FUNGAL ENZYMES AND MICROBIAL SYSTEMS FOR INDUSTRIAL PROCESSING

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Dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Stellenbosch

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MARCH 2008

DECLARATION

I, the undersigned, hereby declare that the work presented in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: _____ Date: _____

ABSTRACT

This study strives to improve two current industrial processes by making them more cost effective through the use of hydrolytic enzymes or microbial systems. The first process targeted is the industrial conversion of starch to ethanol. In the second process, hydrolytic enzymes are applied to the manufacturing of instant coffee.

The engineering of microbial systems to convert starch to bio-ethanol in a one-step process may result in large cost reductions in current industrial processes. These reductions will be due to decreased heating energy requirements, as well as a decrease in money spent on the purchase of commercial enzymes for liquefaction and saccharification. In this study, a recombinant *Saccharomyces cerevisiae* strain was engineered to express the wild-type *Aspergillus awamori* glucoamylase (GA I) and α-amylase (AMYL III) as well as the *Aspergillus oryzae* glucoamylase (GLAA) as separately secreted polypeptides. The recombinant strain that secreted functional GA I and AMYL III was able to utilise raw corn starch as carbon source, and converted raw corn starch into bio-ethanol at a specific production rate of 0.037 grams per gram dry weight cells per hour. The ethanol yield of 0.40 gram ethanol per gram available sugar from starch translated to 71% of the theoretical maximum from starch as substrate. A promising raw starch converter was therefore generated.

In the second part of this study, soluble solid yields were increased by hydrolysing spent coffee ground, which is the waste generated by the existing coffee process, with hydrolytic enzymes. Recombinant enzymes secreted from engineered *Aspergillus* strains (β-mannanase, β-endo-glucanase 1, β-endo-glucanase 2, and β-xylanase 2), enzymes secreted from wild-type organisms (β-mannanases) and commercial enzyme cocktails displaying the necessary activities (β-mannanase, cellulase, and pectinase) were applied to coffee spent ground to hydrolyse the residual 42% mannan and 51% cellulose in the substrate. Hydrolysis experiments indicated that an enzyme cocktail containing mainly β-mannanase increased soluble solids extracted substantially, and a soluble solid yield of 23% was determined using the optimised enzyme extraction process. Soluble solid yield increases during the manufacturing of instant coffee will result in; (i) an increase in overall yield of instant coffee product, (ii) a decrease in amount of coffee beans important for the production of the product, and (iii) a reduction in the amount of waste product generated by the process.

OPSOMMING

Hierdie studie poog om twee huidige industriële prosesse te verbeter deur die prosesse meer koste-effektief met behulp van hidroltiese ensieme en mikrobiese sisteme te maak. Die eerste industrie wat geteiken word, is die omskakeling van rou stysel na etanol, en die tweede om hidrolities ensieme in die vervaardiging van kitskoffie te gebruik.

Die skep van mikrobiese sisteme om rou-stysel in 'n 'een-stap' proses om te skakel na bio-etanol sal groot koste besparing tot gevolg hê. Hierdie besparings sal te wyte wees aan die afname in verhittingsenergie wat tydens die omskakelingsproses benodig word, asook 'n afname in die koste verbonde aan die aankoop van duur kommersiële ensieme om die stysel na fermenteerbare suikers af te breek. In hierdie studie is 'n rekombinante *Saccharomyces cerevisiae*-gis gegenereer wat die glukoamilase (GA I) and α-amilase (AMYL III) van *Aspergillus awamori*, asook die glukoamilase van *Aspergillus oryzae* (GLAA) as aparte polipeptide uit te druk. Die rekombinante gis wat die funksionele GA I en AMYL III uitgeskei het, was in staat om op die rou-stysel as koolstofbron te groei, en het roustysel na bio-etanol teen 'n spesifieke tempo van 0.037 gram per gram droë gewig biomassa per uur omgeskakel. Die etanolopbrengs van 0.40 gram per gram beskikbare suiker vanaf stysel was gelykstaande aan 71% van die teoretiese maksimum vanaf stysel as substraat. 'n Belowende gis wat roustysel kan omskakel na bio-etnaol was dus geskep.

In die tweede deel van hierdie studie is die opbrengs in oplosbare vastestowwe vermeerder deur die koffie-afval wat tydens die huidige industrieële proses genereer word, met hidrolitiese ensieme te behandel. Rekombinante ensieme afkomstig vanaf *Aspergillus*-rasse (β-mannanase, β-endoglukanase 1, β-endo-glukanase 2 en β-xilanase 2), ensieme deur wilde-tipe organismes uitgeskei (β-mannanase), asook kommersiële ensiempreparate wat die nodige ensiemaktiwiteite getoon het (β-mannanase, sellulase en pektinase) is gebruik om die oorblywende 42% mannaan en 51% sellulose in koffie-afval te hidroliseer. Hidrolise eksperimente het getoon dat 'n ensiempreparaat wat hoofsaaklik mannanase bevat, die oplosbare vastestofopbrengs grootliks kan verbeter, met 'n verhoogde opbrengs van 23% tydens geöptimiseerde ensiembehandelings. 'n Verhoogde opbrengs in oplosbare vastestowwe tydens die vervaardiging van kitskoffie sal die volgende tot gevolg hê: (i) 'n toename in totale opbrengs van kitskoffie produk, (ii) 'n afname in die hoeveelheid koffiebone wat vir die produksie ingevoer moet word, en (iii) 'n afname in die hoeveelheid afval wat tydens die vervaardigingsproses produseer word.

ACKNOWLEDGEMENTS

My sincere thanks are extended to:

The Lord Almighty for determination, willpower and strength

Prof. Emile van Zyl, my promoter, and Dr. Johann Görgens, my co-promoter for guidance and encouragement during the course of the study and process of compiling this dissertation

The National Research Foundation and the Department of Microbiology for financial support to complete the study

Dr. Riaan den Haan, Dr. Danie la Grange and Dr. Shaunita Rose for valuable scientific input in designing experiments

Niël van Wyk, Lisa du Plessis, Nicolette Fouche, Dr. Annie Chimphango, Dr. Mariska Lilly and all the members of the Stellenbosch Academy of Dance for emotional support

My parents, sister and friends for encouraging me to complete this important period in my life

My fiancé, Herman Jooste, who supported me through the entire study and encouraged me to complete this dissertation

PUBLICATIONS RESULTING FROM THIS STUDY

Görgens JF, van Zyl WH, Rose S, Setati ME, de Villiers T (2006) Method for producing hemicellulase-containing enzyme compositions and the use thereof. South African Patent 2006/03771.

PUBLICATIONS IN PREPARATION

de Villiers T, Görgens JF, van Zyl WH (2007) Engineered amylolytic yeast for bioethanol production. Prepared for Applied Microbiology and Biotechnology.

de Villiers T, Görgens JF, van Zyl WH (2007) Microbial enzymes for the instant coffee industry. Prepared for Applied Microbiology and Biotechnology.

PREFACE

In this study, two current industrial processes were improved with the use of hydrolytic enzymes, which were sourced from commercial entities or secreted by engineered microbes. This dissertation is therefore presented in two sections (Section I and II) and an Appendix (Appendix A). Section one entails microbes engineered to improve the starch to ethanol industry. The second section involves microbial enzymes used to improve the extraction yield of soluble solids for the instant coffee industry. Section I and II both comprise a literature review and a manuscript (Chapters 2-5). The manuscripts are introduced separately and written according to the style of the journal for which the manuscripts were prepared (Chapters 3 and 5). The registered patent covers the work detailed in Section II and is provided as Appendix A. Chapter 6 contains a general discussion and remarks applying to both Sections I and II.

APPENDIX A: Görgens JF, van Zyl WH, Rose S, Setati ME, de Villiers T (2006) Method for producing hemicellulase-containing enzyme compositions and the use thereof. South African Patent 2006/03771.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

β-CD	β-cyclodextrin
BP	British Petroleum
CBM	Carbohydrate-binding module
CBP	Consolidated Bioprocessing
CGTase	Cyclomaltodextrin glucanotransferase
CSIR	Council for Scientific and Industrial Research
C-termini	Carboxy termini
CO_2	Carbon dioxide
Cp-I	C-terminal peptide
DDGS	Distillers dried grains and solubles
DP	Degree of polymerisation
DW	Dry weight
E10	10% ethanol blend with petroleum
E85	85% ethanol blend with petroleum
EPIC	Ethanol Promotion and Information Council
FFVs	Flex-fuel vehicles
GH	Glycoside hydrolase
Gp-I	Glycopeptide 1
GRAS	Generally regarded as safe
HFCs	Hydrofluorocarbons
HPLC	High performance liquid chromatography
IDC	Industrial Development Corporation
IPCC	Intergovernmental Panel on Climate Change
MP	Mature protein
MTBE	Methyl tertiary butyl ether
MW	Molecular weight
NBL	National Brands Limited
NCP	National Chemical Products
N-termini	Amino termini
OAPEC	Organisation of Arab Petroleum Exporting Countries
OD	Optical density
PFCs	Perfluorocarbons
PG	Polygalacturonase

PL	Pectin lyase
rpm	Revolutions per minute
RSH	Raw starch hydrolysing
RSYP	Raw starch medium
SBD	Starch binding domain
SC	Selective complete
Ser	Serine
SSF	Simultaneous saccharification and fermentation
Thr	Threonine
TIM	Triose phosphate isomerase
UN	United Nations
UNCED	United Nations Commission on Environment and Development
USA	United States of America
3D	Three dimensional

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 1: INTRODUCTION TO FUNGAL ENZYMES AND MICROBIAL SYSTEMS FOR INDUSTRIAL PROCESSING

Enzymes (fungal, bacterial and recombinant) are used in numerous new applications in the food, feed, agriculture, paper, leather, textiles, and fuel ethanol industries, resulting in significant cost reductions, yield improvements, and improvement in product characteristics. Rapid technological developments are further stimulating the chemistry and pharmaceutical industries to embrace enzyme technology, a trend strengthened by concerns regarding health, energy, raw materials, and the environment (van Beilen and Li, 2002). As nature's solution to controlling chemical reactions in all living organisms, enzymes provide a 'green' solution to an industrialised world amid growing environmental concerns. Continued growth of the industrial enzyme market is dependent on identification and characterisation of new enzymes from natural sources, the modification of these enzymes for optimal performance in selected applications, and high-level expression of the enzymes (Cherry and Fidantsef, 2003).

The oil industry may benefit from enzyme technology if the world's dependence on this fossil fuel is decreased, therefore increasing the usage of 'greener' technologies such as the production of biofuels. Furthermore the oil price is constantly increasing, and the drive to find cleaner alternatives is fuelled by the need for energy security (Lynd et al., 2002). Cellulosic biomass is receiving much attention as a result of its abundance and relatively low cost (Lynd et al., 1999). The current process of converting starch to bioethanol is well established, but energy cost is high, and the technology may therefore benefit from the design of microbial systems for the one-step conversion of biomass to ethanol (Gray et al., 2006; Greene, 2004).

Coffee is one of the most important products in world trade, second only to oil as source of foreign exchange (Sivetz and Desrosier, 1979; Smith, 1985). Instant coffee production is dependent on new innovative ways to increase productivity of the process to allow for an increase in profitability and to sustain the growing demand for the product. The instant coffee product is produced by extraction from roasted coffee beans, and residual insoluble material in the beans is discarded as waste product (Adams and Dougan, 1987). This represents a loss of raw material, final product and possible profits to the manufacturer. The cost of waste removal further adds cost to the manufacturing process. The application of enzyme technology represents an effective natural measure for improving productivity without significantly complicating the extraction process or compromising the quality of the product.

This study was undertaken to benefit two industries: the conversion of biomass (raw starch) to fuel ethanol/bioethanol, as well the food industry, in particular improving extraction yield of soluble solids in the instant coffee industry.

1.1 AMYLOLYTIC YEAST FOR STARCH CONVERSION

1.1.1 Introduction: Plant biomass as a renewable energy

The search for a renewable energy to sustain energy consumption worldwide is on. Growing environmental concerns, the need for energy security, utilisation of agricultural surpluses and biomass resources, as well as job creation are only a few reasons feeding this initiative in our industrialised world (Lynd et al., 2002). Plant biomass is a carbon-neutral renewable resource (Ragauskas et al., 2006) and biomass conversion, particularly cellulosic feedstock conversion, is receiving much attention as a result of its abundance and a relatively low cost (Lynd et al., 1999). Converting cellulose to glucose for bioethanol production using a commercially feasible process featuring enzymatic hydrolysis was a vision developed as early as 1971 (Reese and Mandels, 1971). The commercial practice of converting starch to ethanol by an enzymatic process is a fairly mature technology (Gray et al., 2006). The energy cost of converting corn to ethanol is high, and as the commercial conversion process is wide spread, the need to develop a more feasible process is evident. A single step process where production of hydrolytic enzymes to hydrolyse starch and fermentation of the resulting sugars is accomplished via an amylolytic microorganism or consortium of organisms could yield large cost reductions for starch conversion. This process has been designated Consolidated Bioprocessing (CBP) (Greene, 2004; Lynd et al., 2002).

1.1.2 Reasons for developing a CBP process for starch conversion

The industrial process of converting starch to bioethanol involves four steps (Venkatasubramanian and Keim, 1985). These include (i) extraction of starch from the biomass, (ii) the conversion of the starch to yield fermentable sugars, which are then (iii) fermented to ethanol upon the addition of yeast. In the final step ethanol is refined and concentrated by distillation. Extraction of starch is accomplished via wet milling or dry milling, the latter being the procedure most widely used in the United States (USA) (Kwiatkowski et al., 2006; RFA, 2007; Srinivasan et al., 2005). Starch may be converted to fermentable sugars via acid hydrolysis or enzymatic hydrolysis (Robertson et al., 2006).

Enzyme hydrolysis replaced acid hydrolysis in recent years, as the acid hydrolysis procedure presented its own drawbacks such as equipment corrosion and yield losses of fermentable sugars.

Enzymatic hydrolysis is initiated when starch is pre-treated to yield a viscous slurry, which is then liquefied by heat treatment and α-amylase (Fig. 1.1). The starch is cooked and undergoes saccharification after addition of glucoamylase. Yeast is added after cooling the mixture for fermentation of sugars to ethanol. The process includes large temperature changes (32-120°C) using vast amounts of heating energy (Kelsall and Lyons, 2003). Addition of caustic soda, lime, and sulphuric acid to maintain pH levels suitable for the enzymes, as well as urea serving as nitrogen source for the yeast, adds to the end product cost (McAloon et al., 2000).

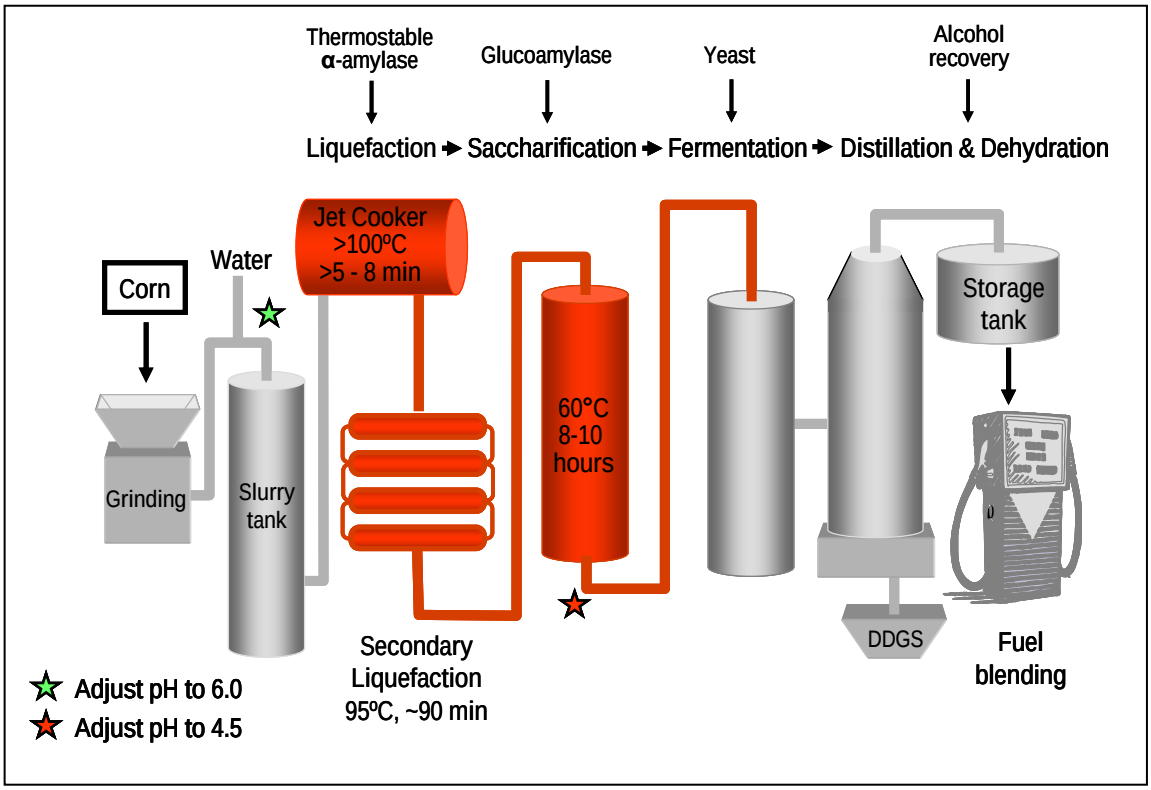

Fig. 1.1 Conventional ethanol production process using corn as feedstock. Adapted from http://www.genencor.com/cms/resources/file/ebf95c076d3afc7/STARGEN%20Backgrounder.pdf.

The energy balance of corn to ethanol has raised some concern in the industry. Reports tackling this balance, however, indicated that the balance is positive, even before subtracting energy which is allocated to coproducts (Srinivasan et al., 2006). This was indicated by an

energy output/input ratio of 1.3 (Farrell et al., 2006). A comparison of six studies reporting on the net energy balance indicated a positive net energy of 4-9 MJ l^{-1} ethanol. Yet another study comparing six 'starch to ethanol' scenarios and four 'cellulose to ethanol' scenarios, reads: "It is safe to say that corn ethanol reduces fossil fuel and oil consumption when used to displace gasoline" (Hammerschlag, 2006).

In order to design a more energy-efficient ethanol production process, the enzymes used for biomass hydrolysis should be more efficient and less expensive (Gray et al., 2006; Nigam and Singh, 1995). With the intention to increase net energy yield, the hydrolysis temperature required to generate glucose could be lowered to that of the fermentation step, therefore carrying out saccharification and fermentation simultaneously (SSF) (Devantier et al., 2005; Lynd et al., 1999). Lowering the temperature when liquefying the starch to match that of saccharification and fermentation also adds the benefit of decreasing the viscosity of the generated slurry (Kelsall and Lyons, 2003). Thermally treated slurries complicate pumping and stirring of the material. An additional benefit would be that lower temperatures minimise the formation of unwanted Maillard reaction coproducts such as fusel oils and glycerol, which could reduce glucose yield for fermentation (Galvez, 2005).

A raw starch hydrolyzing (RSH) enzyme cocktail, Stargen™ 001 (Genencor) was developed, which converts starch into dextrins at low temperatures (<48°C) and hydrolyses dextrins into sugars during SSF. The cocktail contains an acid-stable α-amylase from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger*. Comparable ethanol conversion efficiencies, ethanol yields, and distillers dried grains and solubles (DDGS) yields were reached using the RSH enzyme (Wang et al., 2007). Using the RSH enzyme saves heating energy as jet cooking is eliminated and less water and fewer chemicals are needed for the process. One drawback in converting raw starch to ethanol at a lower temperature is the risk of contamination of the fermentation broth. Contamination is usually kept at bay in a conventional starch to ethanol plant in the jet cooking stage (Shigechi et al., 2004).

To eliminate commercial enzyme purchase costs, SSF has been performed effectively with mixed cultures, where one organism is amylolytic and the other responsible for ethanol production (Dostalek and Haggstrom, 1983; Han and Steinberg, 1987; Kurosawa et al., 1989; Lee et al., 1983; Tanaka et al., 1986). The amylolytic organism acts as the saccharifying agent, therefore replacing the addition of commercial saccharifying enzymes

(Ashikari et al., 1989). Up to 9.7 g l^{-1} ethanol was produced during SSF with *Saccharomycopsis fibuligera* and *Zymomonas mobilis* after 25 hours of cultivation with an initial soluble starch concentration of 30 g l^{-1} (Dostalek and Haggstrom, 1983). The volumetric productivity of ethanol was 0.54 g l^{-1} h^{-1} and the ethanol yield was calculated as 0.48 gram ethanol per gram available sugar from starch (g g^{-1}), which correlates to 86% of the theoretical maximum from starch. A mixed culture of *Aspergillus awamori* and *Zymomonas mobilis* produced up to 21 g l^{-1} and 25 g l^{-1} ethanol at 100 rpm and 220 rpm, respectively, with an initial soluble starch concentration of 100 g l^{-1} (Tanaka et al., 1986). The ethanol yield of 0.33 g g^{-1} was lower at 100 rpm compared to the yield when cultivated at 220 rpm (0.38 g g^{-1}) (calculated as 59% and 68% of theoretical maximum, respectively). The one drawback in these systems is that the amylolytic organism utilises most of the soluble starch for growth, which leaves little sugars for the fermentative organism to convert to ethanol (Nakamura et al., 1997).

Generating an amylolytic fermentative organism may address this shortcoming. A more cost-effective procedure where an organism produces sufficient amounts of amylolytic enzymes to sustain growth on raw unmodified starch as sole carbon source for the production of ethanol as product is depicted in Figure 1.2. Applying a raw starch utilising yeast in the starch conversion process will have all the benefits from an SSF procedure, such as a lowered heating energy requirement and chemical usage. The added benefit will be elimination of the large cost associated with commercial enzyme purchase.

The engineered organism producing amylolytic enzymes and ethanol would be suitable for a Consolidated Bioprocessing (CBP) process (Lynd et al., 1999). In the long term, generation of ethanol and coproducts employing a CBP process will ensure the production of commodity chemicals and animal feeds in a sustainable manner in a biorefinery environment.

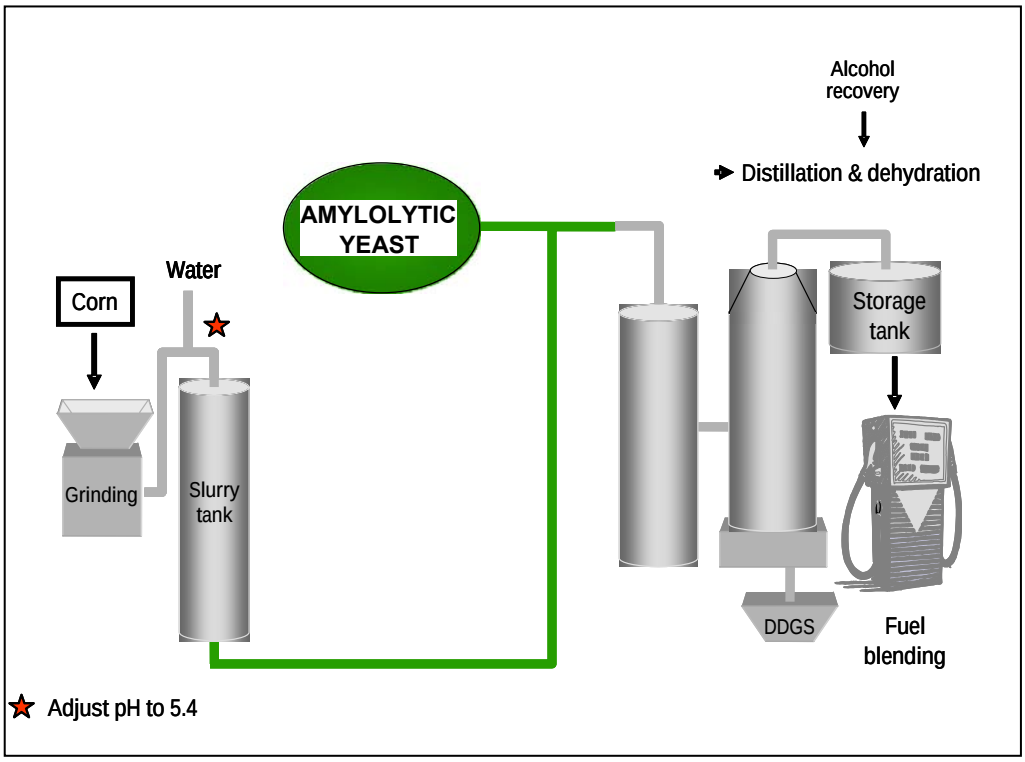

Fig. 1.2 Modification of the conventional ethanol production from corn. Amylolytic yeast is introduced to liquefy, saccharify and ferment raw starch to ethanol in a one-step process. Adapted from http://www.genencor.com/cms/resources/file/ebf95c076d3afc7/STARGEN%20Backgrounder.pdf.

1.1.3 Recombinant expression systems for starch conversion

Genetic engineering is used extensively for producing hosts with desired characteristics for the starch industry (Pandey et al., 2000). Mainly α-amylases and glucoamylases are expressed in heterologous hosts to ensure higher enzyme productivity compared to the native host. Expression of thermostable enzymes as well as the ability to produce more than one desirable enzyme in one host enables the generation of more competitive organisms for the industry.

Yeasts displaying glucoamylases (Kondo et al., 2002; Murai et al., 1997, 1998 and 1999; Ueda and Tanaka, 2000) and α-amylases have been created (Shigechi et al., 2002). Glucoamylase and α-amylase genes have also been integrated into the *Saccharomyces cerevisiae* genome (Eksteen et al., 2003; Knox et al., 2004). Recombinant

S. cerevisiae strains were also generated to secrete separate polypeptides (either glucoamylase or α-amylase) or bi-functional proteins (glucoamylase and α-amylase) (Birol et al., 1998).

Engineering a host strain to express raw starch hydrolysing enzymes will be even more advantageous. Raw starch hydrolysing enzymes that function at elevated temperatures have been identified in *A. awamori*, *Aspergillus foetidus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus*, *Mucor rouxians*, *Mucor javanicus*, *Neurospora crassa*, *Rhizopus delemar*, and *Rhizopus oryzae* (Pandey et al., 2000). Of special interest are the glucoamylases from *A. awamori* and *A. oryzae* (*koji* mold), as well as the α-amylase from *A. awamori*, which hydrolyse raw starch (Hata et al., 1991; Matsubara et al., 2004a and 2004b; Queiroz et al., 1997; Singh and Soni, 2001). The enzymes are important in the industrial production of saké (Japanese rice wine) and miso (Japanese seasoning) (Ueda, 1981; Yokotsuka and Sasaki, 1998; Fleet, 1998). The α-amylases and glucoamylases from these strains display a synergistic effect during raw starch degradation (Abe et al., 1988; Ueda, 1981). These strains however are not ethanol producing strains.

Although wild type strains of *S. cerevisiae* do not have the ability to hydrolyse raw starch (Tubb, 1986), *S. cerevisiae* var. *diastaticus* produces glucoamylase enzymes, which are capable of hydrolysing soluble starch (Adam et al., 2004; Bignell and Evans, 1990). The yeast *S. cerevisiae* is known for its high fermentation capacity, high ethanol productivity (41 g l^{-1} h^{-1}) (Ben Chaabane et al., 2006) and high ethanol tolerance. The yeast has also been utilised extensively to produce and secrete heterologous enzymes (Bitter et al., 1987; Hitzeman et al., 1983a and 1983b; Smith et al., 1985). It would therefore be advantageous to exploit *S. cerevisiae* to secrete the amylolytic enzymes of *A. awamori* and *A. oryzae* for the purpose of generating a raw starch bio-converter for bioethanol production. It is in the scope of this study to understand the design and application of an amylolytic yeast strain for raw starch hydrolysis in a CBP process.

1.1.4 Project Aim

The aim of this study was to engineer an amylolytic *S. cerevisiae* strain capable of utilising raw unmodified starch as sole carbon source for the production of bioethanol.

1.1.5 Objectives identified for this study

Certain objectives were identified that would realise the project aim. These included:

- Identifying fungal amylolytic genes coding for raw starch hydrolysing enzymes appropriate for cloning into *S. cerevisiae*.
- Engineering *S. cerevisiae* strains to express and secrete the identified amylolytic enzymes.
- Demonstrate that functional amylolytic enzymes were secreted by the engineered yeast strains.
- Demonstrate that growth of the engineered yeast strains on the raw starch substrate could be enabled.
- Quantify growth rates of a selected strain on raw starch versus soluble starch versus glucose.
- Determine if ethanol was produced by the selected strain during anaerobic fermentation.
- Benchmark the recombinant yeast to existing raw starch fermenting microbial systems.

1.2 MICROBIAL ENZYMES FOR THE INSTANT COFFEE INDUSTRY

1.2.1 Introduction: Instant coffee and enzyme technology

The large market for instant coffee in South Africa is at present unsaturated and is of large economic importance as a result of new possibilities for export to other South African and Indian Ocean islands. Manufacturers in South Africa are investigating enzyme technology to improve the productivity of their processes to meet the growing demand for instant coffee and increase its profitability. The application of enzyme technology represents an effective natural measure for improving productivity without significantly complicating the extraction process or compromising the quality of the product.

Instant coffee is produced by thermal water extraction of soluble solids from roasted Robusta and Arabica green coffee beans. Approximately 50% of the total coffee bean dry weight can be extracted in this manner and used in the final product. The remainder of the product is called spent ground and is discarded as a waste product. The presence of the insoluble material in the coffee beans therefore represent a loss of raw material, final product and possible profits to the manufacturer, especially since the green coffee beans are imported from abroad. Hydrolytic enzymes may be able to hydrolyse the insoluble matter in coffee spent ground, thereby increasing soluble solid yield extracted from the bean. This will increase the overall yield of instant coffee product, and decrease the amount of coffee beans imported for production. It will also reduce the amount of spent ground waste produced by the process as the economic disposal of large quantities of waste is an important factor in reducing plant operating costs.

Due to the complex structure of roasted coffee beans, it is foreseen that maximal extraction yields will be obtained by using a mixture of hydrolytic enzymes to hydrolyse insoluble components in the Arabica and Robusta coffee beans. Enzyme cost will however play an important role in the decision whether more than one enzyme will be applied. Food industries in South Africa are dependent on imported enzymes and there are currently no known enzymes for specific application to instant coffee production.

1.2.2 A recombinant expression system for enzyme production

Aspergillus niger has several advantages to serve as host for heterologous protein expression. It has a high secretion capacity, a relatively well-studied genetic background, and grows rapidly on inexpensive media (van den Hondel et al., 1992; Verdoes et al., 1995). Furthermore, enzymes produced by *A. niger* have GRAS status (Schuster et al., 2002). *Aspergillus* strains expressing heterologous proteins have been used in various industries for the production of enzymes, which include proteases, catalases, isomerases, α-galactosidases, rennin, lipase, phytase, glucoamylase, pectinase, glucose oxidase, and α-amylase (Ward et al., 1992; Archer, 2000; Gibbs et al., 2000). Xylanase and endoglucanase genes have also been expressed constitutively in an *A. niger* strain (Rose and van Zyl, 2002). Creating *A. niger* strains to express fungal enzymes will greatly benefit this project to ensure a high enzyme secretion yield, as large quantities of the enzymes will be needed for characterisation and extraction experiments.

1.2.3 Project Aim

The aim of this study part of the study was to increase soluble solid yields extracted from coffee spent ground after enzyme treatment for use in the industrial process of manufacturing instant coffee.

1.2.4 Objectives identified for this study

Certain objectives were identified which would realise the project aim. These included:

- Isolating and screening enzyme cocktails from recombinant and wild type fungal strains for enzyme activities that were able to increase soluble solids extracted from coffee spent ground.
- Sourcing commercial enzyme cocktails that could increase soluble solids extracted.
- Characterising the recombinant enzymes and selected enzymes present in the cocktails.
- Analyse polysaccharide content of roasted coffee beans and spent ground.
- Perform and optimise extraction experiments to determine increase in soluble solid yield after enzyme treatment of spent ground.

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SECTION I: AMYLOLYTIC YEAST FOR STARCH CONVERSION

CHAPTER 2: LITERATURE REVIEW: TOWARDS AN UNDERSTANDING OF AMYLOLYTIC YEAST FOR STARCH CONVERSION TO BIOETHANOL

2.1 BIOMASS FOR BIOCONVERSION

2.1.1 Introduction

Biofuels have become the new hot topic in world news. Global warming and the need for energy security drive this movement towards a 'greener' future. Oil prices are on the increase, and countries such as South Africa are joining in on the race to find cleaner alternatives to fossil fuels, as 60% of the country's petroleum is manufactured from imported crude oil (Nassiep KM, personal communication, 2006). Biomass is converted to ethanol in the industry for use in fuels, where the ethanol is blended with petroleum (Hahn-Hägerdal et al., 2006).

The starch bioconversion process is well established, albeit improvements are necessary to render the process more energy-efficient. An energy output/input ratio of 1.3 has been calculated (Farrell et al., 2006). Research groups currently focus on either improving the commercial hydrolysing enzymes applied in the process, or improving microbes producing the hydrolytic enzymes necessary for the process to proceed efficiently.

This chapter will discuss how biomass may serve as a renewable energy for bioethanol production. Current views on bioethanol production expressed by role-players in South Africa as well as the rest of the world are presented. The bioconversion of starch to ethanol in particular is described, with attention being paid to the role enzymatic hydrolysis plays in bioconversion procedures. The final part of this review deals with the development of amylolytic yeasts for the purpose of enzymatic hydrolysis of raw or native starch to realise the vision of CBP.

2.1.2 Biomass conversion for bioethanol production

The history of corn to ethanol goes back to the oil embargo initiated by members of the Organisation of Arab Petroleum Exporting Countries (OAPEC) in the 1970's. The need for a renewable burning fuel such as ethanol was recognised. Ethanol is considered to be a cleaner fuel alternative to fossil fuels (Lin and Tanaka, 2006). It is also the only practical fuel oxygenate substitute for methyl tertiary butyl ether (MTBE), a carcinogen in gasoline (Venkatasubramanian and Keim, 1985). Ethanol is blended with petroleum and most vehicles produced since 1982 can operate on petroleum/ethanol blends of up to 10% ethanol (E10) (The Ethanol Promotion and Information Council (EPIC), 2007). Flex-fuel vehicles (FFVs) or "Ethanol vehicles" are capable of running on a blend containing up to 85% ethanol and 15% petroleum (E85), or any mixture of the two.

Biomass is an excellent source of energy and a 20% greenhouse gas benefit has been calculated for hydrolysis and fermentation of corn to ethanol when compared to petroleum (Lynd L, personal communication, 2006). Bioenergy from biomass has the potential to benefit sustainable development in industrialised and developing countries (Hoogwijk et al., 2003). It has numerous environmental and social benefits, which include employment opportunities, the use of surplus agricultural land in industrialised countries, reduction of carbon dioxide (CO_2) levels, down-scaling of waste generation, and nutrient recycling (Hall, 1997). As biomass resources are locally available and geographically more evenly distributed compared to fossil fuels, large capital investments are not necessary to import material for energy conversion and therefore provides security of supply (Hahn-Hägerdal et al., 2006).

2.1.3 Biomass conversion: International view

Biomass provided 14% of the world's energy in 1991 (Hall, 1991). Although it was the most important source of energy in developing countries (35%), it contributed only 4% to industrial countries such as the USA and 14 % to Sweden. These statistics gave rise to the assumption that biomass was a fuel of the past and perceived as a low status fuel associated with poverty (Hall and Scrase, 1998). This perception was contradicted by influential bodies such as the Intergovernmental Panel on Climate Change (IPCC), Greenpeace, Shell International and the United Nations Commission on Environment and Development (UNCED). They predicted an increase rather than a decline in global use of biomass for

energy in the future (Hall and Scrase, 1998). The Kyoto agreement, which was signed in 1997, is an indication that industrialised countries are politically accepting a transition to a 'greener' future. This is a result of the threat of global climatic change, which is largely due to burning fossil fuels. Countries that ratify the Kyoto protocol have committed to reduce their emissions of greenhouse gasses (CO_2, methane, nitrous oxide, sulphur hexafluoride, hydrofluorocarbons (HFCs), and perfluorocarbons (PFCs)) by 5,2% compared to the year 1990 before 2012 (UNFCCC, 1997).

2.1.4 Biomass conversion: South African view

In South Africa, cellulosic biomass conversion to chemicals and fuels was a high priority from the late 1970's to the early 1990's (Lynd et al., 2003). This was fuelled by the threat of economic sanctions and high oil prices. The Council for Scientific and Industrial Research (CSIR) funded research regarding the conversion of bagasse to ethanol by employing enzyme hydrolysis (Paterson-Jones, 1989). This program later included the production of single-cell protein. The contribution to biomass conversion by the University of Stellenbosch in the 1980's was aimed at developing yeasts that expressed saccharolytic enzymes, which was supported by National Chemical Products (NCP). Several other organisations contributed to the cause of bioethanol production, which included production of ethanol from non-cellulosic feed stocks such as sorghum, and in producing cellulase enzymes on pilot plant scale (Watson and Nelligan, 1983). All these efforts came to an abrupt end by the early 1990's (Lynd et al., 2003). One of the most prevalent reasons was that biomass conversion was of less immediate concern when compared to improving services and opportunities for the majority of the population previously disadvantaged.

The demand for biofuels in South Africa was recently recognised. Sixty percent of South Africa's petroleum is manufactured from imported crude oil and the residual from coal (Nassiep KM, personal communication, 2006). The long-term outlook for crude oil prices is bleak. Three factors have been identified that play a role in a more sustainable mobility solution. These include climatic change, air quality and the security of supply and energy. Biofuels is the only option that is available to address climatic change and security of supply and energy (von Blottnitz et al., 2005). British Petroleum (BP) International therefore supports the responsible introduction of conventional biofuels, e.g. sugar and starch crops hydrolysed and fermented to ethanol for gasoline (Bennet P, personal communication, 2006). Sasol Ltd. is considering biofuel as an additive in petrol, as cleaner fuel specifications

were set by the government in January 2006 in an attempt to ensure protection of the environment (Tait B, personal communication, 2005). It can further act as an octane-enhancing fuel additive and therefore be used as substitute for lead. The large surplus and low market prices of maize/corn has prompted the origin of the Ethanol Africa group. The organisation aims setting up plants for converting surplus corn into ethanol for blending into fuel. The group calculated that 3 million tons of corn converted into ethanol will produce 1.26 billion litres of petrol, which translates to 12% of local consumption (South African Broadcasting Corporation, 2005). A national strategic plan has been developed to produce 1.1 billion litres of ethanol per year in the next decade (Nassiep KM, personal communication, 2006). The Industrial Development Corporation (IDC) is backing this project and will fund between seven and ten ethanol plants. The plants will produce ethanol mainly from sugar cane (50%) and the rest from sugar beet, corn and sorghum (Strumpf, 2006).

2.1.5 Current and future state of ethanol production from biomass

Brazil has been the largest ethanol producer for many years (RFA, 2007a). The USA became the worlds' largest producer at the end of 2006. Ethanol productivity for 2006 is summarised in Table 2.1. Biofuel implementation is being driven forwards by policies in several countries. Targets set for different countries are summarised in Table 2.2.

Table 2.1 Summary of ethanol production from the two leading countries, as well as South Africa (RFA, 2007a).

Country	**Production of ethanol for 2006**
United States	18.4 billion litres
Brazil	17 billion litres
South Africa	386 million litres

Table 2.2 Summary of biofuel targets set by selected countries.

