2013).

Most reports on raw starch conversion involved low substrate loads or low conversion rates that are not economically viable on an industrial scale (reviewed in den Haan et al., 2013). Nevertheless, studies by Viktor et al. (2013) and Shigechi et al. (2002) demonstrated the construction of amyolytic yeast strains capable of effectively liquefying and saccharifying high concentrations of raw starch to bioethanol. The challenge remains to increase the starch to ethanol conversion without the addition of external enzymes. This strategy requires higher extracellular α -amylase and glucoamylase activities, which could be achieved either through increased production of extracellular enzymes or improved specific activities (den Haan et al., 2013). The discovery of more effective raw starch hydrolysing amylases is therefore essential to reduce the production cost and improve the CBP organism for industrial use (van Zyl et al., 2011; Aydemir, 2014).

In this study, raw starch degrading α -amylase encoding genes from *Aureobasidium pullulans* (*ApuA*), *Aspergillus terreus* (*AteA*), *Cryptococcus* sp. S-2 (*CryA*) and *Saccharomycopsis fibuligera* (*SfiA*) were expressed in *S. cerevisiae* Y294 and the amyolytic yeast strains evaluated in terms of extracellular α -amylase activity. The *ApuA* and *AteA* genes were selected for co-expression with the *Aspergillus tubingensis* glucoamylase (*GlaA*) encoding

gene and the strains evaluated for the conversion of raw starch to ethanol (i.e. consolidated bioprocessing) at a high substrate loading (200 g.l^{-1}) using the *S. cerevisiae* Y294[AmyA-GlaA] strain as benchmark strain (Viktor et al., 2013). Fermentation conditions were subsequently optimised for maximum ethanol production.

3.2 Materials and Methods

3.2.1 Media and strain cultivation

Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). The *Escherichia coli* DH5 α strains used were cultured at 37°C in Terrific Broth (12 g.l^{-1} tryptone, 24 g.l^{-1} yeast extract, 4 ml.l^{-1} glycerol, 0.1 M potassium phosphate buffer, pH 7) containing 100 mg.ml^{-1} ampicillin.

The laboratory *S. cerevisiae* Y294 host strain (Table 3.1) was maintained on YPD agar plates (10 g.l^{-1} yeast extract, 20 g.l^{-1} peptone, 20 g.l^{-1} glucose and 12 g.l^{-1} agar) and transformants were selected for and maintained on $\text{SC}^{-\text{URA}}$ agar plates containing 6.7 g.l^{-1} yeast nitrogen base without amino acids (BD-Diagnostic Systems, Sparks, MD, USA), 20 g.l^{-1} glucose and 1.5 g.l^{-1} yeast synthetic dropout medium supplements (Sigma-Aldrich, Germany) and 12 g.l^{-1} agar. Aerobic cultivation of *S. cerevisiae* strains were performed on a rotary shaker (200 rpm) at 30°C in 125 ml Erlenmeyer flasks containing 20 ml double strength $\text{SC}^{-\text{URA}}$ medium ($2\times\text{SC}^{-\text{URA}}$: 13.4 g.l^{-1} yeast nitrogen base without amino acids, 20 g.l^{-1} glucose and 3 g.l^{-1} yeast synthetic drop-out medium supplements). The $2\times\text{SC}^{-\text{URA}}$ medium used for small-scale fermentations was amended to contain 5 g.l^{-1} glucose and 200 g.l^{-1} raw corn starch (Sigma-Aldrich) as carbohydrate source. Ampicillin (100 mg.l^{-1}) and streptomycin (75 mg.l^{-1}) were added to prevent bacterial contamination.

3.2.2 Strains and plasmids

All relevant yeast and bacterial strains as well as the plasmids used in this study are listed in Table 3.1.

Table 3.1: Microbial strains and plasmids used in this study

<i>S. cerevisiae</i> strains:	Genotype	Reference
Y294	<i>α leu2-3,112 ura3-52 his3 trp1-289</i>	ATCC 201160
Y294[BBH4]	<i>URA3 ENO1_P-XYNSEC-ENO1_T</i>	Njokweni et al. (2012)
Y294[AmyA]	<i>URA3 ENO1_P-XYNSEC-AmyA-ENO1_T</i>	Viktor et al. (2013)
Y294[AteA]	<i>URA3 ENO1_P-XYNSEC-AteA-ENO1_T</i>	This study
Y294[ApuA]	<i>URA3 ENO1_P-XYNSEC-ApuA-ENO1_T</i>	This study
Y294[CryA]	<i>URA3 ENO1_P-XYNSEC-CryA-ENO1_T</i>	This study
Y294[SfiA]	<i>URA3 ENO1_P-XYNSEC-SfiA-ENO1_T</i>	This study
Y294[AteA-GlaA]	<i>URA3 ENO1_P-XYNSEC-AteA-ENO1_T; ENO1_P-GlaA-ENO1_T</i>	This study
Y294[ApuA-GlaA]	<i>URA3 ENO1_P-XYNSEC-ApuA-ENO1_T; ENO1_P-GlaA-ENO1_T</i>	This study
Y294[AmyA-GlaA]	<i>URA3 ENO1_P-XYNSEC-AmyA-ENO1_T; ENO1_P-GlaA-ENO1_T</i>	Viktor et al. (2013)
Bacterial strains:		
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook et al. (1989)
Plasmids:		
pMA-RQ-ApuA	<i>bla ApuA</i>	GenScript
pMA-RQ-AteA	<i>bla AteA</i>	GenScript
pMA-RQ-CryA	<i>bla CryA</i>	GenScript
pMA-RQ-SfiA	<i>bla SfiA</i>	GenScript
yBBH4	<i>bla URA3 ENO1_P-XYNSEC-ENO1_T</i>	Njokweni et al. (2012)
yApuA	<i>bla URA3 ENO1_P-XYNSEC-ApuA-ENO1_T</i>	This laboratory
yAteA	<i>bla URA3 ENO1_P-XYNSEC-AteA-ENO1_T</i>	This study
yCryA	<i>bla URA3 ENO1_P-XYNSEC-CryA-ENO1_T</i>	This study
ySfiA	<i>bla URA3 ENO1_P-XYNSEC-SfiA-ENO1_T</i>	This study
yBBH1-GlaA	<i>bla URA3 ENO1_P-GlaA-ENO1_T</i>	Viktor et al. (2013)
yApuA-GlaA	<i>bla URA3 ENO1_P-XYNSEC-ApuA-ENO1_T; ENO1_P-GlaA-ENO1_T</i>	This study
yAteA-GlaA	<i>bla URA3 ENO1_P-XYNSEC-AteA-ENO1_T; ENO1_P-GlaA-ENO1_T</i>	This study

3.2.3 Strain construction

The *ApuA*, *AteA*, *CryA* and *SfiA* genes were designed and synthesised by GenScript (Piscataway, USA) for expression in *S. cerevisiae* based on the native gene sequences (Accession numbers: L15383.1, AEH03024.1, ADN65120.1 and ADU24597.1, respectively). The truncated genes (without a secretion signal) were amplified with TaKaRa Extaq polymerase (Takara Bio Inc., Japan) using the respective pMA-RQ vectors (Table 3.1) as template and the gene-specific primers listed in Table 3.2. Following agarose gel electrophoresis, the amplified DNA was eluted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). Electro-competent *S. cerevisiae* Y294 cells (Cho et al., 1999) were transformed with the linearised yBBH4 plasmid (digested with *NruI*) or amplified *AteA*, *ApuA*, *CryA* or *SfiA* PCR products.

Table 3.2: PCR primers used in the study with the relevant restriction sites underlined (*NruI* = tcgcga; *XhoI* = ctcgag)

Gene ¹	Primer name and sequence	Protein ²
<i>ApuA</i> 1812 bp	ApuA-L: 5'-gaaccctggctgtggagaagcgc <u>tcgcg</u> actcaccctgcacaatggagaagtcaatcg-3' ApuA-R: 5'-gactagaaggcttaatacaaaagct <u>ctcgag</u> tcaccctgccaagtattgctgaccgatgc-3'	67.72 kDa
<i>AteA</i> 1764 bp	AteA-L: 5'-gaaccctggctgtggagaagcgc <u>tcgcg</u> actgacccagcagaatggcgcagccagtca-3' AteA-R: 5'-gactagaaggcttaatacaaaagct <u>ctcgag</u> tcaccccaagtatcagcaactgtcaccgt-3'	66.29 kDa
<i>CryA</i> 1836 bp	CryA-L: 5'-gaaccctggctgtggagaagcgc <u>tcgcg</u> actgtcccctgcccgaatggcgaagccagtcc-3' CryA-R: 5'-gactagaaggcttaatacaaaagct <u>ctcgag</u> ctaggaggaccacgtaaacctgatgtcggc-3'	69.41 kDa
<i>SfiA</i> 1430 bp	SfiA-L: 5'-gaaccctggctgtggagaagcgc <u>tcgcg</u> acaaccagtgactctattcaaaagagaact-3' SfiA-R: 5'-gactagaaggcttaatacaaaagct <u>ctcgag</u> tcatgaacaatgtcagaagcatatttagc-3'	54.34 kDa

¹Native secretion signals are omitted

²Predicted (unglycosylated) size of the secreted protein.