Country	Target	Year	Reference
United States	5% usage	2012	(RFA, 2007c)
United States	10% usage	2017	(Novozymes, 2007)
Europe	5.75% usage	2010	(Novozymes, 2007)
China	15% usage	2020	(Novozymes, 2007)
South Africa	4.5%	2012	(Department of Minerals and Energy, 2006)

2.1.6 Future biomass potential

Renewable forms of energy are considered to be 'green' because little of the Earth's resources are depleted (Hall and Scrase, 1998). Plant growth requires CO_2 utilisation and biomass-based processes and products can therefore be incorporated into nature's carbon cycle with lifecycle greenhouse gas emissions approaching zero in some instances (Lynd, 1996 and 1999). Hoogwijk et al. (2003) identified six crucial factors that will determine biomass availability for energy usage. These are (i) the demand for food by the population, (ii) the type of food production systems that can be adopted, (iii) the productivity of forest and energy crops, (iv) the usage of bio-materials, (v) availability of degraded land, and (vi) competing land use types, e.g. surplus agricultural land used for forestation. As reviewed by Lin and Tanaka (2006), wood residues are the largest current source of biomass for energy conversion. Municipal waste is second in line, and is followed by agricultural residues and dedicated energy crops. Among these resources, dedicated energy crops such as corn and sugarcane are currently utilised fairly well, although crops such as tall grasses seem to be the most promising future source of biomass (Hoogwijk et al., 2003).

2.2 BIOCONVERSION OF STARCH

2.2.1 Starch as biomass

Photosynthesis is the cornerstone of biomass/glucan formation (Kennedy et al., 1987). Glucans are the most abundant polymer in plants where cellulose (β-1-4-glucan) is the major structural component, and starch the major reserve of many storage tissues. Starch granules are deposited in the seeds, fruits, leaves, tubers and bulbs of plants as reserve food supply for periods of dormancy, germination, and growth in varying amounts (up to 75% of biomass). Sources of starch used for commercial preparations include seeds of corn, wheat,

barley, oats, rice, and sorghum, tubers and roots of potato, arrow root, and cassava, as well as piths of the sago palm. Several factors govern the choice of raw material for commercial preparations of starch. These include availability, cost, efficiency of processing and quality of the final product (Galliard and Bowler, 1987). Furthermore, starch may be used in its unmodified state or treated with chemicals, or physical factors such as heat or enzymes. The major commercial source of starch in the USA is corn, while wheat is used in Canada and Australia, and tropical countries tend to use cassava roots. Both wheat and barley are used in Europe as a result of varying climatic conditions.

2.2.2 Starch composition

Starch is abundant in various higher plants, and as the primary source of carbohydrate may account for 20-70% of the dry weight (DW) of some plants (Solomon, 1978). Synthesis of the α-1,4 glucan-linked D-glucopyranose chains is localised in chloroplasts of green photosynthetic tissues, or in amyloplasts of non-green storage tissues (Thomas and Atwell, 1999). Polymerisation of glucose to yield starch results in amylose and amylopectin polymers (Tester et al., 2004). The glycoside linkages between the glucose units are stable under alkaline conditions, but become hydrolysable under acidic conditions (Swinkels, 1985).

Linear amylose chains (molecular weight (MW) of 10^5–10^6 Da; DP 500-5000) are composed of α-1,4-linked D-glucopyranose units. A very small portion of α-1,6-linked branches were identified on the amylose polymer (Curá et al., 1995), and on average 2-8 branch points per molecule were identified where the side-chains range from 4 to >100 glucose units (Hizukuri et al., 1981; Takeda et al., 1984). Amylose chains are organised in helixes (Fig. 2.1). Hydrogen atoms on the inside of the helix make the molecule hydrophobic, which allows amylose to form a clathrate complex with fatty acids, alcohols, and iodine. Amylose forms an intense blue colour when allowed to react with iodine (λ_{max} 640nm), and pure amylose binds 19-20% iodine on weight basis (Solomon, 1978; Tester et al., 2004). Amylopectin binds only a small amount of the iodine (1.25%) and the complex formed turns a reddish brown (λ_{max} 540nm) (Kennedy et al., 1987; Solomon, 1978).

Amylopectin (10^7-10^9 Da) is more complex than amylose as α-1,4 glucan chains are added onto existing α-1,4 glucan-linked chains via α-1,6 linkages at branching points in a "cut-and-paste" fashion (Wasserman et al., 1995). The chains are highly branched with a

tumbleweed-like structure and include helixes, double helices, and packed clusters (Whistler and BeMiller, 1997) (Fig. 2.2). The structure contains 5% α-1,6 linkages, leading to short α-1,4 glucan-linked chains that occur in a bimodal distribution of A-chains and B-chains. A-chains (DP≈15) are side chains linked only via their reducing ends to the rest of the molecule, and B-chains (DP≈45) are the chains to which A-chains attach. The C-chain carries the only reducing group in the molecule (Oates, 1997).

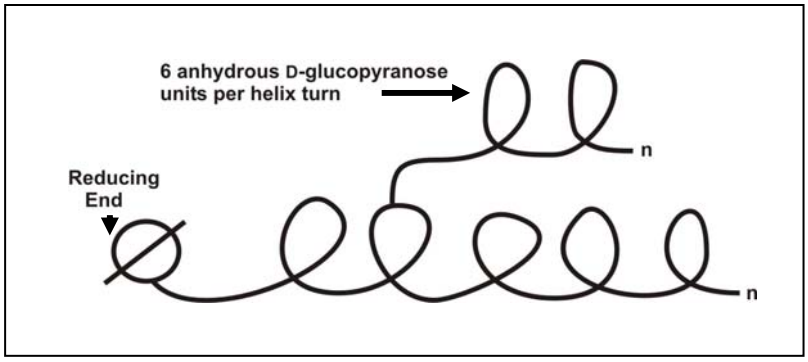

Fig. 2.1 Simplified representation of an amylose helix chain (Thomas and Atwell, 1999).

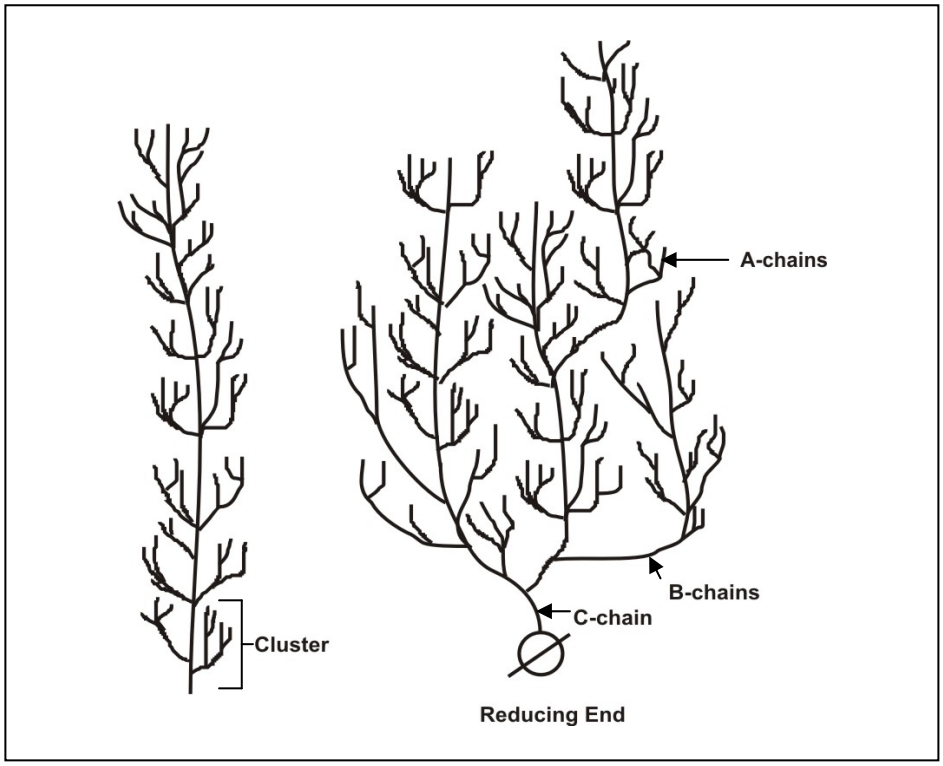

Fig. 2.2 Simplified representation of a portion of an amylopectin molecule (left) and the typical packed clusters of amylopectin (right). Adapted from (Thomas and Atwell, 1999; Whistler and BeMiller, 1997).

2.2.3 Conventional corn starch

Conventional corn starch (corn from dent corn or *Zea mays indenta*) is grouped with cereal starches (Tester et al., 2004). The starch granules are spherical or polyhedral shaped and range from 2-30 μm in size (Fig. 2.3). Corn starch contains on average 62% starch, 8% protein, and 3% lipid, of which approximately 73% of the starch fraction is amylopectin and 27% amylose. The ratio between the amylose and amylopectin fraction in corn starch is known to affect its chemical characteristics (Klucinec and Thompson, 2002; Lii et al., 1996; Oates, 1997; You and Izydorczyk, 2002). As amylose percentage increases, ethanol production from fermentation decreases (Evans and Thompson, 2004; Sharma et al., 2007). Every tonne of corn (15.5% moisture) generally contains about 625 kg of starch, which is present in the endosperm portion of the corn kernel in the form of granules (International Starch Institute, 2007). When hydrolysed, 625 kg of starch theoretically yields 687.5 kg of glucose. The relationship between amylose and amylopectin is complex, and the amylose portion is assumed to exist in the granule as an entity separated from the amylopectin portion. The amylopectin fraction of the starch determines the bulk structure, and amylose can be removed from the structure with hot water leaching (Oates, 1997). The released soluble amylose is responsible for viscosity changes in the solution of leached starch granules. Starch granules contain both ordered, crystalline regions and amorphous regions, which are less ordered and more susceptible to attack by enzymes or acids.

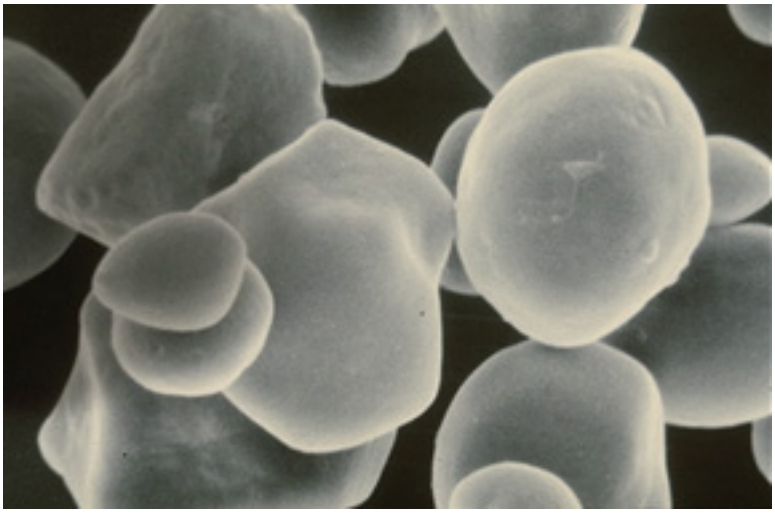

Fig. 2.3 Scanning electron micrograph of raw corn starch granules. Source: http://food.oregonstate.edu/images/starch/cornstarch_raw.jpg.

2.2.4 Raw or unmodified starch

Starch granules remain unmodified (raw) and insoluble in cold water (Daniel et al., 2000). At 25°C, the granules start absorbing water, and as the temperature increases, the granules start to vibrate vigorously. Crystallinity decreases, and when the starch and water suspension is heated above a critical point, designated the pasting or gelatinisation temperature, the granules disintegrate to make a paste (Table 2.3). The amylose portion has a linear structure and this conformation helps aligning adjacent amylose chains when in solution (Kelsall and Lyons, 2003). The extensive hydrogen bonding results in high gel strength. A greater energy requirement is therefore necessary to gelatinise the starch. The more branched amylopectin molecules do not align as easily, resulting in weaker hydrogen bonding and gel strength. As the amylose content in a starch preparation increases, so does the gelatinisation temperature (Ellis et al., 1998). Retrogration occurs when the cooked starch is left to stand, which is mainly due to the amylose fraction in the starch. Retrogration is manifested in the formation of a gel or precipitate (Swinkels, 1985).

Table 2.3 Gelatinisation temperatures (Daniel et al., 2000; Kelsall and Lyons, 2003).

Source	Gelatinisation temperature in °C
Corn standard	62-72
Corn high amylose	67->80
Rice	65-73
White potato	62-68

2.2.5 The role of enzymes in starch degradation

Starch-hydrolysing enzymes originally termed diastases are widely distributed in the animal, microbial and plant kingdoms (Solomon, 1978). Efficient starch hydrolysis calls for the activities of both α-1,4 and α-1,6-debranching activities. Four groups of starch converting enzymes confer this activity and include endo-amylases, exo-amylases, debranching enzymes and transferases. The α-amylases and glucoamylases play the most important role in starch bioconversion to ethanol in the industry, and these enzymes will be described in more detail below. Endo-amylases display α-1,4-cleaving activity and include the α-amylases (EC 3.2.1.1). Exo-amylases such as β-amylases (EC 3.2.1.2) cleave α-1,4 glycosidic bonds only, whereas glucoamylases (EC 3.2.1.3) as well as α-glucosidases

(EC 3.2.133) display both α-1,4 and α-1,6-hydrolysing activities (van der Maarel et al., 2002).

Almost all amylolytic enzymes belong to the glycoside hydrolase family (EC 3.2.1.-), with the exception of cyclomaltodextrin glucanotransferase (CGTase) (EC 2.4.1.19), which is a hexosyltransferase (Enzyme Nomenclature, 1992). Glycoside hydrolases hydrolyse the glucosidic bond between two or more carbohydrates or between a carbohydrate and non-carbohydrate moiety, where as CGTases catalyse the transfer of sugar moieties from activated donor molecules to specific acceptor molecules. Approximately 10% of amylolytic enzymes are able to hydrolyse linkages in raw or unmodified starch (Machovic et al., 2005), and generally contain a Starch Binding Domain (SBD) (Coutinho and Reilly, 1997). Only a few enzymes have been identified to hydrolyse raw starch without the presence of a specialised binding domain. The barley α-amylase is one of the few examples, where binding occurs on the catalytic domain (Robert et al., 2005; Søgaard et al., 1993). The SBD is a carbohydrate-binding module, which enhances the ability of the enzyme to degrade raw starch. The domain is responsible for starch binding. This brings the catalytic site of the enzyme in closer proximity with the substrate, therefore increasing the rate of catalytic activity (Cornett et al., 2003). SBDs are found in carbohydrate-binding module (CBM) Families 20, 21, 25 and 26 in the Carbohydrate Active Enzymes Database (http://www.cazy.org) (Boraston et al., 2004; Coutinho and Henrissat, 1999). Forty percent of the enzymes in CBM20 and 90% of CBM21 cannot degrade raw starch, despite displaying similar structural features as raw starch degrading enzymes. The amylase members belonging to Family 20 all have SBDs at the carboxy termini (C-termini) of the protein and are listed in Table 2.4 (the non-amylases are not listed as they do not comply with the C-terminal SBD rule). The CBM20 module is approximately 90-130 residues long (Machovic et al., 2005). The 3D-structure of the *A. niger* glucoamylase SBD in solution bound to β-cyclodextrin (β-CD), a cyclic starch analogue, has been resolved (Jacks et al., 1995; Sorimachi et al., 1996 and 1997). The structure revealed that the well-defined β-sheet structure seen in the free SBD is maintained in the SBD-βCD complex. The two starch-binding sites of the SBD appear in Figure 2.4. Glucoamylases from *Rhizopus oryzae* belong to CBM21 where the SBDs are situated at the amino terminal (N-terminal) of the protein (Ashikari et al., 1986). Family 25 and 26 contain mostly α-amylases from bacterial origin.

Table 2.4 Classification of starch hydrolysing enzymes with C-terminal SBDs.

Bond hydrolysed	General name	Systemic name	EC number
1,4-linked and 1,6-linked	glucoamylase/glucan 1,4-α-glucosidase	1,4-α-D-glucan glucohydrolase	EC 3.2.1.3
1,6-linked	amylo-α-1,6-glucosidase	glycogen phosphorylase-limit dextrin α-1,6-glucohydrolase	EC 3.2.133
1,4-linked	glucan 1,4-α-maltotetraohydrolase	1,4-α-D-glucan maltotetraohydrolase	EC 3.2.1.60
1,4-linked	α-amylase	1,4-α-D-glucan glucanohydrolase	EC 3.2.1.1
1,4-linked	β-amylase	1,4-α-D-glucan maltohydrolase	EC 3.2.1.2
1,4-linked	cyclomaltodextrin glucanotransferase	1,4-α-D-glucan transferase (cyclising)	EC 2.4.19

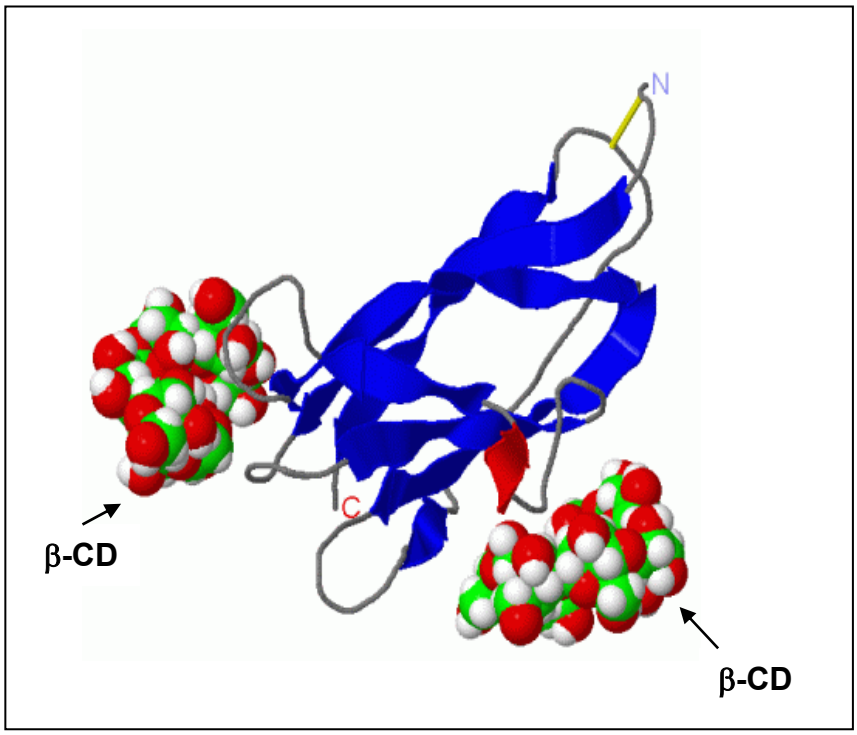

Fig. 2.4 3D-structure of *A. niger* glucoamylase SBD in complex with β-CD at the two binding sites. The C- and N-termini are indicated (Sorimachi et al., 1997).

2.2.5.1 α-Amylase

α-Amylases, also known as liquefying enzymes, are endohydrolases and employ a retaining mechanism for hydrolysis (Enzyme Nomenclature, 1992). The enzymes belong to glycoside hydrolase (GH) Family 13 and clan GH-H (MacGregor et al., 2001). They hydrolyse the 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units (Fig. 2.5). The endo-action occurs in a random manner to liberate reducing groups with the α-configuration. The term 'α' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed (Enzyme Nomenclature, 1992). Hydrolysis reduces the molecular size of starch and therefore the viscosity of the starch solution (Solomon, 1978). Hydrolysis of amylose liberates maltose and maltotriose, but as maltotriose is a poor substrate for α-amylase, the second stage of hydrolysis of maltotriose to maltose and D-glucose is very slow, and only takes place if large amounts of enzyme are available (Fig. 2.5) (Walker and Whelan, 1960). Microbial α-amylases are not able to hydrolyse 1,6-linked units, and therefore a number of α-limit dextrins containing at least one 1,6-linkage are also generated when a polysaccharide such as starch is hydrolysed (Kennedy et al., 1987). Isomaltose is not formed during starch hydrolysis with α-amylase, because the presence of 1,6-linkages confers some stability on some of the adjacent 1,4-linked units, and therefore not all the 1,4-linked units can be hydrolysed (Manners and Marshall, 1971). All α-amylases are dependent on at least one calcium ion per mole enzyme for enzyme activity and conformational stability (Hsiu et al., 1964; Imanishi, 1966; Saboury, 2002; Vallee et al., 1959). Amylases display a typical bell-shaped curve when activity at different pHs is plotted. The maximum activities of the enzymes seem to be in the acidic range of pH 4.5-7.0. α-Amylases tend to be more heat stable when calcium is present, although enzymes from different sources vary in heat stability (Solomon, 1978).

Most α-amylases have a multidomain structure with three major units. The core of the enzyme is referred to as domain A and contains a $(\beta/\alpha)_8$-barrel or a triose phosphate isomerase (TIM) barrel catalytic domain, where the active site is always located at the C-terminal end of the barrel structure (Farber and Petsko, 1990). The core has a highly symmetrical fold of eight inner parallel β-strands, which are surrounded by eight helices (Svensson, 1994). The loops that link the β-strands of the adjacent helices usually carry the active amino acids.

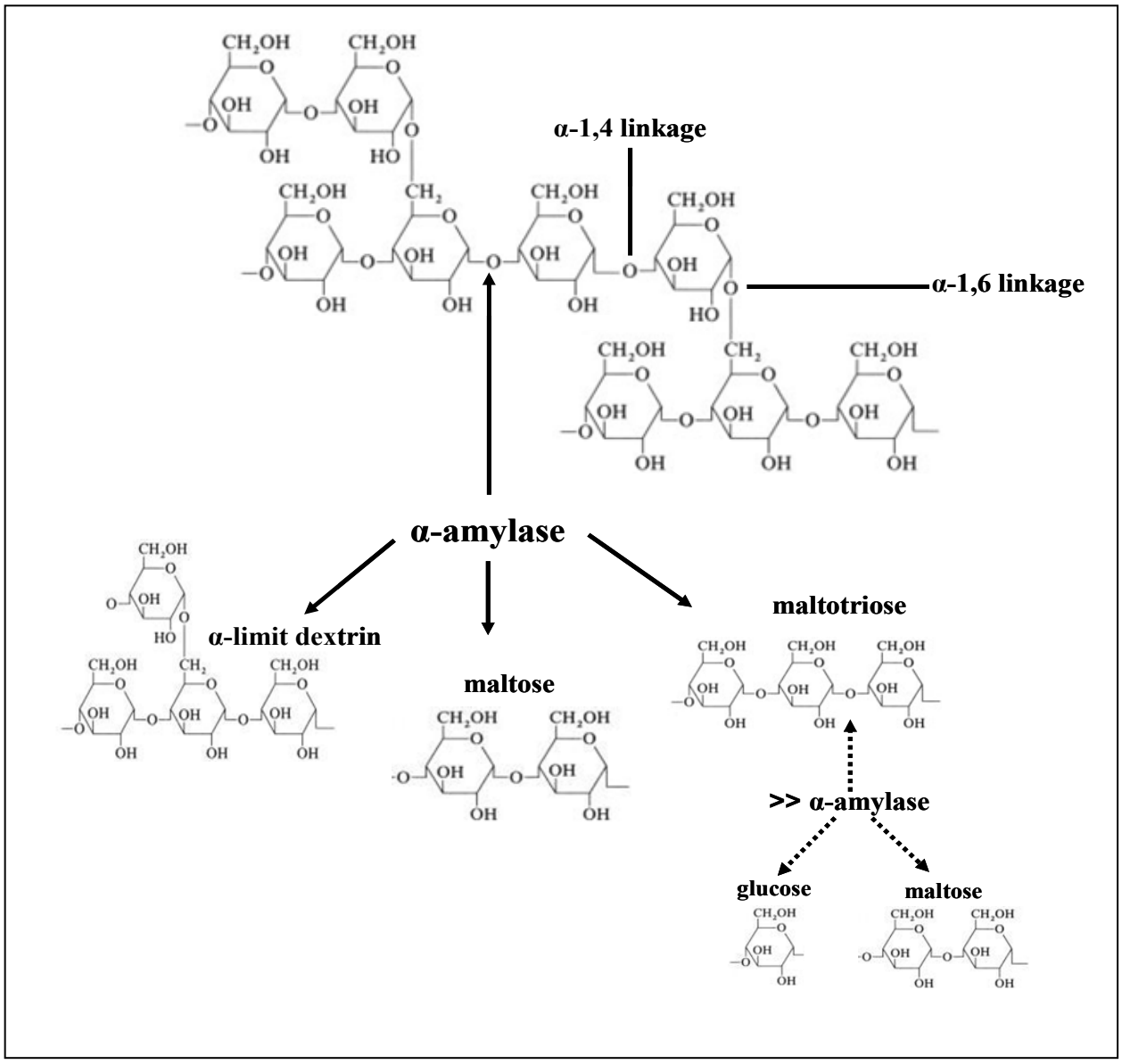

Fig. 2.5 Schematic representation of the action of α-amylase on starch. Broken arrows indicate that only excess amounts of α-amylase will hydrolyse maltotriose into maltose and glucose.

These loops may be long enough to be considered as domains, and domain B is formed by the protrusion formed between the third β-strand and third α-helix of the TIM barrel (Farber and Petsko, 1990) (See Fig. 2.6 for domains A and B). An extended substrate binding site is localised to loops at the C-terminal ends of β-strands in the $(\beta/\alpha)_8$- barrel domain. The third domain (C domain) stabilises the TIM barrel by shielding the hydrophobic residues of domain A from the surrounding solution. The domain displays β-strands in a Greek-key motif (β-sandwich).

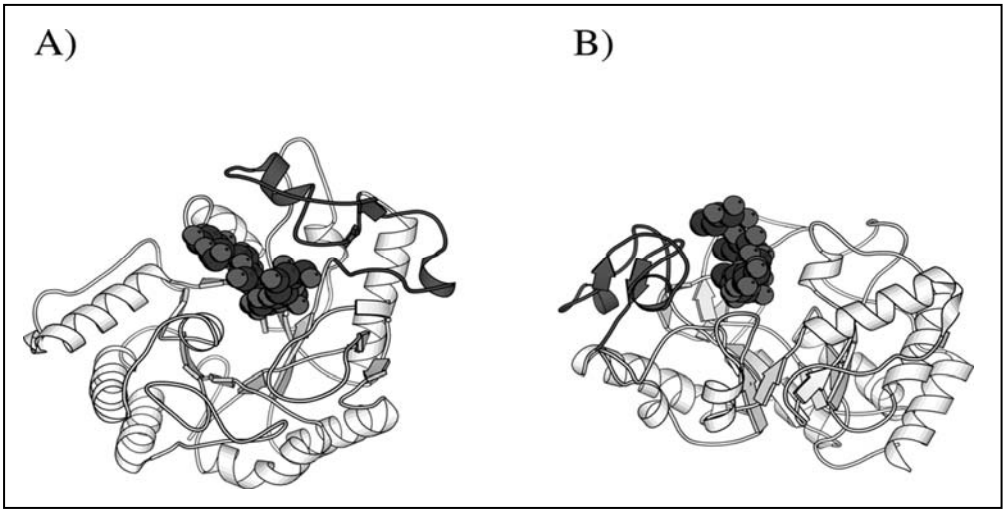

Fig. 2.6 Schematic representation of the structure of the A and B domains of the *B. subtilis* α-amylase. α-Helices are shown as spiral ribbons, whereas β-strands are drawn as arrows from the N-terminal to the C-terminal of the β-strand. The TIM barrel fold corresponding to the A domain is shown in light grey, and the B domain is shown in dark grey. The space fill model of the maltopentaose ligand indicates the position of the active site (at the C-terminal side of the β-sheet in the core of the TIM-barrel fold). A): An end view in which the C-terminal side of the β-sheet is toward the reader. B): A side view in which the C-terminal side of the β-sheet is toward the top of the page (Pujadas and Palau, 2001).

Some α-amylases contain an additional domain D and E, where domain E in the CGTase usually displays activity towards raw starch (Jespersen et al., 1991; Svensson et al., 1989) (Fig. 2.7). Raw starch α-amylases tend to act mainly by surface erosion or by penetration at local points on the surface of a starch granule. This is followed by an inside-out hydrolysis process starting within the granule (Galliard and Bowler, 1987).

The α-amylases have considerably low sequence similarity, although four amino acids are invariant throughout the entire family of EC 3.2.1.1 (Hasegawa et al., 1999; Matsuura et al., 1980 and 1984; Nakamura et al., 1992; Swift et al., 1991; Vihinen et al., 1990). The amino acid sequence of *Bacillus stearothermophilus* α-amylase is fairly homologous (about 60%) with that of a thermostable α-amylase from *Bacillus amyloliquefaciens*. Homology is least among thermolabile α-amylases from *Bacillus subtilis*, *A. oryzae*, plants and animals (Nakajima et al., 1986). The Arg-204 and the three catalytic residues; Asp-206, Glu-230, and Asp-297 (amino acid numbering of TAKA amylase sequence from *A. oryzae*) together with two invariant residues His-122 and His-296, form the basis of conserved regions in the protein. The catalytic residues lie on the C-terminal end loops of the 4^{th}, 5^{th} and 7^{th} β-strands of the barrel, respectively (Matsuura, 2002). A total of seven conserved regions have been reported. The first four are situated at the C-terminus of the β_3, β_4, β_5, and β_7 strands of the TIM barrel (Nakajima et al., 1986). The fifth region is located near the C-terminus of domain B, around the calcium-binding site Asp-175 of TAKA amylase. The sixth and seventh are found at the β_2 and β_8 strands of the TIM barrel (Janecek, 1995 and 2002).

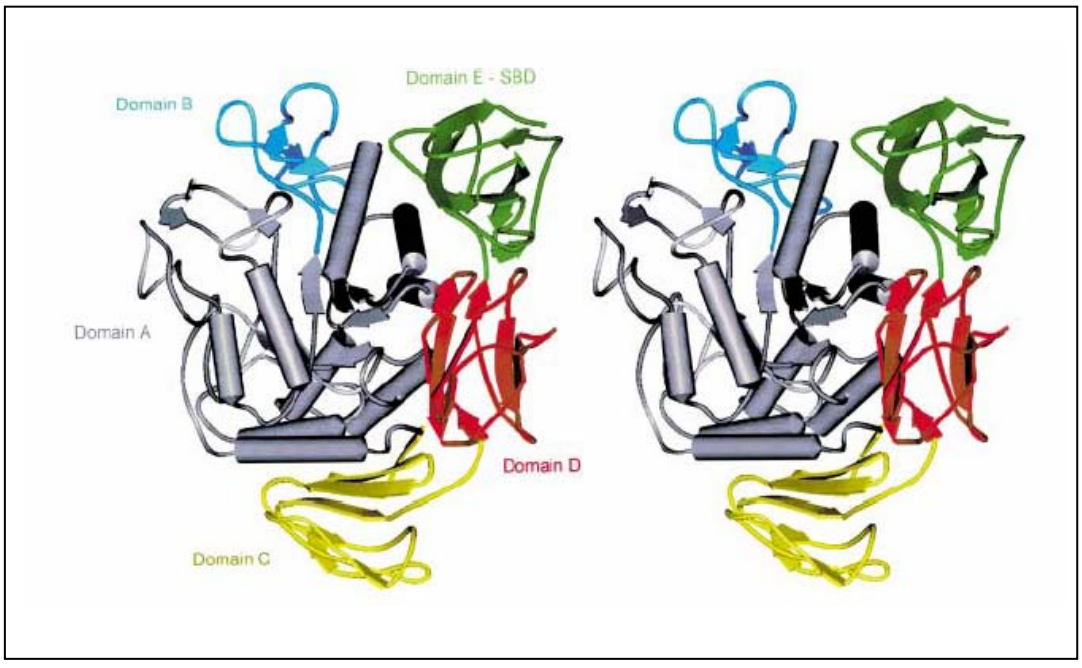

Fig. 2.7 Stereo view of a CGTase as an example of a five-domain member of the α-amylase family having the C-terminal SBD. Domains A-D are also indicated (Janecek et al., 2003).

2.2.5.2 Glucoamylase

Glucoamylases (1,4-α-D-glucan glucohydrolase EC 3.2.1.3) belong to GH Family 15 (Coutinho and Henrissat, 1999). Glucoamylases are exoamylases, also known as saccharifying enzymes, that hydrolyse the terminal 1,4-linked α-D-glucopyranosyl residues successively from non-reducing ends of starch chains to release D-glucose. The products of hydrolysis have the β-configuration due to inversion of the product. The enzyme acts more rapidly on substrates as the degree of polymerisation increases (Belshaw and Williamson, 1993; Reese et al., 1968). Most forms of the enzyme can rapidly hydrolyse 1,6-α-D-glucosidic bonds when the next bond in the sequence is 1,4-linked (Fierobe et al., 1998; Pazur and Ando, 1960). The rate of hydrolysis between linkages depends on the nature of the linkage in the molecule adjacent to that of the linkage being hydrolysed. The specific activity towards the 1,6-linkage is however only 0.2% of that for the 1,4-linkage (Cabral et al., 1983; Fierobe et al., 1996; Frandsen et al., 1995; Hiromi et al., 1966; Kennedy et al., 1987; Sierks and Svensson, 1994). Complete conversion to D-glucose is unattainable when high concentrations of α-limit dextrins are hydrolysed with glucoamylase. The D-glucose yield reaches a maximum and then decreases, as glucoamylases are capable of reforming 1,3-, 1,4-, and 1,6-linkages between α-D-glucopyranosyl residues in the presence of high D-glucose concentrations (Kennedy et al., 1987).

Glucoamylases from *Aspergillus* strains tend to have an optimum activity in pH range 4.5-5.0, and *Rhizopus* glucoamylases in the range of 4.5-5.5. Glucoamylases are relatively temperature stable at higher temperatures, with *Aspergillus* enzymes having greater thermal stability than *Rhizopus* enzymes, and both *Aspergillus* and *Rhizopus* enzymes being more stable than *Endomyces* species (Kennedy et al., 1987).

Fungal glucoamylases contain a catalytic domain near the amino terminus (A-1 to V-469). In enzymes with raw starch degrading ability, the catalytic domain is connected to a raw starch affinity site on the C-terminus (A-470 to R-615) (Fukuda et al., 1992; Hayashida et al., 1991) with an O-glycosylated polypeptide linker. The *A. niger* glucoamylase SBD is approximately 100 amino acids long, and connected to the catalytic domain via a 70 amino acid-long O-glycosylated linker. The raw starch affinity site or motif required for adsorption onto raw starch contains β-strand elements (Tanaka et al., 1986); one parallel and 5-6 antiparallel pairs of β-strands forming two β-sheets (Jacks et al., 1995; Sorimachi

et al., 1996), with an open sided β-barrel (Jacks et al., 1995). When this C-terminal region was compared to glucoamylases and α-amylases of many strains, four areas of sequence similarity were identified (Svensson et al., 1989). The raw starch affinity site is divided into a glycopeptide I (Gp-I) region (A-470 to V-514) and the C-terminal peptide (Cp-I) region (A-515 to R-615) or SBD (Belshaw and Williamson, 1993; Fukuda et al., 1992). The Gp-I region consists of mainly Thr and Ser residues, which promote hydrolysis of raw starch (Fukuda et al., 1992; Hayashida et al., 1989). The Cp-I region or SBD binds to raw starch via formation of an inclusion complex between residues Trp-562 and the hydrophobic cavity in the substrate. The minimal sequence identified around Trp-562 to be essential for hydrolysis of raw starch is PL(W-562)YVTVTLPA (Goto et al., 1994). The second tryptophan residue (Trp-590) has higher affinity for ligands than Trp-562. Trp-562 is proposed to play a role in recognising the substrate, while Trp-590 is involved in tighter binding and preparing the substrate for catalysis (Sorimachi et al., 1997). The SBD plays an active role in hydrolysing raw starch, and enzymes where the SBD was partially or totally removed by proteolytic excision, could hydrolyse soluble starch only (Coutinho and Reilly, 1997). Fusion of the *A. niger* SBD to barley α-amylase 1 displayed enhanced enzyme activity towards barley starch granules, highlighting the role of the SBD in raw starch hydrolysis (Juge et al., 2002). The position of the SBD at the C-terminus of *Aspergillus* glucoamylase was shown to be essential for raw starch hydrolysis in a SBD domain shuffling study (Cornett et al., 2003).

The carbohydrate content of glucoamylases range from 10-20% of the MW (Pazur et al., 1971; Ueda, 1981), which is usually in the range of 48-90 kDa, except the 125 kDa glucoamylases produced by *A. niger*. Glycosylation plays an integral part in stability. Glycosylation does not seem to affect the protein's tertiary structure, however elimination of glycosylation leads to decreased enzyme secretion and thermal stability of the enzyme (Coutinho and Reilly, 1997; Williamson et al., 1992). The stability of the enzyme may be compromised when glycosylation is eliminated, as the protein would then be able to make steric contact with the linker. Glycosylation around the catalytic domain contributes to rigidity of this domain, and rigidifies the long polypeptide linker in solvents, therefore assisting in physical separation of the catalytic domain and SBD. The carbohydrate moiety has the additional benefit of preventing partially and unfolded proteins to aggregate. O-glycosylation further protects the protein from proteolysis (Coutinho and Reilly, 1997; Gal-Coeffet et al., 1995). The addition of small amounts of glucose partly protects glucoamylases at high temperatures (Fukui and Nikuni, 1969).

2.2.6 Industrial ethanol production technologies applied in starch conversion

Several different sources of starch are utilised for bioconversion to ethanol. Corn is the major feedstock in the USA (Devantier et al., 2005). Corn is also one of the feed stocks that may be used to produce ethanol in South Africa (Strumpf, 2006). As reviewed by Venkatasubramanian and Keim (1985), four major steps involved in the conversion of starch biomass to bioethanol include (i) extraction of starch from the biomass, (ii) conversion of starch to fermentable sugars, (iii) fermentation of the fermentable sugars, and (iv) ethanol distillation. Extraction of starch is accomplished via two different approaches namely wet milling and dry milling. The processes differ in two respects. During wet milling, corn component parts such as protein, oil, fibre and minerals are fractionated off, thereby increasing overall coproducts return. Wet milling further involves using fresh water in a counter-current mode, therefore conserving water usage through recycling liquid to the fermentors. Dry milled or shelled corn is converted to fermentable sugars without recovering the individual component parts of corn as separate coproducts. The dry milling procedure is less expensive to set up and operate compared to wet milling. Coproduct-return in wet milling, however, increases revenue generated.

Until the 1970's, conversion of starch to liberate glucose was accomplished using dilute acid and high temperatures (120-150°C) (Robertson et al., 2006). Acid hydrolysis required the use of expensive high-grade stainless steel equipment, which could withstand the corrosive power of strong and concentrated aqueous solutions of mineral acids. Production of maximum fermentable sugars was further hindered by exposure of the sugars to the harsh conditions in the procedure, as amylose was broken down at an earlier time compared to amylopectin. The costly process was replaced by a high-temperature, liquid-phase enzymatic starch hydrolysis procedure.

The high-temperature hydrolysis requires vast amounts of heating energy. Net energy yield can be increased by utilising SSF (Devantier et al., 2005; Lynd et al., 1999). Lowering the temperature of hydrolysis and fermentation decreases the viscosity of the generated slurry, therefore lowering complications of pumping and stirring of the material. Lower temperatures further minimise the formation of unwanted Maillard reaction coproducts such as fusel oils and glycerol, which could reduce glucose yield for fermentation (Galvez, 2005). A RSH enzyme Stargen 001 (Genencor) was therefore developed for processes where starch is not cooked. Broin and Novozymes have also collaborated to produce the BPX (Broin

project X) enzymes to hydrolyse raw starch in a non-cooking fermentation scenario (Berven, 2005).

2.2.6.1 Wet milling procedure

In the wet milling procedure (Fig. 2.8), corn is treated with a warm sulphuric acid solution in a process called steeping for 24-48 hours (RFA, 2007b). In this step soluble ash, protein and carbohydrates are leached out. The oil fraction (hull and germ) is toughened and the protein matrix, which holds the starch granules, is softened. The former ash, protein and carbohydrate solution is either used as soluble animal feed, or dried with the fibre to be sold as dried feed. Hydroclone separation, counter-current washing, dewatering, drying, and extraction with solvents recover the germ oil fraction. Fibre is then removed to produce corn gluten feed from the degermed slurry after further milling, screening, counter-current washing, dewatering and drying. The feed contains 21% protein if steep water is dried with the fibre. In the next step, insoluble protein is removed by centrifugation, concentration and drying to produce corn gluten meal, which contains 60% protein. In the final step, the slurry is washed with fresh water in a counter-current fashion to remove solubles and protein, and the solution leaves the system as a slurry consisting of refined starch containing 35-40% solids. The slurry is then treated with α-amylase and glucoamylase to produce fermentable sugars for the yeast to ferment to ethanol (Venkatasubramanian and Keim, 1985). The dilute alcohol solution, referred to as 'beer', is passed through a beer still that strips the fermented mash and concentrates it to approximately 60-70% by volume. A distillation step is then used to concentrate the alcohol to 95% by volume.