An in-frame fusion of the respective *AteA*, *ApuA*, *CryA* and *SfiA* genes with the secretion signal of the *Trichoderma reesei xyn2* gene (*XYNSEC* on yBBH4) was obtained via yeast-mediated ligation (Cho et al., 1999). The plasmids were isolated from the *S. cerevisiae* transformants using the High Pure Plasmid Isolation kit (Roche Applied Science, Germany) and transferred to chemically competent *E. coli* cells for amplification. Plasmids were isolated and the final vector constructs were verified with restriction analysis (Figure 3.1). Standard protocols were followed for DNA manipulation (Sambrook et al., 1989). Restriction endonucleases and ligase were sourced from Roche Applied Science (Germany).

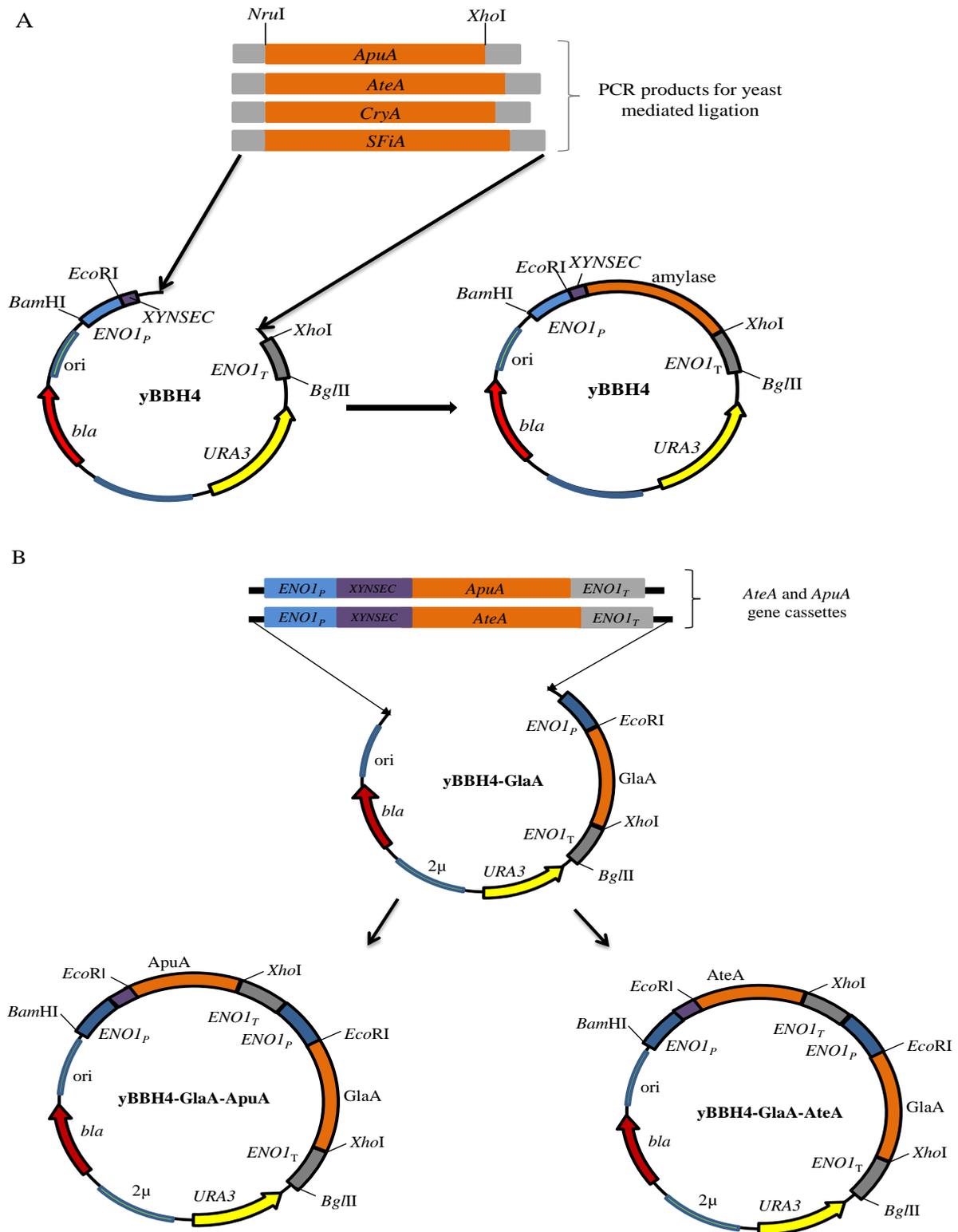


Figure 3.1: (A) Schematic representation of the final vector constructs for the multi-copy expression of the *ApuA*, *AteA*, *CryA* and *SfiA* genes. (B) The *ApuA* and *AteA* gene cassettes were isolated and cloned onto the *yBBH1-GlaA* vector for co-expression with the *A. tubingensis* *GlaA* gene. All amylase genes were expressed under the transcriptional control of the *ENO1* promoter and terminator sequences.

The $ENOI_P$ -ApuA- $ENOI_T$ and $ENOI_P$ -AteA- $ENOI_T$ cassettes were excised from yApuA and yAteA with *Bam*HI and *Bgl*III restriction endonucleases and cloned into the *Bam*HI site of yBBH1-GlaA, yielding yApuA-GlaA and yAteA-GlaA, respectively (Figure 3.1). The yApuA-GlaA and yAteA-GlaA vector constructs were transformed to *S. cerevisiae* Y294 to yield Y294[yApuA-GlaA] and Y294[yAteA-GlaA], respectively.

3.2.4 Protein analysis

Recombinant *S. cerevisiae* Y294 strains were cultivated in 20 ml $2\times SC^{-URA}$ medium for 3 days. The cells were harvested by centrifugation and 20 μ l of the supernatant was denatured at 100°C for 3 min in the presence of a protein denaturing loading dye (Sambrook et al., 1989). The protein species were separated on an 8% SDS-PAGE using a 5% stacking gel and Tris-glycine buffer. Electrophoresis was carried out at 100 V for \pm 90 minutes at ambient temperature. Protein species were visualised using the silver staining method (O'Connell and Stults, 1997).

3.2.5 Amylase assays and enzyme characterisation

All recombinant *S. cerevisiae* strains were cultivated on SC^{-URA} plates containing 2% soluble corn starch at 30°C for 48 hours. The plates were transferred to 4°C, resulting in the development of clear zones around colonies displaying extracellular α -amylase activity (Viktor et al., 2013). The yeast transformants were inoculated to a concentration of 1×10^6 cells. ml^{-1} in 20 ml $2\times SC^{-URA}$ medium using 125 ml Erlenmeyer flasks and cultured at 30°C with agitation at 200 rpm. The supernatant was harvested at regular intervals and α -amylase activity quantified with the reducing sugar assay (Miller, 1959), which involved a 5 min hydrolysis of a 0.2% soluble corn starch solution (in 0.05 M citrate-phosphate buffer at pH 5, 450 μ l substrate and 50 μ l of enzyme used in assays) at 30°C. Enzyme activity was expressed as U. ml^{-1} supernatant, with one unit defined as the amount of enzyme required to release one μ mole of glucose per minute.

The amylase assays were conducted for the ApuA and AteA enzymes at different pH values (varying between pH 3 and 8). The relative activity was expressed as a percentage, with the highest level of activity taken as 100%. Temperature stability of the enzymes was determined

by incubating the respective enzymes at 30°C in 0.05 M citrate-phosphate buffer (pH 5) for up to 120 hours before conducting the activity assay.

3.2.6 Fermentation studies

Fermentation experiments were performed in 120 ml glass serum bottles as described by Viktor et al. (2013). The 10% pre-cultures (10 ml of a stationary culture) of *S. cerevisiae* Y294[yAteA-GlaA], Y294[yApuA-GlaA] and Y294[AmyA-GlaA] strains were inoculated into 2×SC^{-URA} media containing 200 g.l⁻¹ raw corn starch and 5 g.l⁻¹ glucose as carbohydrate source. Agitation and incubation were performed on a magnetic multi-stirrer at 30°C, with regular sampling through a syringe needle pierced through the rubber stopper.

The *S. cerevisiae* Y294[AteA-GlaA] strain was cultivated as described above either in the presence of 2.83 µl STARGENTM 002 enzyme (Genencor, Finland) (equivalent to 10% of its recommended loading: 1.42 ml enzyme per kg starch), or with the pH of the growth medium adjusted to pH 6 using 1 M NaOH. For varied substrate loading, fermentation experiments contained either 200 or 100 g.l⁻¹ raw corn starch. The supernatant of the *S. cerevisiae* Y294[AteA] strain was harvested after 3 days of cultivation in SC^{-URA} medium and lyophilised using the Virtis 6K benchtop freeze dryer (United Scientific, South Africa) to concentrate the AteA enzyme activity to 0.036 U.mg⁻¹ enzyme. The lyophilised enzyme was kept at -20°C until it was needed. The *S. cerevisiae* Y294[AteA-GlaA] strain was cultivated in the presence of 0, 50, 100 and 150 mg lyophilised AteA. The co-fermentation experiments contained an initial inoculum of 10 ml of the *S. cerevisiae* Y294[AteA-GlaA] pre-culture and either 10 ml of *S. cerevisiae* Y294[BBH4], 10 ml of *S. cerevisiae* Y294[GlaA] or 5 ml of each of the latter pre-cultures.