2.2.6.2 Dry milling procedure

The dry milling procedure is the most widely used method in the USA for generating fuel ethanol, with 82% of all ethanol produced employing this method (Kwiatkowski et al., 2006; RFA, 2007a; Srinivasan et al., 2005). Dry milling is preferred over wet milling as it requires less equipment and has lower capital investment (Belyea et al., 2004). The process is summarised in Figure 2.9. Granules of corn are milled into flour or meal and suspended in water to form a mash (Kelsall and Lyons, 2003). The mash is pre-mixed with α-amylase at 40-60°C, and then cooked at 90-120°. The starch is then gelatinised or liquefied by high-pressure steam at 90°C and a thermostable α-amylase.

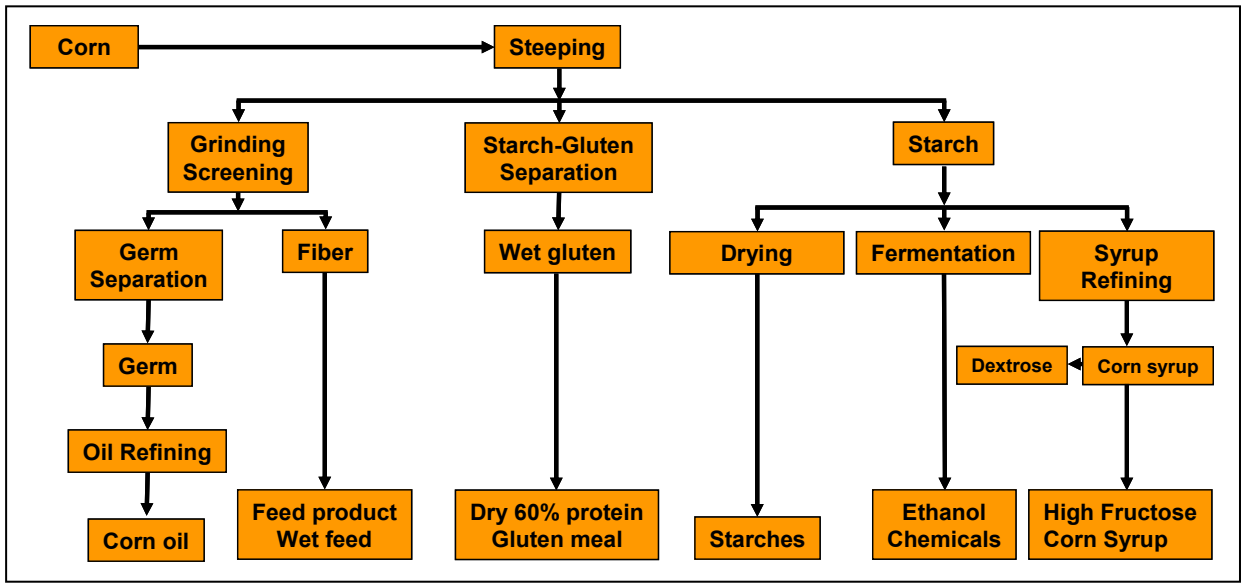

Fig. 2.8 Flow diagram depicting the recovery of coproducts from corn in the wet milling process (RFA, 2007b).

Subsequently starch is cooked and transferred to the saccharification tank where glucoamylase is added to hydrolyse the starch to fermentable sugars at 60°C. After cooling the mixture to 32°C, yeast is added and sugars are fermented in a batch mode for 40-50 hours (RFA, 2007b). As with wet milling, the beer is passed through a beer still that strips the fermented mash and concentrates it to approximately 60-70% by volume. A distillation step follows to concentrate the alcohol. The stillage is then centrifuged to separate the solids form the solubles, and solubles are concentrated to 30% by evaporation. The concentrated solubles together with the residual material comprising yeast, insolubles from corn (protein, fat and fibre), and unconverted starch are then dried and concentrated to produce DDGS, which is sold as animal feed for ruminant animals as a result of the high fibre content of the material.

DDGS has a low market value and as ethanol production increases, so will the production of DDGS. Almost one kilogram of DDGS is produced per kilogram of ethanol produced in a dry-milling plant (Schilling et al., 2004). Demand and supply balance play an important role, and in order to sustain this balance, DDGS volumes need to be decreased and the market for this product has to be diversified. With this in mind, modifications have been proposed to recover coproducts (germ and pericarp fibre) at the beginning of the process (Singh et al., 1999; Singh and Eckhoff, 1996 and 1997; Wahjudi et al., 2000).

The additional use of enzymes in a so-called E-Mill extends the coproducts range with endosperm fibre (Singh et al., 2005).

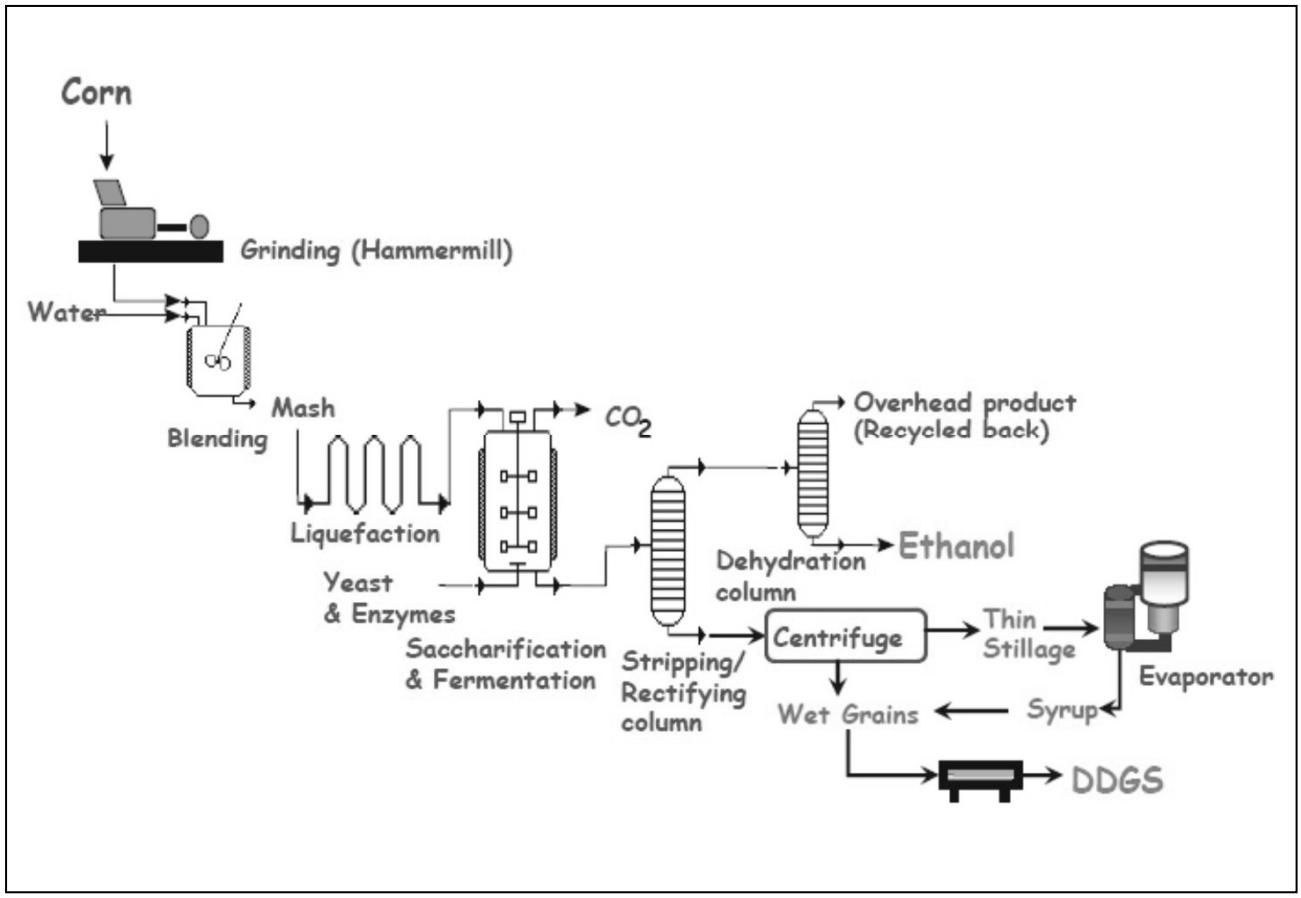

Fig. 2.9 Simplified representation depicting the dry milling process for ethanol production from corn (Srinivasan et al., 2006).

Removal of the mentioned coproducts increased fermentation capacity by increasing ethanol concentration by 8-27%. It reduced the DDGS fibre from 11 to 2%, and increased protein content from 28 to 58%. The DDGS could therefore be sold as monogastric foodstuff. Separation of fibre from DDGS at the end of the process using sieving and elutriation can further improve DDGS product quality (Srinivasan et al., 2005), and yield fibre which can be used to make corn fibre oil, corn fibre gum, bioethanol, and xylitol (Grohmann and Bothast, 1997; Srinivasan et al., 2006).

2.2.7 Unmodified or raw starch hydrolysing enzymes in dry milling

The use of raw starch hydrolysing enzymes as replacement of conventional liquefaction and saccharification enzymes in a dry-grind corn process has proven to be very valuable

(Robertson et al., 2006). In a dry-milling plant, energy is used in jet cooking, liquefaction, distilling, dehydration, as well as drying operations. By using RSH enzymes, process energy usage will be reduced substantially. In a recent comparison between conventional amylase enzymes used in the industry and Stargen 001 (Genencor International), the RSH enzyme revealed comparable ethanol conversion efficiencies (Wang et al., 2007). Ethanol yields as well as DDGS production were also similar. The only noticeable difference between the processes was sugar profiles, where the RSH enzyme released only 7% glucose (w/v) compared to 19% (w/v) for conventional enzymes. Substrate was treated as recommended by the enzyme suppliers, whereby the starch mash was treated with Stargen 001 for 2 hours at 48°C before cooling the mixture down to 30°C for yeast addition. These results therefore strengthen the notion that raw starch utilising yeasts may decrease costs for ethanol production even further by cutting out commercial enzyme purchase costs.

2.3 GENETIC ENGINEERING OF YEASTS FOR STARCH HYDROLYSIS

2.3.1 *Saccharomyces cerevisiae*: the ideal eukaryotic model for heterologous protein production

The flexible yeast host *S. cerevisiae* has been studied extensively as a model to understand eukaryotic biology at cellular and molecular level. Their simple biology allows for uncomplicated manipulation, as with bacteria, and they grow very rapidly in relatively cheap media (Watson et al., 1996). Genetic and molecular analysis is simplified as a result of their small genomes and sequencing of the entire 12 500-kb genome was completed in 1996 (Dujon, 1996; Levy, 1994). The cells are organised similarly to cells in more complex eukaryotic organisms, and many yeast proteins are closely related in structure and function to their mammalian counterparts (Watson et al., 1996). The mechanism of protein synthesis is furthermore conserved between *S. cerevisiae* and more complex organisms (Altmann and Trachsel, 1994). Strains of *S. cerevisiae* have often been used as a host for heterologous gene expression (Bitter et al., 1987; Hitzeman et al., 1983a; Hitzeman et al., 1983b; Smith et al., 1985). The yeast is known for its high fermentation capacity, high ethanol productivity (41 g l^{-1} h^{-1}), high ethanol tolerance and 'generally regarded as safe' (GRAS) status (Ben Chaabane et al., 2006; Ibragimova et al., 1995; Knox et al., 2004). The yeast *S. cerevisiae* therefore represents a flexible host system which can be manipulated to produce/secrete the necessary enzymes to function as an amylolytic microorganism utilised for starch metabolism.

2.3.2 Heterologous amylase expression in yeast

The amylase genes from certain microbes have been expressed in *S. cerevisiae* to yield amylolytic yeasts (de Moraes et al., 1995; Eksteen et al., 2003a; Knox et al., 2004; Kondo et al., 2002; Ma et al., 2000). These include secretion of the heterologous enzymes, and/or anchoring the enzymes on the cell wall of the yeast. When the enzymes are displayed on the cell wall, a biocatalyst organism is generated, as the yeast can be re-used for consecutive fermentations. Secretion of enzymes ensures that the enzyme moves freely in the fermentation, therefore not hindering the enzyme in locating its substrate. Displaying the enzymes on the cell wall is disadvantaged in this way, as the cells need to be in close proximity to the substrate in order for it to be hydrolysed. Mixing therefore always plays an important role during fermentation. The whole-cell biocatalysts, however, have the advantage of lowering the risk of contamination during fermentation, as glucose is generated in close proximity to the cell wall and is utilised immediately by the host. The biocatalyst fermentation may also be used for selection of a fitter host, as the cells displaying a more effective enzyme will be at an advantage. Furthermore, biocatalysts may be recycled by recovering the enzymes together with the yeast (Khaw et al., 2006).

2.3.2.1 Engineered yeast strains able to utilise soluble or cooked starch

Several strains have been engineered in the last 20 years that have proven successful in converting soluble or cooked starch to ethanol. Calculated ethanol yields, volumetric productivities or specific productivities are listed in Table 2.5. Values were calculated from available data presented in the different studies.

In the quest for the development of a novel whole-cell biocatalyst, yeasts for cell surface display of glucoamylase were created (Murai et al., 1997, 1998 and 1999; Ueda and Tanaka, 2000). The *R. oryzae* glucoamylase was displayed on the surface of *S. cerevisiae*, which enabled the yeast to grow on soluble starch during aerobic cultivation (Murai et al., 1997). The *R. oryzae* glucoamylase was also displayed on the cell wall of a flocculent yeast strain, which produced ethanol very effectively in soluble starch medium under anaerobic conditions with an ethanol yield of 0.59 gram ethanol per gram fermentable sugar (g g^{-1}) (Kondo et al., 2002). The strain was further improved by the addition of the *Bacillus stearothermophilus* α-amylase. The α-amylase was either co-displayed with the glucoamylase, or secreted into the medium. An ethanol concentration of 60 g l^{-1} was

reached after a 100 hour fed-batch soluble starch fermentation (Shigechi et al., 2002). The strain co-displaying the two enzymes produced up to 18 g l^{-1} ethanol after 36 hours of fermentation from 50 g l^{-1} low temperature cooked corn starch (80°C) at an ethanol yield of 0.51 g g^{-1}. The ethanol concentration increased to 30 g l^{-1} when using 90 g l^{-1} cooked corn starch (Shigechi et al., 2004a). It has to be noted however that a very high cell load was added to yield these results (OD600 = 60 or ± 30 g l^{-1} DW cells). These strains also do not have the ability to utilise raw corn starch (Shigechi et al., 2004b).

The *LKA1* and *LKA2* α-amylase genes from *Lipomyces kononenkoae* were integrated into the *S. cerevisiae* genome and heterologous enzymes were secreted from the host (Eksteen et al., 2003b). The strain produced 6.1 g l^{-1} ethanol after 156 hours of fermentation in a 2% starch medium with an ethanol yield of 0.38 g g^{-1}. The strain was improved by the Knox et al. (2004) group by the addition of a glucoamylase to the system. The SFG1 glucoamylase from *S. fibuligera* and LKA1 α-amylase from *L. kononenkoae* were integrated into the yeast genome for secretion. Up to 21 g l^{-1} ethanol was produced after 120 hours of fermentation from soluble starch with a volumetric productivity of 0.562 g l^{-1} h^{-1} and yield of 0.40 g g^{-1} (Knox et al., 2004).

S. cerevisiae strains were also generated to secrete separate polypeptides of *A. awamori* glucoamylase, *B. subtilis* α-amylase and mouse α-amylase (Birol et al., 1998). A bifunctional fusion protein constructed from the *B. subtilis* α-amylase and *A. awamori* glucoamylase was also expressed. The strain secreting the *A. awamori* glucoamylase and *B. subtilis* α-amylase as separate polypeptides from a double expression cassette performed the best and displayed the highest ethanol production of 43.8 g l^{-1} in 100 g l^{-1} cooked starch media after 120 hours of fermentation. The specific ethanol productivity was 0.045 g (g DW cells)$^{-1}$ h^{-1} and ethanol the yield was 0.44 g g^{-1}. In a follow-up study, the ethanol production of the strain increased to 47.5 g l^{-1} in fed-batch experiments and the yield increased to 0.47 g g^{-1} (Ülgen et al., 2002).

Table 2.5 Ethanol concentration, production and yield from amylolytic yeast strains cultivated in soluble starch or cooked corn starch.

Source of gene/s [a]	**Inoculum and media** [b]	**[Ethanol]** [c]	**Ethanol productivity** [d] **or specific productivity** [e]	**Ethanol yield** [f]	**Reference**
R. oryzae glucoamylase gene fused to α-agglutinin for cell surface display.	5 g l^{-1} DW cells in 10 g l^{-1} soluble starch	1.5 g l^{-1} after 70 h. aerobic	0.046 g l^{-1} h^{-1} 0.004 g (g DW cells)$^{-1}$ h^{-1}	0.19	(Murai et al., 1997)
R. oryzae glucoamylase gene fused to α-agglutinin for cell surface display on flocculent yeast strain.	13 g l^{-1} DW cells in 40 g l^{-1} soluble starch with 5 g l^{-1} glucose	25 g l^{-1} after 30 h	0.71 g l^{-1} h^{-1} 0.190 g (g DW cells)$^{-1}$ h^{-1}	0.59	(Kondo et al., 2002)
R. oryzae glucoamylase displayed on yeast surface.	30 g l^{-1} DW cells in 50 g l^{-1} cooked corn starch	13 g l^{-1} after 48 h	0.42 g l^{-1} h^{-1}	0.50	(Murai et al., 1997; Shigechi et al., 2004a)
R. oryzae glucoamylase and *B. stearothermophilus* α-amylase displayed on yeast surface.	30 g l^{-1} DW cells in 50 g l^{-1} cooked corn starch	18 g l^{-1} after 36h	1.25 g l^{-1} h^{-1}	0.51	(Shigechi et al., 2004a)
R. oryzae glucoamylase displayed on yeast surface and *B. stearothermophilus* α-amylase secreted.	30 g l^{-1} DW cells in 50 g l^{-1} cooked corn starch	17 g l^{-1} after 48h	0.64 g l^{-1} h^{-1}	0.49	(Shigechi et al., 2004a)
S. diastaticus glucoamylase secreted from yeast.	50 g l^{-1} soluble starch	14.3 g l^{-1} after 240 h	0.16 g l^{-1} h^{-1} 0.007 g (g DW cells)$^{-1}$ h^{-1}	0.53	(Nakamura et al., 1997)

[a] Recombinant host was *S. cerevisiae* in all cases, [b] batch fermentation, [c] ethanol concentration (g l^{-1}), [d] ethanol volumetric productivity (g l^{-1} h^{-1}),

[e] Ethanol specific productivity (g (g DW cells)$^{-1}$ h^{-1}), [f] ethanol yield as g (g consumed sugar)$^{-1}$. A blank space indicates that not enough data was presented to determine the value.

Table 2.5 Ethanol concentration, production and yield from amylolytic yeast strains cultivated in soluble starch or cooked corn starch (continued).

Source of gene/s [a]	Inoculum and media [b]	[Ethanol] [c]	Ethanol productivity [d] or specific productivity [e]	Ethanol yield [f]	Reference
LKA1 and LKA2 α-amylases from *Lipomyces kononenkoae* integrated into yeast genome for secretion.	2 g l^{-1} DW cells in 20 g l^{-1} soluble starch	6.1 g l^{-1} after 156 h		0.38	(Eksteen et al., 2003b)
SFG1 glucoamylase from *S. fibuligera* and LKA1 α-amylase from *L. kononenkoae* integrated into yeast genome for secretion.	5% v/v inoculum in 55 g l^{-1} soluble starch	21 g l^{-1} after 120 h	0.562 g l^{-1} h^{-1} 0.042 g (g DW cells)$^{-1}$ h^{-1}	0.40	(Knox et al., 2004)
A. awamori glucoamylase secreted from yeast.	1% v/v inoculum in 100 g l^{-1} soluble starch	44.8 g l^{-1}	0.030 g (g DW cells)$^{-1}$ h^{-1}	0.48	(Inlow et al., 1987)
A. awamori glucoamylase and *B. subtilis* α-amylase secreted as separate polypeptides from yeast.	10% v/v inoculum in 100 g l^{-1} or 50 g l^{-1} soluble starch	43.8 g l^{-1} in 100 g l^{-1} starch 18.8 g l^{-1} in 50 g l^{-1} starch, both after 120 h	0.045 g (g DW cells)$^{-1}$ h^{-1} in 100 g l^{-1} and 0.0429g (g DW cells)$^{-1}$ h^{-1} in 50 g l^{-1} soluble starch	0.44 for 100 g l^{-1} and 0.38 for 50 g l^{-1} soluble starch	(Birol et al., 1998; de Moraes et al., 1995)
A. awamori glucoamylase and *B. subtilis* α-amylase secreted as separate polypeptides from *S. cerevisiae.*	10% v/v inoculum in 40 g l^{-1} soluble starch with 4 g l^{-1} glucose at pH 5.6	21.5 g l^{-1} after 70 h	0.31 g l^{-1} h^{-1}	0.54	(Ülgen et al., 2002)

[a] Recombinant host was *S. cerevisiae* in all cases, [b] batch fermentation, [c] ethanol concentration (g l^{-1}), [d] ethanol volumetric productivity (g l^{-1} h^{-1}),

[e] Ethanol specific productivity (g (g DW cells)$^{-1}$ h^{-1}), [f] ethanol yield as g (g consumed sugar)$^{-1}$. A blank space indicates that not enough data was presented to determine the value

2.3.2.2 Raw starch utilising yeast strains

Very few groups have reported results on yeast strains which are able to utilise raw starch as carbon source. A summary of various strains cultivated in raw starch to date is presented in Table 2.6. All approaches to date utilise the *R. oryzae* glucoamylase, which is secreted or displayed on the surface of the yeast. Several different strains have been created where different α-amylases have been combined with the glucoamylase in the hope to improve the amylolytic activity and therefore the ethanol productivity of the generated strains.

The first group reporting on a raw starch fermenting yeast engineered a yeast strain which heterologously secreted the *Rhizopus* glucoamylase (Ashikari et al., 1989b; Ashikari et al., 1989a). Up to 51 g l^{-1} ethanol was produced at an ethanol yield of 0.20 g g^{-1}after 120 hours of fermentation using ground raw corn as carbon source (250 g l^{-1}). The volumetric ethanol productivity was 0.675 g l^{-1} h^{-1}.

In order to increase the ability of strains to convert starch to ethanol, α-amylase genes were introduced to assist the glucoamylase. The yeast developed to display glucoamylase from the cell surface by Murai et al. (1997) served as basis for the production of a yeast strain with high fermentation ability. The generated strain was able to grow on ground raw corn and produce ethanol at a concentration of 23.4 g l^{-1} at a yield of 0.13 g g^{-1}. The fermentation efficiency of this strain was, however, lower than that of strains secreting the glucoamylase. The fermentation efficiency was increased with the addition of a α-amylase preparation from *Bacillus licheniformis* to liquefy the starch material before fermentation (Murai et al., 1998).

Shigechi et al. (2004b) replaced the *B. stearothermophilus* α-amylase from a previously engineered strain with the α-amylase from *Streptococcus bovis*. The newly generated strain displayed the α-amylase together with the *R. oryzae* glucoamylase on the surface of the yeast and produced up to 61.8 g l^{-1} ethanol after 72 h of fermentation in raw starch medium where the total sugars corresponded to 200 g l^{-1}. An ethanol yield of 0.44 g g^{-1} sugar consumed was calculated. In these experiments, a very high cell load was used as inolculum to reach the high ethanol yields, as 100 g of wet weight cells were added per litre medium (corresponds to 15 g l^{-1} DW).

Khaw et al. (2006) used the yeast engineered to display the *R. oryzae* glucoamylase created by Murai et al. (1997) and either co-displayed (Shigechi et al., 2004b) or secreted a *Streptococcus bovis* α-amylase from either nonflocculent or flocculent yeast strains. The α-amylase was responsible for the rate-limiting factor of starch hydrolysis to oligosaccharides in all strains created, whether the strains secreted or displayed the enzyme from either flocculent or nonflocculent strains. The nonflocculent strain displaying the glucoamylase and secreting α-amylase produced up to 51 g l^{-1} ethanol in media containing 10% raw corn starch and 1% glucose. It showed a specific ethanol productivity of 0.18 g (g DW cells)$^{-1}$ h^{-1}, which was three-fold higher, than the nonflocculent strain displaying both the glucoamylase and α-amylase, or the flocculent strain displaying the glucoamylase and secreting the α-amylase (Khaw et al., 2006). Furthermore, the specific ethanol productivity was 4.5-fold higher than for the flocculent strain displaying both the glucoamylase and α-amylase. The ethanol yield for the flocculent and nonflocculent strains displaying the glucoamylase and secreting the α-amylase was similar (0.45 and 0.46 g g^{-1} respectively). Ethanol yields were lower for the flocculent and nonflocculent strains displaying both the glucoamylase and α-amylase (0.20 and 0.38 g g^{-1} respectively). It should be noted that the limiting factor presented by the displayed α-amylase may be lessened when contact between starch granules and yeast displaying the enzyme is increased (Khaw et al., 2007).

From these studies, it is evident that both glucoamylase and α-amylases are required for efficient raw starch hydrolysis. It is also apparent that strains secreting the enzymes perform better at producing ethanol than strains where the enzymes are displayed on the cell wall.

Table 2.6 Ethanol production by strains cultivated in raw starch.

Glucoamylase	α-amylase	Monomeric sugar equivalent used in medium	[Ethanol] [a]	Ethanol productivity [b] or specific productivity [c]	Ethanol yield [d]	Reference
Rhizopus enzyme secreted.	None	250 g l^{-1}	51 g l^{-1} after 120 h	0.675 g l^{-1} h^{-1}	0.20	(Ashikari et al., 1989b)
Rhizopus enzyme displayed on surface.	None	230 g l^{-1}	23.4 g l^{-1} after 168 h		0.13	(Murai et al., 1998)
Rhizopus enzyme displayed on surface.	*Streptococcus bovis* enzyme displayed on surface	200 g l^{-1}	61.8 g l^{-1} after 72 h	1.008 g l^{-1} h^{-1} 0.069 g (g DW cells)$^{-1}$ h^{-1}	0.44	(Shigechi et al., 2004b)
Rhizopus enzyme displayed on surface of nonflocculent strain.	*Streptococcus bovis* enzyme secreted	120 g l^{-1}	51 g l^{-1} after 60 h	1.283 g l^{-1} h^{-1} 0.18 g (g DW cells)$^{-1}$ h^{-1}	0.46	(Khaw et al., 2006)
Rhizopus enzyme displayed on surface of nonflocculent strain.	*Streptococcus bovis* enzyme displayed on surface	120 g l^{-1}	23 g l^{-1} after 60 h	0.305 g l^{-1} h^{-1} 0.06 g (g DW cells)$^{-1}$ h^{-1}	0.38	(Khaw et al., 2006)
Rhizopus enzyme displayed on surface of flocculent strain.	*Streptococcus bovis* enzyme secreted	120 g l^{-1}	24 g l^{-1} after 60 h	0.321 g l^{-1} h^{-1} 0.06 g (g DW cells)$^{-1}$ h^{-1}	0.45	(Khaw et al., 2006)
Rhizopus enzyme displayed on surface of flocculent strain	*Streptococcus bovis* enzyme displayed on surface	120 g l^{-1}	20 g l^{-1} after 60 h	0.208 g l^{-1} h^{-1} 0.04 g (g DW cells)$^{-1}$ h^{-1}	0.20	(Khaw et al., 2006)

[a] Ethanol concentration (g l^{-1}), [b] ethanol volumetric productivity (g l^{-1} h^{-1}), [c] ethanol specific productivity (g (g DW cells)$^{-1}$ h^{-1}), [d] ethanol yield as g (g consumed sugar)$^{-1}$. A blank space indicates that not enough data was presented to determine the value.

2.3.3 *Aspergillus* amylases in yeast

The glucoamylases from *A. awamori* and *A. oryzae* (*koji* mold), and the α-amylase from *A. awamori* hydrolyse raw starch (Hata et al., 1991b; Matsubara et al., 2004a and 2004b; Queiroz et al., 1997; Singh and Soni, 2001). The enzymes are industrially important in the production of saké (Japanese rice wine) and miso (Japanese seasoning) (Ueda, 1981; Yokotsuka and Sasaki, 1998; Fleet, 1998). The fungal strains *A. awamori* and *A. oryzae* produce both α-amylase and glucoamylase (Gines et al., 1989; Hata et al., 1991b; Tada et al., 1989), which have exhibited a synergistic effect during raw starch degradation (Abe et al., 1988; Ueda, 1981). All yeasts currently engineered to hydrolyse raw starch, however, utilise the *R. oryzae* glucoamylase and α-amylase from *S. bovis* (Khaw et al., 2006) or a α-amylase preparation from *B. licheniformis* (Murai et al., 1998). Utilising the amylase genes from *Aspergillus* for raw starch conversion in *S. cerevisiae* could therefore contribute to generating a novel CBP organism.

Two glucoamylases are produced by *A. oryzae* depending on growth conditions. The enzyme encoded by the *glaA* gene is produced in liquid/submerged culture, and has shown raw starch degrading activity, whereas the enzyme produced in solid-state culture degrades only soluble starch (Hata et al., 1997). The glucoamylase and α-amylase genes from *A. oryzae* and the glucoamylase from *A. awamori* have been expressed in *S. cerevisiae* (Hata et al., 1991a; Lin et al., 1998; Randez-Gil and Sanz, 1993). Some groups created polyploid strains and used the δ-integration system to increase heterologous protein production of the host strain (Ekino et al., 2002; Saito et al., 1996). The *A. awamori* α-amylase shown to hydrolyse raw starch (Matsubara et al., 2004a and 2004b) has not been expressed in *S. cerevisiae* yet. Neither have combinations of both glucoamylases and α-amylases from *Aspergillus* species.

It is believed that developing a biorefinery concept for biomass conversion with CBP organisms is a viable way to ensure a 'greener' future. The project aim for this study was therefore to engineer an amylolytic *S. cerevisiae* strain capable of utilising raw unmodified starch as sole carbon source for the production of bioethanol. Objectives were identified to realise the goal of this study, which include: (i) identify fungal amylolytic genes coding for raw starch hydrolysing enzymes appropriate for cloning into *S. cerevisiae*; (ii) engineer *S. cerevisiae* strains to express and secrete the identified amylolytic enzymes; (iii) demonstrate that functional amylolytic enzymes were secreted by the engineered yeast

strains; (iv) demonstrate that growth of the engineered yeast strains on the raw starch substrate is enabled; (v) quantify growth rates of a selected strain on raw starch versus soluble starch versus glucose; (vi) determine if ethanol is produced by the selected strain during anaerobic fermentation; and (vii) evaluate whether it would be beneficial to improve enzyme/microbe performance.

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CHAPTER 3: Engineered amylolytic yeast for bioethanol production

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Document prepared for submission to Applied Microbiology and Biotechnology.

Abstract

The cost associated with converting starch to ethanol is high, and the need to develop a more feasible process is evident. A raw starch hydrolysing and fermenting yeast could yield large cost reductions in Consolidated Bioprocessing (CBP), a one-step hydrolysis and fermentation procedure. The yeast *Saccharomyces cerevisiae* was engineered to secrete separate polypeptides of A*spergillus awamori* glucoamylase (GA I), *Aspergillus oryzae* glucoamylase (GLAA) and α-amylase (AMYL III) in different combinations. The GA I displayed an affinity (K_m) of 3.574 (μg ml^{-1}) and a maximum specific activity (V_{max}) of 11.604 towards raw corn starch. A specific raw starch hydrolysing activity of 140 nanokatals per gram dry weight cells (nkat (g DW cells)$^{-1}$) was produced by the best strain secreting the GA I and AMYL III proteins. During anaerobic cultivation, an ethanol yield of 0.40 gram ethanol per gram available sugar (g g^{-1}) was produced from raw starch (71% of theoretical maximum) at a specific ethanol productivity of 0.037 gram ethanol per gram dry weight cells per hour (g (g DW cells)$^{-1}$ h^{-1}). Preliminary serum bottle fermentations indicate that the recombinant strain is a promising raw starch converter when compared to previously generated strains.

Introduction

The commercial practice of converting starch to ethanol by an enzymatic process is a fairly mature technology (Gray et al., 2006). The energy cost of converting corn to ethanol is high, and as the commercial conversion process is wide spread, the need to develop a more energy-efficient process is evident. A single step process where production of hydrolytic enzymes to hydrolyse raw starch and fermentation of the resulting sugars could be accomplished via an amylolytic microorganism or consortium of organisms, and thereby yield large cost reductions and improve the energy balance for starch conversion (Lynd et al., 2002).

Benefits of using raw starch hydrolysing enzymes include (i) a decrease in required heating energy, (ii) a reduction in osmotic stress during fermentation or a reduction in by-product formation, (iii) a reduction in yeast growth inhibitors such as high concentrations of glucose and products formed through Maillard reactions, (iv) an increase in capacity as a higher density fermentation leads to higher ethanol yields, (v) an increase in the quality of distiller's dried grains and solubles (DDGS) as a result of higher protein content, and (vi) a saving in capital cost as capacity increases or when a new plant is built without additional heating equipment.

Several strains have been engineered in the last 20 years which have proven successful in converting soluble or cooked starch to ethanol (Birol et al., 1998; de Moraes et al., 1995; Eksteen et

al., 2003; Inlow et al., 1987; Knox et al., 2004; Kondo et al., 2002; Ma et al., 2000; Murai et al., 1997; Nakamura et al., 1997; Shigechi et al., 2004a; Ülgen et al., 2002). Very few groups have reported results on yeast strains that are able to utilise raw starch as carbon source. All approaches to date utilise the *Rhizopus oryzae* glucoamylase, which is secreted or displayed on the surface of the yeast (Ashikari et al., 1989; Murai et al., 1998). Several different strains have been created where the α-amylases from *Streptococcus bovis* has been combined with the *R. oryzae* glucoamylase in the hope to improve the amylolytic activity and therefore the ethanol productivity of the generated strains (Khaw et al., 2006; Shigechi et al., 2004b).

The glucoamylase and α-amylase genes from *A. oryzae* and the glucoamylase from *A. awamori* have been expressed in *S. cerevisiae* (Ekino et al., 2002; Hata et al., 1991; Lin et al., 1998; Randez-Gil and Sanz, 1993; Saito et al., 1996). The glucoamylases and α-amylases are characterised by their synergistic action during starch hydrolysis (Abe et al., 1988; Ueda, 1981). The *A. awamori* α-amylase shown to hydrolyse raw starch (Matsubara et al., 2004a; Matsubara et al., 2004b) has not been expressed in *S. cerevisiae* yet, neither has combinations of both the glucoamylases and α-amylases from *Aspergillus* species. Utilising combinations of glucoamylase and α-amylase genes from *Aspergillus* for raw starch conversion in *S. cerevisiae* could therefore contribute to generating a novel CBP organism.

In the present investigation, we have engineered *Saccharomyces cerevisiae* strains that secrete *Aspergillus* raw starch hydrolysing enzymes to warrant utilisation of raw starch as sole carbon source for the production of bioethanol. Thereby the conversion of raw starch to ethanol in a one-step process could be demonstrated.

Materials and methods

Strains and media

All chemicals, media components and supplements were of analytical grade standard. The genotypes and sources of the plasmids, yeast and bacterial strains used in the experiments are summarised in Table 1. Recombinant plasmids were constructed and amplified in *Escherichia coli* XL1-Blue. The bacterial strains were cultivated at 37°C on a rotating wheel in Terrific Broth or on Luria-Bertani agar (Sambrook et al., 1989). Ampicillin for selection of resistant bacteria was added to a final concentration of 100 μg ml^{-1}. Fungal strains were cultivated at 30°C on a rotary shaker set at 100 rpm. *Aspergillus* strains were cultivated in maltodextrin medium (5% maltodextrin, 0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl, and trace elements). The fungal strains were maintained at 30°C on minimal medium (1% glucose, 0.6% $NaNO_3$, 0.2% neopeptone,

0.15% KH_2PO_4, 0.1% yeast extract, 0.1% casamino acids, 0.05% $MgSO_4$, 0.05% KCl, 2% agar and trace elements). Strains of *S. cerevisiae* were cultivated in either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or selective complete medium (SC) (2% glucose, and 0.17% yeast nitrogen base without amino acids) with additional growth factors and amino acids as necessary (without uracil (SC^{-ura}) or leucine (SC^{-leu})) at 30°C on a rotary shaker set at 100 rpm unless otherwise stated.

Table 1 Summary of plasmids and strains used in this study.

Plasmids/Strains	Relevant genotype	Source or Reference
pDRIVE	*bla*	QIAGEN (Valencia, CA, USA)
pAZ1	*bla URA3 ENO1$_P$-XYN2-ENO1$_T$*	This laboratory
pBS-XYNSEC	*bla XYNSEC*	(Den Haan et al., 2007)
yXYNSEC	*bla URA3 PGK1$_{PT}$*	(van Rooyen et al., 2005)
ySFI	*bla URA3 PGK1$_P$-BGL1-PGK$_T$*	(van Rooyen et al., 2005)
E. coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F'proAB lacq ZΔM15 Tn10(tet)]*	Stratagene (La Jolla, CA, USA)
S. cerevisiae Y294	*α leu2-3, 112 ura3-52 his3 trp1-289*	ATCC 201160
A. awamori	*Wild type strain*	CBS 115.52
A. oryzae var. *oryzae*	*Wild type strain*	CBS 819.72

DNA manipulations

Standard protocols were followed for DNA manipulation (Sambrook et al., 1989). Restriction endonucleases and T_4 DNA polymerase were supplied by either Roche diagnostics (Randburg, South Africa) or Fermentas Inc. (Maryland, USA). DNA fragments were eluted from agarose gels using phenol (Benson, 1984).

PCR amplification

Sequence specific primers were designed for the *Trichoderma reesei* xylanase 2 secretion signal (*XYNSEC*) (xynsec-L, xynsec-R2), *A. oryzae* glucoamylase (*glaA*) (Aoryglu-left, Aoryglu-right), *A. awamori* α-amylase (*amyl III*) (Aawaamy-left, Aawaamy-right), and *A. awamori* glucoamylase (*GA I*) (Aawagluc-L, Aawagluc-R) (Table 2). The primers were designed to exclude the native signal peptides of the amylase genes. The PCR reaction mixture was as follows: 200 ng template, 100 pmol of each primer, 0.2 mM each of deoxynucleoside triphosphate, reaction buffer supplied by the manufacturer, and *TaKaRa Ex Taq*^{TM} (Takara Bio Inc., Shiya, Japan). Total RNA was

isolated from *A. oryzae* and *A. awamori* after cultivation in maltodextrin medium (Plüddemann and van Zyl, 2003). mRNA was isolated from total RNA using the Fast Track mRNA isolation kit (Invitrogen Corporation, Carlsbad, CA, USA). A first strand cDNA mix was amplified from the mRNA and used as template for the amylase gene sequence PCR.

Table 2 Summary of primers. Gene origin and Genbank accession numbers are listed.

Gene (Genbank)	**Organism**	**PCR primer sequence**
XYNSEC	*T. reesei*	**Xynsec-L:** 5'-CTGAATTCAGGCCTCAACATGGTCTCCTTCACC-3'
(QM6a)		**Xynsec-R2:** 5'-AGATCTTTTAAATACGTATCGCGAGCGCTTCTCCACAGCC-3'
glaA	*A. oryzae*	**Aoryglu-left:** 5'-GATATCTACGTACAACCTGTCCTTAGACAGG-3'
(D10698)		**Aoryglu-right:** 5'-TCGCGACTCGAGTTACCGCCAAACATCGC-3'
GA I	*A. awamori*	**Aawagluc-L:** 5'—AGGCCTTACGTAACCTTGGATTCGTGGTTG-3'
(AB083161)		**Aawagluc-R:** 5'-AAGCTTCTCGAGTTACCGCCAGGTGTCAGT-3'
		Syngluc-L: 5'-CCCATCTGGTGACTTGTCT-3'
		Syngluc-R: 5'-ACCGGTGGTAGTAGCAGTAG-3'
amyl III	*A. awamori*	**Aawaamy-left:** 5'-AGGCCTTACGTACTGTCAGCTGCAGAATGG-3'
(AB083160)		**Aawaamy-right:** 5'-GGATCCAGATCTTTACCTCCACGTATCAACCA-3'
		Synalpha-L: 5'-GCTGAATGGAGAACTCAATC-3'
		Synalpha-R: 5'-TAGAGGTGGCAGTACAGGAG-3'
FUR1	*S. cerevisiae*	**FUR1-left:** 5'-ATTTCTTCTTGAACCATGAAC-3'
		FUR1-right: 5'-CTTAATCAAGACTTCTGTAGCC-3'
ENO1	*S. cerevisiae*	**ENO1-L:** 5'-GGATCCACTAGTCTTCTAGGCGGGTTATC-3'
		ENO1-R: 5'-AAGCTTGCGGCCGCAAAGAGGTTTAGACATTGG-3'
PGK1	*S. cerevisiae*	**PGKbeginprom:** 5'-ACTGAAGCTTGGATCCTTAAAGATGCCG-3'
		PGKendterm: 5'-ACTGAAGCTTGGCCAAGCTTTAACGAAC-3'
		PGK1-left: 5'-CGGGATCCTTAAAGATGCCGATTTGG-3'
		PGK1-right: 5'-CGGAATTCTATTTGTTGTAAAAAGTAGATAATTAC-3'

Restriction enzyme sites are underlined

Design of codon optimised synthetic gene sequences

Sequences of the *A. awamori GA I* and *A. awamori amyl III* were used as template for design of sequences using only codons that are favoured by *S. cerevisiae* (Sharp and Cowe, 1991). The codon adaptation index (CAI) for the wild type *GA I* and *amyl III* genes when expressed in *S. cerevisiae*, was calculated as 0.115 and 0.132 respectively (Sharp and Li, 1987). The optimised *GA I* and *amyl III* genes had CAI values of 0.921 and 0.923 respectively. A codon optimised version of the *T. reesei XYNSEC* was designed and used as secretion signal. All endonuclease restriction sites used frequently for cloning procedures in our laboratory were removed from within the designed genes

(*BamH*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Nru*I, *Sac*I, *Spe*I, *Stu*I and *Xho*I), and specific sites necessary for cloning purposes were attached to the 5'- (*Stu*I and *Eco*RI) and 3'-ends (*Xho*I and *Bgl*II) of the sequence. The designed sequences were used as template to synthetically produce the optimised genes (GenScript Corporation).

Construction of plasmids for secretion of glucoamylase and α-amylase

The 132-bp PCR amplified *XYNSEC* sequence fragment (amplified using the pBS-XYNSEC plasmid as template) was ligated into the commercial pDRIVE plasmid, and designated pDxynsec5. The yeast expression plasmid, designated yEXC2 was constructed as follows: the pAZ1 *Eco*RI-*Bgl*II *T. reesei xyn2* fragment was replaced with the *Eco*RI-*Bgl*II *XYNSEC* fragment from pDxynsec5 (Fig. 1). Glucoamylase and α-amylase gene sequence fragments obtained by PCR were cloned into commercial plasmid pDRIVE. Sequences were verified as described elsewhere.

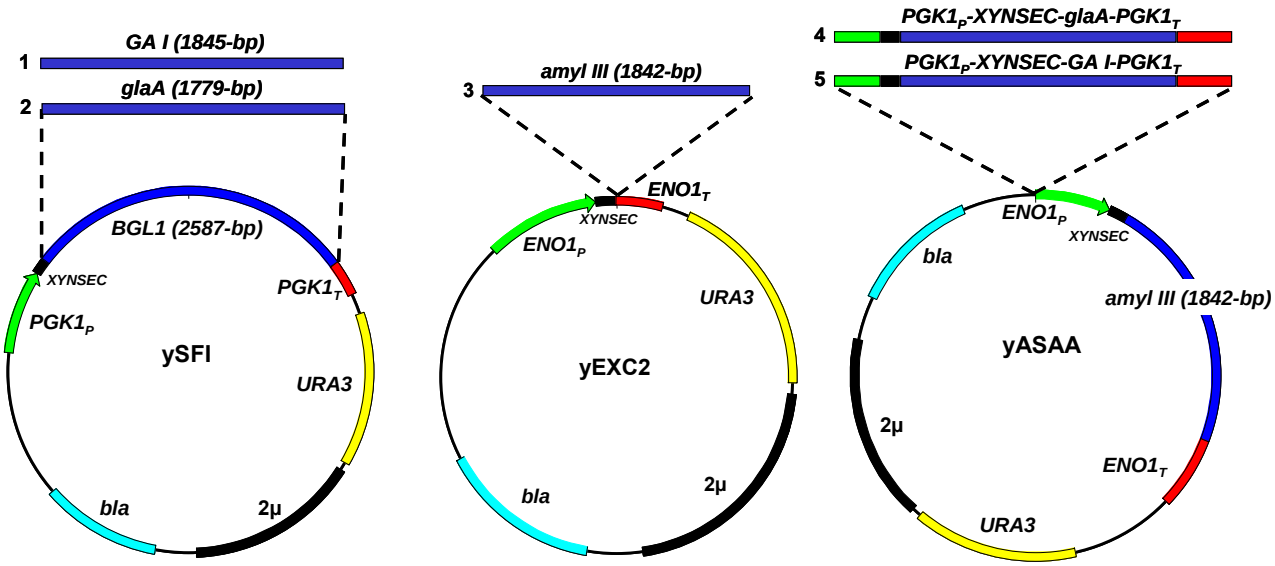

Fig. 1 A schematic summary of the plasmids used to generate amylase expression cassettes in this study. The *BGL*1 gene from ySFI was replaced with *A. awamori GA I* (1) or *A. oryzae glaA* (2) in frame with *XYNSEC*. *A. awamori amyl III* (3) was inserted in frame with *XYNSEC* in yEXC2. The *A. oryzae glaA* (4) or *A. awamori GA I* (5) *PGK1* promoter and terminator cassettes were inserted into yASAA. The synthetic codon optimised constructs were made similarly. The *S. cerevisiae* 2 micron autonomous replicating sequence (*ARS2*) is responsible for episomal replication of the plasmid and the bacterial β-lactamase (*bla*), and *S. cerevisiae* orotidine-5'-phosphate decarboxylase (*URA3*) were used as selectable markers.

The gene fragments were sub-cloned into yeast expression plasmids as follows: The *Nru*I-*Xho*I β-glucosidase (*BGL1*) fragment of ySFI (Fig. 1) was replaced with either the *SnaB*I-*Xho*I *glaA* fragment from pDOgluc13, or the *Stu*I-*Xho*I *GA I* fragment from pDawagluc1. The resulting plasmids were designated yASOG and yASAG, respectively. The *Eco*RI-*Xho*I *XYNSEC-GA I* fragment of yASAG was replaced with the synthetic codon optimised *Eco*RI-*Xho*I s*XYNSEC*-s*GA I* fragment to yield plasmid ySYAG. The *SnaB*I-*Xho*I *amyl III* fragment from pDAawa2alpha was ligated in frame with the *XYNSEC* in yEXC2 (plasmid digested with *Nru*I and *Xho*I), resulting in plasmid yASAA. The *Eco*RI-*Xho*I *XYNSEC-amyl III* fragment of yASAA was replaced with the synthetic codon optimised *Eco*RI-*Xho*I s*XYNSEC*-s*amyl III* fragment to yield plasmid ySYAA. The *PGK1*$_P$-*XYNSEC-glaA-PGK1*$_T$ construct was removed from yASOG with *Kpn*I and *Hind*III. The recessed 3' *Hind*III site was filled with Klenow fragment and the construct was then sub-cloned into yASAA (plasmid digested with *Kpn*I and *BamH*I, recessed 3' *BamH*I site filled with Klenow fragment) to generate plasmid yOGAA. The *PGK1*$_P$-*XYNSEC-GA I-PGK1*$_T$ cassette was removed from yASAG by digesting with *Hind*III and filling the recessed 3' terminus with Klenow fragment, followed by a partial digestion with *Kpn*I to release the cassette from the plasmid. The cassette was then subcloned into yASAA (plasmid digested with *Kpn*I and *BamH*I, recessed 3' *BamH*I site filled with Klenow fragment) to generate plasmid yAGAA. In a similar way, the codon optimised *PGK1*$_P$-*sXYNSEC-sGA I-PGK1*$_T$ cassette was inserted into ySYAA to yield ySYAGAA. Also, the *Eco*RI-*Xho*I *XYNSEC-GA I* fragment of yASAG was inserted into ySYAA to yield, yASAGSYAA.

DNA sequencing

The nucleotide sequences and open reading frames of the amylase fragments were determined with the dideoxy chain termination method using fluorescently labelled nucleotides on an ABI PRISMTM 3100 Genetic analyser. Sequence fragments were assembled manually in a word processing program. Sequence data was analysed with the PC based BLAST program (www.ncbi.nih.gov/BLAST) and protein sequences and restriction sites predicted and identified with the DNAMAN (version 4.1) software package (Lynnon Biosoft). Primer sequences are listed in Table 2.

Yeast transformation

The *S. cerevisiae* strain Y294 was transformed with the individual recombinant yeast expression plasmids using the dimethyl sulfoxide-lithium acetate method (Hill et al., 1991). Transformants were confirmed to be *S. cerevisiae* strains with PCR using *PGK1*, *ENO1* or *FUR1* sequence specific

primers (Table 2). The presence of amylase genes in transformants was confirmed with PCR using gene specific primers (Table 2). Disruption of the uracil phosphoribosyltransferase (*FUR*1) gene in the *S. cerevisiae* transformants containing episomal plasmids was performed to ensure autoselection of the *URA3*-bearing expression plasmids in non-selective medium (Kern et al., 1990). Autoselective transformants (*fur1::LEU2*) were screened for on selection agar deficient in uracil and leucine ($SC^{-ura-leu}$) and confirmed by PCR (Table 2).

Enzyme assays

Raw starch hydrolysis by transformants was tested on raw starch agar (2% raw corn starch [Sigma], 2% peptone, and 0.1% glucose). Yeast cells were spotted onto the agar and incubated at 30°C for 4 days. The plates were stained afterwards with an iodine solution (3% KI, 0.3% iodine) to visualise clear hydrolysis zones.

Supernatant harvested from cultures cultivated in 250 ml baffled Erlenmeyer flasks containing 100 ml YPD medium for 3 days served as amylase source for liquid assays and was concentrated twenty times by freeze-drying. All buffers used in determining enzyme activity had a pH of 5.4 unless otherwise mentioned; it was previously determined that the yeast laboratory strain used preferred fermentation at pH 5.4-5.5 and 30°C.

Total amylase activity on soluble starch was determined in liquid assays using the DNS method (Miller, 1959). The substrate used was 0.1% soluble potato starch in citrate-phosphate buffer. Sodium azide (NaN_3) was added to a final concentration of 0.02% to inhibit microbial growth. The hydrolysing reaction was carried out at 30°C for 30 min. and the reducing glucose units produced were expressed as nanokatals per gram dry weight biomass (nkat (g DW cells)$^{-1}$), which is defined as the enzyme activity needed to produce 1 nmol of glucose equivalents per second under the given assay conditions.

Amylase activity on raw starch was determined using a modified version of the DNS method. The substrate used was 2% raw corn starch in citrate-phosphate buffer. NaN_3 (0.02%) was added to inhibit microbial growth. The hydrolysing reaction was carried out at 30°C for 90 min. The hydrolysis reaction was terminated after addition of DNS reagent (1% 3,5-dinitro-salicyclic acid, 20% potassium sodium tartrate, 1% NaOH, 0.2% phenol, 0.05% Na_2SO_3) and boiled at 100°C for 15 min. The cooled reaction solution was centrifuged at 5000 rpm for 5 min. to remove the retrograded starch gel. The reducing glucose units produced were expressed as nkat (g DW cells)$^{-1}$.

Optimum temperature for raw starch hydrolysis by glucoamylase on raw starch at pH 5.4 was determined using an adapted protocol (De Mot and Verachtert, 1985). One hundred micro litres of enzyme preparation (enzyme diluted in citrate-phosphate buffer with 20 mM $CaCl_2$) was incubated

with 2% raw corn starch in 0.5 ml citrate-phosphate buffer at temperatures 30, 40, 50, 60, and 65°C for 60 min. NaN_3 (0.02%) was added to inhibit microbial growth. The hydrolysis reaction was terminated by boiling in a water bath for 5 min. Glucose in a cooled sample was determined using the peroxidase-glucose oxidase method from a glucose assay kit [Sigma]. One glucoamylase unit of activity (U) is defined as the amount of enzyme producing 1 μmol of glucose per minute under the specified conditions.

α-Amylase activity was determined using the Red Starch assay from Megazyme. Activity was expressed as Ceralpha Units per gram dry weight biomass (CU (g DW cells)$^{-1}$). The optimum temperature for α-amylase hydrolysis at pH 5.4 was determined by applying the enzyme to the red starch substrate at temperatures 30, 40, 50, 60, and 70°C. The preferred pH for α-amylase hydrolysis at 30°C was also determined by diluting enzyme in buffers with pH 4.0, 4.5, 5.0, 5.5, and 6.0.

To determine the specific activity of the GA I protein, an extracellular protein fraction of recombinant *A. awamori* glucoamylase was purified from a 4 day old YPD culture (3 litres) inoculated with Y294[yASAG]. The culture was centrifuged, then filtered and concentrated ten-fold in the Diaflo Ultrafilter (Amicon Inc., Beverly, MA, USA). Recombinant glucoamylase supernatant (50 ml) was bound to 30% (w/v) raw corn starch by stirring for 18 hours, and recovered via centrifugation (10 min., 13,000 rpm). The glucoamylase was removed from the starch by stirring in 50 ml 1% (v/v) triethylamine and subsequent centrifugation (10 min., 13,000 rpm). The supernatant was concentrated to 5 ml in the Diaflo Ultrafilter PM10 concentrator and dialysed with citrate-phosphate buffer (pH 4.0). The entire process was performed at 4-8°C.

Kinetic parameters were determined for the purified recombinant GA I. Up to four different raw starch (1, 2, 5, and 10% K_m) and maltose concentrations (0.2, 0.6, 1.0 and 1.5 mM K_m) were used for rate hydrolysis. Glucose liberated as a result of hydrolysis of the substrate by the enzyme over time was determined using the peroxidase-glucose oxidase method from a glucose assay kit [Sigma].

Electrophoresis and Zymogram analysis

Recombinant enzymes were characterised by running the protein fractions on 7.5% acrylamide gels (7.5% acrylamide, 1.5 mM Tris (pH 8.8), 10% SDS, 10% ammonium persulfate and 0.1% TEMED) with 0.1% soluble starch for SDS-PAGE and overlay Zymogram analysis. The 5% stacking gel contained 30% acrylamide, 1.5 mM Tris (pH 6.8), 10% SDS, 10% ammonium persulfate and TEMED. Loading buffer contained 60 mM Tris-HCL (pH 6.8), 25% glycerol, 2% SDS, 14 mM β-mercaptoethanol, and 0.1% bromophenol blue. The proteins were either left untreated (no

boiling), denatured by boiling for 10 min. or de-N-glycosylated with PNGaseF (Biolabs Inc., Merck, Wadeville, South Africa) and then loaded onto the gels. SDS-PAGE was carried out at 4°C and 150V for 90 min. in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). Gels were washed with citrate-phosphate buffer (pH 4.5) for 30 min. at room temperature with gentle agitation to remove SDS. Gels were stained with Coomassie Brilliant Blue R250 in methanol, and de-stained with 10% acetic acid. For overlay gels, the de-stained gels were stained with an iodine solution (3% KI, 0.3% iodine) for 5 min.

Anaerobic cultivation

Recombinant amylolytic yeast strains were cultivated in raw starch medium (RSYP) (2% raw corn starch [Sigma], 0.67% yeast nitrogen base with amino acids [Difco], 2% peptone, and 0.05% glucose), soluble starch medium and glucose medium where the equivalent amount of raw starch was replaced with either soluble starch or glucose. The raw starch was sterilised with ethanol and dried at 30°C overnight before adding to autoclaved medium. To prevent flocculation of yeast, 1 mM Aspirin (Sigma) was added to the raw starch before ethanol sterilisation (Strauss, 2005). Streptomycin (0.5 g l^{-1}) was added to prevent bacterial contamination under non-sterile raw starch conditions. Pre-cultures of Y294[yxynsec] and Y294[yAGAA] grown to stationary phase in YPD medium were used as inoculum. Cells were washed with a salt solution (0.9% NaCl) to prevent medium and enzyme carry-over.

The cells were inoculated at 10% v/v (±0.3 g l^{-1}) in quadruplicate experiments using 120 ml glass serum bottles sealed with rubber stoppers containing 100 ml medium supplemented with 0.01 g l^{-1} ergosterol and 0.42 g l^{-1} Tween 80 (Yu et al., 1995). The contents of the serum bottles were mixed on a magnetic stirrer at 30°C. Samples were taken through a capped syringe needle pierced through the bottle stopper. Samples were periodically taken and yeast cells in the media were counted in duplicate on a haemocytometer for raw starch and soluble starch fermentations. The OD_{600} was determined for cells grown in glucose.

Analytical methods

Aerobic growth on glucose (YPD medium) was measured in triplicate in shake flask cultures as absorbance at OD_{600}. A calibration chart was prepared to correlate dry weights (DWs) with optical densities at OD_{600} as well as cell counts determined using a haemocytometer. DWs were determined from 10 ml culture samples. Cells were collected on glass filters after filtration, washed several

times with deionised sterile water, and dried in a microwave to constant weight (approximately 15 min. at 30% power).

Residual fermentable sugars present during anaerobic cultivations were determined in duplicate for each culture with the phenol-sulphuric method using glucose to create a linear standard (Dubois et al., 1956). Maltose, glycerol, acetate and ethanol concentrations were determined by high performance liquid chromatography (HPLC), with a Waters 717 injector (Milford, MA, USA) and Agilent 1100 pump (PaloAlto). The compounds were separated on an Aminex HPX-87H column (Bio-Rad Inc., CA, USA) at a column temperature of 45°C with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml $min.^{-1}$ and subsequently detected with a Waters 410 refractive index detector.

Calculations

Maximum specific growth rates were calculated at specific time points on a growth curve in YPD and glucose medium as ln OD_{600} versus time (h^{-1}). In soluble starch and RSYP medium the g DW cells ml^{-1} was converted to ln OD_{600} versus time (h^{-1}). A minimum of four adjacent points was used to calculate the value at a particular point on the curve during exponential growth phase.

Results

Plasmid construction and amylolytic yeast strain generation

Strains of *A. awamori* and *A. oryzae* were cultivated in maltodextrin medium, mRNA isolated and used for first strand cDNA synthesis. The *glaA* gene of *A. oryzae*, *GA I* gene of *A. awamori*, and *amyl III* gene of *A. awamori* were amplified by PCR from the cDNA and cloned into commercial plasmid pDRIVE before sub-cloning into yeast expression plasmids (Fig. 1 and Table 3). The glucoamylase and α-amylase genes were inserted in frame with the *XYNSEC* secretion signal for constitutive expression under the transcriptional control of the *S. cerevisiae PGK1* and *ENO1* promoters and terminators respectively. The resulting recombinant plasmids were transformed into *S. cerevisiae* Y294 (Table 4). A reference strain was constructed by transforming *S. cerevisiae* Y294 with a plasmid containing the *PGK1* promoter and terminator, as well as the *T. reesei* secretion signal, but without an amylolytic gene. The strains constructed to harbour episomal plasmids were uracil (Ura^+) prototrophic. Genomic DNA isolated from the transformants served as template for PCR to confirm the presence of recombinant genes (data not shown). Autoselective strains were generated by *FUR1* disruption and confirmed by PCR (data not shown).

Table 3 Summary of plasmids generated in this study.

Plasmids	Relevant genotype
pDOgluc13	*bla glaA*
pDawagluc1	*bla GA I*
pDAawa2alpha	*bla amyl III*
yXYNSEC	*bla URA3 PGK1$_{PT}$*
yASOG	*bla URA3 PGK1$_P$-XYNSEC-glaA-PGK1$_T$*
yASAG	*bla URA3 PGK1$_P$-XYNSEC-GA I-PGK1$_T$*
yASAA	*bla URA3 ENO1$_P$-XYNSEC-amyl III-ENO1$_T$*
yOGAA	*bla URA3 PGK1$_P$-XYNSEC-glaA-PGK1$_T$ ENO1$_P$-XYNSEC-amyl III-ENO1$_T$*
yAGAA	*bla URA3 PGK1$_P$-XYNSEC-GA I-PGK1$_T$ ENO1$_P$-XYNSEC-amyl III-ENO1$_T$*
ySYAG	*bla URA3 PGK1$_P$-sXYNSEC-sGA I-PGK1$_T$*
ySYAA	*bla URA3 ENO1$_P$-sXYNSEC-samyl III-ENO1$_T$*
ySYAGAA	*bla URA3 PGK1$_P$-sXYNSEC-sGA I-PGK1$_T$ ENO1$_P$-sXYNSEC-samyl III-ENO1$_T$*
yASAGSYAA	*bla URA3 PGK1$_P$-XYNSEC-GA I-PGK1$_T$ ENO1$_P$-sXYNSEC-samyl III-ENO1$_T$*

Table 4 Summary of recombinant strains generated in this study.

Strains	Strain designation	Amylase/s secreted
Y294[*fur1::LEU2* yXYNSEC]	Y294[yXYNSEC]	none
Y294[*fur1::LEU2* yASOG]	Y294[yASOG]	GLAA
Y294[*fur1::LEU2* yASAG]	Y294[yASAG]	GA I
Y294[*fur1::LEU2* yASAA]	Y294[yASAA]	AMYL III
Y294[*fur1::LEU2* yOGAA]	Y294[yOGAA]	GLAA and AMYL III
Y294[*fur1::LEU2* yAGAA]	Y294[yAGAA]	GA I and AMYL III
Y294[*fur1::LEU2* ySYAG]	Y294[ySYAG]	sGA I
Y294[*fur1::LEU2* ySYAA]	Y294[ySYAA]	sAMYL III
Y294[*fur1::LEU2* ySYAGAA]	Y294[ySYAGAA]	sGA I and sAMYL III
Y294[*fur1::LEU2* yASAGSYAA]	Y294[yASAGSYAA]	GA I and sAMYL III

Sequence analysis

Open reading frames for all the amylolytic genes were confirmed by sequencing. Furthermore, predicted protein sequences were analysed for previously reported conserved regions and specific amino acids with specific functions in the genes (Fig. 2 and 3). The sGA I protein sequence conformed to all sites (Fig. 2), including the minimal sequence identified around the W-597 (W-562 in mature protein (MP)) found to be essential for hydrolysis of raw starch (PLWYVTVTLPA) (Goto et al., 1994). The second conserved W-624 (W-590 in MP) residue involved in tighter binding and preparing the substrate for catalysis was also identified (Sorimachi et al., 1997).

General acid and base catalysts E-213 (E-179 in MP) and E-434 (E-400 in MP) (Frandsen et al., 1994; Harris et al., 1993) as well as the Y-85, W-87, R-89, D-90, W-154, E-214, R-339, D-343, and W-351 residues that play a role in substrate transition-state stabilisation and/or ground-state binding were conserved (Fierobe et al., 1996; Frandsen et al., 1995). The Gp-I region, which is heavily O-glycosylated and TS rich, was also identified. The glycosylation is responsible for enzyme stability and enhanced activity on raw starch (Goto et al., 1995 and 1999). The Gp-I region is crucial for correct folding of the enzyme (Goto et al., 2004).

```
1              GAATTCCAACATGGTTTCCTTCACTTCTTTGTTGGCCGGTGTCGCTGCCATTTCTGGTGT
1                       M  V  S  F  T  S  L  L  A  G  V  A  A  I  S  G  V

61             CTTGGCTGCTCCAGCTGCTGAAGTTGAACCAGTTGCCGTCGAAAAGAGAACTTTGGATTC
21              L  A  A  P  A  A  E  V  E  P  V  A  V  E  K  R  T  L  D  S

121            CTGGTTGTCTAACGAAGCCACTGTTGCTAGAACTGCTATTTTGAACAACATCGGTGCCGA
41              W  L  S  N  E  A  T  V  A  R  T  A  I  L  N  N  I  G  A  D

181            CGGTGCTTGGGTTTCCGGTGCTGACTCCGGTATTGTTGTTGCTTCCCCATCCACCGATAA
61              G  A  W  V  S  G  A  D  S  G  I  V  V  A  S  P  S  T  D  N

241            CCCAGACTACTTCTACACCTGGACTAGAGACTCCGGTTTGGTCATTAAGACTTTGGTTGA
81              P  D  Y  F  Y  T  W  T  R  D  S  G  L  V  I  K  T  L  V  D

301            CTTGTTCAGAAACGGTGATACTGACTTGTTGTCTACCATTGAACACTACATTTCTTCCCA
101             L  F  R  N  G  D  T  D  L  L  S  T  I  E  H  Y  I  S  S  Q

361            AGCTATCATCCAAGGTGTTTCCAACCCATCTGGTGACTTGTCTTCCGGTGGTTTGGGTGA
121             A  I  I  Q  G  V  S  N  P  S  G  D  L  S  S  G  G  L  G  E

421            ACCAAAGTTCAACGTCGATGAAACCGCTTACACTGGTTCTTGGGGTAGACCACAAAGAGA
141             P  K  F  N  V  D  E  T  A  Y  T  G  S  W  G  R  P  Q  R  D

481            TGGTCCAGCCTTGAGAGCTACTGCCATGATCGGTTTCGGTCAATGGTTGTTGGATAACGG
161             G  P  A  L  R  A  T  A  M  I  G  F  G  Q  W  L  L  D  N  G

541            TTACACTTCCGCTGCTACCGAAATCGTTTGGCCATTGGTTAGAAACGACTTGTCCTACGT
181             Y  T  S  A  A  T  E  I  V  W  P  L  V  R  N  D  L  S  Y  V

601            CGCTCAATACTGGAACCAAACCGGTTACGACTTGTGGGAAGAAGTTAACGGTTCTTCTTT
201             A  Q  Y  W  N  Q  T  G  Y  D  L  W  E  E  V  N  G  S  S  F
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661          CTTCACCATCGCCGTCCAACACAGAGCCTTGGTTGAAGGTTCCGCTTTCGCTACCGCTGT
221           F  T  I  A  V  Q  H  R  A  L  V  E  G  S  A  F  A  T  A  V

721          CGGTTCCTCTTGTTCCTGGTGTGATTCTCAAGCTCCACAAATCTTGTGTTACTTGCAATC
241           G  S  S  C  S  W  C  D  S  Q  A  P  Q  I  L  C  Y  L  Q  S

781          TTTCTGGACCGGTTCTTACATCTTGGCTAACTTCGATTCCTCTAGATCCGGTAAGGACAC
261           F  W  T  G  S  Y  I  L  A  N  F  D  S  S  R  S  G  K  D  T

841          CAACACTTTGTTGGGTTCTATCCACACCTTCGATCCAGAAGCTGGTTGTGACGACTCTAC
281           N  T  L  L  G  S  I  H  T  F  D  P  E  A  G  C  D  D  S  T

901          TTTCCAACCATGTTCTCCAAGAGCTTTGGCTAACCACAAGGAAGTCGTTGACTCTTTCAG
301           F  Q  P  C  S  P  R  A  L  A  N  H  K  E  V  V  D  S  F  R

961          ATCCATCTACACCTTGAACGACGGTTTGTCCGATTCTGAAGCTGTTGCTGTCGGTAGATA
321           S  I  Y  T  L  N  D  G  L  S  D  S  E  A  V  A  V  G  R  Y

1021         CCCAGAAGATTCCTACTACAACGGTAACCCATGGTTCTTGTGTACTTTGGCTGCTGCTGA
341           P  E  D  S  Y  Y  N  G  N  P  W  F  L  C  T  L  A  A  A  E

1081         ACAATTGTACGACGCTTTGTACCAATGGGATAAGCAAGGTTCCTTGGAAATTACTGACGT
361           Q  L  Y  D  A  L  Y  Q  W  D  K  Q  G  S  L  E  I  T  D  V

1141         CTCCTTGGACTTCTTCAAGGCTTTGTACTCTGGTGCTGCTACTGGTACTTACTCCTCTTC
381           S  L  D  F  F  K  A  L  Y  S  G  A  A  T  G  T  Y  S  S  S

1201         CTCTTCTACCTACTCCTCCATTGTTTCCGCTGTTAAGACCTTCGCTGATGGTTTCGTTTC
401           S  S  T  Y  S  S  I  V  S  A  V  K  T  F  A  D  G  F  V  S

1261         TATCGTCGAAACCCACGCTGCTTCCAACGGTTCCTTGTCCGAACAATTCGACAAGTCTGA
421           I  V  E  T  H  A  A  S  N  G  S  L  S  E  Q  F  D  K  S  D

1321         CGGTGATGAATTGTCTGCTAGAGACTTGACCTGGTCTTACGCTGCTTTGTTGACCGCTAA
441           G  D  E  L  S  A  R  D  L  T  W  S  Y  A  A  L  L  T  A  N

1381         CAACAGAAGAAACTCTGTTGTTCCACCATCTTGGGGTGAAACTTCCGCTTCTTCCGTTCC
461           N  R  R  N  S  V  V  P  P  S  W  G  E  T  S  A  S  S  V  P

1441         AGGTACTTGTGCTGCCACTTCTGCTTCCGGTACTTACTCTTCCGTCACTGTTACCTCCTG
481           G  T  C  A  A  T  S  A  S  G  T  Y  S  S  V  T  V  T  S  W
```

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1501        GCCATCCATCGTCGCTACCGGTGGTACTACCACTACTGCTACTACCACCGGTTCTGGTGG
501          P  S  I  V  A  T  G  G  T  T  T  T  A  T  T  T  G  S  G  G

1561        TGTCACCTCCACTTCCAAGACCACCACCACTGCTTCTAAGACCTCCACCACTACTTCTTC
521          V  T  S  T  S  K  T  T  T  T  A  S  K  T  S  T  T  T  S  S


1621        CACTTCTTGTACCACCCCAACTGCTGTTGCCGTCACTTTCGATTTGACTGCCACTACCAC
541          T  S  C  T  T  P  T  A  V  A  V  T  F  D  L  T  A  T  T  T

1681        CTACGGTGAAAACATTTACTTGGTCGGTTCCATTTCTCAATTGGGTGACTGGGAAACCTC
561          Y  G  E  N  I  Y  L  V  G  S  I  S  Q  L  G  D  W  E  T  S

1741        CGACGGTATCGCTTTGTCTGCCGACAAGTACACCTCTTCTAACCCATTGTGGTACGTTAC
581          D  G  I  A  L  S  A  D  K  Y  T  S  S  N  P  L  W  Y  V  T

1801        TGTTACTTTGCCAGCTGGTGAATCTTTCGAATACAAGTTCATCAGAGTTGAATCTGATGA
601          V  T  L  P  A  G  E  S  F  E  Y  K  F  I  R  V  E  S  D  D

1861        TTCTGTTGAATGGGAATCTGACCCAAACAGAGAATACACCGTTCCACAAGCCTGTGGTGA
621          S  V  E  W  E  S  D  P  N  R  E  Y  T  V  P  Q  A  C  G  E

1921        ATCCACCGCTACCGTTACTGACACCTGGAGATAA
641          S  T  A  T  V  T  D  T  W  R  *
```

Fig. 2 Predicted protein sequence of the *sGA I* gene of *A. awamori* expressed in *S. cerevisiae* (Y294[ySYAG]). The ***XYNSEC*** secretion signal is indicated in blue. The sequence identified in glucoamylases essential for raw starch hydrolysis (Goto et al., 1994) was conserved (**PL(W-597)YVTVTLPA**), as well as the second tryptophan (**W**) residue and is double underlined in purple text. The **Gp-I region** is indicated as text in red. The **Cp-I region** or SBD is indicated in green text (Belshaw and Williamson, 1993; Fukuda et al., 1992). The general acid and base catalysts **E-213** and **E-434**, as well as **Y-85**, **W-87**, **R-89**, **D-90**, **W-154**, **E-214**, **R-339**, **D-343**, **W-351** sites which play a role in substrate transition-state stabilisation and or ground-state binding are indicated in orange text. Possible **N-glycosylation sites** are underlined by a broken line, although only the first and third sites were found to be glycosylated when expressed in yeast (Chen et al., 1994).

The sequence identified in glucoamylases essential for raw starch hydrolysis was also present in the sAMYL III, although not perfectly conserved (PEWSVTVSLPV versus PLWYVTVTLPA) (Fig. 3). The second conserved tryptophan was also present. Furthermore the TS rich region, which

promotes raw starch hydrolysis, was also identified (Fukuda et al., 1992; Hayashida et al., 1989). The α-amylases have considerably low sequence similarity, although four amino acids are invariant (Hasegawa et al., 1999; Matsuura et al., 1980 and 1984; Nakamura et al., 1992; Swift et al., 1991; Vihinen et al., 1990). These are the R-242 (R-204 in MP) and the three catalytic residues; D-244 (D-206 in MP), E-268 (E-230 in MP), and D-335 (D-297 in MP) (MP numbering of TAKA amylase sequence from *A. oryzae*). A further two invariant residues namely the H-160 and H-334 form the basis of conserved regions in the protein. These residues were also conserved in the AMYL III.

```
1              AGGCCTGAATTCCAACATGGTTTCCTTCACCTCCTTGTTGGCCGGTGTCGCTGCTATCTC
1                              M  V  S  F  T  S  L  L  A  G  V  A  A  I  S

61             CGGTGTCTTGGCTGCTCCAGCTGCTGAAGTTGAACCAGTCGCTGTCGAAAAGAGATTGTC
21              G  V  L  A  A  P  A  A  E  V  E  P  V  A  V  E  K  R  L  S

121            TGCCGCTGAATGGAGAACTCAATCTATCTACTTCTTGTTGACCGACAGATTCGGTAGAAC
41              A  A  E  W  R  T  Q  S  I  Y  F  L  L  T  D  R  F  G  R  T

181            TGATAACTCTACCACCGCCACCTGTAACACCGGTGACCAAATCTACTGTGGTGGTTCCTG
61              D  N  S  T  T  A  T  C  N  T  G  D  Q  I  Y  C  G  G  S  W

241            GCAAGGTATCATCAACCACTTGGACTACATTCAAGGTATGGGTTTCACTGCTATCTGGAT
81              Q  G  I  I  N  H  L  D  Y  I  Q  G  M  G  F  T  A  I  W  I

301            CTCTCCAATTACTGAACAATTGCCACAAGATACCTCTGACGGTGAAGCCTACCACGGTTA
101             S  P  I  T  E  Q  L  P  Q  D  T  S  D  G  E  A  Y  H  G  Y

361            CTGGCAACAAAAGATTTACAACGTCAACTCCAACTTCGGTACTGCTGATGACTTGAAGTC
121             W  Q  Q  K  I  Y  N  V  N  S  N  F  G  T  A  D  D  L  K  S

421            TTTGTCTGACGCTTTGCACGCCAGAGGTATGTACTTGATGGTTGACGTCGTCCCAAACCA
141             L  S  D  A  L  H  A  R  G  M  Y  L  M  V  D  V  V  P  N  H

481            CATGGGTTACGCCGGTAACGGTAACGACGTTGACTACTCCGTTTTCGACCCATTCGATTC
161             M  G  Y  A  G  N  G  N  D  V  D  Y  S  V  F  D  P  F  D  S

541            TTCCTCCTACTTCCACCCATACTGTTTGATTACCGACTGGGACAACTTGACTATGGTCCA
181             S  S  Y  F  H  P  Y  C  L  I  T  D  W  D  N  L  T  M  V  Q

601            AGACTGTTGGGAAGGTGATACTATTGTCTCCTTGCCAGACTTGAACACCACTGAAACTGC
201             D  C  W  E  G  D  T  I  V  S  L  P  D  L  N  T  T  E  T  A
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661   TGTCAGAACCATCTGGTACGATTGGGTCGCTGACTTGGTTTCCAACTACTCTGTTGATGG
221    V  R  T  I  W  Y  D  W  V  A  D  L  V  S  N  Y  S  V  D  G

721   TTTGAGAATTGACTCCGTCGAAGAAGTCGAACCAGATTTCTTCCCAGGTTACCAAGAAGC
241    L  R  I  D  S  V  E  E  V  E  P  D  F  F  P  G  Y  Q  E  A

781   TGCCGGTGTTTACTGTGTCGGTGAAGTTGACAACGGTAACCCAGCTTTGGATTGTCCATA
261    A  G  V  Y  C  V  G  E  V  D  N  G  N  P  A  L  D  C  P  Y

841   CCAAAAGTACTTGGACGGTGTTTTGAACTACCCAATTTACTGGCAATTGTTGTACGCTTT
281    Q  K  Y  L  D  G  V  L  N  Y  P  I  Y  W  Q  L  L  Y  A  F

901   CGAATCCTCTTCTGGTTCTATCTCCAACTTGTACAACATGATTAAGTCCGTTGCCTCCGA
301    E  S  S  S  G  S  I  S  N  L  Y  N  M  I  K  S  V  A  S  D

961   CTGTTCTGATCCAACCTTGTTGGGTAACTTCATTGAAAACCACGACAACCCAAGATTCGC
321    C  S  D  P  T  L  L  G  N  F  I  E  N  H  D  N  P  R  F  A

1021  TTCTTACACTTCCGACTACTCTCAAGCTAAGAACGTCTTGTCTTACATCTTCTTGTCTGA
341    S  Y  T  S  D  Y  S  Q  A  K  N  V  L  S  Y  I  F  L  S  D

1081  TGGTATCCCAATCGTTTACGCTGGTGAAGAACAACACTACTCTGGTGGTGACGTTCCATA
361    G  I  P  I  V  Y  A  G  E  E  Q  H  Y  S  G  G  D  V  P  Y

1141  CAACAGAGAAGCTACTTGGTTGTCCGGTTACGACACCTCCGCTGAATTGTACACTTGGAT
381    N  R  E  A  T  W  L  S  G  Y  D  T  S  A  E  L  Y  T  W  I

1201  CGCTACTACCAACGCCATCAGAAAGTTGGCCATCTCCGCTGATTCTGACTACATCACTTA
401    A  T  T  N  A  I  R  K  L  A  I  S  A  D  S  D  Y  I  T  Y

1261  CGCTAACGACCCAATCTACACCGATTCTAACACTATCGCCATGAGAAAGGGTACTTCCGG
421    A  N  D  P  I  Y  T  D  S  N  T  I  A  M  R  K  G  T  S  G

1321  TTCTCAAATTATCACCGTCTTGTCCAACAAGGGTTCCTCTGGTTCTTCCTACACCTTGAC
441    S  Q  I  I  T  V  L  S  N  K  G  S  S  G  S  S  Y  T  L  T

1381  TTTGTCCGGTTCTGGTTACACCTCTGGTACTAAGTTGATCGAAGCCTACACCTGTACTTC
461    L  S  G  S  G  Y  T  S  G  T  K  L  I  E  A  Y  T  C  T  S

1441  TGTTACTGTTGACTCTAACGGTGACATTCCAGTCCCAATGGCTTCTGGTTTGCCAAGAGT
481    V  T  V  D  S  N  G  D  I  P  V  P  M  A  S  G  L  P  R  V
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1501          TTTGCCACCAGCTTCTGTTGTCGACTCTTCTTCTTTGTGTGGTGGTTCTGGTAACACTAC
501            L  P  P  A  S  V  V  D  S  S  S  L  C  G  G  S  G  N  T  T