3.2.6.1 HPLC analysis

Ethanol, glycerol, acetic acid and glucose levels were quantified with high performance liquid chromatography (HPLC), using a Surveyor Plus liquid chromatograph (ThermoScientific, South Africa) consisting of a LC pump, autosampler and Refractive Index Detector. The compounds were separated on a Rezex RHM Monosaccharide 7.8×300 mm column (00H0132-K0, Phenomenex) at 60°C with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.6 ml.min⁻¹. The theoretical CO₂ yield was calculated based the ethanol concentrations as per HPLC analyses.

3.3 Results

3.3.1 Strain construction and evaluation

The *ApuA*, *AteA*, *CryA* and *SfA* genes were cloned onto the yBBH4 vector by means of yeast-mediated ligation, whereas the *ApuA* and *AteA* gene cassettes were sub-cloned onto pBBH1-GlaA for co-expression with *GlaA* in *S. cerevisiae* Y294 (Figure 3.1). The *S. cerevisiae* Y294[BBH4] strain was used as reference strain for all experiments. The *S. cerevisiae* strains expressing α -amylases developed clearing zones on solid SC^{-URA} media containing 2% soluble starch (Figure 3.2), with no zones produced by *S. cerevisiae* Y294[BBH4].

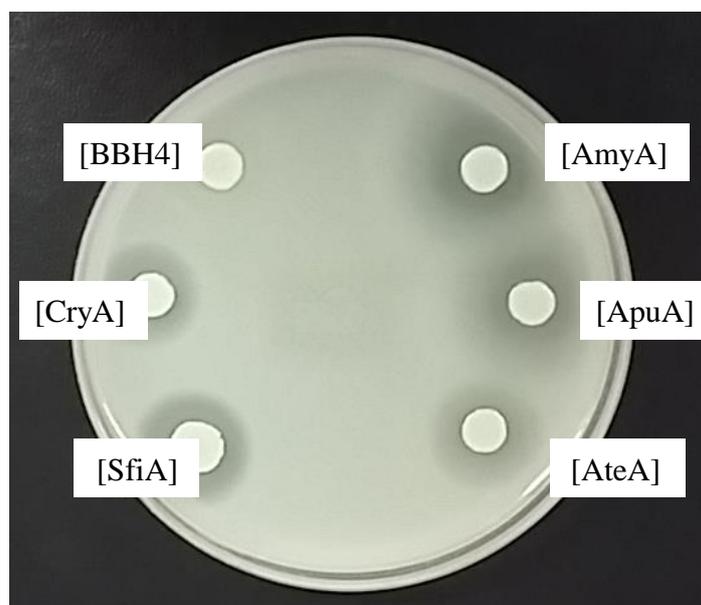


Figure 3.2: Recombinant *S. cerevisiae* Y294 strains displaying extracellular α -amylase activity on SC^{-URA} agar plates containing 2% (w/v) corn starch after 48 hours of incubation at 30°C. The presence of zones surrounding the colonies is indicative of α -amylase activity.

The yeast strains were cultivated in 2×SC^{-URA} medium and daily samples of the supernatant were taken for quantitative analysis on soluble starch. The *S. cerevisiae* Y294[AmyA] strain (Viktor et al., 2013) was used as the benchmark strain and had an activity of 2.16 U.ml⁻¹ (36.05 nkat.ml⁻¹). The *S. cerevisiae* Y294[AteA] and *S. cerevisiae* Y294[ApuA] strains produced α -amylase activities of 3.20 U.ml⁻¹ (53.16 nkat.ml⁻¹) and 2.57 U.ml⁻¹

(42.69 nkat.ml⁻¹) on soluble starch, respectively, after 72 hours of cultivation (Figure 3.3). The other strains produced low levels of extracellular α -amylase activities.

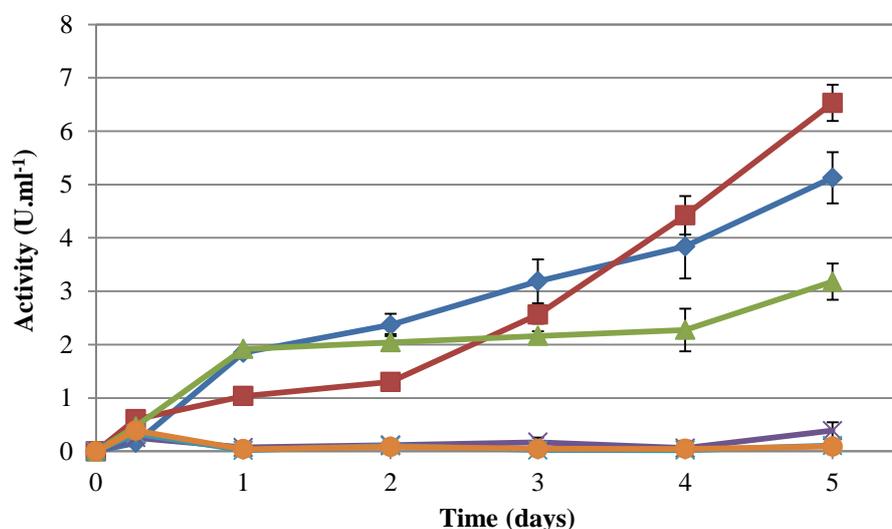


Figure 3.3: The extracellular α -amylase activity was quantitatively determined for (■) *S. cerevisiae* Y294[ApuA], (◆) *S. cerevisiae* Y294[AteA], (×) *S. cerevisiae* Y294[CryA], (×) *S. cerevisiae* Y294[SfiA], (▲) *S. cerevisiae* Y294[AmyA] and (●) *S. cerevisiae* Y294[BBH4] over time. Values represent the mean of three repeats and error bars indicate the standard deviation from the mean value.

3.3.2 Enzyme characterisation

All yeast strains were cultivated in $2\times\text{SC}^{-\text{URA}}$ medium and the supernatant obtained after 3 days of cultivation. Separation of the proteins in the supernatant by SDS-PAGE (Figure 3.4) revealed that both the ApuA and AteA enzymes were bigger than the predicted theoretical protein size (Table 3.2), indicating that the proteins were glycosylated. The CryA protein was visible as a heterogeneous band whereas SfiA displayed a prominent band of the predicted size. The ApuA and AteA enzymes were selected for further characterisation due to the superior levels of α -amylase activity displayed by the corresponding recombinant strains (Figure 3.3).

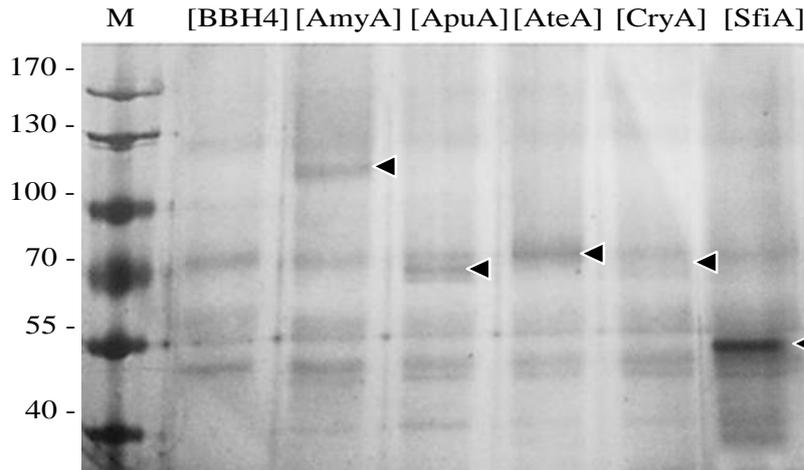


Figure 3.4: The supernatant was harvested and the proteins separated by SDS-PAGE. The triangles indicate the additional protein species that are lacking in the reference strain, *S. cerevisiae* Y294[BBH4].

The pH preferences of ApuA and AteA were determined by performing the reducing sugar assay at different pH values (Figure 3.5A). Both enzymes performed best between pH 4 and 5, which is consistent with the pH typically used for fermentation studies. Temperature stability of ApuA and AteA was determined by incubating the enzymes (strain supernatant) at 30°C without substrate for up to 5 days prior to performing the reducing sugar assay. More than 75% of the enzyme activity was retained after 5 days indicating their stability at fermentation temperature (Figure 3.5B).