1561          CACTACTACTACCGCTGCTACTTCTACTTCTAAGGCCACTACCTCTTCCTCCTCCTCTTC
521            T  T  T  T  A  A  T  S  T  S  K  A  T  T  S  S  S  S  S  S

1621          TGCTGCTGCTACCACTTCTTCCTCCTGTACTGCCACCTCTACTACCTTGCCAATTACTTT
541            A  A  A  T  T  S  S  S  C  T  A  T  S  T  T  L  P  I  T  F

1681          CGAAGAATTGGTTACCACTACTTACGGTGAAGAAGTTTACTTGTCTGGTTCCATCTCTCA
561            E  E  L  V  T  T  T  Y  G  E  E  V  Y  L  S  G  S  I  S  Q

1741          ATTGGGTGAATGGGATACCTCCGACGCTGTTAAGTTGTCTGCTGACGATTACACCTCCTC
581            L  G  E  W  D  T  S  D  A  V  K  L  S  A  D  D  Y  T  S  S

1801          TAACCCAGAATGGTCTGTCACTGTTTCTTTGCCAGTTGGTACTACCTTCGAATACAAGTT
601            N  P  E  W  S  V  T  V  S  L  P  V  G  T  T  F  E  Y  K  F

1861          CATTAAGGTTGATGAAGGTGGTTCTGTCACCTGGGAATCTGACCCAAACAGAGAATACAC
621            I  K  V  D  E  G  G  S  V  T  W  E  S  D  P  N  R  E  Y  T

1921          TGTTCCAGAATGTGGTTCCGGTTCCGGTGAAACTGTCGTCGACACTTGGAGATAA
641            V  P  E  C  G  S  G  S  G  E  T  V  V  D  T  W  R  *
```

Fig. 3 Predicted protein sequence of the *samyl III* gene of *A. awamori* expressed in *S. cerevisiae* (Y294[ySYAA]). The ***XYNSEC*** secretion signal is indicated in green. **Regions 1-4** are underlined and indicate conserved regions in the sequence as identified by the authors who characterised the gene for the first time (Matsubara et al., 2004b). The sequence identified for raw starch hydrolysis was also present in the AMYL III, although not perfectly conserved (**PEWSVTVSLPV** versus PLWYVTVTLPA), and is double underlined in purple text. The second **W** was also present. Furthermore the **TS linker** characterised by O-glycosylation was also identified and is indicated as text in red (Matsubara et al., 2004). Conserved amino acids namely **R-242**, **D-244**, **E-268**, and **D-335** (amino acid numbering of TAKA amylase sequence from *A. oryzae*), as well as **H-160** and **H-334** are indicated in blue text.

Recombinant amylase production and characterisation

The ability of the amylolytic strains to produce functional amylases was visualised as cleared hydrolysis zones or halos in raw starch agar stained with iodine (Fig. 4). Amylolytic activity was

confirmed in liquid assays. Raw starch and soluble starch activity was determined at the pH (pH 5.4) and temperature (30°C) preferred by yeast during cultivation (Fig. 5). The Y294[yASAG] and Y294[ySYAG] strains secreting GA I and sGA I, respectively, produced the highest soluble as well as raw starch hydrolysing activity. The cocktails containing GA I and AMYL III or sGA I and sAMYL III secreted by Y294[yAGAA] and Y294[ySYAGAA] respectively, displayed very similar raw starch hydrolysing activities.

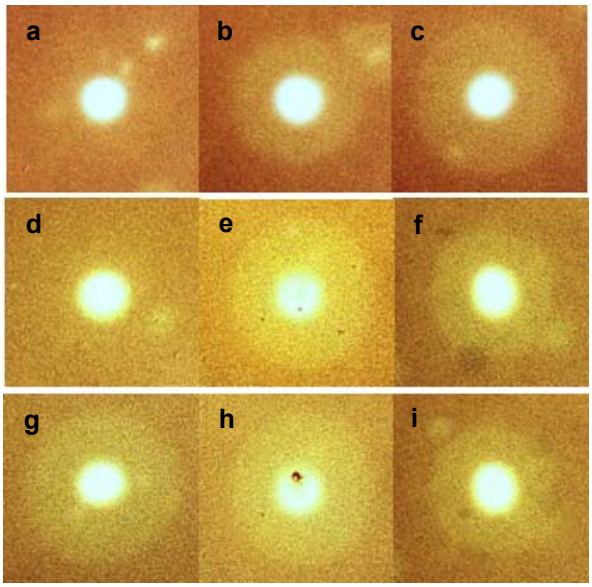

Fig. 4 Raw starch hydrolysis appears as clear zones around colonies secreting functional amylases. Strain (a) and (d) Y294[yxynsec] (reference strain), (b) Y294[yASOG] secreting GLAA, (c) Y294[yASAG] secreting GA I, (e) Y294[ySYAG] secreting sGA I, (f) Y294[OGAA] secreting GLAA and AMYL III, (g) Y294[yAGAA] secreting GA I and AMYL III, (h) Y294[ySYAGAA] secreting sGA I and sAMYL III, and (i) Y294[yASAGSYAA] secreting GA I and sAMYL III were grown for 4 days on agar containing raw starch and then stained with an iodine solution.

All α-amylase activity levels were very low compared to the amount of glucoamylase activity produced per gram DW cells. The strain secreting AMYL III (y294[yASAA]) produced more α-amylase activity per gram DW cells (30.1 CU (g DW cells)$^{-1}$ (± 5.77)) than the Y294[ySYAA] strain secreting the optimised sAMYL III (13.1 CU (g DW cells)$^{-1}$ (± 1.85)). The GA I and sGA I cocktails produced by Y294[yASAG] and Y294[ySYAG] respectively, showed α-amylase activity which was comparable to or higher than the level of α-amylase activity of the AMYL III cocktail secreted by Y294[yASAA].

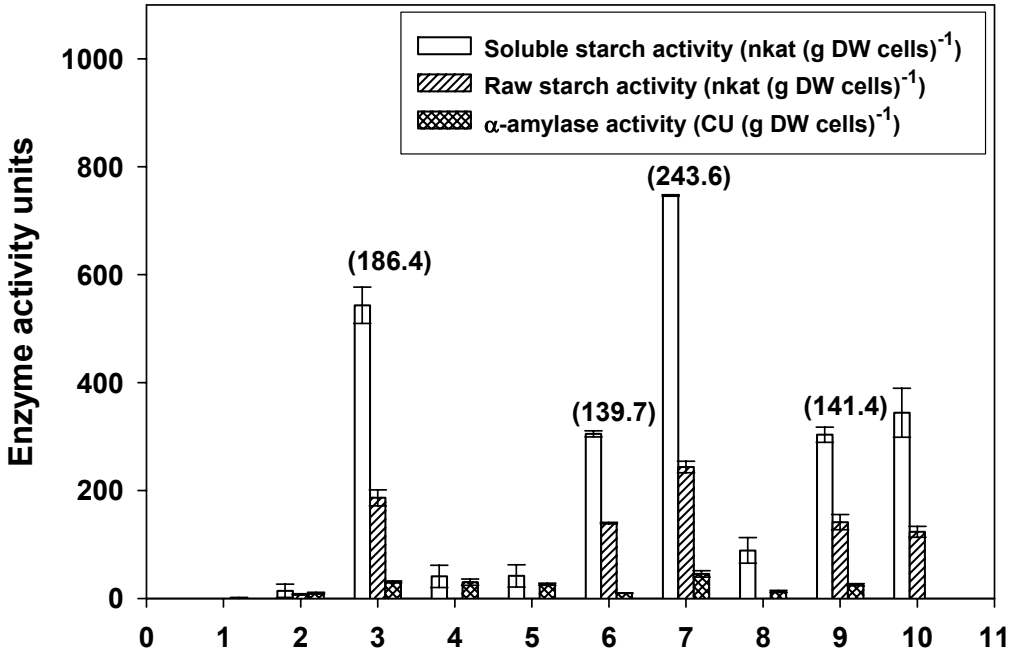

Fig. 5 Total amylase activity expressed as nkat g DW cells^{-1} and α-amylase activity expressed as CU g DW cells $^{-1}$ for concentrated enzyme cocktails produced by the reference strain (1) Y294[yxynsec] and recombinant amylolytic yeast strains (2) Y294[yASOG], (3) Y294[yASAG], (4) Y294[yASAA], (5) Y294[yOGAA], (6) Y294[yAGAA], (7) Y294[ySYAG], (8) Y294[ySYAA], (9) Y294[ySYAGAA], and (10) Y294[yASAGSYAA]. Raw starch hydrolysing activity values are indicated for samples (3) Y294[yASAG], (6) Y294[yAGAA], (7) Y294[ySYAG] and (9) Y294[ySYAGAA]. Soluble and raw starch hydrolysing activity was determined at 30°C and pH 5.4, and α-amylase activity at 50°C and pH 5.4.

The optimum temperature at pH 5.4 for raw starch activity was determined for the following enzyme cocktails; GA I secreted by Y294[yASAG], AMYL III secreted by Y294[yASAA], GA I and AMYL III secreted by Y294[yAGAA], and sGA I and sAMYL III secreted by Y294[ySYAGAA] (Table 5). An optimum pH of 4.5 was measured at 30°C for the AMYL III secreted by Y294[yASAA]. All the enzyme cocktails tested were stable at 30°C and pH 5.4 for 52 hours as they retained more than 99% activity under these conditions.

Table 5 Summary of enzyme cocktail temperature preferences.

Strain	Enzymes	Temp.[a] at pH 5.4
Y294[yASAG]	GA I	60
Y294[yASAA]	AMYL III	50
Y294[yAGAA]	GA I and AMYL III	40
Y294[ySYAGAA]	sGA I and sAMYL III	40

[a] Optimum temperature at pH 5.4

The recombinant GA I was purified applying the adsorption characteristic of the enzyme, as the SBD will adhere to the starch granule. Kinetic parameters were determined and results are summarised in Table 6. The affinity for maltose was two-fold higher than for raw starch, and maximum specific activity towards raw starch was five-fold higher than for maltose.

Table 6 Kinetic parameters of GA I.

K_m	V_{max}	pH	Temperature	Reference
[a]3.574 μg ml^{-1}	11.603	4.0	50°C	This study
[b]1.648 mM	2.410	4.0	50°C	This study
[b]1.82 mM	NR	4.5	45°C	(Fierobe et al., 1997)
[b]1.09 mM	NR	4.5	35°C	(Allen et al., 1998)

[a] Raw corn starch substrate

[b] Maltose substrate

NR Not reported

Characterisation of protein species by SDS-PAGE gel electrophoresis indicated that the GA I and AMYL III proteins were both hyper-glycosylated by the yeast and displayed broad protein species of 135-150 kDa (Chen et al., 1995; Jacks et al., 1995; Kovaleva et al., 1989; Romanos et al., 1992) (Fig. 6 and 7). After de-glycosylation, the protein species were approximately 115 kDa. The size of the glucoamylase protein species was comparable to previously published data (Table 7). De-glycosylation was performed using an enzyme which removes only N-glycosylated groups. The difference in fragment size was therefore not substantial on an SDS-PAGE gel, as the proteins might contain O-glycosylated groups as well (Chen et al., 1995). Overlay Zymogram analysis indicated that both GA I and AMYL III were active and showed clear hydrolysis zones after iodine staining (Fig. 6b, 7b and 8b).

Table 7 Summary of glucoamylase and α-amylase protein species secreted by native and *S. cerevisiae* hosts.

Enzyme	Host	Mol mass (kDa)	Reference
A. awamori var. *kawachi* glucoamylase	*S. cerevisiae*	120	(Goto et al., 1997)
A. awamori glucoamylase	*S. cerevisiae*	82[a] (66[b])	(Khan et al., 2000)
A. awamori glucoamylase	*S. cerevisiae*	84[a]	(Fierobe et al., 1997)
A. awamori glucoamylase	*S. cerevisiae*	120 (115 [c])	(Chen et al., 1995)
A. awamori glucoamylase	*S. cerevisiae*	120 (115 [c])	(Chen et al., 1994)
A. awamori α-amylase III	*A. awamori*	90 (67[b])	(Matsubara et al., 2004b)

[a] Determined by mass spectrometry

[b] Carbohydrate free MW in kDa

[c] De-N-glycosylated MW in kDa

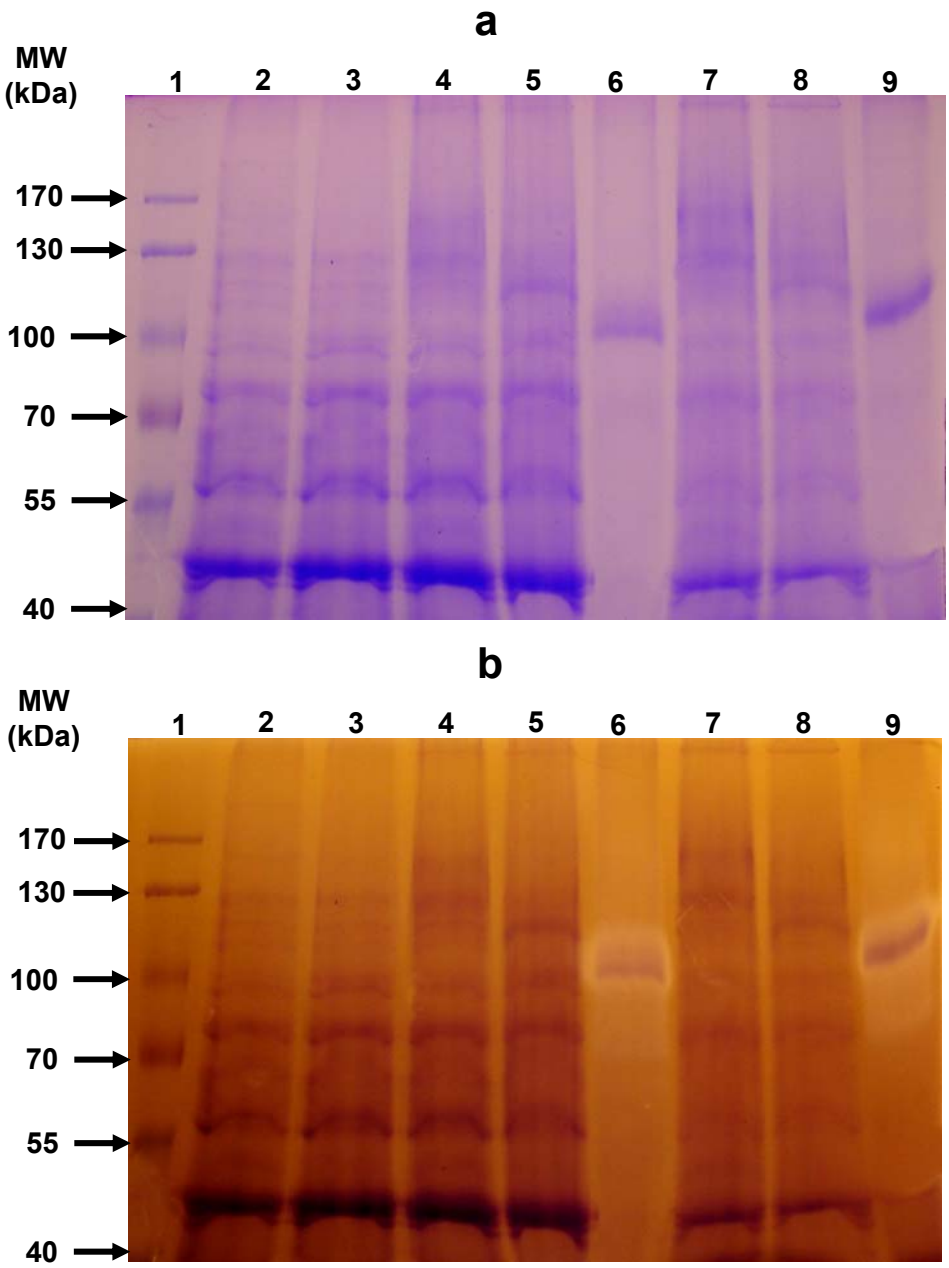

Fig. 6 (a) SDS-PAGE (7.5% acrylamide, 0.1% soluble starch) analysis showing protein species stained with Coomassie Brilliant Blue R250 and (b) overlay Zymogram stained with iodine showing starch hydrolysing activity. Samples were loaded as follows: lane 1, molecular weight marker (Fermentas); lane 2, denatured Y294[yxynsec] proteins; lane 3, denatured Y294[yxynsec] proteins treated with PNGaseF; lane 4, denatured GA I secreted by Y294[yASAG]; lane 5, denatured GA I secreted by Y294[yASAG] treated with PNGaseF; lane 6, untreated GA I secreted by Y294[yASAG]; lane 7, denatured AMYL III secreted from Y294[yASAA]; lane 8, denatured AMYL III secreted from Y294[yASAA] treated with PNGaseF; lane 9, untreated AMYL III secreted from Y294[yASAA].

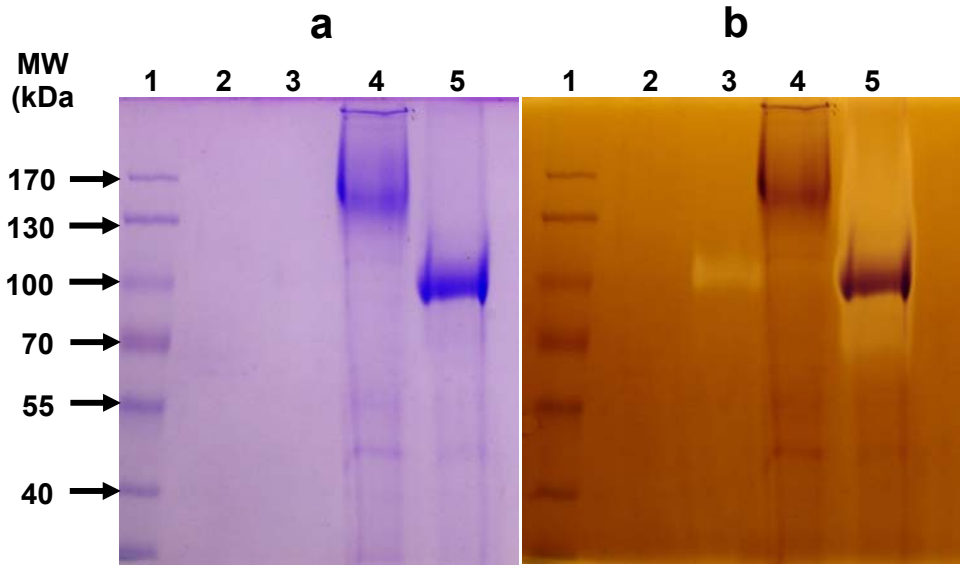

Fig. 7 SDS-PAGE (7.5% acrylamide, 0.1% soluble starch) analysis showing protein species stained with Coomassie Brilliant Blue R250 on the left (a), and (b) overlay Zymogram stained with iodine showing starch hydrolysing activity on the right. The enzyme cocktail secreted by Y294[yASAG] was adsorbed to raw starch at 4°C and then released by triethylamine. The protein was dialysed with buffer and concentrated with acetone. The purified protein was loaded as follows: lane 1, molecular weight marker (Fermentas); lane 2, denatured GA I (2 µl); lane 3, untreated GA I (2 µl); lane 4, denatured GA I (20 µl); lane 5, untreated GA I (20 µl).

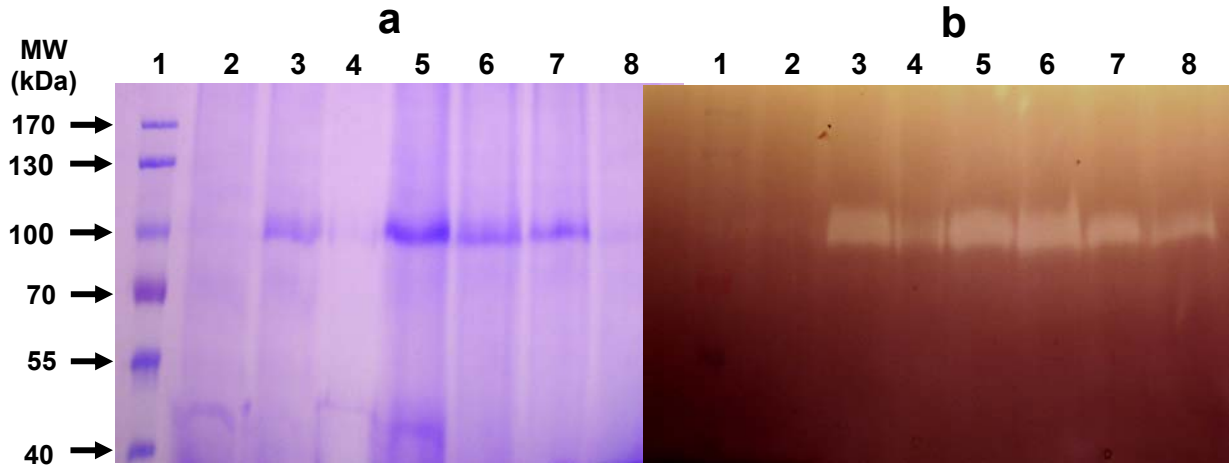

Fig. 8 (a) SDS-PAGE analysis (7.5% acrylamide, 0.1% soluble starch) showing untreated protein species of selected enzyme cocktails and (b) Zymogram showing untreated protein species with starch hydrolysing activity. Samples were loaded on the gels as follows: lane 1, molecular weight marker from Fermentas; lane 2, proteins produced by reference strain Y294[yxynsec]; lane 3, GA I secreted by Y294[yASAG]; lane 4, purified GA I from Y294[yASAG]; lane 5, AMYL III secreted from Y294[yASAA]; lane 6, GA I and AMYL III secreted by Y294[yAGAA]; lane 7, sGA I and sAMYL III secreted by Y294[ySYAGAA]; lane 8, GA I and sAMYL III secreted by Y294[yASAGSYAA].

Growth kinetics of amylolytic strains grown on raw starch, soluble starch and glucose

The anaerobic maximum specific growth rate of Y294[yAGAA] on raw starch (0.003 h^{-1}) was almost 30-fold lower than on soluble starch (0.085 h^{-1}). The anaerobic maximum specific growth rate of Y294[yAGAA] on glucose (0.248 h^{-1}) gave an indication of the expected growth rate when all the starch is converted to glucose for fermentation in a non-limiting step. The maximum specific growth rates for all the engineered strains determined during aerobic cultivation on glucose (YPD medium) in shake flasks ranged between 0.365-0.386 h^{-1} and all strains reached an OD_{600} of 6.2-7.1, which corresponded to 3.2-3.7 g l^{-1} DW cells.

Ethanol, biomass and by-product yields during anaerobic growth on starch and glucose

The anaerobic fermentation profiles of the Y294[yAGAA] strain in glucose, soluble starch, and raw starch are presented in Figures 9a-c. The different yields of by-products from and carbon balances for anaerobic fermentation by the Y294[yAGAA] strain obtained in raw starch and glucose media are indicated in Table 8. The carbon balance for the fermentation in soluble starch medium could not be calculated accurately as glucose produced by enzyme hydrolysis interfered with total residual fermentable sugar determined with the phenol-sulphuric acid assay. Levels of CO_2 were estimated from the ethanol produced based on the assumption that one mol CO_2 is produced per mol ethanol. Y294[yAGAA] sustained growth on raw starch as sole carbon source and produced a maximum ethanol concentration of 5.5 g l^{-1} after 312 hours of fermentation. A yield of 0.40 g of ethanol per gram available sugar from raw starch was calculated, which corresponds to 71% of the theoretical maximum of 0.56 g ethanol per g starch (Table 9). The maximum ethanol concentration from soluble starch was 3.0 g l^{-1} after 46 hours. Y294[yAGAA] produced up to 7.1 g l^{-1} ethanol after 19.5 hours cultivation in glucose.

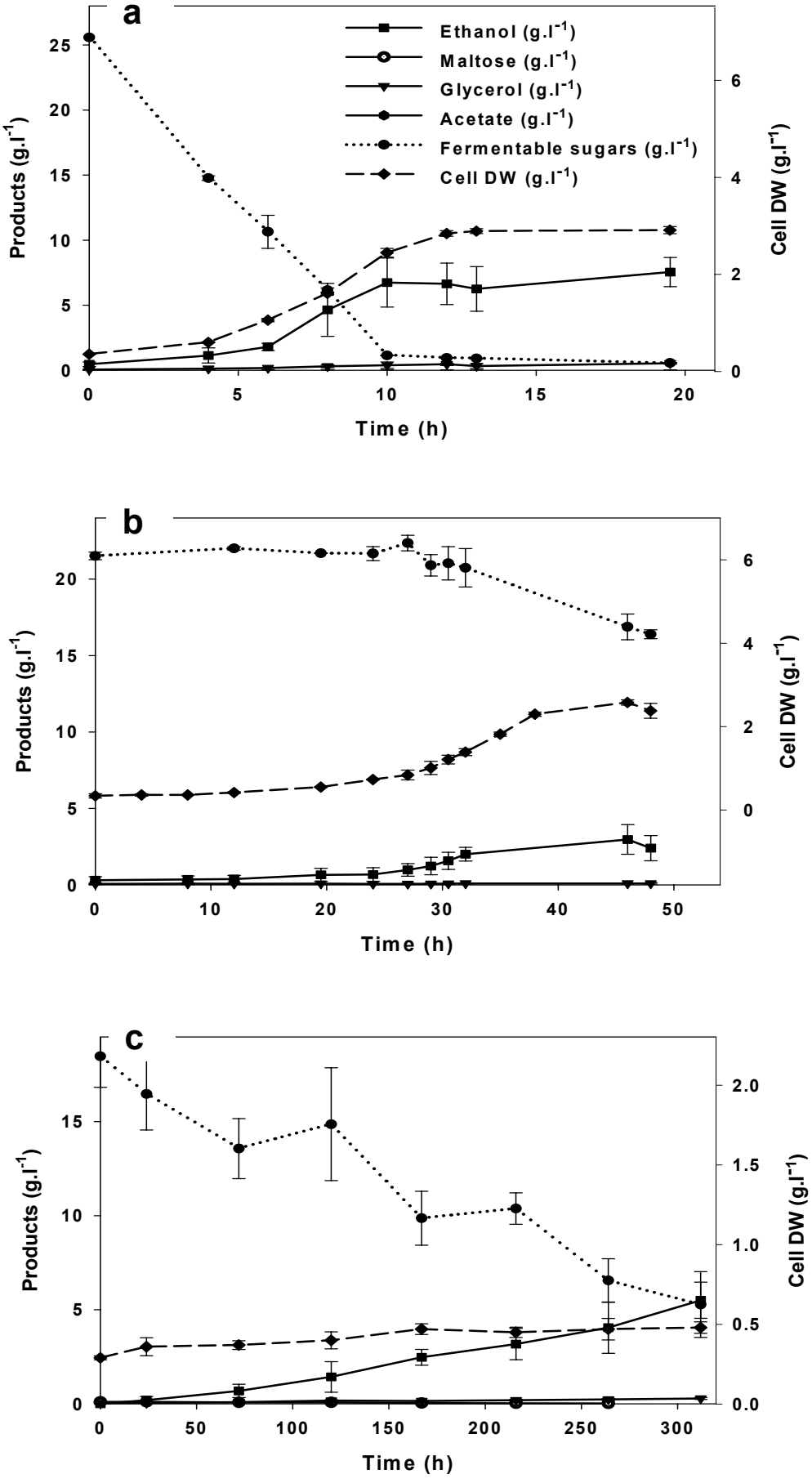

Fig. 9 Fermentation profiles of the Y294[yAGAA] strain cultivated anaerobically in serum bottles in a) 2.2% glucose medium, b) 2% soluble potato starch medium and c) 2% RSYP medium. The soluble and raw starch media were supplemented with 0.05% glucose. Ethanol, maltose, glycerol and acetate levels (g l^{-1}) determined with HPLC, as well as fermentable sugars (g l^{-1}) determined with the phenol sulphuric assay method are indicated on the y-axis. Cell DW (g l^{-1}) level is indicated on the secondary y-axis.

Table 8 Product yields for anaerobic batch cultures of *S. cerevisiae* recombinant strains in serum bottles.

Strain	μ_{max} (h^{-1})	**Product Yields (g (g glucose)$^{-1}$)**				**Carbon balance** $Cmol_{in}/Cmol_{out}$.
		Biomass	**Ethanol**	**Glycerol**	**Acetate**	
Y294[yxynsec] [a]	0	-	-	-	-	-
Y294[yAGAA] [a]	0.003	0.02 [c]	0.40	0.02	-	0.83
Y294[yAGAA] [b]	0.248	0.13 [d]	0.47	0.02	0	1.12

Strains cultivated in [a] 20 g l^{-1} raw starch and 0.5 g l^{-1} glucose, and [b] 22.5 g l^{-1} glucose.

[c] inoculum of 0.29 g l^{-1} DW cells increased to 0.49 g l^{-1} DW cells during fermentation

[d] inoculum of 0.36 g l^{-1} DW cells increased to 2.92 g l^{-1} DW cells during fermentation

– indicates not determined.

Table 9 Ethanol production by strains cultivated in starch.

Strains	Sugar	Ethanol concentration	Ethanol productivity	Specific ethanol productivity	Ethanol Yield	Reference
Raw starch medium						
G-5315-2	250 g l^{-1}	51 g l^{-1} after 120 h	0.675 g l^{-1} h^{-1}		0.20 (36%) [b]	(Ashikari et al., 1989)
YF207/pGA11/pUFLA	200 g l^{-1}	61.8 g l^{-1} after 72 h	1.008 g l^{-1} h^{-1}	0.069 g (g DW cells)$^{-1}$ h^{-1}	0.44 (79%) [b]	(Shigechi et al., 2004b)
YF237 (nf)/ pGA11 (sd)/ pSBAA2 (se)	120 g l^{-1}	51 g l^{-1} after 60 h	1.283 g l^{-1} h^{-1}	0.180 g (g DW cells)$^{-1}$ h^{-1}	0.46 (82%) [b]	(Khaw et al., 2006)
YF237 (nf)/ pGA11 (sd)/ pSBAA2 (sd)	120 g l^{-1}	23 g l^{-1} after 60 h	0.305 g l^{-1} h^{-1}	0.060 g (g DW cells)$^{-1}$ h^{-1}	0.38 (68%) [b]	(Khaw et al., 2006)
YF207 (f)/ pGA11 (sd)/ pSBAA2 (se)	120 g l^{-1}	24 g l^{-1} after 60 h	0.321 g l^{-1} h^{-1}	0.060 g (g DW cells)$^{-1}$ h^{-1}	0.45 (80%) [b]	(Khaw et al., 2006)
YF207 (f)/ pGA11 (sd)/ pSBAA2 (sd)	120 g l^{-1}	20 g l^{-1} after 60 h	0.208 g l^{-1} h^{-1}	0.040 g (g DW cells)$^{-1}$ h^{-1}	0.20 (36%) [b]	(Khaw et al., 2006)
Y294[yAGAA]	22.5 g l^{-1}	5.5 g l^{-1} after 312 h	0.018 g l^{-1} h^{-1}	0.037 g (g DW cells)$^{-1}$ h^{-1}	0.40 (71%) [b]	This study
Soluble starch medium						
YPG/AB	48 g l^{-1}	21.5 g l^{-1} after 70 h	0.31 g l^{-1} h^{-1}		0.54 (96%) [b]	(Ülgen et al., 2002)
Aspergillus awamori and *Zymomonas mobilis*	110 g l^{-1}	21 g l^{-1}			0.33 (59%) [b]	(Tanaka et al., 1986)
Saccharomycopsis fibuligera and *Zymomonas mobilis*	33 g l^{-1}	9.7 g l^{-1} after 25 h	0.54 g l^{-1} h^{-1}		0.48 (86%) [b]	(Dostalek and Haggstrom, 1983)
Y294[yAGAA]	22.5 g l^{-1}	3.0 g l^{-1} after 312 h	0.257 g l^{-1} h^{-1}	0.022 g (g DW cells)$^{-1}$ h^{-1}	[c]	This study
Glucose medium						
Y294[yAGAA]	22.5 g l^{-1}	7.1 g l^{-1} after 312 h	1.237 g l^{-1} h^{-1}	0.125 g (g DW cells)$^{-1}$ h^{-1}	0.47 (92%) [d]	This study

[a] Monomeric sugar equivalent determined from the sum of starch and glucose in medium. [b] Ethanol yield as g (g consumed sugar)$^{-1}$ and % of theoretical maximum (0.56 g g^{-1} from starch) indicated in brackets. [c] Ethanol yield was not determined as glucose produced by enzyme hydrolysis interfered with measured total residual fermentable sugars. [d] Ethanol yield as g g^{-1} and % of theoretical maximum (0.51 g g^{-1} from glucose) indicated in brackets.

(nf) denotes nonflocculent, (f) denotes flocculent, (sd) denotes surface displayed, (se) denotes secreted. A blank space indicates that not enough data was presented to determine the value.

Discussion

A more efficient and cost effective conversion of starch to ethanol requires organisms producing enzymes that are capable of converting raw starch in a one-step process. Applying a raw starch utilising yeast in the starch conversion process will have all the benefits from a simultaneous saccharification and fermentation (SSF) procedure, such as a lowered heating energy requirement and chemical usage (Devantier et al., 2005; Lynd et al., 1999). The added benefit will be the elimination of the large cost associated with commercial enzyme purchase.

In this study, yeast was engineered to secrete both a raw starch hydrolysing glucoamylase and α-amylase from *Aspergillus awamori* origin, which allowed the yeast to sustain growth on raw starch as sole carbon source and ferment the hydrolysed substrate to ethanol, albeit with a low maximum specific growth rate (0.003 h^{-1}). The recombinant strain (Y294[yAGAA]) produced a maximum ethanol concentration of 5.5 g l^{-1} after 312 hours of fermentation in 20 g l^{-1} raw starch with 0.5 g l^{-1} glucose. Although the volumetric ethanol productivity of the Y29[yAGAA] strain (0.018 g l^{-1} h^{-1}) was much lower than the productivity determined for previously generated strains (0.208-1.283 g l^{-1} h^{-1}) (Table 9), a yield of 0.40 g of ethanol per gram available sugar from raw starch was calculated, which corresponds to 71% of the theoretical maximum from starch. The yield compared well to calculated yields of strains previously engineered for raw starch conversion (Table 9). Furthermore, the specific ethanol productivity of 0.037 g (g DW cells)$^{-1}$ h^{-1} was comparable to a flocculent yeast strain co-displaying the *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase (0.04 g (g DW cells)$^{-1}$ h^{-1}) (Khaw et al., 2006). The flocculent strain produced up to 20 g l^{-1} ethanol after 60 hours in a medium containing 100 g l^{-1} raw starch with 10 g l^{-1} glucose at an ethanol yield of 0.20 g g^{-1} (36% of theoretical maximum), using an inoculum of 3.33 g cells per litre medium. In our study, only 20 g l^{-1} raw starch and 0.5 g l^{-1} glucose was used in the medium and the cell inoculum was only 0.3 g l^{-1}. It is therefore speculated that by increasing the starch and glucose concentration in the medium and using a higher cell inoculum, the overall ethanol production of the Y294[yAGAA] strain will improve in a batch or even fed-batch fermentation system.

When the Y294[yAGAA] strain was cultivated in 20 g l^{-1} soluble starch (with additional 0.5 g l^{-1} glucose), an ethanol concentration of 3 g l^{-1} was reached after 46 hours of fermentation. A higher ethanol concentration has been measured in a previous study, where up to 21.5 g l^{-1} ethanol was produced after 70 hours in a controlled batch fermentation by a *S. cerevisiae* strain secreting separate polypeptides of *A. awamori* glucoamylase and *B. subtilis* α-amylase (Ülgen et al., 2002) (Table 9). The Ülgen group's fermentation medium contained 40 g l^{-1} soluble starch with additional 4 g l^{-1} glucose, once again indicating that a higher starch and glucose concentration could increase

ethanol concentration. Their strain showed a volumetric ethanol productivity of 0.310 g l^{-1} h^{-1}, which is only 1.2-fold higher than the volumetric productivity of the Y294[yAGAA] strain (0.257 g l^{-1} h^{-1}). Up to 9.7 g l^{-1} ethanol was recorded during SSF with *Saccharomycopsis fibuligera* and *Zymomonas mobilis* after 25 hours of cultivation with an initial soluble starch concentration of 30 g l^{-1} (Dostalek and Haggstrom, 1983) (Table 9). The volumetric productivity of ethanol was 0.54 g l^{-1} h^{-1}, which is 1.7-fold higher than for the Y294[yAGAA] strain.

The Y294[yAGAA] strain was cultivated in 22.5 g l^{-1} glucose to determine ethanol produced in a non-limiting step as an indication whether enzyme production was a limiting factor in the generated strain. An ethanol concentration of up to 7.1 g l^{-1} was measured after 12 hours of fermentation. The volumetric ethanol productivity of the strain (1.237 g l^{-1} h^{-1}) was well in line with the 1 g l^{-1} h^{-1} preferred by the industry for ethanol fermentation (Dien et al., 2003). The ethanol yield of 0.47 g g^{-1} corresponded to 92% of the theoretical maximum from glucose. Enzyme production was thus too low to warrant high ethanol productivities in medium containing soluble or raw starch.

Various factors may contribute to a deficiency in adequate amounts of heterologously secreted enzymes or non-functional protein species. To prevent inefficient secretion of enzymes, the genes were fused to the *T. reesei* xylanase 2 secretion signal (Den Haan et al., 2007). Inefficient secretion of heterologous proteins has been observed in yeast (Lee et al., 1999), and de Moraes et al. (1995) showed that when using the native leader peptide from *A. awamori* glucoamylase, 5-12% of the activity was left within cells. The classical *S. cerevisiae* system for secretion of heterologous recombinant proteins utilising the *S. cerevisiae* α-factor leader sequence for secretion, and the endoprotease Kex2p (Lys-Arg) cleavage site for protein maturation was therefore utilised (Germain et al., 1992 and 1993; Julius et al., 1984; Kurjan and Herskowitz, 1982; Romanos et al., 1992).

A second factor which may affect enzyme production is whether the engineered strain showed a metabolic burden as a result of heterologous enzyme secretion. A deleterious affect on growth was however not evident as the maximum specific growth rate for the Y294[yAGAA] determined during aerobic cultivation on glucose (YPD medium) was very similar to the maximum specific growth rate for the reference strain Y294[yxynsec] (0.37 h^{-1} versus 0.38 h^{-1}).

Gene sequence may affect the functionality of expressed and secreted enzymes, as truncated genes or mutations may affect regions essential for enzyme activity and functionality. Sequencing confirmed open reading frames for the cloned gene sequences. Conserved residues in protein sequences indicated that translated proteins should be functional and effectively hydrolyse raw starch. Functionality of enzymes was visually confirmed by raw starch hydrolysis zones around yeast transformants in agar plates strained with iodine. Clear hydrolysis zones for secreted enzymes were visible in overlay Zymogram analysis, although protein species were hyper-glycosylated by

the yeast. The specific soluble and raw starch activities of the enzymes were furthermore determined in liquid assays.

The characteristics of the enzymes were determined as the preferred temperature and pH of enzymes may indicate why their activity is less than optimal at the cultivation conditions preferred by the yeast. The GA I cocktail produced by Y294[yASAG] had an optimum temperature of 60°C at pH 5.4 and the activity dropped to only 67% of the maximum at 30°C (Table 5). The optimum pH for raw starch activity at 30°C could not be determined accurately, as the glycoside linkages between the glucose units become hydrolysable under acidic conditions (Swinkels, 1985). The strain producing both the GA I and AMYL III (y294[yAGAA]) had a lower temperature optimum of 40°C, which would be advantageous in a one-step fermentation scenario, as the cocktail showed 84% of the maximum activity at 30°C (Table 5). Furthermore, the enzymes all remained very stable at 30°C and pH 5.4 over 52 hours (>99%). The AMYL III showed the highest activity at 50°C and pH 5.4, and only 33% activity was recorded at 30°C. The pH optimum of the enzyme was pH 4.5 at 30°C, and only 30% active at pH 5.4. These findings could indicate why low enzyme activity was measured during liquid assays or why no hydrolysis zones were visible for the strains secreting AMYL III or sAMYL III alone.

The affinity and maximum specific activity towards a substrate will give a further indication of how effective an enzyme will be in hydrolysing the substrate. The kinetic parameters of the purified GA I was therefore determined to calculate the enzyme's affinity (K_m) and maximum specific activity (V_{max}) for raw starch and maltose as substrate (Table 6). The affinity for maltose was two-fold higher than for raw starch, and maximum specific activity towards raw starch was five-fold higher than for maltose. The affinity of the GA I for maltose compared well with affinities reported in previous studies. To our knowledge, this is the first study reporting on the affinity and specific activity of an enzyme for raw starch as substrate.

The rate of starch fermentation may be increased by increasing the engineered strain's performance, therefore increasing the level of glucoamylase and α-amylase expression (Inlow et al., 1987). For this reason, glucoamylase and α-amylase sequences were designed using codons preferred by *S. cerevisiae* (Sharp et al., 1988; Sharp and Cowe, 1991). The optimised *GA I* and *amyl III* genes had CAI values of 0.921 and 0.923 respectively. Codon optimised genes offer the advantage of making gene expression less laborious for an organism harbouring the recombinant genes, as codons not frequently used by the organism are removed from the genes. This should increase gene expression. Optimised gene sequences for the *GA I* and *amyl III* genes from *A. awamori* were synthesised and expressed in *S. cerevisiae*. Unfortunately applying the concept of codon adaptation did not improve recombinant protein production in the strain secreting both the sGA I and sAMYL III (Fig. 5). The Y294[yAGAA] and Y294[ySYAGAA] strains displayed similar raw

starch hydrolysing activities at 30°C per gram DW cells. The strains secreting the sGA I enzyme alone (Y294[ySYAG]) showed a 31% increase in raw starch hydrolysing activity per gram DW cells compared to the GA I secreted by Y294[yASAG]. The Y294[ySYAA] strain secreting the sAMYL III however displayed 56% less α-amylase activity per gram DW cells compared to AMYL III secreted from Y294[yASAA]. It can therefore be speculated that the negative effect of the sAMYL III counteracts the positive effect of the sGA I in Y294[ySYAGAA]. As a high yield expression of heterologous proteins is usually a matter of "trial and error", another avenue to be followed would be to express the sGA I and wild-type AMYL III as separate secreted polypeptides to increase gene expression and therefore the rate of starch fermentation.

In conclusion, this study proved the concept that engineering yeast to secrete glucoamylase and α-amylase of *Aspergillus* origin alone yields an organism able to utilise raw starch as carbon source. The specific ethanol production rate of this strain on raw starch as carbon source (0.037 g (g DW cells)$^{-1}$ l^{-1}) was comparable to a previously engineered strain that was cultivated in a controlled batch fermentation with higher carbon source and ten-fold larger inoculum (Khaw et al., 2006). It is therefore possible to increase ethanol production of the current strain in a more optimised fermentation system. An ethanol yield corresponding to 71% of the theoretical yield was observed. These preliminary serum bottle fermentations indicate that the generated strain is a promising raw starch converter. The GA I displayed an affinity (K_m) of 3.574 (μg ml^{-1}) and a maximum specific activity (V_{max}) of 11.604 towards raw corn starch. To our knowledge, this is the first study reporting on the affinity and specific activity of an enzyme for raw starch as substrate. Up to 140 nkat (g DW cells)$^{-1}$ raw starch hydrolysing activity was produced by the generated strain secreting both GA I and AMYL III. The Y294[yAGAA] and Y294[ySYAGAA] strains displayed similar raw starch hydrolysing activities at 30°C per gram DW cells. This could be due the lower expression of α-amylase, which counteracts the effect of the glucoamylase expressed at a higher level.

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SECTION II: MICROBIAL ENZYMES FOR THE INSTANT COFFEE INDUSTRY

CHAPTER 4: LITERATURE REVIEW: ENZYME TECHNOLOGY FOR THE INSTANT COFFEE INDUSTRY

4.1. COFFEE AS INTERNATIONAL COMMODITY

4.1.1 Introduction

Coffee is not bought or consumed for nutrition. Its weight has value only insofar as it has acceptable flavour. Coffee has only one value: to give the consumer pleasure and satisfaction through flavour, aroma, and desirable physiological and psychological effects (Sivetz and Foote, 1963).

The coffee tree originated in the province of Kaffa in Ethiopia (Sivetz and Desrosier, 1979; Smith, 1985). It is one of the most important products in world trade, second only to oil as source of foreign exchange. World consumption of coffee is increasing annually (International Coffee Organization, 2007b). Coffee consumption for 2007 is estimated at 119-120 million bags, whereas the estimated figure for 2006 was 118 million (Fig. 4.1). Coffee exports account for up to 80% of foreign exchange earnings in developing countries. The cultivation of coffee, its processing, trading and transportation provide job opportunities for millions of people world wide.

Instant coffee was invented by Satori Katyo in 1901 (Sivetz and Desrosier, 1979). Nestlé developed a process for preparing soluble coffee in percolators in 1934, and in 1939, the Nescafe or spray-dried coffee was invented. Instant coffee has several advantages over ground coffee. Preparation time is lessened dramatically, no specialised brewing equipment is necessary, volumes are reduced and weigh less, and the instant version has a longer shelf life. It is also more cost effective to produce instant coffee, as the lowest quality beans are used. Better quality beans are rather sold whole.

In this chapter, a general background of coffee processing will be presented. Coffee bean composition and structure is described so as to show why certain methods are used in coffee preparation. Attention is given to hydrolytic enzymes used in the industry for bean

preparation, as well as enzymes shown to have an effect on certain sugars present in the coffee bean itself.

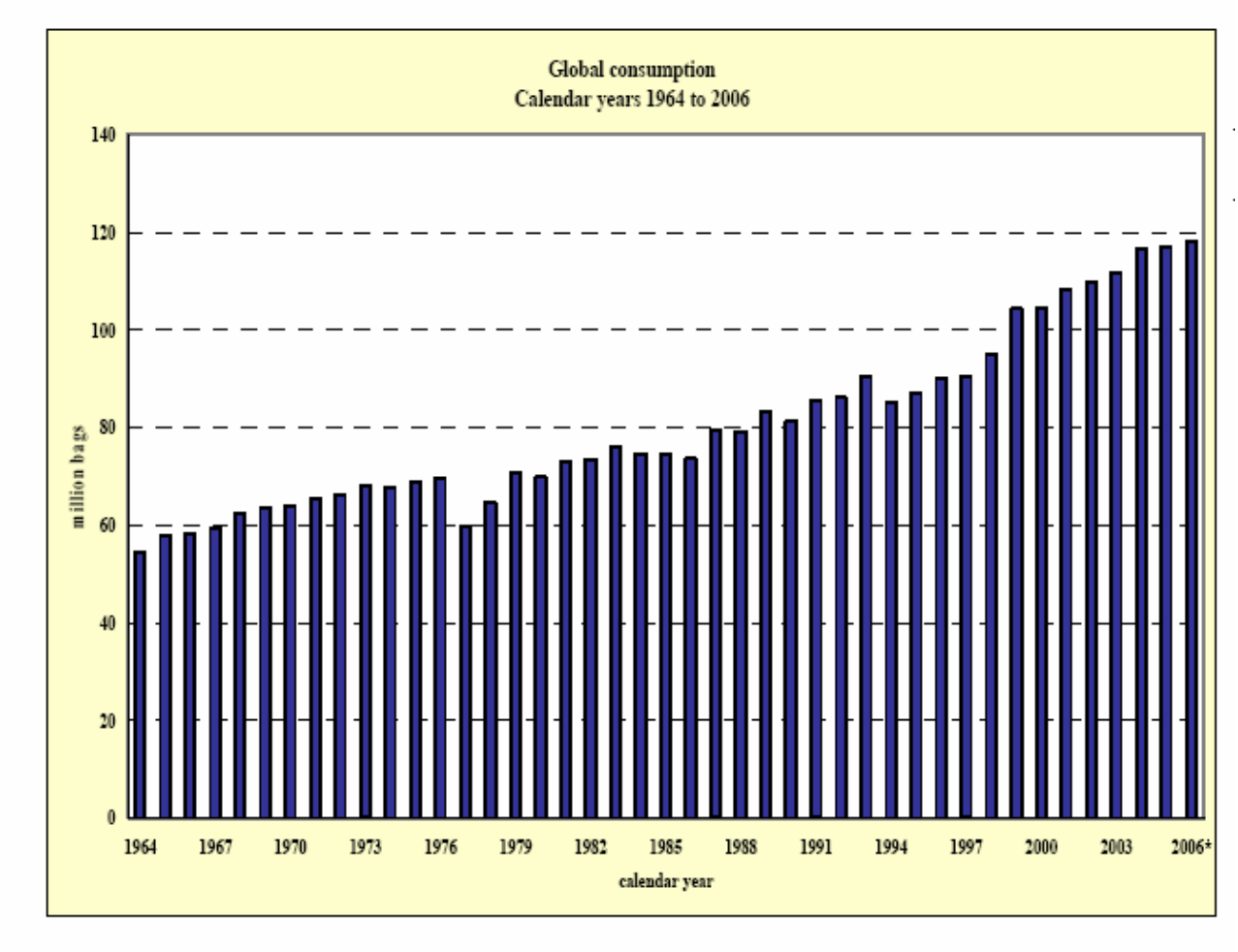

Fig. 4.1 Global consumption of coffee shown as millions of bags consumed annually from 1964 to 2006 (Osorio, 2007).

4.2 THE COFFEE PLANT

Coffee belongs to the botanical family *Rubiaceae* of which most species are tropical trees and shrubs (Clifford and Wilson, 1985; Wrigley, 1988). The most important species for the industry are *Coffea arabica* (Arabica coffee) and *Coffea canephora* (Robusta coffee) (Berthaud and Charrier, 1988). Arabica coffee accounts for more than 70% of the world's production, and is grown throughout Latin America, Central and East Africa, India, as well as Indonesia. The term 'Robusta' is actually a name given to the widely grown variety of the *Coffea canephora* species. Robusta coffee is grown in West and Central Africa, throughout South-East Asia and in Brazil. Main differences between Robusta and Arabica include that Robusta will grow at lower altitudes, tolerate higher temperatures and heavier rainfall, and

demand higher soil humus content compared to Arabica (Smith, 1985). Robusta is also more resistant to disease. Furthermore, the Robusta shrub produces rounded brown fruits which take up to 11 months to mature, whereas Arabica coffee fruits are green to pale green, oval and mature in 7 to 9 months (International Coffee Organization, 2007a). The Robusta seeds are also smaller than the Arabica variety.

Coffee beans are the seeds housed in the fruit produced by the evergreen coffee plant (Clifford and Wilson, 1985; International Coffee Organization, 2007c; Smith, 1985; Wrigley, 1988) (Fig. 4.2). Two flat seeds (coffee beans) are usually formed in the fruit. If one seed fails to develop, a more rounded seed is formed and designated a 'peaberry'. The fruit resemble cherries when ripe as the exocarp (skin) has a red colour. Two beans lie flat sides together, and are covered individually by a seed coat (silverskin) and then by the yellowish endocarp (parchment). The endocarp is surrounded by an inner mesocarp (mucilage) and outer mesocarp (pulp). When the fruit ripen, a thin layer of mucilage is formed around the endocarp. In order to roast the coffee beans, the layers have to be removed from the coffee cherry and dried. Beans are processed either by a wet method or dry method, the latter being more cost effective as less machinery is necessary for the process. Dried beans ready for roasting are designated green coffee.

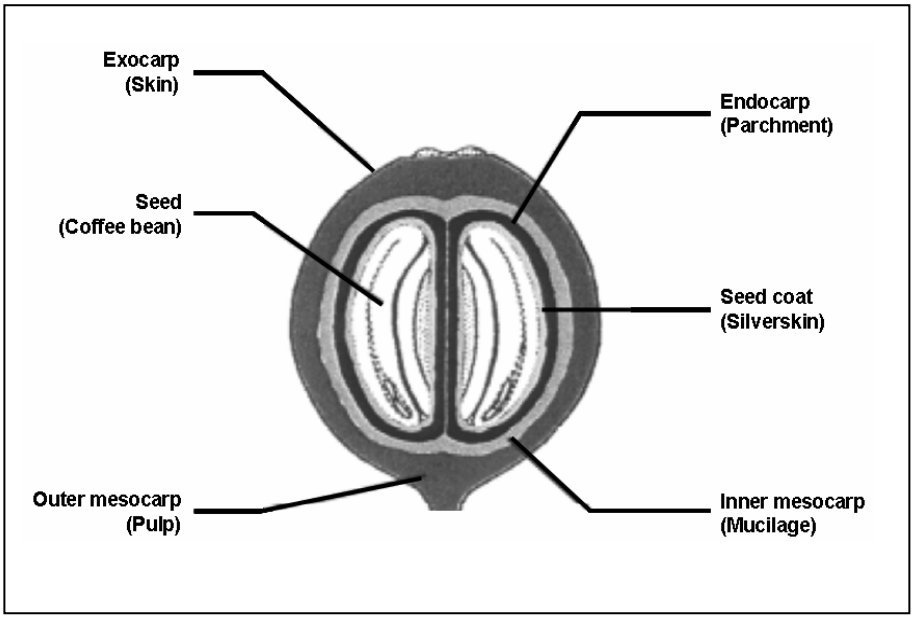

Fig. 4.2 Longitudinal section of a coffee cherry (Avallone et al., 2000).

4.3 PROCESSING OF COFFEE BEVERAGE

4.3.1 Preparation of green beans

The dry method is technologically simpler than the wet method and is the method of choice for drying Robusta beans (Gonzalez-Rios et al., 2007b). However the method chosen for drying depends on the conditions and resources in a production region. At least 95% of the Arabica coffee produced in Brazil and most coffee produced in Ethiopia, Haiti and Paraguay employ the dry method due to cost factors (Smith, 1985). The climate in these regions is constantly warm, allowing easier drying of the entire cherry.

The wet method produces a product that is of better quality compared to the dry method, and the beans command higher prices. This process ensures that the intrinsic qualities of the coffee are preserved. This results in green coffee with less defective beans. The process is however more expensive as specific equipment and large quantities of water are used. Almost all Arabica coffees are produced with this method, with the exception of those produced in Brazil and countries mentioned above.

4.3.1.1 Dry method:

In the dry method or 'natural' method the whole coffee cherry is dried (International Coffee Organization, 2007c; Smith, 1985). The process is comprised of a (i) cleaning, (ii) drying and (iii) hulling step. As the cherries are harvested by a 'stripping' method, cleaning involves separating unripe, overripe and damaged cherries (Vincent, 1987). It is not unusual to find up to 80% of unripe fruit when cherries are harvested in this manner. The separation of the fruit and removing of dirt is performed by winnowing, which is done by hand using a large sieve (Smith, 1985). Washing channels may also be used to separate floating overripe cherries. This concept works on the fact that overripe and dry cherries have a moisture content of 20-50%, whereas unripe and ripe cherries (green, yellow and red) sink with 50-70% moisture content (Vincent, 1987). The cherries are dried in the sun on concrete slabs or raised trestles to 12.0-12.5% moisture content. Drying takes up to 4 weeks and is weather dependent. To ensure even drying, the cherries are raked or turned by hand. This process is the most important step as it affects the end product, namely the green coffee. Over dried beans will be brittle and break during hulling, whereas too moist beans will stand the risk of becoming contaminated by fungi

and bacteria. Dried beans are stored until curing can take place, usually at a different location to where the beans are processed (Vincent, 1987).

4.3.1.2 Wet method:

Beans produced by the wet method are referred to as 'washed' coffee (International Coffee Organization, 2007c; Smith, 1985). As with dry milling, cleaning and sorting of the beans are performed, although in this case in tanks filled with water. The floating cherries are removed and processed using the dry method. The residual ripe cherries are used in the wet method (Vincent, 1987). The key difference between the wet and dry method is the removal of the exocarp and outer pulp or mesocarp before drying of the bean is commenced (Smith, 1985). During depulping, cherries are squeezed by a moving and a fixed surface under water to separate the beans surrounded by the endocarp from the mesocarp and exocarp. It is essential to perform this process as soon as possible after harvesting to ensure that the fruit does not deteriorate, as this will affect the quality of the beans. Pulped beans are then subjected to vibration on screens to separate them from imperfectly pulped beans, followed by water-washing in channels where flotation is used to separate the beans once more. The floating beans consist of very few healthy beans and are discarded (Vincent, 1987). Pulping is done mechanically, and therefore not all of the pulp is removed. The residual flesh is removed from beans in a fermentation step for up to 72 hours by inherent mucilage enzymes. These include pectinases, but fermentation may be accelerated by different micro organisms such as *Saccharomyces* (Vincent, 1987). Additional pectolytic enzymes may be added to speed up the fermentation process even further (Sivetz and Desrosier, 1979) and will be discussed below. Fermentation in water has furthermore proven to generate coffees with more fruity, floral and caramel attributes, whereas dry mucilage removal resulted in more neutral coffees (Gonzalez-Rios et al., 2007a). The fermentation step is closely monitored to ensure that the coffee does not acquire undesirable flavours. The beans are then dried either mechanically in ovens, or on concrete slabs in the sun as described for the dry method. Beans are stored until the curing process.

4.3.2 Curing

The first step in curing usually involves a second drying process of the bean to 11% moisture content. This enables the husk and parchment to be removed more easily (Vincent, 1987). Cleaning then commences to remove dirt, which may include pebbles, dust, metal pieces and other foreign bodies. The beans are then sent for hulling. In this process the entire mesocarp and endocarp outer layers of the beans prepared using the dry method are removed by decortication. The beans prepared by the wet method are de-husked from their parchment. The beans are then sorted, graded and bagged ready for export (Rothfos, 1980).

4.3.3 Roasted coffee

Between green coffee and the finished product of coffee beverage there are several stages: (i) roasting, (ii) grinding, (iii) infusion with water or brewing, and (iv) in the case of soluble or instant coffee beverage, drying followed by reconstitution (Clarke, 1987c; Smith, 1985). During roasting, the characteristic aroma, flavour and colour of the coffee bean are generated and the polysaccharide content is rendered more extractable (Trugo, 1985). Roasting is necessary as green coffee has no desirable taste or aroma. The extracted polysaccharides contribute to organoleptic properties of the coffee brew such as viscosity, mouth-feel and foam stability in espresso coffee as well as retaining volatile substances (Nunes et al., 1997). The increase in ease of extractability is in part due to changes in the microstructure of the bean, as the beans increase in volume and large micropores appear in the cell wall (Schenker et al., 2000). Accompanying these physical changes, amounts of polysaccharide are degraded or structurally modified to a form different from that in the green bean (Redgwell et al., 2002b). Roasting involves the application of considerable heat to the beans, which is kept in motion so as to ensure an even roast. It is a time-temperature-dependent process whereby chemical and physical changes are induced in the green beans (Clarke, 1987c). A loss of dry matter is evident, mainly as CO_2 and water and volatile products as a result of pyrolysis. Roasting proceeds until the colour developed is satisfactory, at which stage the coffee is rapidly cooled down by an air current with or without the addition of water spray or ‘quench’, so as to ensure that the coffee is not burnt (Franca et al., 2005). The roasting is performed in batch or a continuous application.

The roast degree affects the colour of the bean, as well as the taste of the beverage (Clarke, 1987c). The degree of roast is linked to weight loss of the bean, which is an additional

means of determining the degree of roast apart from visual determination. As much as 12-18% weight loss is standard for a dark roast preferred in the European continent, whereas a light roast incurs a 1-5% weight loss only. A lightly roasted washed Arabica will show maximum acidity, thinnish body and possibly insufficiently developed flavour. As the roast increases, the acidity will subside, and the strength or body of the cup will increase. The flavour, however, will improve to a certain point, and then deteriorate into bitterness.

The bean undergoes a two stage transformation during the roasting process. In the first step, the 12.0-12.5% free moisture of the beans is driven off. This stage encompasses up to 80% of the roasting time, and the beans change from a straw like (100°C) to pale brown colour (120-150°C) in a gradual process (Fowler et al., 1998). The second stage of the roasting involves pyrolysis where the beans swell and rapidly change to a darker colour (230°C), which is followed by the emission of oily smoke and crackling sounds. The beans turn brown due to sugar caramelisation coupled to Maillard reactions (Franca et al., 2005). Chemical composition of the beans also change rapidly, which needs to be halted immediately by cooling when the desired grade of roast has been reached.

Consumers requiring a fresh characteristic flavour and aroma should purchase whole roasted beans to be ground when required, as the beans retain their freshness for up to one week in normal atmospheric pressure (Clarke, 1987c). When the beans have been ground, freshness and aroma will start disappearing after 2-3 days and become stale, unless the product has been specially treated and packed (Fowler et al., 1998).

4.4 INSTANT OR SOLUBLE COFFEE PRODUCTION

The first instant product or dried soluble extract was made in the USA and was expensive in relation to its quality (Smith, 1985). In 1938, Nestlé started producing a light coloured powder comprising 50% soluble coffee solids and 50% maltodextrins. The product became known to a larger extent when the product was included in the rations given to soldiers of the USA army during the Second World War. General Products developed a 100% coffee solids product in the 1950s and freeze-dried products were introduced in the 1960s. This was followed with agglomeration of spray-dried soluble coffee and aromatisation. The latter being the adding back of aromatic constituents isolated during early stages of instant coffee production to the final product (Fowler et al., 1998). Factors contributing to the success of instant coffee include (i) the quality of a branded soluble coffees remains constant from one

purchase to the next, (ii) it is easy to prepare and very little time is required for the process of resuspension, (iii) the quality remains stable once the packaging is opened, and (iv) the price per cup is not expensive (Smith, 1985). The manufacturing of soluble or instant coffee is summarised in Figure 4.3.

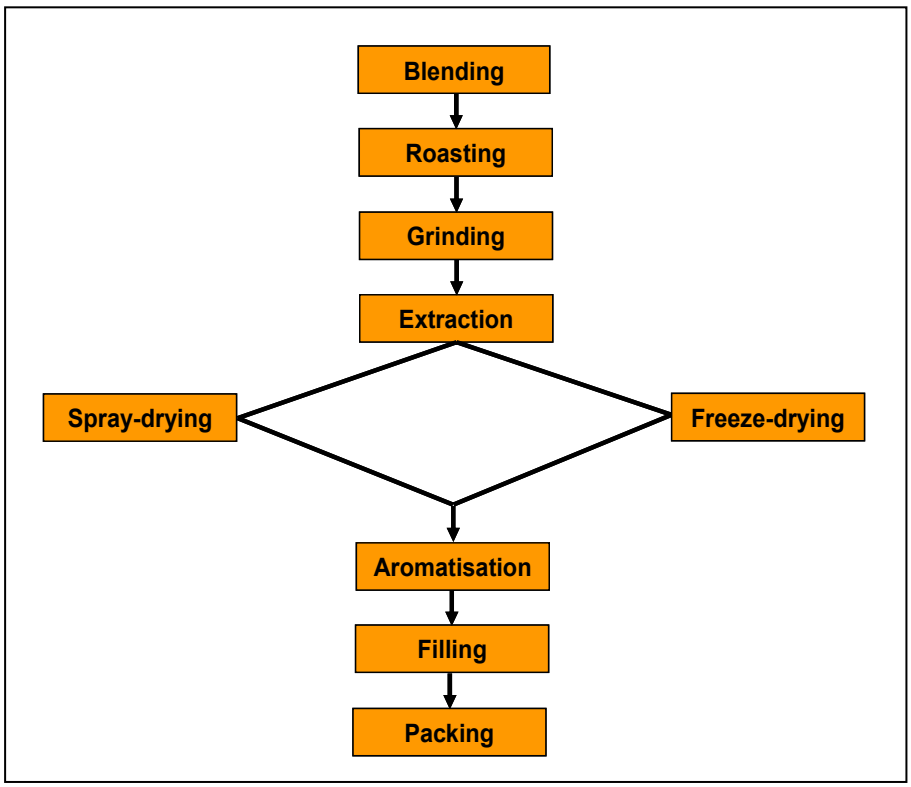

Fig. 4.3 Flow diagram of the instant coffee process (Smith, 1985).

4.4.1 Roasting and grinding

The first step involves producing a coffee extract by roasting. The beans are roasted in rotating cylinders at 165°C or higher. After roasting, the beans are ground to between 0.5 and 1.1 mm. Specialised rollers produce a cutting action rather than a crushing action. In this step the grind size is very important as a too fine powder will impede the passage of the coffee liquor in the extraction columns (Smith, 1985).

4.4.2 Extraction

Coffee is extracted after addition of water to the ground beans at 100-180°C (Clarke, 1987b; Smith, 1985). National Brands Ltd. (NBL) performs the extraction at 170°C. This extraction percolation takes place in a battery of approximately six to seven percolators using a counter-current principle. In this step the optimum extraction of soluble solids is required, without damaging the quality of the product. The optimum yield is determined by the extraction temperature, and as the temperature increases, the cup quality decreases. Manufacturers however employ different temperatures for this procedure depending on the quality of coffee produced. The soluble solids extracted with hot water at 100°C range from 18-25%, whereas the yield is increased to 28-30% with exhaustive extraction. These previous figures relate to Arabica coffee, with figures for Robusta being generally 2-3% higher. When the extraction temperature is increased to 180°C, extraction yield of soluble solids is increased as more proteins, polysaccharides and melanoidins are solubilised.

The counter-current method is employed for extraction, the summary of which appears in Figure 4.4. The hot water at 180°C is introduced to percolator 1, which houses the least fresh coffee beans that have been extracted the most (Smith, 1985). The liquor collects soluble solids and then enters percolator 2, which has some fresher coffee where less extraction has taken place. Solubles are collected and the liquor is then pumped into percolator 3, with coffee slightly fresher than the coffee in percolator 2. The process is repeated until the water reaches percolator 6, which in this case has been charged with fresh coffee. At this stage the liquor passing through the percolator is only around 100°C, and the least damage is therefore done to the delicate flavours essential to the quality of the end-product. The liquor then leaves the percolation system and is cooled and transferred to a storage tank. The spent coffee from percolator 1 is then emptied and charged with fresh coffee, to become percolator 6 in the next extraction cycle. After extraction the liquor in the storage tank is prepared for drying. The liquid is either centrifuged or filtered to remove colloidal tars and other insoluble matter. The liquid is then passed through an evaporator for concentration of the soluble solids. After concentration the liquor is dried. Either spray-drying or freeze-drying is applied (Clarke, 1987a).

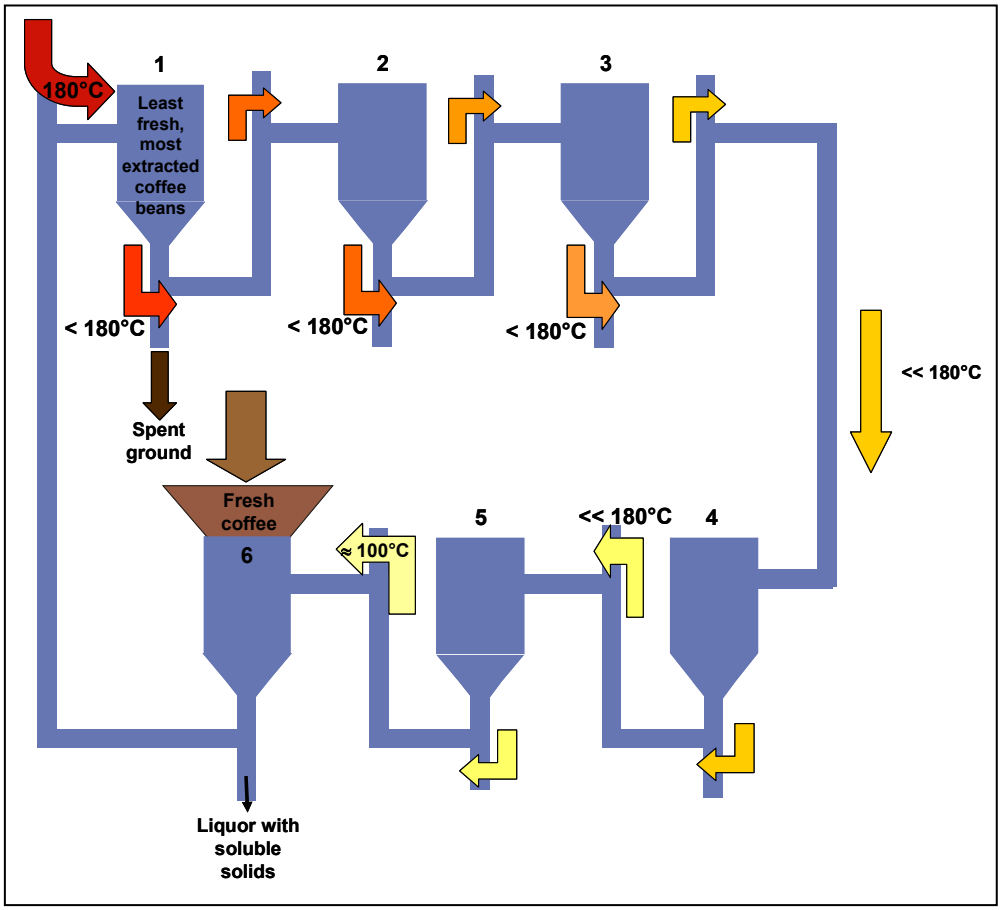

Fig. 4.4 Diagram showing extraction of soluble solids from roasted coffee beans.

4.4.3 Liquor drying

4.4.3.1 Spray-drying:

Spray-drying is performed in a 'cone-shaped' tower (Clarke, 1987a). The liquor enters at the top of the tower under pressure concurrently with a hot air jet at approximately 250°C. The particles drop to the bottom of the cone and lose water to become dried particles, which collect at the bottom of the apex in the cone. The exhaust air leaves at the side of the tower, and residual coffee particles are removed from the air by passage through the cyclone equipment. The objective of the exercise is to produce larger particles, as the 'fines' tend to adhere to the sides of the tower, therefore generating the need for more cleaning. A higher 'fines' concentration in the end product also decreases the quality. Fines are kept to a minimum by the pre-concentration step in the evaporator before drying commences.

Many soluble coffee products contain agglomerated or steam-fused particles rather than a fine powder. Consumers tend to prefer a larger particle than the powder product which tends to produce more foam on the beverage surface at resuspension. Agglomeration is acquired through wetting the powder particles, allowing them to combine, and then re-drying the end-product. Several patents have been complied on the agglomeration of instant coffee.

4.4.3.2 Freeze-drying

Freeze-drying inflicts less damage to food products when drying compared to spray-drying (Clarke, 1987a). The process is however more expensive than spray-drying. During freeze drying, water is removed by sublimation. The concentrated liquor is frozen either by (i) spraying the liquid onto the surface of a rotating refrigerated drum, (ii) filling trays with the liquid and applying cold blasts of air, or (iii) feeding the liquid onto the surface of an endless belt housed in a cold room. The frozen liquor is then grinded to a specific particle size and subjected to a vacuum chamber where the frozen water sublimes from the sample, leaving a dried product.

4.4.3.3 Spray freezing

An alternative to spray-drying and freeze-drying of coffee has recently been proposed (MacLeod et al., 2006). During spray-freeze crystallisation, a liquid or solution is solidified by atomisation in a cold, low humidity environment. This ensures reduced volatility and loss of aroma compounds. When applying the new technique to coffee, a free-flowing product was generated, which retained more aroma and flavour compounds while maintaining qualities of good colour, resuspension and appearance (Mumenthaler and Leuenberger, 1991). The cost of the cold, dry gas which is used during the process is however very high and this process route will therefore rather be preferred for higher value products such as pharmaceutical, biotechnological or functional material applications (Moritz and Nagy, 2002).

4.4.4 Aromatisation, filling and packing

The final freeze-dried or spray-dried product will present an acceptable coffee flavour when reconstituted with water, but the dry product does not have any distinct flavour (Fowler et

al., 1998). For this reason, manufacturers aromatise the product by spraying volatile coffee oil onto the product before packaging. This process is usually performed under a blanket of inert gas, such as CO_2 to ensure that the volatiles in the oil do not oxidise.

4.4.5 Spent ground

The spent ground emptied from the percolators are collected and blown to silos (Adams and Dougan, 1987). This insoluble waste material has a moisture content of 75-80%. The slurry is subjected to a screen press to remove excess process water to produce a cake with approximately 50% moisture content. Residual solids remaining in the screw press effluent is removed by continuous centrifugation. The waste product is then removed from a coffee plant by waste disposal companies. The residual insoluble, but potentially soluble solids in the spent ground are therefore lost. It is difficult to obtain information on exactly how much waste is generated by coffee manufacturers, as there is a reluctance to provide any information which may lead to a procedure to calculate production levels and specific process yields. It is however possible to calculate an approximate value of 480kg (dry basis) of spent ground released from 1 tonne of dry green coffee beans, if it is assumed that 20% is removed by roasting and 40-50% by extraction.

4.5 COFFEE COMPOSITION

The quantity and nature of the extracted soluble solids (including carbohydrates) are of great importance for the instant coffee industry (Trugo, 1985). Polysaccharides, which are the major constituents of green and roasted coffee, play an important role in the generation of soluble solids. Their solubilisation plays a critical role in determining the yield of soluble solids used for instant coffee production (Redgwell et al., 2003). As water temperature for extraction increases, the soluble solid yield increases as well. Home brews with water temperatures between 80-100°C do not extract the coffee exhaustively. Yields may vary from 15% to approximately 28%, dependent on the grind size of the product and the filter machine used. Data for residual carbohydrates in the coffee after extraction has therefore not been determined. Commercial samples of instant coffee generally contain small amounts of arabinose, galactose and mannose, together with trace amounts of sucrose, ribose and xylose. Glucose and fructose present in instant coffees are generally added when chicory is used in the specific blend of instant coffee.

4.5.1 Green coffee

Green coffee contains a large range of different carbohydrates. These are subdivided into polysaccharides and low molecular weight sugars, which include monosaccharides, disaccharides and trisaccharides (Trugo, 1985). The green beans contain approximately 50% polysaccharides, which play an important role in the organoleptic properties of the coffee brew (Bradbury, 2001; Sachslehner et al., 2000; Smith, 1985). These properties include viscosity, mouth-feel and retention of volatile substances (Redgwell et al., 2002b).

Particular polysaccharides are often associated with the cell wall or endosperm (reserve polysaccharide) of the plant material (Trugo, 1985). Lignin is highly insoluble and tends to associate with cell walls, whereas pectins are based on mainly uronic acids and closely related to carbohydrates. In 1960, the constituent monosaccharides of green coffee were confirmed after hydrolysis, and included mannose, arabinose, galactose and glucose, although the polysaccharide make-up was not known (Wolfrom et al., 1960). The polysaccharide content of the green bean has since been the subject of several investigations (Bradbury and Halliday, 1990; Wolfrom et al., 1961; Wolfrom and Patin, 1964 and 1965). Several different percentages of monosaccharide units in green coffee have been published, although mannose always constitutes the largest percentage (Trugo, 1985). The most recent data on precise monosaccharide content in green coffee of Arabica and Robusta varieties are shown in Table 4.1 (Redgwell et al., 2002a).

The structural features of galactomannans present in green coffee have recently been elucidated (Oosterveld et al., 2004). The polysaccharides were extracted from *Coffea arabica* with water (90°C, 1 hour). Anion-exchange chromatography was used to extract the galactomannans, which eluted in two neutral populations. The high molecular weight fraction (2 000 kDa) was highly substituted with both galactose residues (30%) and acetyl groups (9%). The low molecular weight fraction (20 kDa) was much less substituted (11% acetyl groups and 4% galactose residues). The acetyl groups hindered hydrolytic degradation by endo-mannanase from *Aspergillus niger*.

Table 4.1 Monosaccharide composition of total polysaccharides in Robusta and Arabica varieties (Redgwell et al., 2002a).

Variety	Monosaccharide composition (mole %)								Total
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA [a]	(% DW)
Robusta									
Conillon	0.8	0.3	10.7	0.7	42.1	26.4	15.0	3.9	50.6
Cote d'Ivoire	0.9	0.3	9.5	0.4	42.0	27.5	14.4	5.0	45.4
Indes	0.9	0.3	9.5	0.4	42.0	27.5	14.4	5.0	42.9
Arabica									
Catimor	0.6	0.3	8.8	0.6	47.0	23.7	14.7	4.4	45.7
Sarchimor	0.5	0.3	8.2	0.5	48.2	23.3	14.8	4.2	46.0
Yellow Caturra	0.6	0.3	8.2	0.6	47.2	23.0	15.3	4.9	45.0

[a] Approximately two thirds of the uronic acid was galacturonic acid and one third glucuronic acid

4.5.2 Roasted Coffee

Knowledge of the effect of roasting on polysaccharides was based on studies performed in the 1960-1970s (Thaler, 1979). Reviews of the subject have interpreted the data of Thaler in different ways, making it difficult to obtain a clear idea of the actual extent of degradation of individual polysaccharides for a given set of roasting conditions. A summary presented by Trugo et al. (1985) appears in Table 4.2. Soon after, studies showed that coffee arabinogalactans are particularly susceptible to degradation during roasting while the mannans are only moderately degraded (Leloup and Liardon, 1993; Redgwell et al., 2002b). The cellulose remains largely undegraded even at longer roasting times.

Table 4.2 Calculated percentage retention of polysaccharides (determined as monosaccharides) on roasted Arabica coffee (Trugo, 1985).

Monosaccharide	Absolute amount in green coffee	Green (%)	Light Roast (%)	Medium Roast (%)	Dark Roast (%)
Arabinose	1.7	100	40	40	40
Galactose	9.3	100	72	70	70
Mannose	20.8	100	79	65	66
Glucose	6.8	100	87	81	90

The effect of roasting on degradation and structural features of the polysaccharides in Arabica coffee beans using a set of described roasting conditions has been determined

(Redgwell et al., 2002b). In each of three varieties of Arabica coffee beans, between 12 and 24% of the polysaccharide content was degraded after a light roast and after a dark roast, this increased to 35–40%. Arabinogalactans were more susceptible to degradation than the mannans. No significant decrease in cellulose was measured. A comparison of the calculated monosaccharide percentages in a dark roast from the Thaler data and corresponding percentage determined in the Redgwell data are presented in Table 4.3. The percentages for the monosaccharide degraded in the Redgwell data were higher for arabinose and galactose, but similar results were obtained for mannose.

Table 4.3 Figures presented as a comparison between data from 1985 and 2002. (Redgwell et al., 2002b; Thaler, 1979). Percentage monosaccharides that were degraded in dark roasted Arabica coffee is displayed.

Monosaccharide	Thaler data	Redgwell data
Arabinose	60	80
Galactose	30	50
Mannose	34	35

The most comprehensive coverage of data on the effect that roasting has on carbohydrate composition of the coffee bean concluded that carbohydrates are present as polysaccharides which are either extractable or unextractable (Oosterveld et al., 2003). Galactomannans are unextractable in green beans, but become more soluble as the degree of roast increases. Furthermore, the degree of polymerisation and degree of branching of galactomannans decrease with an increase in degree of roast (Nunes and Coimbra, 2002). Arabinogalactans and pectins were the most susceptible to degradation at more severe roasting conditions (Oosterveld et al., 2003). Only the portion of individual sugars from the differentially roasted beans that could not be extracted with water at 90°C for 1 hour, 170°C for 30 min. or 0.05 M NaOH (0°C, 1 hour) is presented in Table 4.4. The amount of individual sugar in the green coffee bean was set to 100%.

Table 4.4 Percentage of individual sugars that was recovered as unextractable polymer (Oosterveld et al., 2003).

Roast	Rha [a]	Ara [b]	Xyl [c]	Man [d]	Gal [e]	Glc [f]	UA [g]
Light	6	3	32	54	3	64	13
Medium	30	4	29	49	3	67	10
Dark	16	2	30	34	3	68	5

[a] Rhamnose, [b] Arabinose, [c] Xylose, [d] Mannose, [e] Galactose, [f] Uronic acid

4.5.3 Spent ground

Residual polysaccharide content in spent ground has as yet not been reported in literature. It is difficult to calculate these values from published data on polysaccharide content removed during extraction for instant coffee production, as almost all manufacturers use different extraction temperatures and do not report the methods that are employed to extract the soluble solids. It would be safe to speculate, however, that spent ground contains mostly cellulose and a large portion of mannan, as only small quantities of mannose has been recorded in instant coffees, and cellulose is undegraded during roasting and extraction.

4.6 ENZYMES AND COFFEE

4.6.1 Enzyme technology in instant coffee manufacturing

To date the majority of publications reporting on enzyme usage in coffee manufacturing are dedicated to the fermentation process (Fowler et al., 1998; Kashyap et al., 2001) and treatment of the liquid coffee extract (Nicolas et al., 1998). During fermentation the mucilage coat is removed from the bean with the aid of pectic enzymes (Fowler et al., 1998; Kashyap et al., 2001). Pectic enzyme preparations are added to speed up the fermentation process as 25-30% of the pulpy layer of the beans constitutes pectic substances (Castelein and Verachtert, 1983). The pectins contain 60% uronic acids with a high degree of methyl esterification and a moderate degree of acetylation. The additional side activities of cellulases and hemicellulases in the pectic enzyme preparation aid in the digestion of the mucilage layer. This is due to approximately 8-9% cellulose and 15-18% neutral noncellulosic polysaccharides present in the mucilage layer (Avallone et al., 2000 and 2001). A diluted commercial enzyme preparation is added at 2-10 gram per ton beans at 15-20°C. The fermentation step is accelerated as a result of the addition of the commercial

enzyme over and above inherent enzymes in the fermenting pulp, which include pectin esterase, galacturonase, α-galactosidase, peroxidase and polyphenol oxidase (Amorim and Amorim, 1977). The enzyme-assisted fermentation is shortened up to 20-40 hours compared to the usual 40-80 hours needed when natural fermentation takes place. As commercial enzyme preparations are costly, the inoculated waste mucilage is used as source of microbial pectic enzymes. The fermentation liquid is washed, filtered and then sprayed onto a new set of beans ready for fermentation, thereby recycling the commercial preparation of pectic, cellulolytic and hemicellulolytic enzymes.

Galactomannan contains 94% mannose residues and provides the hardiness to coffee beans (Smith, 1985). It is composed of a linear chain of ß-D-1,4-linked mannose units with single unit galactose side chains at C-6 (Bradbury and Halliday, 1990; Navarini et al., 1999; Sachslehner et al., 2000; Wolfrom et al., 1961). It is not surprising then that endo-β-1,4-mannanase has been shown to reduce the viscosity in coffee extracts (Wong and Saddler, 1993). Mannanases from *Sclerotium rolfsii* also hydrolyse coffee mannan and reduce viscosity (Sachslehner et al., 2000). Furthermore, *Aspergillus awamori* is induced to produce β-mannanase and β-mannosidase when grown on coffee waste as carbon source (Kurakake and Komaki, 2001). In the industry, immobilised β-mannanases are used to hydrolyse galactomannan in coffee extract, thereby decreasing viscosity and preventing formation of a gel during freezing of the product (Nicolas et al., 1998).

4.7 SHORTCOMINGS IDENTIFIED IN THE CURRENT INSTANT COFFEE MANUFACTURING PROCEDURE

Instant coffee is produced by the extraction of soluble solids from roasted beans by using thermal extraction. Only half of the total coffee bean dry weight can be extracted in this manner and used in the final product. The remainder of the product is discarded as coffee spent ground. The presence of the insoluble material in the coffee beans therefore represents a loss of raw material, final product and possible profits to the manufacturer. Hydrolytic enzymes may be able to hydrolyse the insoluble matter, therefore increasing soluble solid yield extracted from the bean. This will increase overall yield of instant coffee product, and decrease the amount of coffee beans that are imported for production. It will also reduce the amount of spent ground waste produced by the process, which is a substantial operational cost.

The goal of this study was therefore to increase soluble solid yields extracted from coffee spent ground after enzyme treatment for use in the industrial process of manufacturing instant coffee. Objectives were identified to realise the goal of this study, which include: (i) isolate and screen enzyme cocktails from recombinant and wild type fungal strains for enzyme activities, which could increase soluble solids extracted from coffee spent ground; (ii) acquire commercial enzyme cocktails which could increase soluble solids extracted; (iii) characterise the recombinant enzymes and selected enzymes present in the cocktails; (iv) analyse the polysaccharide content of roasted coffee beans and spent ground; and (v) perform and optimise extraction experiments to quantify the increase in soluble solid yield after enzyme treatment of spent ground.

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CHAPTER 5: Microbial enzymes for the instant coffee industry

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Document prepared for submission to Applied Microbiology and Biotechnology.

Abstract

Instant coffee is produced by thermal water extraction from roasted Robusta and Arabica coffee beans. Approximately half of the total coffee bean dry weight can be extracted in this manner and used in the final product, and residual insoluble material in the beans is discarded as waste product. Hydrolytic enzymes may be able to hydrolyse the insoluble matter in the waste product (coffee spent ground), thereby increasing soluble solid yield, decreasing the amount of coffee beans imported for production, and reducing the amount of coffee spent ground generated. Monosaccharide analysis of coffee spent ground revealed that the coffee spent ground comprised mainly of cellulose and mannan. Mannanases, cellulases, xylanases and pectinases were applied to coffee spent ground to increase the soluble solid yield. Yield increases of up to 16.6% were obtained with the *Trichoderma aculeatus* mannanase (Man1) secreted by a recombinant *Aspergillus niger* strain. This figure was increased to 23% when the enzyme hydrolysis procedure was optimised using the Shin Nihon mannanase enzyme. Cellulases did not perform as well as the mannanase enzymes due to the recalcitrant nature of the polysaccharide. Synergism was not detected between any of the enzyme combinations tested. Applying a mannanase enzyme to coffee spent ground will increase soluble solid yield generated and benefit the current instant coffee production process substantially.

Introduction

Instant coffee production is dependent on new innovative ways to increase productivity of the process, to allow for an increase in profitability and to sustain the growing demand for the product. The instant coffee product is produced by thermal water extraction from roasted Robusta and Arabica coffee beans. Approximately half of the total coffee bean dry weight can be extracted in this manner and used in the final product, and residual insoluble material in the beans is discarded as waste product (Adams and Dougan, 1987). The presence of the insoluble material in the coffee beans represents a loss of raw material, final product and possible profits to the manufacturer. The cost of waste removal further adds cost to the manufacturing process. The technology to increase soluble solids extracted from roasted coffee beans for instant coffee processing without unnecessarily changing the extraction process is therefore in high demand.

Polysaccharides, which are the major constituents of green and roasted coffee, play an important role in the generation of soluble solids (Trugo, 1985). Their solubilisation determines the yield of soluble solids used for instant coffee production (Redgwell et al., 2003). Polysaccharides become

soluble or remain unextractable during roasting (Oosterveld et al., 2003b). The polysaccharides which remain unextractable will constitute the residual material in coffee spent ground.

Cellulose remains unextractable during roasting and the solubility of this polysaccharide is not affected by the degree of roast. Galactomannans are unextractable in green beans, but become more soluble as the degree of roast increases. Arabinogalactans and xylan are easily degraded during extraction and become soluble. It would however be safe to speculate that spent ground contains mostly cellulose and a large portion of mannan, as only small quantities of mannose has been recorded in instant coffees, and cellulose is undegraded during roasting and extraction.

The degree of polymerisation and degree of branching of galactomannans decrease with an increase in degree of roast (Nunes and Coimbra, 2002). Heat administered during the water extraction for instant coffee production breaks some of the interchain hydrogen bonding by opening the cell-wall matrix (Leloup and Liardon, 1993; Oosterveld et al., 2003b). This has proven to be the case where the extractability of galactomannans from green beans using water was very low (Fischer et al., 2001; Oosterveld et al., 2003a), and improved dramatically after roasting (Nunes and Coimbra, 2001; Oosterveld et al., 2003b). It can therefore be argued that the polysaccharides remaining in spent ground should be more readily hydrolysable by hydrolytic enzymes.

To date the majority of publications reporting on enzyme application in coffee manufacturing are dedicated to the fermentation process (Fowler et al., 1998; Kashyap et al., 2001) and treatment of the liquid coffee extract (Nicolas et al., 1998). During fermentation, the mucilage coat is removed from the bean with the aid of pectic enzymes (Fowler et al., 1998; Kashyap et al., 2001). Pectic enzyme preparations are added to speed up the fermentation process as 25-30% of the pulpy layer of the beans constitutes pectic substances (Castelein and Verachtert, 1983). In the industry, immobilised β-mannanases are used to hydrolyse galactomannan in coffee extract, thereby decreasing viscosity (Wong and Saddler, 1993) and preventing formation of a gel during freezing of the product (Nicolas et al., 1998). Mannanases from *Sclerotium rolfsii* also hydrolyse coffee mannan and reduce viscosity (Sachslehner et al., 2000). Furthermore, *Aspergillus awamori* is induced to produce β-mannanase and β-mannosidase when grown on coffee waste as carbon source (Kurakake and Komaki, 2001).

In the present investigation, soluble solid yields extracted from coffee spent ground were increased after enzyme treatment with mannanases, cellulases, xylanases and/or pectinases. Enzymes from wild-type and recombinant organisms, as well as commercial enzyme cocktails, were applied to the coffee spent ground during hydrolysis experiments. The parameters of the hydrolysis experiments were optimised for use in the industrial process of manufacturing instant coffee.

Materials and Methods

Chemicals

All chemicals, media components and supplements were of analytical grade standard. Different batches of coffee spent ground (screw-press treated) as well as water drained from spent ground were acquired from National Brands Limited (NBL) (Isando, South Africa). Commercial enzymes were acquired from enzyme distributing companies and are listed in Table 1.

Strains and media

The genotypes and sources of recombinant fungal strains used in the experiments are summarised in Table 2. Recombinant fungal strains were maintained at 30°C on minimal medium (1% glucose, 0.6% $NaNO_3$, 0.2% neopeptone, 0.15% KH_2PO_4, 0.1% yeast extract, 0.1% casamino acids, 0.05% $MgSO_4$, 0.05% KCl, trace elements, and 18 g l^{-1} bacteriological agar). Fungal strains were cultivated in liquid cultures at 30°C with agitation at 100 rpm in double-strength minimal medium (2 x MM) containing 0.4% casamino acids, 0.08% $MgSO_4$, 10% glucose, 1.2% $NaNO_3$, and trace elements.

Wild-type fungal strains were maintained at room temperature on malt extract agar (MEA) (2% malt extract, 2% bacteriological agar). The strains were cultivated at 30°C and 100 rpm in minimal medium with spent ground (2% spent ground, 0.5% glucose, 0.6% $NaNO_3$, 0.2% neopeptone, 0.15% KH_2PO_4, 0.1% yeast extract, 0.1% casamino acids, 0.05% $MgSO_4$, 0.05% KCl, and trace elements) or locust bean gum (2% locust bean gum, 0.5% glucose, 0.6% $NaNO_3$, 0.2% neopeptone, 0.15% KH_2PO_4, 0.1% yeast extract, 0.1% casamino acids, 0.05% $MgSO_4$, 0.05% KCl, and trace elements) when screening for enzyme activities.