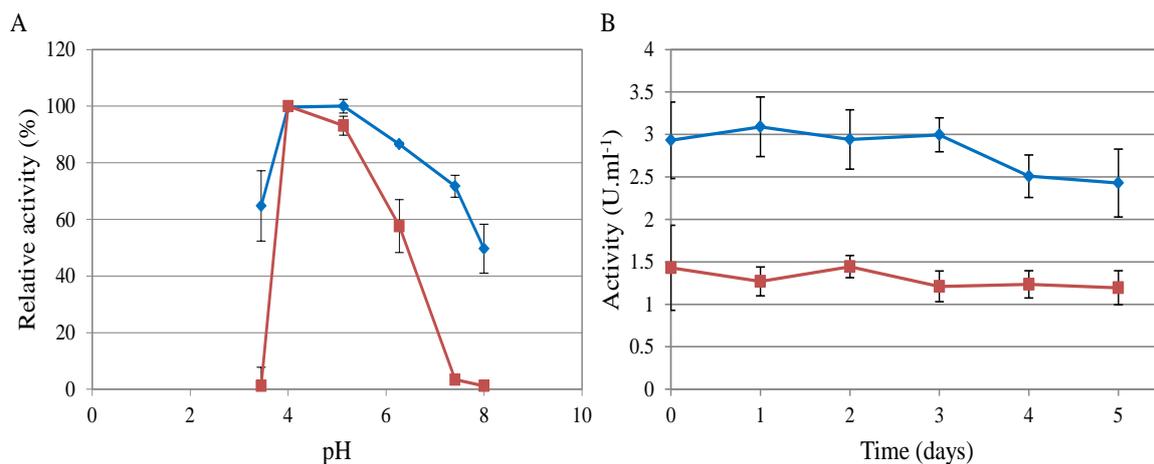


Figure 3.5. (A) The effect of pH on relative activity of (■) ApuA and (◆) AteA was determined using 0.2% corn starch as substrate. (B) Temperature stability of (■) ApuA and (◆) AteA were determined following incubation at 30°C without substrate. Values represent the mean of three repeats and error bars indicate the standard deviation from the mean value.

3.3.3 Fermentation of raw starch

The *S. cerevisiae* Y294[ApuA-GlaA] and Y294[AteA-GlaA] strains were constructed for the co-expression of *GlaA* with *ApuA* and *AteA*, respectively. The strains were evaluated for ethanol production from raw corn starch at high substrate loading using the *S. cerevisiae* Y294[AmyA-GlaA] strain as benchmark strain. Initially, the *S. cerevisiae* Y294[ApuA-GlaA] strain performed better than *S. cerevisiae* Y294[AmyA-GlaA], but the final ethanol concentration was approximately 20% lower (Figure 3.6) after 8 days of cultivation. The *S. cerevisiae* Y294[AteA-GlaA] strain performed similar to the benchmark strain, producing a maximum of 44 g.l⁻¹ ethanol. Glycerol accumulation in the growth medium typically followed the same trend as ethanol production with *S. cerevisiae* Y294[ApuA-GlaA], Y294[AmyA-GlaA] and Y294[AteA-GlaA] respectively producing 2.78, 3.55 and 3.79 g.l⁻¹ glycerol after 8 days of cultivation. Since the ethanol concentrations did not increase further, the fermentations were terminated after 8 days.

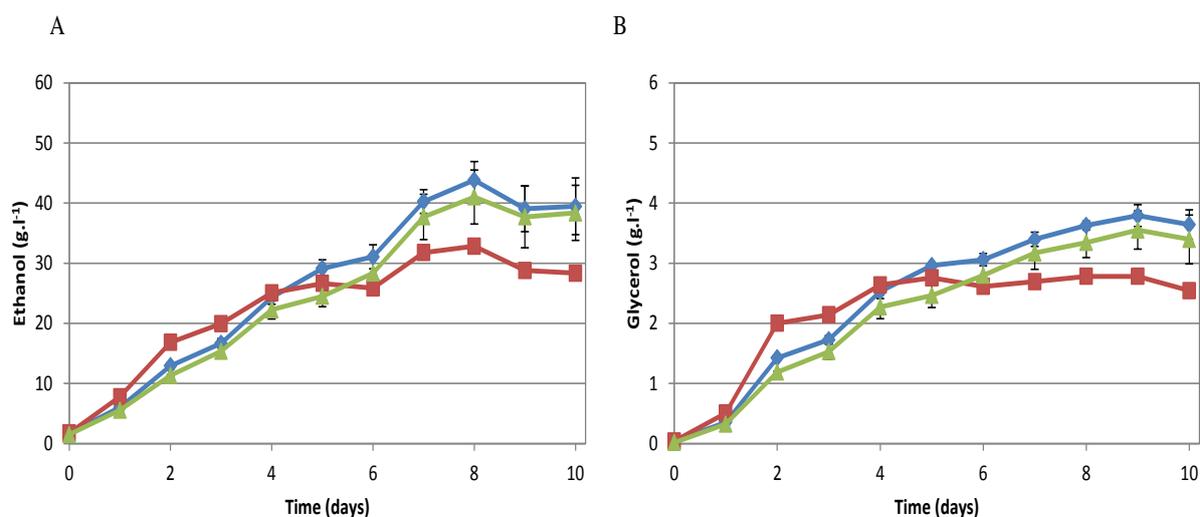


Figure 3.6: The (A) ethanol and (B) glycerol production by (◆) *S. cerevisiae* Y294[AteA-GlaA], (▲) *S. cerevisiae* Y294[AmyA-GlaA], (■) *S. cerevisiae* Y294[ApuA-GlaA] and (●) *S. cerevisiae* Y294[BBH4] over time were monitored under oxygen-limited conditions in 2×SC^{-URA} media containing 200 g.l⁻¹ raw corn starch and 5 g.l⁻¹ glucose as carbohydrate sources. Values represent the mean of three repeats and error bars represent the standard deviation.

When *S. cerevisiae* Y294[AteA-GlaA] was cultivated on 100 and 200 g.l⁻¹ raw corn starch (supplemented with 5 g.l⁻¹ glucose), less ethanol was produced on 100 g.l⁻¹ raw corn starch (41.92 g.l⁻¹) compared to 45.47 g.l⁻¹ on 200 g.l⁻¹ raw starch (Figure 3.7) after 8 days.

Nevertheless, the ethanol production on 200 g.l^{-1} starch was much less than the expected theoretical yield, indicating that starch conversion is hampered at high substrate loading, whereas almost complete conversion was obtained at lower starch concentrations.

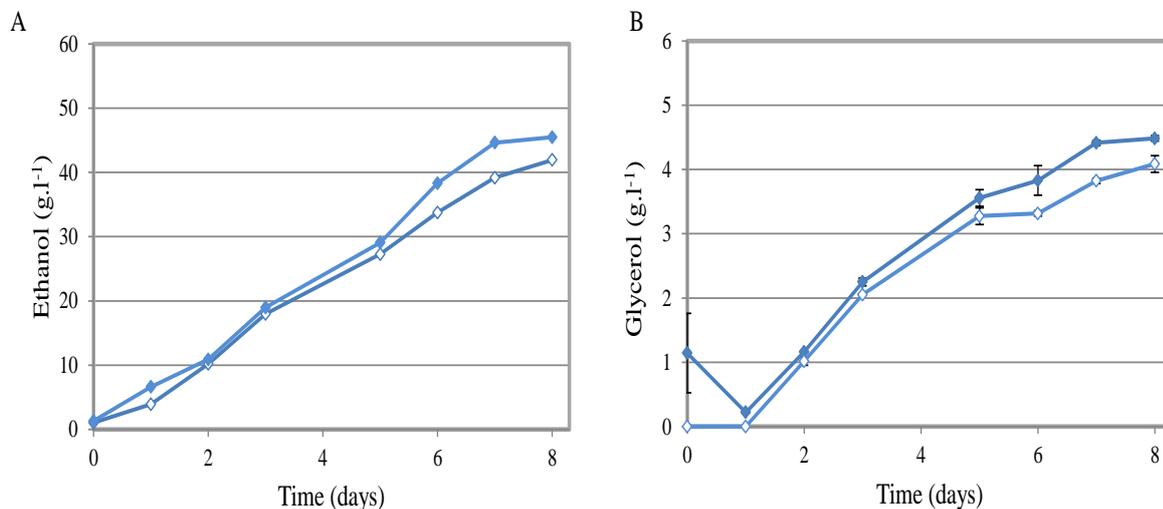


Figure 3.7: The (A) ethanol and (B) glycerol production by *S. cerevisiae* Y294[AteA-GlaA], when cultivated on (◇) 100 g.l^{-1} and (◆) 200 g.l^{-1} raw corn starch and 5 g.l^{-1} glucose as carbohydrate sources. Values represent the mean of three repeats and error bars represent the standard deviation.

A set of fermentations were designed to elucidate the incomplete conversion of starch at high solids loading. Several medium modifications were investigated, including the addition of $\text{NH}_4(\text{SO}_4)_2$, citrate buffer (pH 6), dH_2O and spiking with fresh culture (at day 7), none of which had a significant impact on the ethanol production or the starch conversion rate by *S. cerevisiae* Y294[AteA-GlaA] (data not shown). The *S. cerevisiae* Y294[AteA-GlaA] strain was also evaluated for ethanol production under increased pH values and amylase supplementation. The addition of STARGENTM 002 resulted in increased production of glucose, glycerol, acetic acid and ethanol (Figure 3.8), while an initial pH of 6 only affected glycerol and acetic acid levels, with little effect on the glucose and ethanol concentrations.

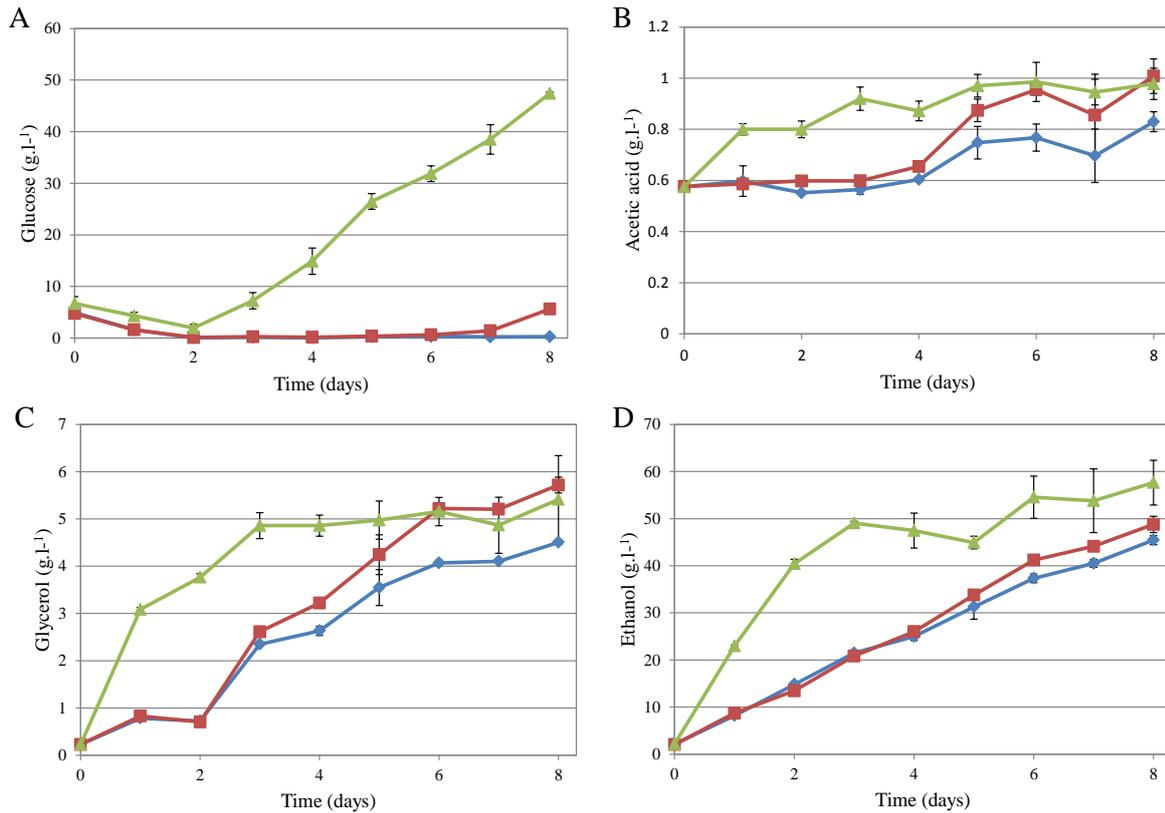


Figure 3.8: (A) Glucose, (B) acetic acid, (C) glycerol and (D) ethanol production by (♦) *S. cerevisiae* Y294[AteA-GlaA], with additional (▲) 2.83 μl STARGEN™ 002 and (■) medium set at pH 6, under oxygen limited conditions in $2\times\text{SC}^{\text{URA}}$ media containing 200 g.l^{-1} raw corn starch and 5 g.l^{-1} glucose as carbohydrate sources. Values represent the mean of three repeats and error bars represent the standard deviation.

When STARGEN™ 002 was added to the medium, *S. cerevisiae* Y294[AteA-GlaA] produced 49 g.l^{-1} ethanol after 3 days (Figure 3.8), after which fermentation slowed down to reach a final ethanol concentration of 57 g.l^{-1} . The contribution of STARGEN™ 002 is evident in the elevated ethanol production rate during the first 3 days of cultivation; in the absence of STARGEN™ 002, the strain took three times longer to produce similar ethanol levels. After 3 days, the starch was steadily hydrolysed as evident from the glucose accumulation, but the fermentation rate nevertheless slowed down.

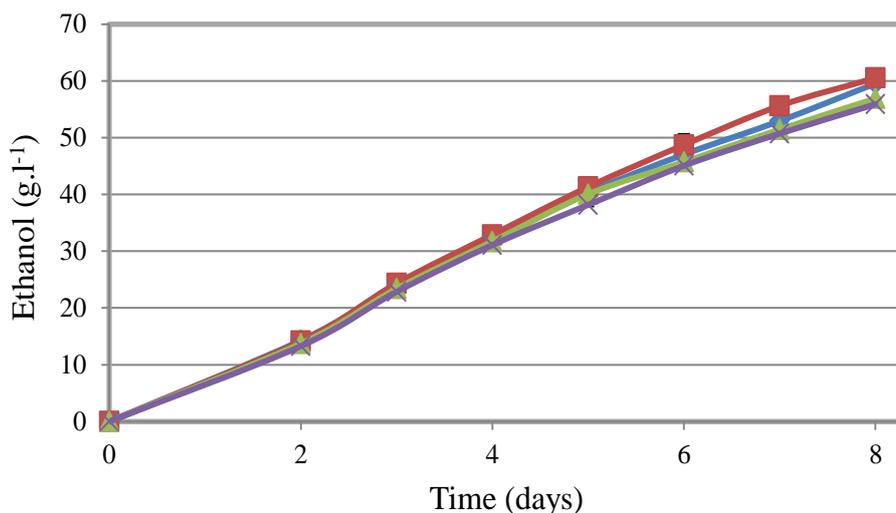


Figure 3.9: Ethanol and glucose production by *S. cerevisiae* Y294[AteA-GlaA] containing (◆) 0 mg, (■) 50 mg, (▲) 100 mg or (×) 150 mg of lyophilised AteA under oxygen limited conditions in $2\times\text{SC}^{-\text{URA}}$ medium containing 200 g.l^{-1} raw corn starch and 5 g.l^{-1} glucose as carbohydrate sources. Values represent the mean of three repeats and error bars represent the standard deviation.

The *S. cerevisiae* Y294[AteA-GlaA] strain was cultivated under oxygen-limited conditions supplemented with exogenous α -amylase (lyophilised AteA) to simulate the effect of STARGENTM 002 (shown in Figure 3.8) and optimise the addition of amylase. The addition of exogenous AteA α -amylase had no significant effect on the ethanol production rate or the ethanol concentration (Figure 3.9). Since the GlaA enzyme could not be lyophilised, co-cultivation was employed to increase the extracellular glucoamylase concentration. The *S. cerevisiae* Y294[AteA-GlaA] strain was co-cultivated with *S. cerevisiae* Y294[GlaA] and *S. cerevisiae* Y294[BBH4], respectively, to determine if an increase in glucoamylase activity would benefit the fermentation process (Figure 3.10).

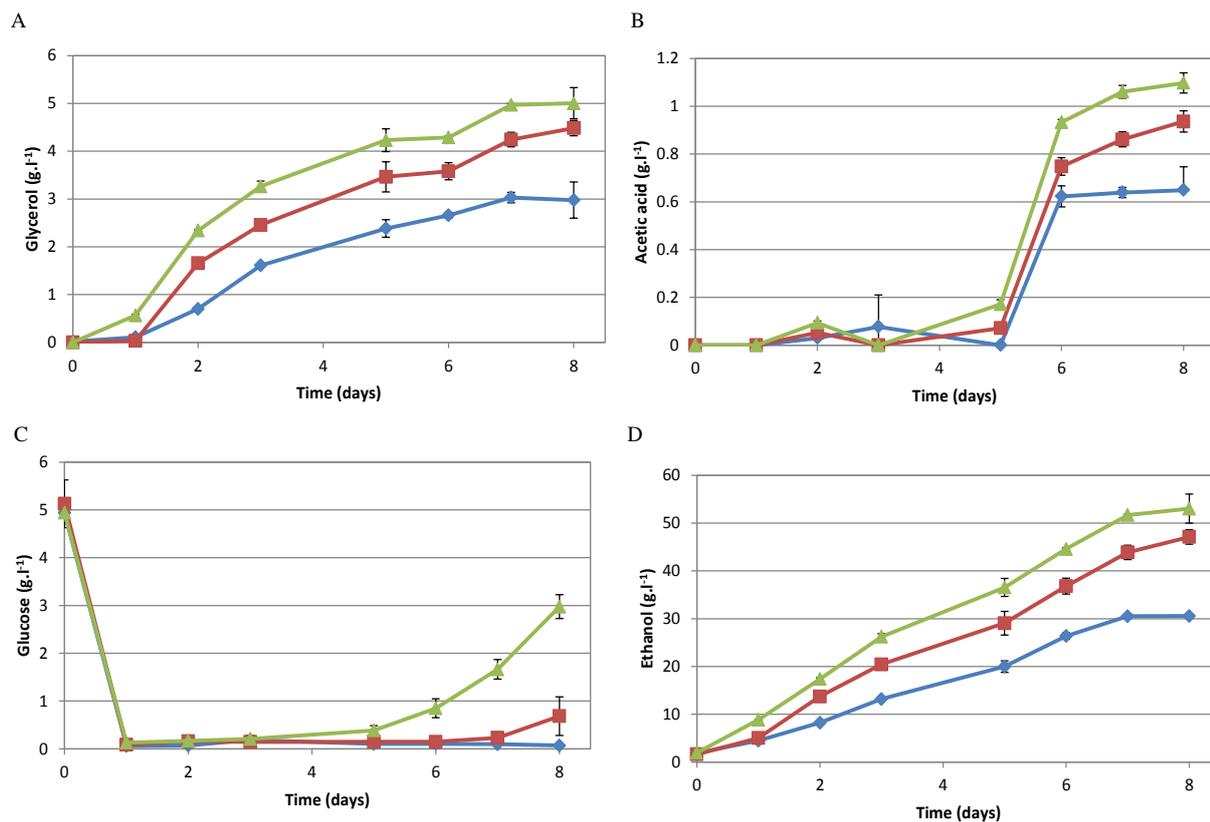


Figure 3.10: (A) Glucose, (B) acetic acid, (C) glycerol and (D) ethanol production by (\blacklozenge) 10 ml *S. cerevisiae* Y294[AteA-GlaA] + 10 ml *S. cerevisiae* Y294[BBH4], (\blacktriangle) 10 ml *S. cerevisiae* Y294[AteA-GlaA] + 10 ml *S. cerevisiae* Y294[GlaA] and (\blacksquare) 10 ml *S. cerevisiae* Y294[AteA-GlaA] + 5 ml *S. cerevisiae* Y294[GlaA] + 5 ml *S. cerevisiae* Y294[BBH4] under oxygen-limited conditions in $2\times\text{SC}^{-\text{URA}}$ media containing 200 g.l^{-1} raw corn starch and 5 g.l^{-1} glucose as carbohydrate sources. Values represent the mean of three repeats and error bars represent the standard deviation.

To ensure a consistent initial total inoculum size in the fermentations, *S. cerevisiae* Y294[BBH4] was added to experiments with less than 20 ml of recombinant cultures. The *S. cerevisiae* Y294[AteA-GlaA] + *S. cerevisiae* Y294[BBH4] co-culture served as benchmark (Figure 3.10). The combination of *S. cerevisiae* Y294[AteA-GlaA] + *S. cerevisiae* Y294[GlaA] produced more glucose, acetic acid, glycerol and ethanol than co-cultures with *S. cerevisiae* Y294[BBH4], with the increase in acetic acid and glycerol typically following the increase in ethanol. The *S. cerevisiae* Y294[AteA-GlaA] + *S. cerevisiae* Y294[BBH4] co-culture produced less ethanol than *S. cerevisiae* Y294[AteA-GlaA] on its own (Figures 3.6, 3.7, 3.8 and 3.9), which is due to the addition of the non-amyolytic *S. cerevisiae* Y294[BBH4] cells to standardise the inoculum size.

Table 3.3: Conversion of raw starch to ethanol and by products by recombinant *S. cerevisiae* strains

<i>S. cerevisiae</i> Y294[AteA-GlaA]	--	--	+ Y294 [GlaA]	pH 6 (NaOH)	STARGEN™ 002	
Fermentation time (h)	192	192	192	192	72	240
<u>Substrate (g.l⁻¹)</u>						
Raw starch	100	200	200	200	200	200
Glucose	5	5	5	5	5	5
Carbohydrate (DW*)	92.5	185	185	185	185	185
<i>Glucose equivalent</i>	<i>106.75</i>	<i>208.5</i>	<i>208.5</i>	<i>208.5</i>	<i>208.5</i>	<i>208.5</i>
<u>Products (g.l⁻¹)</u>						
Glucose	0.92	0.86	2.97	5.62	7.23	57.76
Glycerol	4.08	4.57	5	5.72	4.86	5.04
Acetic acid	0.85	0.96	1.1	1.01	0.92	0.97
Ethanol	41.92	45.47	53.03	48.77	49.06	65.02
CO ₂	40.10	43.49	50.72	46.65	46.93	62.19
Total	87.87	95.35	112.82	107.77	109.00	190.98
Product yield	82%	46%	54%	52%	52%	92%
% Ethanol (theoretical yield)	79%	44%	51%	47%	47%	62%
Ethanol productivity	0.22	0.24	0.28	0.20	0.68	0.27

* DW, dry weight

The ethanol produced by *S. cerevisiae* Y294[AteA-GlaA] + *S. cerevisiae* Y294[GlaA] (Figure 3.10) is only exceeded by the STARGEN™ 002 assisted fermentation (Figure 3.8, Table 3.3), which displayed the highest ethanol production rate over the first 3 days. Nevertheless, the final ethanol concentration (expressed as percentage of theoretical yield) outperformed the STARGEN™ 002 assisted fermentation of 200 g.l⁻¹ starch by 17% on day 8 (Figure 3.10). The carbon conversion by *S. cerevisiae* Y294[AteA-GlaA] + *S. cerevisiae* Y294[GlaA] was much lower than expected (Table 3.3), due to the higher inoculum that

utilised more glucose to generate biomass (which could not be quantified due to the insolubility of the raw starch).

3.4 Discussion

Starch-based production of bioethanol can be more economical if raw starch can be converted to ethanol at a faster rate and at a lower cost. This would entail the construction of raw starch utilising strains that perform better than the laboratory strains developed to date, such as *S. cerevisiae* Y294[AmyA-GlaA] that was used as a benchmark for this study (Viktor et al., 2013). In this study, a number of alternative α -amylase encoding genes (*AteA*, *ApuA*, *CryA* and *SfiA*) were cloned and expressed in *S. cerevisiae* Y294 and the strains evaluated for their ability to hydrolyse starch. The *ENO1* promoter and terminator sequences were used to enhance transcription of the α -amylase encoding genes, while the *T. reesei xyn2* secretion signal (XYNSEC) was used to direct secretion of the enzymes.

The recombinant *S. cerevisiae* strains displayed the ability to hydrolyse soluble starch as indicated by the zones surrounding the microbial colonies (Figure 3.2) on starch plates. The presence of these zones is a qualitative indicator of extracellular hydrolytic activity (Nangin and Sutrisno, 2014). Since zone size is influenced by protein size, protein diffusion rates in agar, agar concentration and protein glycosylation patterns (O'Connell and Stults, 1997), extracellular amylase activities were quantified with reducing sugar assays (Figure 3.3). While the zones on the plates were similar in size, the liquid assays indicated that the best performing α -amylase strains were *S. cerevisiae* Y294[AteA] and *S. cerevisiae* Y294[ApuA], expressing the α -amylases of *A. terreus* and *A. pullulans*, respectively. At 72 hours, the extracellular α -amylase activity of *S. cerevisiae* Y294[AteA] exceeded that of *S. cerevisiae* Y294[AmyA] by 32%, whereas the other strains exhibited much lower levels (Figure 3.3). The zones on the starch plates indicate that the enzymes from these strains were active, but the low levels of extracellular activity indicate that the enzymes might be cell wall associated.

The production of α -amylases by the amylolytic *S. cerevisiae* Y294 strains was confirmed by SDS-PAGE revealing the presence of additional protein species (Figure 3.4) with sizes within the expected range (Kaneko et al., 2005; Robertson et al., 2006; Arikan, 2008). The protein size of the CryA and SfiA proteins corresponded to the predicted sizes (Table 3.2), whereas the larger molecular weights for AteA (72 kDa) and ApuA (70 kDa) indicated possible glycosylation. Glycosylation can contribute to the protein structure, thermostability,

protection against proteolytic attack as well as protein secretion (Kumar and Satyanarayana, 2009), which could explain the higher levels of activity observed for AteA and ApuA compared to that of the CryA and SfiA. However, despite similar protein intensities on SDS-PAGE, SfiA displayed a lower activity compared to AteA, ApuA and AmyA, suggesting a lower specific activity for SfiA.

Temperature and pH are vital parameters for optimal microbial growth and thus the production of extracellular metabolites. Studies on fungal α -amylases indicate pH optima generally fall within the acid to neutral range (Saha et al., 1993; Robertson et al., 2006; Gupta et al., 2003; Sethi et al., 2016). In this study, the pH optima for both ApuA and AteA were between pH 4 and 5 (Figure 3.5A), which is consistent with previous reports. The ApuA and AteA enzymes maintained most of their activity for up to 120 hours at 30°C (Figure 3.5B), indicating enzyme stability at standard fermentation temperatures (Sethi et al., 2016) and thus highlighting potential application for ethanol production (Carrasco et al., 2016).

Based on the results discussed above, the *AteA* and *ApuA* genes were selected for co-expression with the *GlaA* glucoamylase in the recombinant *S. cerevisiae* Y294[AteA-GlaA] and Y294[ApuA-GlaA] strains, with *S. cerevisiae* Y294[AmyA-GlaA] serving as benchmark (Figure 3.6). All strains were able to hydrolyse raw starch and convert the resultant sugars to ethanol and CO₂. An interesting observation was that *S. cerevisiae* Y294[ApuA-GlaA] performed better during the first 4 days (Figure 3.6), but became sluggish over the last 4 days of fermentation. Since all the strains have the same genetic background, it suggested that the ApuA enzyme itself might be negatively affected by the increase in ethanol concentration.

The *S. cerevisiae* Y294[AteA-GlaA] strain produced more ethanol (43.81 g.l⁻¹) than the *S. cerevisiae* Y294[AmyA-GlaA] benchmark strain (41.02 g.l⁻¹ ethanol) after 8 days (Figure 3.6), which corresponds to a 40% starch conversion relative to 37% by *S. cerevisiae* Y294[AmyA-GlaA]. In contrast, *S. cerevisiae* Y294[ApuA-GlaA] only produced 32.83 g.l⁻¹ ethanol, equivalent to a 30% starch conversion. The *S. cerevisiae* Y294[AmyA-GlaA] strain performed slightly better than reported previously (Viktor et al., 2013), which could be ascribed to differences in the inoculum size and batch of starch used.

A lower substrate concentration was used to determine whether a decrease in the substrate loading could result in increased starch conversion by *S. cerevisiae* Y294[AteA-GlaA] strain (Figure 3.7). As expected, less ethanol (41.9 g.l^{-1}) was produced by the strain with the lower substrate loading. Khaw et al. (2006) obtained a yield of 51 g.l^{-1} ethanol with 100 g.l^{-1} starch from a *S. cerevisiae* YF237 strain, which is a higher yield than obtained in this study. The variations could be due to differences in experimental procedure such as a higher glucose concentration used and the yeast expression system used by Khaw et al. (2006). However, after 192 hours of fermentation, the respective starch conversion was 46% and 82% for 200 and 100 g.l^{-1} raw starch (Table 3.3), which corresponds to a theoretical yield of 44% and 79% ethanol, respectively.

A set of experiments was designed to elucidate factors that may have a negative effect on the strain's ability to ferment high concentrations of starch (Figure 3.8). When *S. cerevisiae* Y294[AteA-GlaA] was supplemented with 10% of the recommended STARGENTM 002 enzyme loading, the strain produced 75% of the theoretical ethanol yield on 200 g.l^{-1} corn starch within 72 hours due to the abundance of hydrolytic enzymes at the onset of fermentation. Fermentation controlled at pH 6 was slightly better than *S. cerevisiae* Y294[AteA-GlaA] (with an initial pH of 4.21), which indicates the importance of pH control in enzyme systems. The residual glucose at the end of the fermentation indicates that the amylolytic enzymes remained active, resulting in continued saccharification of the remaining starch. The *S. cerevisiae* Y294[AteA-GlaA] strain displayed minimal residual glucose as the glucose was fermented upon release. This also suggested that the rate of ethanol production could be improved with an increase in amylase activity, which would result in a quicker release of glucose. The accumulation of glucose towards the end of the fermentation indicates that the increased ethanol concentration did not affect the ability of AteA and GlaA to hydrolyse the starch, i.e. poor starch conversion is not due to enzyme inhibition (by ethanol) or substrate recalcitrance.

An increase in the α -amylase concentration had no effect on the rate or final ethanol yield (Figure 3.9), which indicates that the α -amylase was already produced at sufficient levels by *S. cerevisiae* Y294[AteA-GlaA]. As the glucoamylase could not be lyophilised (it would result in loss of activity), *S. cerevisiae* Y294[GlaA] was co-cultured with *S. cerevisiae* Y294[AteA-GlaA] to provide additional glucoamylase (Figure 3.10). The increase in ethanol levels and rate of ethanol production in the presence of additional glucoamylase indicates that

S. cerevisiae Y294[AteA-GlaA] did not produce enough GlaA for optimal starch conversion and as a result, would benefit from increased expression of *GlaA*. It is predicted that increased expression of the *GlaA* in the same host will result in a higher rate of ethanol production than recorded in Figure 3.10. This is because co-culturing *S. cerevisiae* Y294[AteA-GlaA] with the *S. cerevisiae* Y294[GlaA] required a larger inoculum and the additional biomass diverted some of the glucose towards yeast maintenance and cell growth. Therefore, elimination of this co-culture system by improved secretion of the glucoamylase by the recombinant strain could result in more glucose available for conversion to ethanol.

During the course of fermentation, yeast cells are subjected to different stress conditions (including low water activity and accumulation of cytotoxic compounds) (Sousa et al., 2012). In order to adapt to the new environment, the cells trigger stress responses (such as production of glycerol and acetic acid) that enable them to proliferate despite these conditions. The increase in glycerol concentration (Figure 3.6, 3.7, 3.8 and 3.10) and acetic acid (Figure 3.8 and 3.10) correspond to the increase in ethanol production. Glycerol and acetic acid were produced as stress responses to increased levels of ethanol, and consequently resulted in lower ethanol yields as less substrate was converted to ethanol. Glycerol generally acts as an osmotic stabiliser, whereas acetic acid inhibits yeast growth, ultimately inhibiting the fermentation process (leading to sluggish fermentations) and limiting process productivity (Arneborg et al., 1995; Phowchinda et al., 1995; Lee et al., 1999; Palmqvist and Hahn-Hägerdal, 2000; Sousa et al., 2012).

Ethanol concentration can be inhibitory to fermentations. The ethanol concentration reaches equilibrium despite the increase in glucose in the medium (Figure 3.8) suggesting that other factors also influenced ethanol production, such as the host strain's sensitivity to high ethanol concentrations. Although it is not well understood, ethanol inhibition can be either directed towards hampering cell growth, inducing cell death as well as inhibiting fermentations. Ethanol negatively affects the activity of the enzymes in the glycolytic pathway (particularly hexokinases), which consequently reduces fermentation rate (Bai et al., 2008). The fluidity of the plasma membrane is also affected by the ethanol levels resulting in the disruption of nutrient transport into cell (Nagodawithana and Steinkraus, 1976; Baeyans et al., 2015). Different strains display different levels of ethanol tolerance and it is therefore worthwhile to evaluate different host strains for ethanol production.

In conclusion, this study identified a raw starch hydrolysing α -amylase (AteA) that performed better than the benchmark α -amylase (AmyA) and demonstrated its efficacy and stability under fermentative conditions. The recombinant *S. cerevisiae* Y294[AteA-GlaA] strain was developed with the ability to convert raw starch to ethanol at levels that are comparable to those from a similar study by Viktor et al. (2013). This work supports an energy-efficient, single-step conversion of raw starch to ethanol process, which represents significant progress towards the realisation of a CBP yeast for starch-based biofuel production that does not require the addition of heat (for gelatinisation) or exogenous amylases.

3.5 Competing interests

The authors declare that they have no competing interests.

3.6 Acknowledgements

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Chapter 4

General discussion and conclusion

4.1 Introduction

The increasing concern over detrimental environmental effects of greenhouse gases, the depletion of fossil fuel resources and energy security have accelerated the development of alternative fuel sources. The use of biomass to produce fuel could potentially provide a sustainable alternative with minimal production and release of poisonous gases into the atmosphere. Bioethanol is one of the most predominant biofuels, with up to 90% being produced from biomass. It has many applications including its use in the beverage, pharmaceutical and fuel industries (Najafpour, 2006). The use of bioethanol results in economic and environmental benefits such as improved efficiency of combustion and a low carbon footprint (Alzate and Toro, 2006; Marchetti et al., 2007).

Starch is an abundant and relatively low cost substrate that comprises of amylose and amylopectin, which can be hydrolysed by a combination of two enzymes: α -amylase and glucoamylase (Burrell, 2003). Starch conversion to ethanol is a mature technology, but its use in fuel production has been viewed as a threat for food security. However, the Renewable Fuels Association (2016) has stated that there is enough food to both feed and fuel the world, highlighting the importance of proper land use management. Understanding the complex interactions of food security and bioenergy sustainability will aid in ensuring an adequate feedstock supply for both applications (Delapaz, 2016; Kline et al., 2016). Nevertheless, starchy materials for bioethanol production should not be limited to food crops, but also include starch-rich wastes from both agricultural and industrial residue and waste streams (such as potato peels, corn straw and rice husks) (Sarkar et al., 2012; Gupta and Verma, 2015).

Ethanol production from starch should be viewed as part of a biorefinery concept as this increases both biomass conversion and the overall production efficiency. The preferred microbe for most ethanol fermentation industries is *S. cerevisiae*, which is well characterised and the ideal host for heterologous protein expression as it is relatively easy to manipulate, has GRAS status and is able to perform post-translational protein modifications (Byrne et al., 2005). However, it is unable to utilise starch, as it does not produce amylolytic enzymes. Research groups have thus prioritised the use of genetic engineering and recombinant techniques in the construction of yeasts that are able to produce amylolytic enzymes (Sarris and Papanikolaou, 2016).

The construction of a raw starch-hydrolysing *S. cerevisiae* strain expressing both α -amylase and glucoamylase genes could reduce ethanol production cost as it eliminates the need for gelatinisation and decreases the exogenous enzyme requirement, which are major cost contributors (den Haan et al., 2007; Prasad et al., 2007; van Zyl et al., 2007). Several consolidated bioprocessing (CBP) strains for starch have been constructed; however, their efficiency in raw starch hydrolysis remains low. This necessitated the search for novel raw starch hydrolysing enzymes that can convert starch effectively, thereby improving the overall ethanol yield. This study focused on evaluating the hydrolysis efficiency of several α -amylases on starch and their impact on ethanol production from raw starch when co-expressed with a glucoamylase encoding gene in the *S. cerevisiae* Y294 laboratory strain.

4.2 Discussion and conclusions

The main findings of this study can be summarised as follows:

- The *A. terreus* AteA, *A. pullulans* ApuA, *Cryptococcus* sp. S-2 CryA and *S. fibuligera* SfiA genes were successfully expressed on episomal plasmids under the transcriptional control of the constitutive *ENO1* promoter and terminator sequences. Secretion of the proteins was directed by the *T. reesei xyn2* secretion signal sequence
- Clear zones on starch plates surrounding colonies of the *S. cerevisiae* Y294[ApuA], Y294[AteA], Y294[CryA] and Y294[SfiA] strains confirmed the presence of extracellular α -amylase activity.
- The *S. cerevisiae* Y294[CryA] and Y294[SfiA] strains displayed minimal α -amylase activity in liquid assays, which might indicate that the enzymes are cell wall associated or have low levels of specific activity. The *S. cerevisiae* Y294[AteA] and Y294[ApuA] strains displayed similar levels of α -amylase activity to that of the *S. cerevisiae* Y294[AmyA] benchmark strain.
- The AteA and ApuA protein species were larger than their predicted size when separated by SDS-PAGE. The increased molecular size might be attributed to glycosylation, which can contribute to protein stability. The CryA and SfiA proteins were not glycosylated.
- The AteA and ApuA amylases were partially characterised and displayed a pH preference of pH 4 to 5 and a minimum of 75% stability over 5 days at 30°C, suggesting that they should work well under fermentative conditions.

- The *AteA* and *ApuA* genes were respectively co-expressed with the *A. tubingensis* *GlaA* glucoamylase gene in the *S. cerevisiae* Y294 laboratory strain.
- The *S. cerevisiae* Y294[*AteA-GlaA*] strain produced a higher ethanol concentration on raw starch compared to the Y294[*ApuA-GlaA*] strain and compared well with the Y294[*AmyA-GlaA*] benchmark strain.
- The ethanol production by the *S. cerevisiae* Y294[*AteA-GlaA*] strain was limited 45.47 g.l⁻¹ at high substrate loadings with much of the glucose not being utilised.
- The addition of exogenous amylases improved the starch conversion rate, but with minimal effect on ethanol production.
- In an effort to optimise enzyme ratios, it was determined that the *S. cerevisiae* Y294[*AteA-GlaA*] strain produced sufficient α -amylase activity. Additional *GlaA* resulted in an increase in starch conversion, indicating that the *S. cerevisiae* Y294[*AteA-GlaA*] strain would benefit from the addition of extra glucoamylase.

The successful CBP of raw starch was demonstrated by the construction of recombinant amyolytic strains. The *S. cerevisiae* Y294[*AteA-GlaA*] strain compared well with the *S. cerevisiae* Y294[*AmyA-GlaA*] benchmark strain in hydrolysing raw starch and converting the resulting sugars to ethanol. This study highlighted the requirement for an optimal α -amylase:glucoamylase ratio to improve the starch conversion rate. The *S. cerevisiae* Y294[*AteA-GlaA*] and *S. cerevisiae* Y294[*GlaA*] co-culture improved the rate of starch conversion (54%) as compared to the *S. cerevisiae* Y294[*AteA-GlaA*] with a starch conversion of 46%, but the larger inoculum size resulted in some of the glucose being channelled to biomass rather than ethanol. The improved conversion rate would be beneficial to increase the overall ethanol yield. Therefore, it will be worthwhile to investigate strategies to increase glucoamylase activity in the same host to eliminate the use of co-cultures.

Starch conversion at high solids loading was lower than at low substrate loading, indicating a limiting factor at high solids loading. The accumulation of glucose towards the end of the fermentation confirmed that the amylases remained active and are therefore not the limiting factor in this starch-to-ethanol conversion. Different strategies were investigated to optimise the media composition, all of which made little difference to the level of ethanol. It is more likely that the limiting factor is related to the strain background, with *S. cerevisiae* Y294 being a laboratory strain that has not been optimised for ethanol production.

In conclusion, the *AteA* and *ApuA* genes were successfully expressed in the *S. cerevisiae* Y294 laboratory strain. The enzymes were partially characterised and the amylase activity determined over time. The best candidates, the *AteA* and *ApuA* genes, were co-expressed with the *A. tubingensis* *GlaA* gene in *S. cerevisiae* Y294 strain. These recombinant strains were able to hydrolyse raw starch to ethanol, thereby showing potential for use in a CBP process. The *S. cerevisiae* Y294[AteA-GlaA] strain was the best ethanol producing strain in the study, resulting in an ethanol yield of 45.47 g.l⁻¹ that corresponded to a theoretical yield of 44% and productivity of 46%. Direct comparison to other strains is difficult as different methods are used and there are variations in substrate, inoculum size and fermentation conditions used. Significant progress was therefore made in the realisation of an efficient CBP process, which neither requires heating for gelatinisation nor addition of exogenous enzymes for raw starch conversion to ethanol.

4.3 Future work

Enzyme Ratio: The specific activity of enzymes differs; therefore, the ideal enzyme ratio depends on the volumetric activity of the individual enzymes (and not the enzyme concentration). Since the glucoamylase activity is limiting in this particular AteA-GlaA combination, future research should focus on improving the glucoamylase activity in the CBP yeast strain. A number of different approaches can be followed of which the search for novel glucoamylases with high specific activity is essential. However, studies that require metagenomics, proteomics, screening and library development are labour-intensive and time consuming. Robotics are being employed to perform part of the repetitive work, but it is costly to maintain and as such is not a practical option for most research laboratories.

Codon-optimisation is a valuable tool used to increase the expression of foreign genes (Li et al., 2002; Kim and Lee, 2006). The *GlaA* used in this study still contains the native *A. tubingensis* DNA sequence and therefore expression in *S. cerevisiae* might benefit from codon optimisation of the *GlaA*. The XYNSEC (from the *T. reesei xyn 2*) had been used with success for the expression of many genes in *S. cerevisiae*, including the α -amylases used in this study (den Haan et al., 2007; Favaro et al., 2013). However, the secretion of *GlaA* is directed by its native secretion signal and changing the secretion signal might increase the levels of extracellular enzyme (and activity) (Liu et al., 2012). Different promoters can also be evaluated for increased levels of expression. The plasmid used in this study is present at

high copy numbers (± 20 copies) using the *URA3* marker gene. Therefore, the host *S. cerevisiae* Y294[AteA-GlaA] strain can still be transformed with another multicopy vector (using the *LEU2* and *TRP1* markers) carrying the *ENO1_P-GlaA-ENO1_T* cassette to increase the *GlaA* copy number. An increase in copy number could result in an increase in glucoamylase activity.

Strain limitations: The search for native CBP microorganisms is of principal significance as they could serve as new gene sources for incorporation into current engineered industrial CBP microbes (Ali et al., 2016). It will be worthwhile to investigate the use of different yeast strains, especially strains with a history in the ethanol production industry (wine, brewing and whiskey industries). The strains will be more suitable for high ethanol production and may have tolerance to inhibitors associated with the fermentation process. These strains are generally used at lower fermentation temperature; hence it might be worth investigating the starch conversion rate at lower temperature. Although there might be some merit in investigating the possible use of wild isolates as host for amylase expression, it is highly unlikely that they will be tolerant to high ethanol levels if they are not exposed to these conditions in nature.

Once the ideal host strain has been identified, the amylase encoding genes will have to be transferred to the strain, which will represent other challenges. Industrial strains are not as easily manipulated as laboratory strains and do not contain auxotrophic markers. Therefore, the gene cassettes will have to be transferred to another vector that contains an industrial marker (dominant marker). Episomal plasmids require constant selective pressure for maintenance and therefore integration vectors are more desirable. Integration vectors, however, are only maintained in low copy numbers, which leads to low levels of expression. This highlights the importance of finding amylase enzymes with high specific activities that therefore do not require high copy numbers.

Other approaches to improving ethanol production would include the elimination of certain competing pathways to channel more carbon towards ethanol production. This includes the knockout of the glycerol and acetic acid pathways (secondary metabolites that reduce ethanol yield). Ultimately, as the strains will be exposed to harsh industrial fermentative conditions, the final strains should be adapted for tolerance to high ethanol concentration and other fermentation by-products and inhibitors.

4.4 References

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