Isolation of wild-type fungal strains

Wild type fungal strains producing β-mannanases were isolated in our laboratory from coffee beans and coffee spent ground. A substrate consisting of coffee beans or spent ground, or chicory spent-grain, was incubated in a moisture chamber at 22°C for a period of two weeks. During this time fungal growth was periodically transferred to MEA. The fungal isolates growing on MEA were purified by preparing single-spore cultures, followed by successive cultivation on MEA at 22°C.

Table 1 Summary of commercial enzymes used in this study.

Enzyme	Code	Distributor	Microbial source	Specific activities	pH	Temp
Depol^{TM} 40L	D040L	Biocatalysts	*Trichoderma* sp. and *Aspergillus* sp.	1 200 U g^{-1} cellulase, 800 U g^{-1} endo-galacturonase	4.0-6.0	40-60°C
Depol^{TM} 670L	D670L	Biocatalysts	Blend of fungal enzymes	1 200 U g^{-1} cellulase, 800 U g^{-1} pectinase (galacturonase)	4.0-6.0	50-65°C
Depol^{TM} 667P	D667P	Biocatalysts	*Trichoderma* sp.	12 000 U g^{-1} β-glucanase	5.0-7.0	45-55°C
Depol^{TM} 697P	D697P	Biocatalysts	Blend of *Rhizopus* and *Trichoderma* sp.	90 U g^{-1} β-glucanase, 280 U g^{-1} endo-galacturonase (PG), 540 U g^{-1} cellulase	4.0-7.0	20-60°C
Depol^{TM} 112L	D112L	Biocatalysts	*Trichoderma* sp.	7 000 U g^{-1} β-glucanase	3.5-6.0	50-65°C
Cellulase 13L	C013L	Biocatalysts	*Trichoderma* sp.	1 500 U g^{-1} cellulase	3.5-6.0	50-70°C
Macer8^{TM} FJ	M263L	Biocatalysts	*Aspergillus* sp.	1 500 U g^{-1} endo-galacturonase (high levels of pectin lyase (PL), polygalacturonase (PG), and arabanase side-activities)	3.0-5.0	40-60°C
Macer8^{TM} FJ	M282L	Biocatalysts	*Aspergillus* sp.	1 000 U g^{-1} Pectinase	4.5-5.5	10-60°C
Pectinase 690L	P690L	Biocatalysts	*Aspergillus* sp. and *Trichoderma* sp.	300 U g^{-1} polygalacturonase (PG), 300 U g^{-1} cellulase (high levels of PG and low levels of PL)	3.0-5.5	35-45°C
Pectinase 62L	P062L	Biocatalysts	*Aspergillus* sp.	2 200 U g^{-1} endo-galacturonase (high levels of PG, lower levels PL, and has arabanase side-activities)	3.0-5.0	10-50°C
Pectinase 162L	P162L	Biocatalysts	*Aspergillus* sp.	900 U g^{-1} endo-galacturonase g^{-1}, 900 U g^{-1} cellulase	3.0-5.5	35-45°C
Pectinase 444L	P444L	Biocatalysts	*Aspergillus* sp.	600 U g^{-1} endo-galacturonase (lower levels of PG, higher levels PL, and has arabanase side-activities, and low level of pectin esterase)	2.5-5.5	45-55°C
Pectinex Ultra SP-L		Novozymes	*Aspergillus aculeatus*	26 000 U g^{-1} polygalacturonase, 51 U g^{-1} cellulase, 84 U g^{-1} cellobiase, 12 U g^{-1} β-glucosidase (higher levels of PL)	4.5-5.0	30-40°C

Table 1 Summary of commercial enzymes used in this study (continued).

Enzyme	Code	Distributor	Microbial source	Specific activities	pH	Temp.
Mannanase	SNM	Shin Nihon	NR	NR	NR	60°C
Mannanase	GM	Genencor International	NR	1 220 000 MNU g^{-1} mannanase	NR	50°C
Cellulosin GM5		Anchor Bio-technologies	*Aspergillus niger*	10 000 U g^{-1} galactomannanase	3.0-9.0	30-75°C
Mannanase-L		River Biotech	*A. niger*	10 000 U g^{-1} mannanase with cellulase and hemicellulase side activities	3.0-6.0	60-70°C
Cellulase complex	NS50013	Novozymes	NR	700 U g^{-1} endo-glucanase	4.5-6.5	45-60°C
Enzyme complex	NS50012	Novozymes	NR	100 U g^{-1} fungal β-glucanase	3.5-5.5	25-55°C
β-Glucanase	NS50029	Novozymes	NR	200 U g^{-1} β-glucanase	5.5-8.0	30-60°C
Gamanase		Novozymes	*Aspergillus* sp.	1 000 000 viscosity U g^{-1} hemi-cellulase	3.0-6.0	60-70°C
Glucanase 5XL	G015L	Biocatalysts	*Trichoderma longibrachiatum*	12 500 U g^{-1} β-glucanase	3.0-6.5	50-65°C
	TP668L	Biocatalysts	NR	Trial product		
	TP692L	Biocatalysts	NR	Trial product		

NR indicates not reported

Table 2 Summary of recombinant strains used in this study.

Plasmids	Relevant genotype	Recombinant enzyme and source	Reference
A. niger D15 [pGT]	*A. niger* D15 with gpd_P-$glaA_T$	None	(Rose and van Zyl, 2002)
A. niger D15 [*man1*]	*A. niger* D15 with gpd_P-*man1*-$glaA_T$	β-mannanase from *Aspergillus aculeatus* (MRC11624)	Our laboratory
A. niger D15 [*eg1*]	*A. niger* D15 with gpd_P-*eg1*-$glaA_T$	β-endo-glucanase 1 from *Trichoderma reesei* (QM6a)	(Rose and van Zyl, 2002)
A. niger D15 [*eg2*]	*A. niger* D15 with gpd_P-*eg2*-$glaA_T$	β-endo-glucanase 2 from *T. reesei* (QM6a)	Our laboratory
A. niger D15 [*xyn2*]	*A. niger* D15 with gpd_P-*xyn2*-$glaA_T$	β-xylanase 2 from *T. reesei* (QM6a)	(Rose and van Zyl, 2002)

The pure cultures were deposited in the fungal culture collection of the Department of Microbiology at the University of Stellenbosch. The isolates were maintained in this culture collection until they were tested for production of β-mannanase. Other fungal strains in the departmental culture collection mentioned above, which were originally selectively isolated from the natural environment using complex plant materials (e.g. lignocellulose, mannan or xylan) as carbon source in selective media, were also screened for enzyme activities that could increase soluble solid yield from coffee spent ground.

Inoculum preparation

Spores from fungal strains growing on minimal medium were harvested by aspiration with a physiological salt solution (FSO) (0.9% NaCl) and stored at 4°C. Spores were produced on large scale in flasks containing brown rice. Flasks (250 ml) were autoclaved containing 20 g brown rice with 8 ml of a 0.1% urea solution. The rice cake was inoculated with a spore solution and incubated at 30°C for 4 days. Spores were harvested with FSO and stored at 4°C until further use.

Determination of monosaccharide fractions in coffee spent ground

Monosaccharide fractions were determined by Dr. Paul Weimer (USA Dairy Forage Research Centre, University of Wisconsin-Madison). Polysaccharides were hydrolysed by subjecting 0.25 g of sample to two-stage sulphuric acid hydrolysis (Moore and Johnson, 1967). After neutralisation with $CaCO_3$ to pH~5.5, samples were supplemented with 20 mg of myo-inositol (internal standard), then centrifuged (1,500 x g, 15 min.), and 10 ml of supernatant was lyophilised. The lyophilised samples were resuspended in 1000 µl deionised water, and subsequently centrifuged at 12,000 x g for 5 min. Supernatants were dried under an air stream and then subjected to reduction with sodium borodeuteride, and acetylation with acetic anhydride (Blakeney et al., 1983). Gas-liquid chromatography of alditol acetates was performed using a Hewlett-Packard 6890 Plus GC fitted with a flame ionisation detector and a Supelco SPB-225 capillary column (30 m x 0.25 mm, with 0.25 µm film thickness). Helium was used as carrier gas. The temperature program was as follows: 215°C for 2 min., then increased at 4°C per minute for 3.75 min., and finally held at 230°C for 11.25 min.

Enzyme assays

For enzyme production, 2 x MM medium was inoculated with 1 X 10^6 spores ml^{-1}. For non-spore generating organisms, agar squares (10 mm X 10 mm) of freshly grown mycelium were removed from the agar using a scalpel and inserted into a 3 ml syringe. The agar was dispersed from the syringe into growth medium for liquid culturing. The cultures were cultivated for 6 days at 30°C and 100 rpm. Afterwards the supernatant (SN) was harvested by filtration through miracloth (Calbiochem, EMD Biosciences Inc., Darmstadt, Germany) to remove the fungal biomass and centrifuged at 10,000 rpm for 45 min. to remove spores and debris. The SN was concentrated by ultrafiltration at 100 kPa using the Minitan system (Millipore Corporation, Bedford, MA, USA) housing membranes with a cut-off of 10 kDa.

Enzyme activity was determined in liquid assays using the DNS method (Miller, 1959). Conditions are listed in Table 3. Commercial enzyme preparations or samples from the culture SN served as enzyme source and were diluted with 50 mM sodium citrate buffer set at pH~5.0 as the pH of the spent ground was in the range of 4.8-5.2. Diluted enzyme samples were added to substrate solutions (indicated in Table 3) for hydrolysis which was terminated after 5 min. by the addition of DNS solution (1% 3,5-dinitro-salicyclic acid, 20% potassium sodium tartrate, 1% NaOH, 0.2% phenol, 0.05% Na_2SO_3). The reactions were boiled at 100°C in a water bath for 15 min. and colour development in cooled samples was measured as absorbance at 540 nm. The reducing sugars released in 5 min. were determined from linear standard curves, which were plotted using mannose, glucose and xylose as standards. One nanokatal (nkat) of enzyme activity is defined as the amount of enzyme producing 1 nmol of reducing sugars (mannose, glucose, or xylose) per second under the given conditions. Total protein concentration of a sample was determined using the Bio-Rad protein assay (Bio-Rad Inc., CA, USA).

Table 3 Substrate and assay conditions used to determine enzyme activity.

Enzyme activity	Substrate	Assay conditions	Standard curve equivalent
β-mannanase	0.25% locust bean gum galactomannan	60°C and pH 5	mannose
β-glucanase	1% carboxymethylcellulose (CMC)	60°C and pH 5	glucose
β-xylanase	1% birchwood xylan	55°C and pH 5	xylose
polygalacturonase	0.05% polygalacturonic acid	See Table 6	glucose
amylase	0.1% soluble potato starch	See Table 6	glucose

The activity of the different hydrolase enzymes (Man1, Eg1, Eg2 and Xyn2) was measured in duplicate at temperatures ranging from 30-100°C with 5°C increments to determine the temperature at which the enzymes showed the highest activity. The enzymes were incubated for up to 5 hours at specific temperatures to determine stability, and the effect of bovine serum albumin (BSA) on the activity and stability was also tested in triplicate. Stability was further assayed using water drained from the spent ground instead of the 50 mM sodium citrate buffer (pH~5.0).

Hydrolysis experiments

a) Assessment of thermal treatment methods

Three different thermal treatment methods were compared to determine which method would yield the highest increase in soluble solids without enzyme treatment. Four flasks containing 30 g of spent ground were pre-wet at 50°C with agitation for 5 hours using 100 ml sodium citrate buffer (50 mM, pH~5.0). Sodium azide (NaN_3) (0.02% w/v) was added to all flasks to prevent bacterial growth. The content of the first flask was filtered through 150 mm diameter filter papers (Whatman International Ltd, Maidstone, England) to remove spent ground from SN. Fifty millilitres of the SN was freeze-dried as control for the pre-wet stage. BSA was added to the other three flasks at a final concentration of 0.1 mg ml^{-1} and incubated with agitation at 60°C for 18 hours to mimic the hydrolysis stage. The contents from all three flasks were filtered as described above and 50 ml from each flask was freeze-dried. The spent-ground was returned to the flasks and re-suspended with sodium citrate buffer (50 mM, pH~5.0) to a final volume of 100 ml for the thermal treatments. The first of the three flasks was incubated with agitation at 80°C for 18 hours. The second flask was subjected to a liquid autoclave cycle for 15 min. at 100 kPa, followed by incubation with agitation at 80°C for 18 hours. The third flask was subjected to a liquid autoclave cycle for 30 min. at 100

kPa. After thermal treatment, the contents of all 3 flasks were filtered as described earlier and 50 ml SN from each reaction was freeze-dried. All the freeze-dried samples were weighed to determine the yield increase of soluble solids, which was expressed as a percentage of soluble solids generated per dry weight spent ground (w/w %). The experiment was done in triplicate.

b) Hydrolysis of spent ground: Freeze-dry method

Thirty grams of spent ground was pre-wet at 50°C for 5 hours with agitation using 100 ml sodium citrate buffer (50 mM, pH~5.0). Enzyme was added for hydrolysis at different enzyme activity levels and protein weights. BSA (0.1 mg ml^{-1}) was added to certain hydrolysis reactions to stabilise the recombinant enzymes. Spent ground was hydrolysed by incubation with agitation at temperatures of 35°C-60°C for 18 hours. Supernatant was filtered through 150 mm diameter filter papers (Whatman) to remove spent ground and 50 ml supernatant was freeze-dried for soluble solid determination. The spent ground was re-suspended to a final volume of 100 ml with sodium citrate buffer (50 mM, pH~5.0) and subjected to a liquid autoclave cycle for 30 min. per at 100 kPa (thermal treatment). Supernatant was filtered and soluble solids determined after freeze-drying and weighing the sample. The soluble solid yield was expressed as a percentage of soluble solids generated per dry weight spent ground (w/w %).

c) Hydrolysis of spent ground: Total sugar method

As described in point b), spent ground was treated with enzyme and filtered supernatant was used for total sugar determination. Total sugars were determined using the phenol-sulphuric acid method (Dubois et al., 1956). In short, samples were mixed with 5% phenol and mixed. Concentrated sulphuric acid was added and the optical density measured at 490 nm. Total sugars were then determined from a linear standard curve, which was plotted using glucose as standard. Soluble solids were calculated from a standard curve taking total sugars and soluble solids produced from control experiments (no enzymes added) into account. The spent ground was re-suspended with deionised water and subjected to a liquid autoclave cycle for 30 min. at 100 kPa (thermal treatment). Supernatant was filtered and soluble solids calculated after total sugar determination.

d) Hydrolysis optimisation

River Biotech (Cape Town, South Africa) optimised the conditions used for enzyme hydrolysis. Different parameters were tested and yield increases were measured by total sugar assays as described in point c). The Mannanase-L was added to the hydrolysis reactions at different enzyme dosages (0.07, 0.09, 0.11, and 0.15% w/w spent ground). Different hydrolysis temperatures (56, 60, 64, and 70°C) and spent ground to water ratios (15:100, 25:100, 30:100 and 45:100) were also tested. Samples were taken at 0, 1, 2, 3, 5 and 16 hours during the hydrolysis reactions to determine total sugars. The values determined were normalised by subtracting total sugar present in control reactions where no enzymes were added and samples taken at time 0 hours.

Additional laboratory-scale, semi-commercial and pilot-plant trials were performed by River Biotech. Parameters tested included enzyme type, enzyme dosage, contact time for enzyme with spent ground, and particle size of spent ground. The Shin Nihon Mannanase (SNM) enzyme (commercial Japanese enzyme), and Genencor Mannanase (GM) enzyme (experimental European enzyme) were applied to spent ground at different enzyme dosages which included 0.2, 0.5, 1.0, and 2.0 nkat mg^{-1} spent ground. Enzyme hydrolysis was performed at the optimum temperature of the enzymes, which were 60°C and 50°C for the SNM and GM enzymes, respectively. Sampling was performed at intervals to determine optimum contact time of the enzyme on the substrate. Spent ground was added to the experiments 'as-is' or finely milled to increase surface area of the particles.

Results

Monosaccharide analysis

A large portion of the arabinogalactan and almost all of the xylan present in the roasted beans are hydrolysed during the coffee extraction. The residual monosaccharides present in the spent ground are largely mannan and cellulose (Fig. 1).

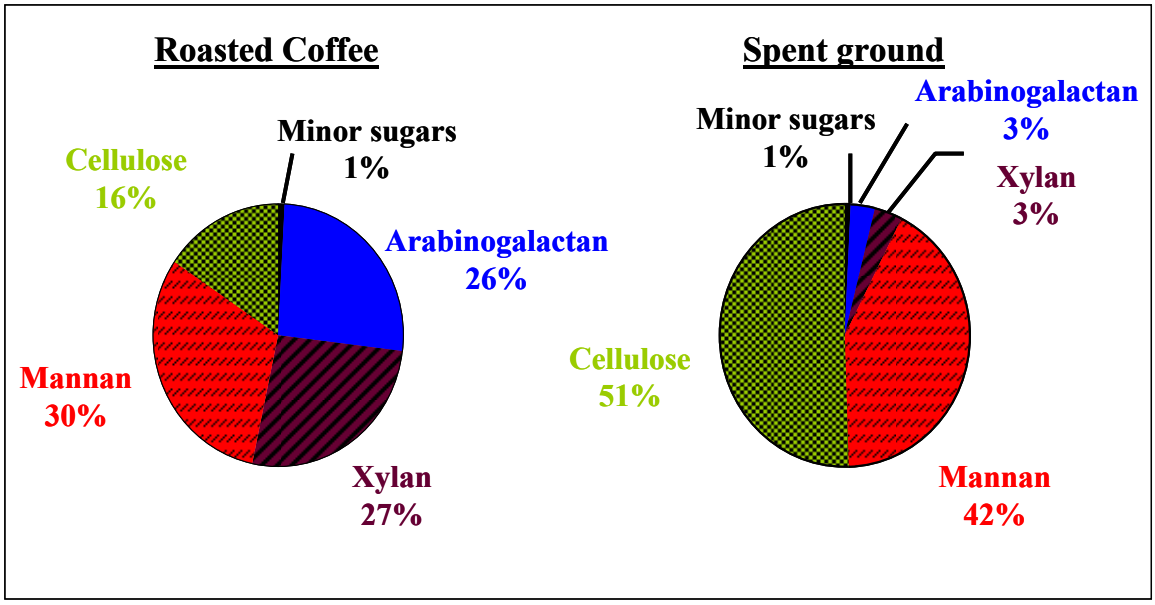

Fig. 1 Pie charts indicating the fraction of monosaccharides in roasted ground coffee and spent ground.

Enzyme production and activities

Fifty-two wild-type fungal strains that were screened for mannanase, endo-glucanase and xylanase activities included two *Penicillium*, three *Aspergillus*, eleven *Pleurotus*, two *Schizophyllum*, four *Lentinus*, one *Laetiporus*, one *Rhizopus*, one *Aureobasidium*, nine *Thermomyces*, fourteen *Sclerotium* and three unidentified strains. The strains that showed levels of activity are summarised in Table 4. Of these, ABO500 and ABO503 showed the highest mannanase activities in the enzyme cocktails secreted into the growth media.

Table 4 Summary of hydrolase activity produced by wild-type strains.

Strain #	Source	Identification	Mannanase [a]	Mannanase [b]	Endo-glucanase	Xylanase
ABO 499	Isolated from contaminated coffee	*Penicillium* variable	ND	+	-	-
ABO500	Vietnam Robusta (WH Van Zyl)	*Aspergillus*	ND	++	-	-
ABO503	Indian Robusta (WH Van Zyl)	*Penicillium purpurogenum*	ND	+++	+	++
ST3-3	Stellenbosch collection (mushroom)	*Pleurotus ostreatus*	ND	-	-	+
S11-1-2	Stellenbosch collection (mushroom)	*Pleurotus pulmonarius*	ND	+	-	-
S10-2	Stellenbosch collection (mushroom)	*Pleurotus florida*	ND	-	-	++
ABO374	Unknown	Unidentified	++	-	ND	ND

[a] Cultivation in locust bean gum medium

[b] Cultivation in spent ground medium

- no detectable activity (0-30 nkat ml^{-1}), + low activity (30-100 nkat ml^{-1}), ++ medium activity (100-1000 nkat ml^{-1}), or +++ high activity (1000 and higher nkat ml^{-1})

The respective activities of Man1, Eg1, Eg2, Xyn2 (recombinant enzymes), and native mannanases from ABO503 and ABO500 produced in 200 ml liquid culture were assayed on day 6 before harvest of the cultures. The mannanase activity for Man1, ABO503 and ABO500 (nkat ml^{-1}), endo-glucanase activity for Eg1 and Eg2 (nkat ml^{-1}), and xylanase activity for Xyn2 (nkat ml^{-1}) as well as total protein content (mg l^{-1}) of the concentrated enzymes were determined. These results are summarised in Table 5.

Table 5 Summary of activities and crude protein concentrations of enzymes.

Enzyme	**Activity (nkat ml^{-1})**	**Conc. Activity [a] (nkat ml^{-1})**	**Conc. protein [b] (mg l^{-1})**	**Specific activity (nkat mg^{-1} protein)**
Man1	1 700	8 000	400	20 000
Eg1	2 000	11 000	800	13 750
Eg2	3 500	13 500	2 100	6 429
Xyn2	3 000	14 500	100	145 000
ABO503 mannanase	2 100	35 000	3000	11 667
ABO500 mannanase	350	3 100	3000	1 333

[a] Concentrated enzyme activity

[b] Total/crude protein content in concentrated enzyme sample

Xyn2 displayed the highest specific activity for al the enzymes tested. The total protein concentration obtained from the concentrated SN from *A. niger* D15 [pGT] (reference strain) was 82 mg l^{-1} and no measurable enzyme activity was detected in the cocktail. Enzyme activities in commercial enzyme cocktails are indicated in Table 6.

Table 6 Enzyme activities present in commercial enzyme cocktails, activities are the average of three determinations.

Enzyme	Code	Protein [a]	Assay conditions	Mannanase [b]	EG [c]	PG [d]	Amylase [e]
Pectinex Ultra SP-L		11 300	pH 5.0 and 35°C	280	377	11 200	169
Cellulosin GM5		6 750	pH 5.0 and 60°C	1 764	225	ND	ND
Macer8™ FJ	M263L	8 000	pH 5.0 and 50°C	3 738	1 795	2 407	3 000
Macer8™ FJ	M282L	22 300	pH 5.0 and 55°C	312	ND	ND	ND
	TP668L	25 500	pH 5.0 and 60°C	48 439	2 597	26	71
	TP692L	21 540	pH 5.0 and 55°C	502	ND	ND	ND
Depol™ 667P	D667P	ND	pH 5.5 and 50°C	7	ND	ND	ND
Depol™ 697P	D697P	ND	pH 5.5 and 50°C	7	ND	ND	ND
Depol™ 40L	D040L	27 960	pH 5.0 and 55°C	512	ND	ND	ND
Depol™ 112L	D112L	29 860	pH 5.0 and 55°C	246	ND	ND	ND
Depol™ 670L	D670L	32 270	pH 5.0 and 55°C	559	ND	ND	ND
Pectinase 690L	P690L	7 254	pH 5.0 and 55°C	1 562	ND	ND	ND
Glucanase 5XL	G015L	39 347	pH 5.0 and 55°C	212	ND	ND	ND
Cellulase 13L	C013L	33 277	pH 5.0 and 55°C	330	ND	ND	ND

[a] Total protein content in enzyme sample (mg l^{-1})

[b] Specific mannanase, [c] endo-glucanase, [d] polygalacturonase, and [e] amylase activities determined in nkat mg^{-1} protein with DNS assays. Assay conditions are indicated in the table.

ND indicates not determined

Enzyme characterisation

a) Temperature optima

The temperatures at which the enzymes displayed optimum activity are shown in Figure 2. The Man1, Eg2 and mannanase enzyme from ABO503 displayed optimum temperatures of 80°C, 75°C and 75°C, respectively. Eg1 showed maximum activity at 60°C, whereas Xyn2 had the lowest optimum of 55°C.

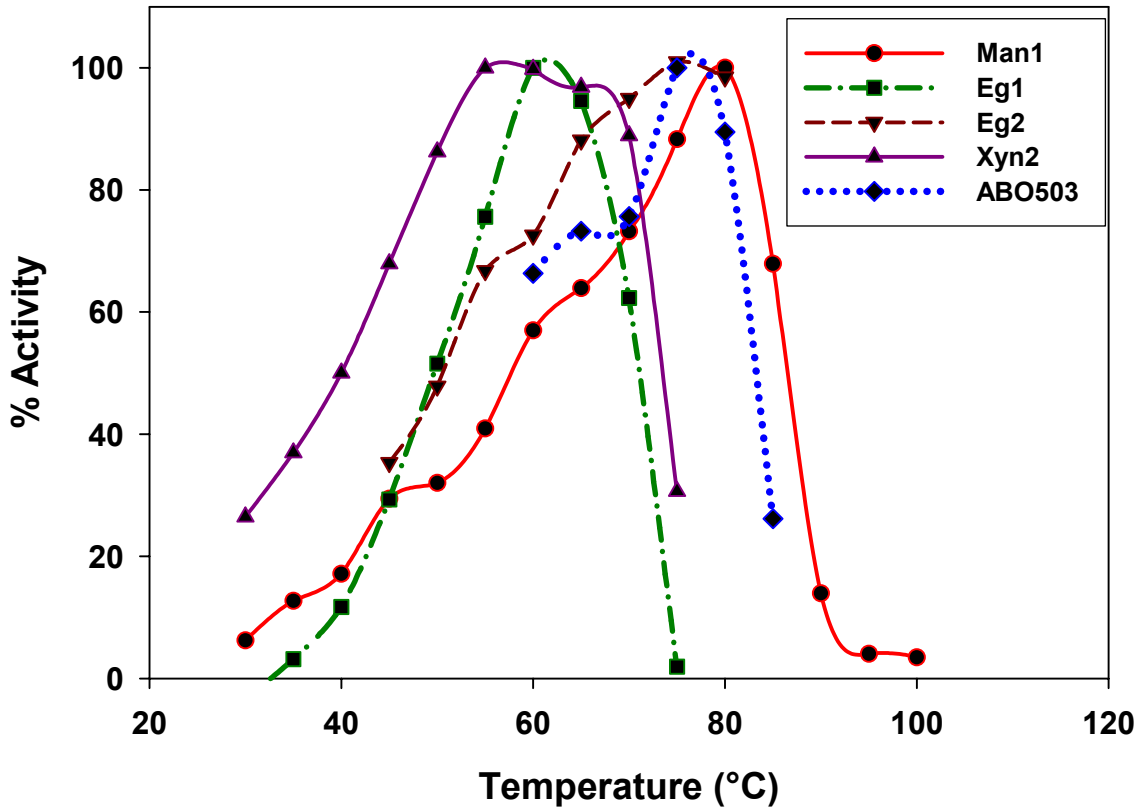

Fig. 2 Graph indicating the temperature optima for enzyme activity at pH 5.0. The activities were determined on 0.25% locust bean gum (Man1 and mannanase of ABO503), 1% CMC (Eg1, and Eg2), and 1% birchwood xylan (Xyn2) substrates.

b) Enzyme stability

The Man1 enzyme retained 80% mannanase activity after 10 min. incubation and more than 50% activity after 5 hours at 60°C (Fig. 3). It lost all activity after 2 min. at 80°C and after only 1 minute at 90 °C. BSA increased enzyme activity and stability at 60°C, but had no effect at 80°C and 90°C.

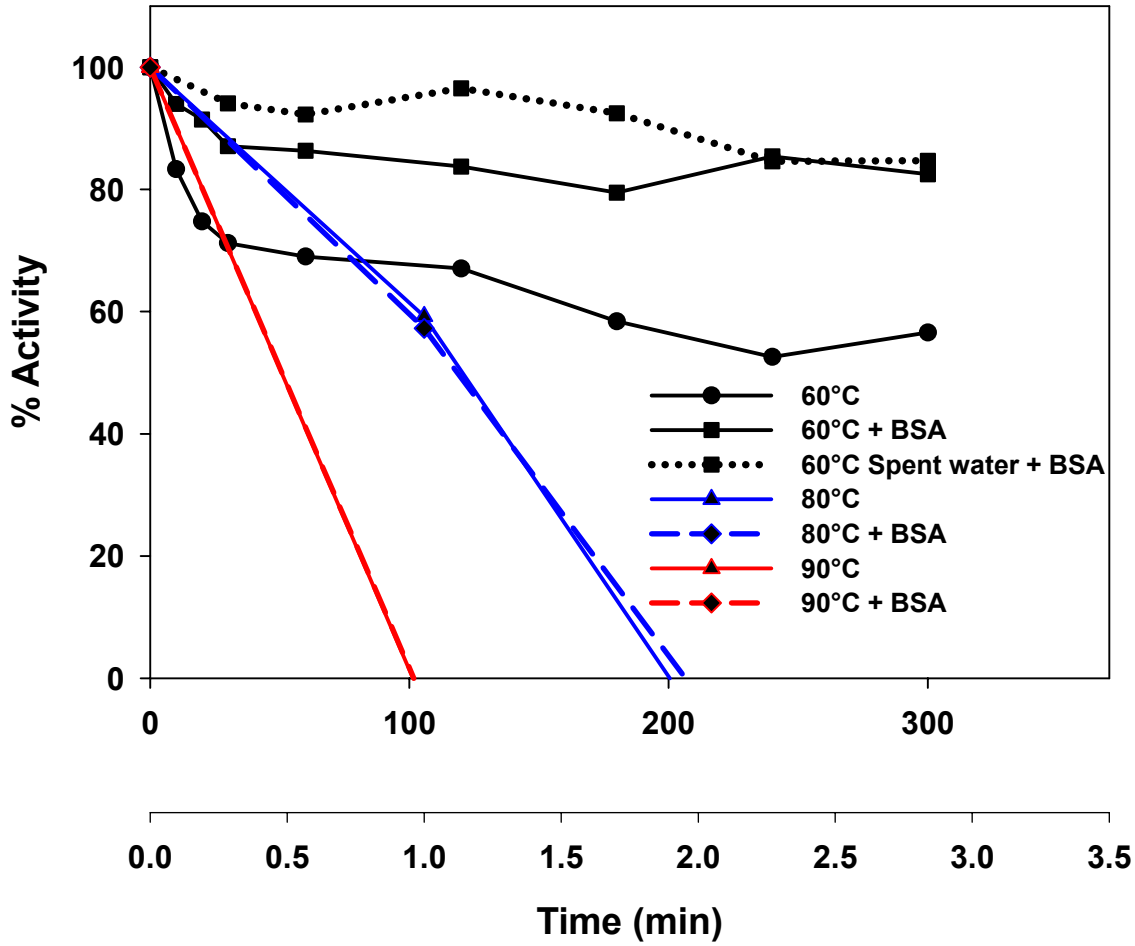

Fig. 3 Enzyme stability of the Man1 enzyme over time at different temperatures. Samples where BSA was added to the enzyme dilution are indicated by + BSA in the legend. All enzyme dilutions were made with citrate buffer (pH 5.0) except for the indicated sample where water drained from spent ground was used as the diluent. All reactions carried out at 60°C are plotted using the time indicated on the x-axis. The reactions carried out at 80°C and 90°C are plotted using the time indicated on the secondary x-axis.

The ABO503 mannanase enzyme cocktail displayed a similar stability as the Man1 at 60°C, but was slightly more stable at higher temperatures (data not shown). The enzyme lost all mannanase activity after 10 min. incubation at 80°C and 2 min. incubation at 90°C.

The Eg1 retained 80% endo-glucanase activity after 30 min. and more than 50% activity after 5 hours incubation at 60°C (Fig. 4). BSA increased enzyme activity and stability at 60°C.

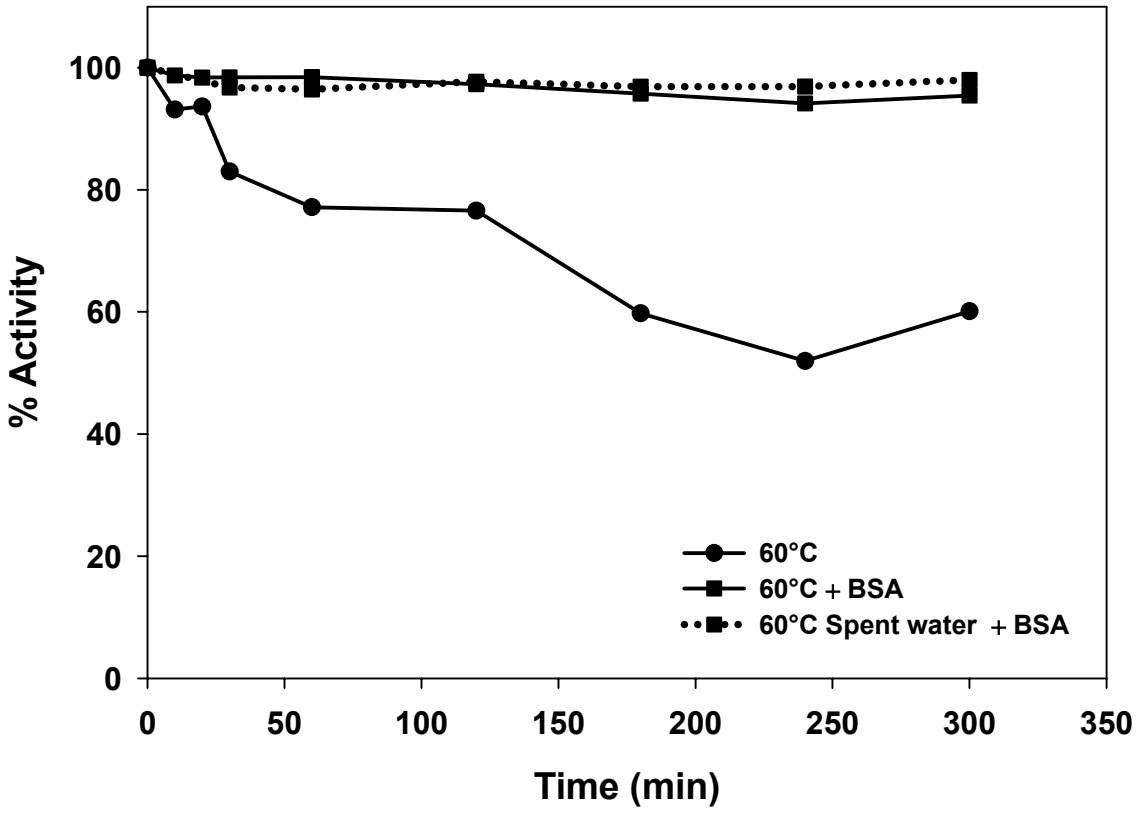

Fig. 4 Enzyme stability of the Eg1 enzyme over time at 60°C. Samples where BSA was added to the enzyme dilution are indicated by + BSA in the legend. All enzyme dilutions were made with citrate buffer (pH 5.0) except for the indicated sample where water drained from spent ground was used as the diluent.

The Eg2 enzyme had a higher optimum temperature compared to the Eg1, and the enzyme was therefore more stable at 60°C. It retained more than 90% activity after 240 hours incubation at 60°C (data not shown).

The Xyn2 enzyme showed only 40% xylanase activity after 5 min. and 10% after 10 min. incubation at 60°C (Fig. 5). The enzyme however retained more than 80% activity after 2 hours and 70% activity after 4 hours at 50°C. It performed even better at 40°C where more than 90% activity was retained after 4 hours incubation.

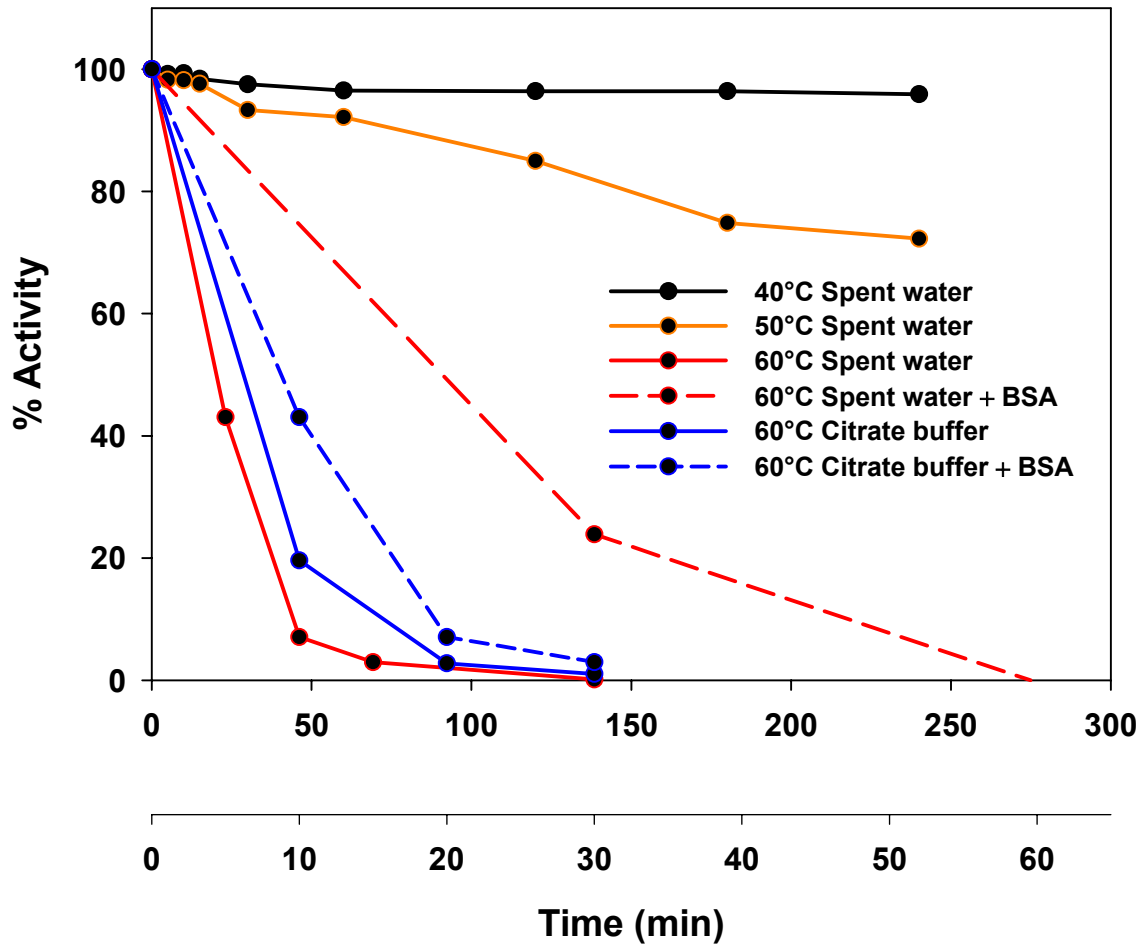

Fig. 5 Enzyme stability of the Xyn2 enzyme over time at different temperatures. Samples where BSA was added to the enzyme dilution are indicated by + BSA in the legend. Enzyme dilutions were made with either water drained from spent ground or citrate buffer (pH 5.0). All reactions carried out at 40°C and 50°C are plotted using the time indicated on the x-axis. The reactions carried out at 60°C are plotted using the time indicated on the secondary x-axis.

Hydrolysis experiments

a) Assessment of thermal treatment methods

Three different thermal treatments were tested for percentage yield increase of soluble solids to add as post-treatment after enzyme hydrolysis. The first treatment involving incubation of the flask with spent ground at 80°C for 18 hours yielded a 2.6% (±0.05) increase. The second method entailed the 18 hour step at 80°C with an additional autoclave cycle at 121°C and 100 kPa for 15 min. This treatment showed an increase of 3.4% (±0.02). The final treatment method entailed an autoclave cycle for 30 min. and this method yielded the highest increase in soluble solids (3.5 % (±0.03)). The latter treatment was used after enzyme hydrolysis to denature the enzyme remaining in the hydrolysis mixture, as well as increase the overall yield of soluble solids.

b) Hydrolysis of spent ground

Percentage yield increases in soluble solids were determined for the recombinant, native and commercial enzyme cocktails individually. The amount of enzyme added was quantified as enzyme activity per weight spent ground (nkat mg^{-1}) or protein weight per weight spent ground (g $(30\ g)^{-1}$). A thermal treatment was administered after enzyme hydrolysis by autoclaving at 121°C and 100 kPa for 30 min. to denature remaining active enzymes. The yield increases determined for recombinant and native enzyme cocktails are presented in Figures 6 and 7. The Man1 enzyme performed the best when compared at enzyme activity level with Eg1, Xyn2, ABO503 and ABO300 (Fig. 6). As the Xyn2 enzyme has very high specific activity, this enzyme performed better than the Man1 when low concentrations of enzyme were used (Fig. 7). Yield increases as a result of treatment with Man1 and commercial enzyme cocktails are shown in Figure 8. Man1 was once again the best enzyme tested. A close second was the trial product TP668L (which contained mainly mannanase), followed by Cellulosin GM5 and the maceration enzyme M263L. The effect of adding higher enzyme concentrations was tested for selected commercial enzymes (Fig. 9). Very high yields of soluble solids were obtained when using large amounts of Pectinex Ultra SP-L.

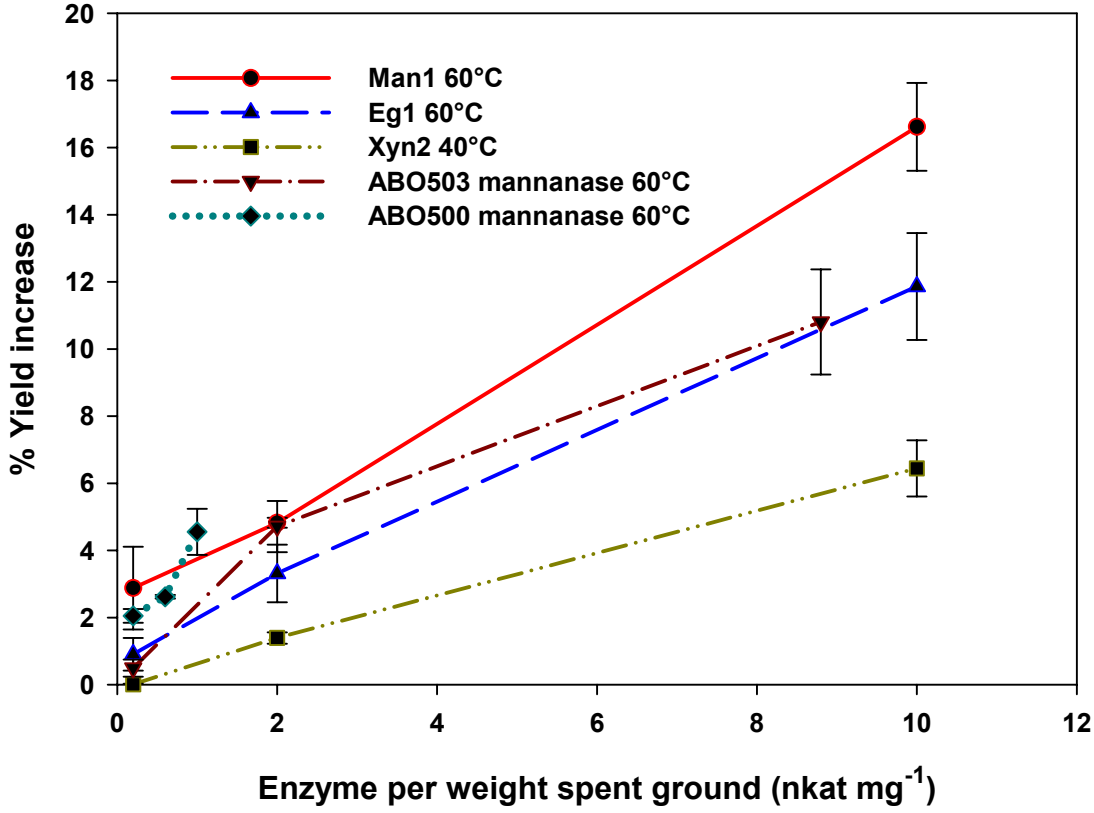

Fig. 6 Total soluble yield increases measured in spent ground (Freeze-dry method). The spent ground was treated with recombinant cocktails Man1 (●), Eg1 (▲) and Xyn2 (■), as well as native mannanases from ABO503 (▼) and ABO500 (♦), and then subjected to a thermal treatment at 121°C and 100 kPa for 30 min. Man1, Eg1 and Xyn2 were added at concentrations of 0.2, 2.0 and 10.0 nkat mg^{-1} spent ground in triplicate. ABO503 was added at concentrations of 0.2, 2.0 and 8.8 nkat mg^{-1} spent ground in triplicate. ABO500 was added at concentrations of 0.2, 0.6 and 1.0 nkat mg^{-1} spent ground in triplicate. Temperature of enzyme hydrolysis is indicated in the figure legend.

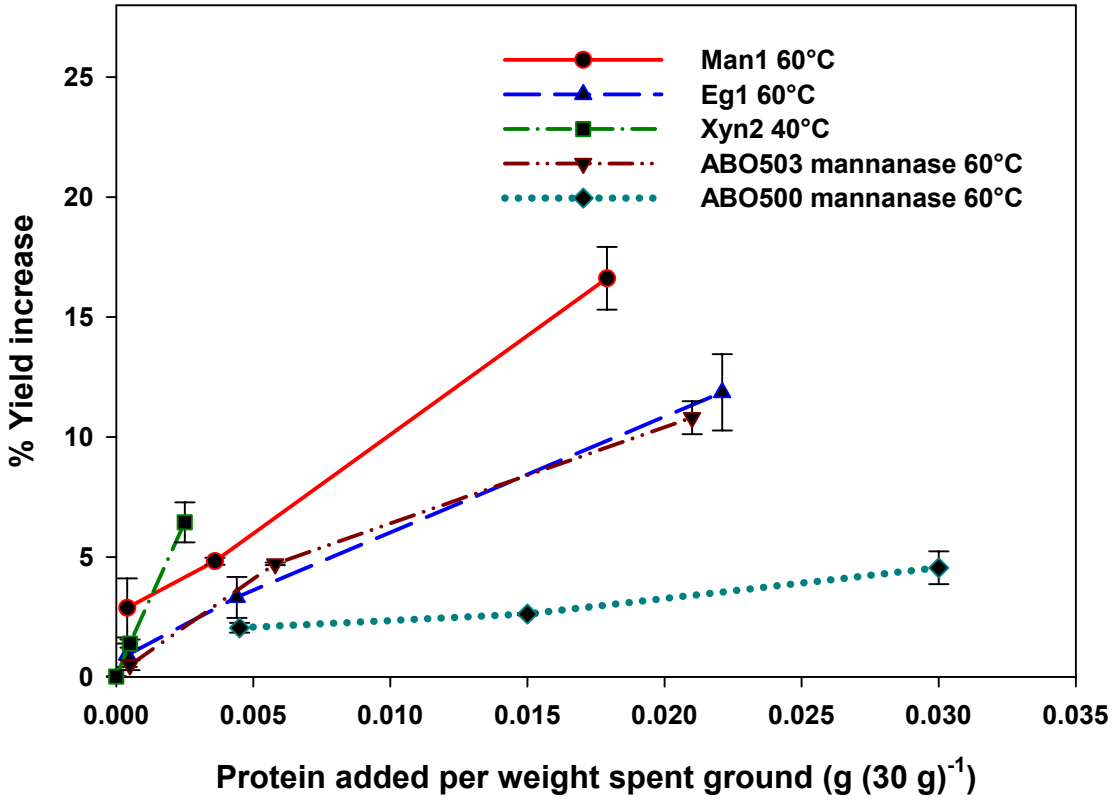

Fig. 7 Total soluble yield increases measured in spent ground (Freeze-dry method). The spent ground was treated with recombinant cocktails Man1 (●), Eg1 (▲) and Xyn2 (■), as well as native mannanases from ABO503 (▼) and ABO500 (♦) and then subjected to a thermal treatment at 121°C and 100 kPa for 30 min. Enzymes were added at different protein weights per 30 g spent ground in triplicate, and temperatures of enzyme hydrolysis are indicated in the figure legend.

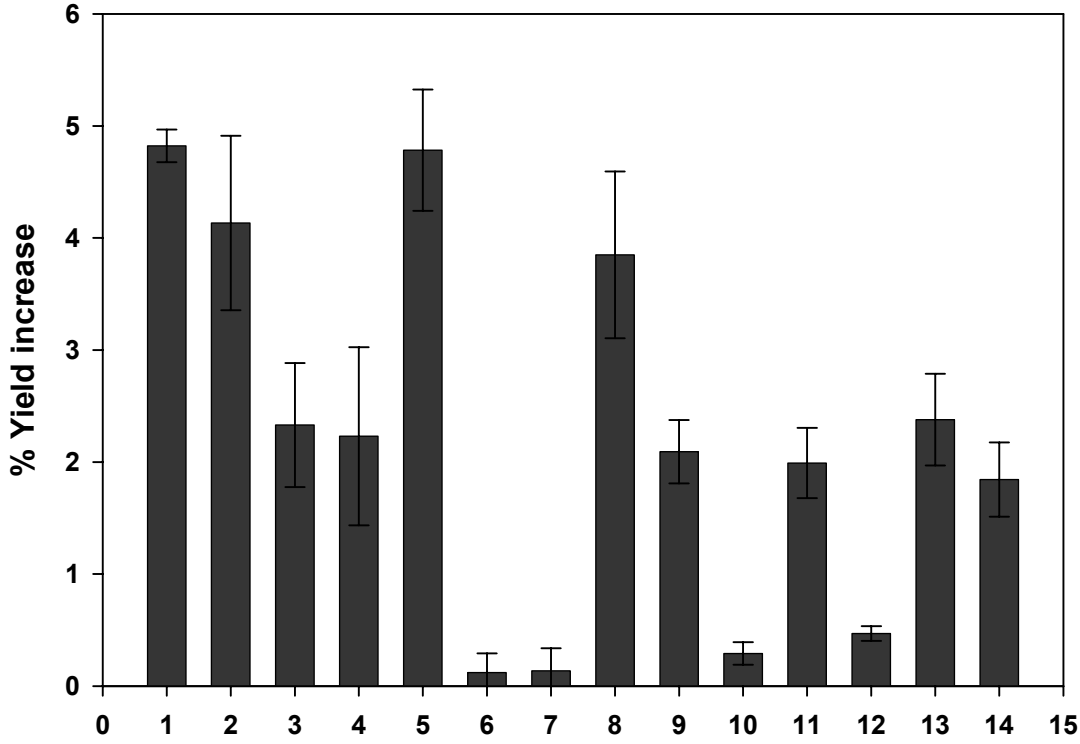

Fig. 8 Total soluble yield increases measured in spent ground (Freeze-dry method). The spent ground was treated with the recombinant cocktail (1) Man1, and commercial cocktails (2) Cellulosin GM5, (3) Pectinex Ultra SP-L, (4) Gamanase, (5) TP668L, (6) D040L, (7) D112L, (8) M263L, (9) D670L, (10) G015L, (11) C013L, (12) M282L, (13) TP692L, and (14) P690L. No thermal treatment was administered. Enzymes were added at 0.004 g enzyme protein per 30 g spent ground in triplicate. The enzyme hydrolysis was performed at 60°C for Man1, Gamanase, and TP668L. The Pectinex Ultra SP-L enzyme hydrolysis was performed at 35°C. The remaining hydrolysis reactions were performed at 55°C.

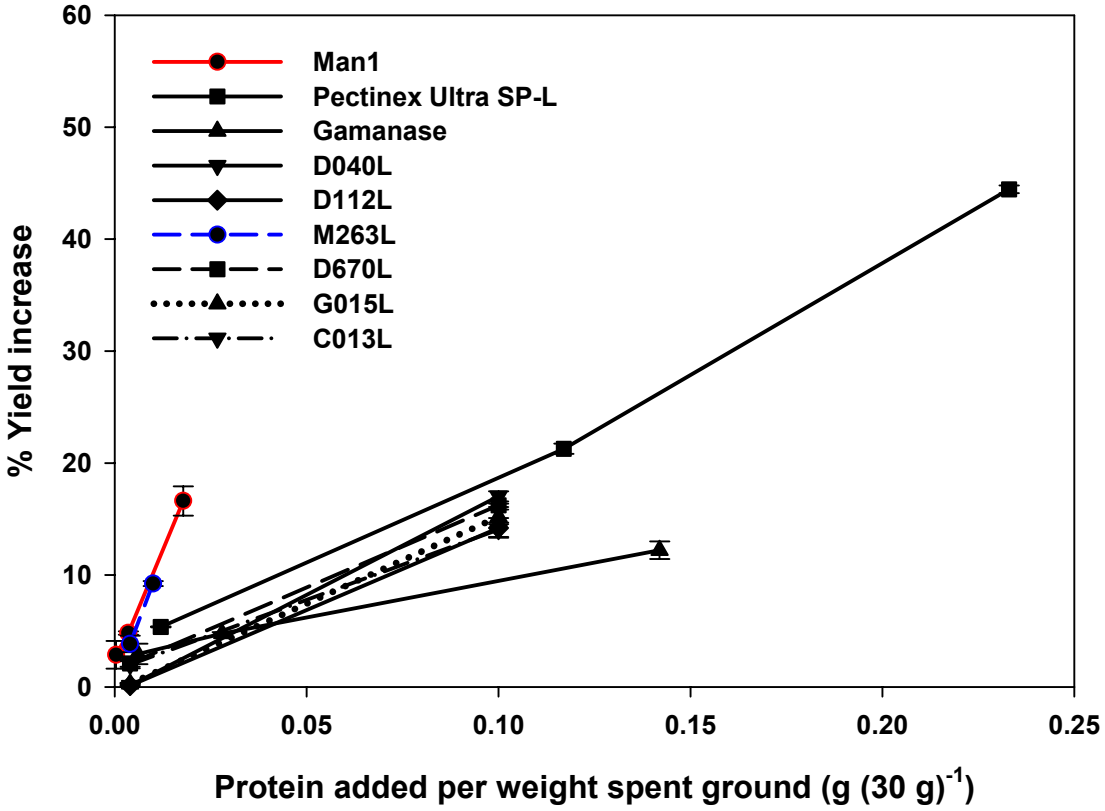

Fig. 9 Total soluble yield increases measured in spent ground (Freeze-dry method). The spent ground was treated with the recombinant cocktail Man1 and commercial cocktails as indicated in the legend and then subjected to a thermal treatment at 121°C and 100 kPa for 30 min. Enzymes were added at different protein weights in g per 30 g spent ground. All treatments were done in triplicate. The enzyme hydrolysis was performed at 60°C for Man1 and Gamanase. The Pectinex Ultra SP-L enzyme hydrolysis was performed at 35°C. The residual hydrolysis reactions were performed at 55°C.

To determine whether synergism occurred between combinations of enzymes, enzymes were added together in different ratios during enzyme hydrolysis. These results are listed in Tables 7 and 8.

In cross reference Table 7, the average of three soluble solid yield percentages (Freeze-dry method) is shown for combinations of Man1, Pectinex Ultra SP-L, Eg1 and the mannanase from ABO503. The standard deviations were below 7% for all values. Enzymes were added based on mannanase enzyme activity per weight spent ground (nkat mg^{-1}) for Man1, Pectinex Ultra SP-L and the mannanase from ABO503. Eg1 was added based on endo-glucanase activity per weight spent ground (nkat mg^{-1}). Spent ground batch B was used for the experiments. Temperatures used for the enzyme hydrolysis are indicated in the table. Enzyme hydrolysis was followed by a thermal treatment at 121°C for 30 min.

In cross reference Table 8, the average of three soluble solid yield percentages (Freeze-dry method) is shown for combinations of Man1, Eg2, Pectinase 062L (P062L), Pectinase 444L (P444L), Cellulase complex (NS50013), Enzyme complex (NS50012), and β-glucanase (NS50029). Enzymes were added as enzyme protein per weight spent ground (g 30 g^{-1}). The standard deviations were below 10% for all values. Varying batches of spent ground were used and are indicated. Temperatures used for the enzyme hydrolysis are indicated in the table. Enzyme hydrolysis was followed by a thermal treatment at 121°C for 30 min. None of the enzymes tested however reacted synergistically with Man1, as all reactions showed an additive percentage yield increase in soluble solids.

Table 7 Soluble solid yield increases obtained when combinations of enzymes were added to test synergism based on enzyme activity.

Temp [a]				**35°C**		**60°C**			
	Enzyme			**Pectinex Ultra SP-L**		**Eg1**		**ABO503**	
		Activity [b]		**1.0 nkat mg^{-1}**	**2.0 nkat mg^{-1}**	**1.0 nkat mg^{-1}**	**1.4 nkat mg^{-1}**	**0.5 nkat mg^{-1}**	**2.0 nkat mg^{-1}**
			% Yield [c]	1.711	6.617	1.466	1.997	1.056	2.184
35°C	**Man1**	**0.08 nkat mg^{-1}**	0.084		6.495				
		1.0 nkat mg^{-1}	0.559	2.200					
60°C	**Man1**	**0.5 nkat mg^{-1}**	0.588						2.325
		2.0 nkat mg^{-1}	2.856			4.397	4.509	3.133	
		3.2 nkat mg^{-1}	3.730			4.009			

[a] Temperature of hydrolysis reaction

[b] Enzyme activity added per weight spent ground (nkat mg^{-1})

[c] % Yield increase in soluble solids for respective enzyme

Table 8 Soluble solid yield increases obtained when combinations of enzymes were added to test synergism based on enzyme protein.

Temp [a]					50°C				55°C		60°C	
	Enzyme				P444L		P062L	NS50012	NS50013	NS50029	Eg2	
		Batch [b]			A	C		A		B		
			Weight [c]		0.004 g	0.004 g	0.004 g	0.004 g	0.004 g	0.004 g	0.004 g	0.008 g
				% Yield [d]	3.705	2.240	1.905	1.257	2.986	ND	0.388	0.597
50°C	Man1	A	0.004 g	3.271	6.387			4.266				
		C	0.004 g	0.798		3.199	2.477					
55°C		A	0.004 g	3.396					7.202			
		B	0.004 g	2.568						2.890		
60°C			0.004 g	3.221							4.305	4.013

[a] Temperature of hydrolysis reaction

[b] Batch of spent ground used for the experiment

[c] Enzyme protein added per weight spent ground (g 30 g^{-1})

[d] % Yield increase in soluble solids for respective enzyme

ND indicates no increase in soluble solids detected

A variation between different batches of spent ground received from NBL was detected during enzyme hydrolysis reactions performed to determine synergism. A test enzyme hydrolysis was performed by treating different batches with Man1 enzyme. These results are shown in Figure 10. A clear variation in yield was evident.

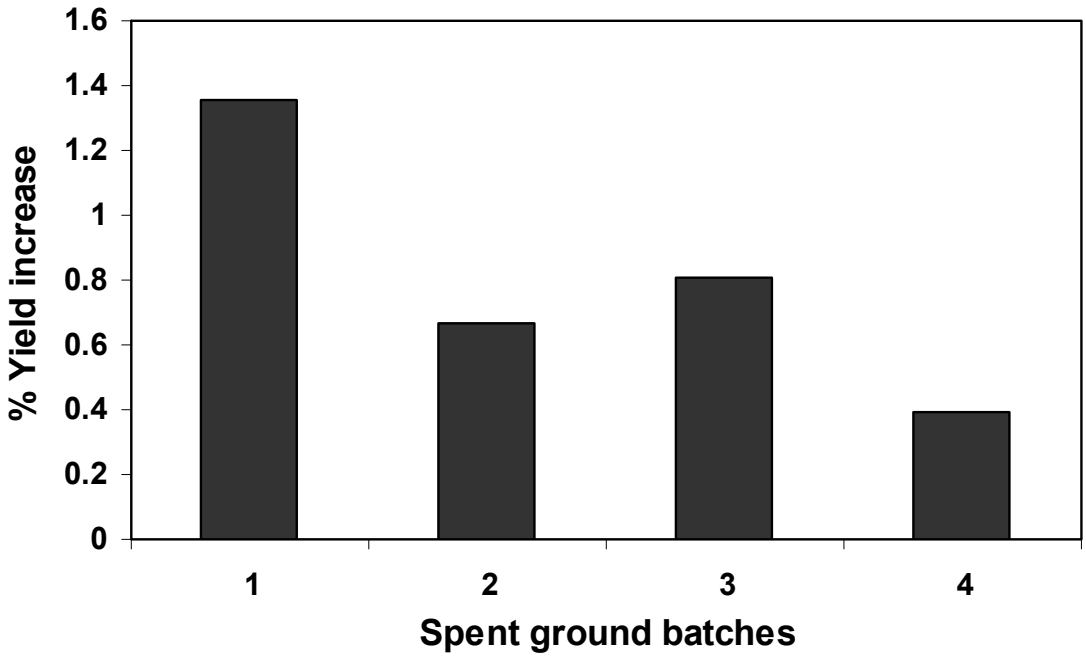

Fig. 10 Total soluble yield increases measured in spent ground (Freeze-dry method) to test the effect of different spent ground batches on yield increase when treated with Man1. The spent ground batches (1) Batch A, (2 and 3) Batch B and (4) Batch C were treated with Man1 at 1.0 nkat mg^{-1} spent ground. The same sample of enzyme was used to treat the different batches of spent ground. Enzyme hydrolysis was performed at 60°C. No thermal heat extraction was administered.

As it was shown that BSA played a role in increasing enzyme activity and stability, the effect was also tested during enzyme hydrolysis of spent ground with Man1. The result of this experiment is shown in Figure 11. BSA however did not notably increase the percentage yield obtained when added to hydrolysis reactions.

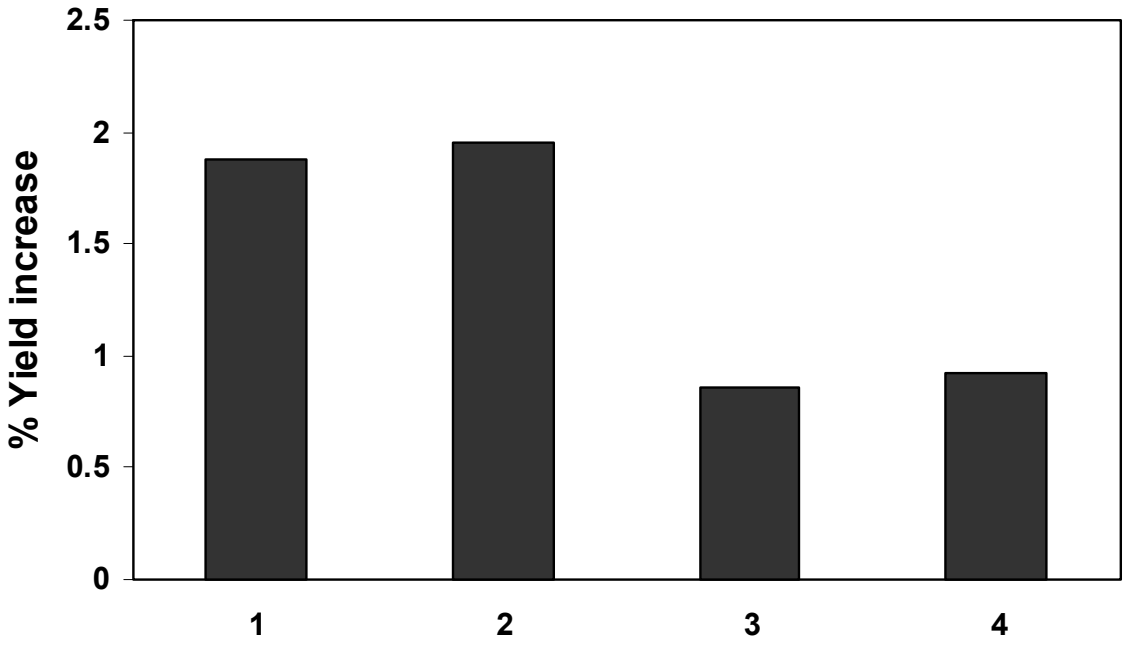

Fig. 11 Total soluble yield increases measured in spent ground (Freeze-dry method) to test the effect of BSA on yield increase when treated with 1 nkat Man1 mg^{-1} spent ground. (1) Batch A spent ground treated with Man1, (2) Batch A spent ground treated with Man1 with addition of 0.1 mg ml^{-1} BSA, (3) Batch B spent ground treated with Man1, (4) Batch B spent ground treated with Man1 with addition of 0.1 mg ml^{-1} BSA. Enzyme hydrolysis at 60°C was followed by a thermal treatment at 121°C for 30 min.

To determine whether soluble solid extraction would reach a plateau when treated with a mannanase enzyme, increasing amounts of Man1 was added to spent ground for hydrolysis. The yields in these experiments were calculated by determining the increase in total soluble sugars. No saturation was observed for Man1 up to 50 nkat enzyme mg^{-1} spent ground (Fig. 12). As the most concentrated Man1 enzyme samples did not allow for reactions where more than 50 nkat mg^{-1} spent ground could be added, the Mannanase-L enzyme from River Biotech was added to spent ground for hydrolysis with higher enzyme activity. The sugars generated started reaching a plateau for enzyme activities larger than 50 nkat mg^{-1} spent ground.

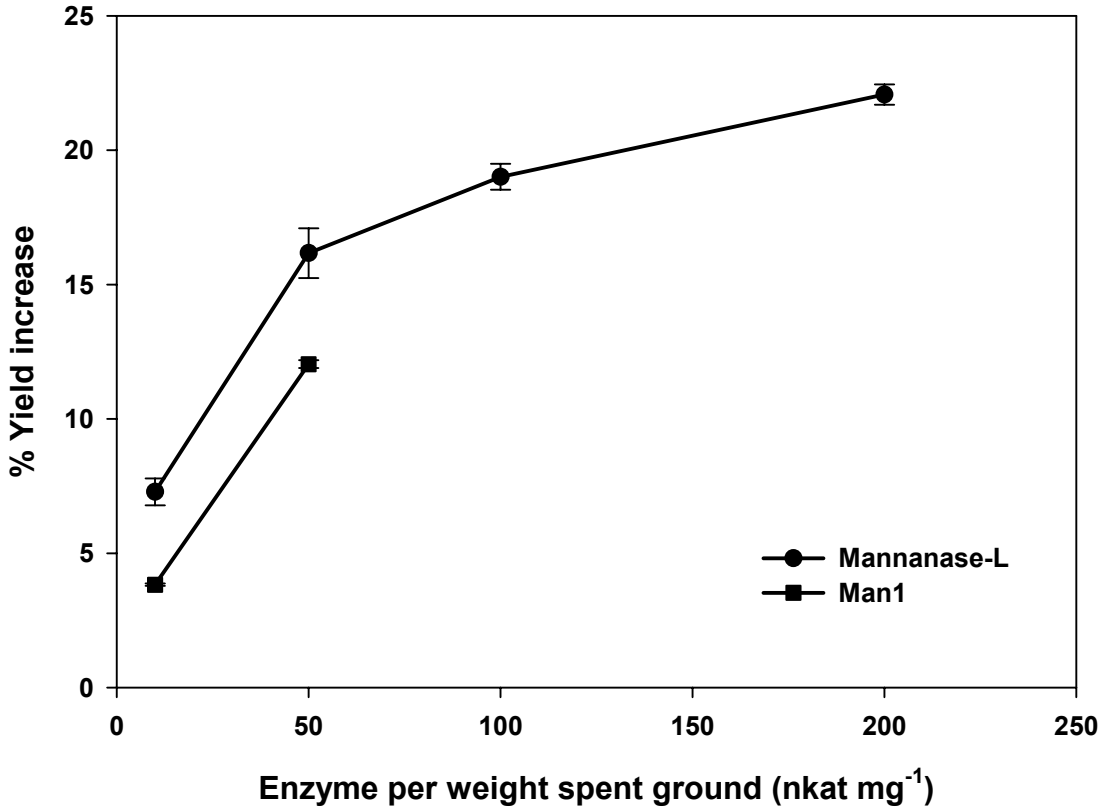

Fig. 12 Total soluble yield increases measured in spent ground (Total sugar method) to test enzyme and substrate saturation. The spent ground was treated with Man1 and Mannanase-L from River Biotech as a large quantity of this enzyme was available for assays. The spent ground was treated with 10 and 50 nkat Man1 mg^{-1} spent ground, and 10, 50, 100 and 200 nkat Mannanase-L mg^{-1} spent ground. Enzyme hydrolysis at 60°C was followed by a thermal treatment at 121°C for 30 min.

c) Enzyme hydrolysis optimisation

Enzyme hydrolysis optimisation was outsourced to River Biotech. Different parameters were tested, which entailed (i) Mannanase-L enzyme concentration added, (ii) temperature of hydrolysis, and (iii) spent ground to water ratio. The results generated were published in a confidential report. Their results indicated that a hydrolysis reaction where 0.9% w/w enzyme was added to 45 gram spent ground in a total of 100 ml water yielded the highest increase in soluble solid yield. The hydrolysis was performed at 60°C for 16 hours.

Further optimisation was performed at laboratory, semi-commercial and pilot-plant scale by River Biotech. Parameters tested included enzyme type, enzyme dosage, contact time for enzyme with spent ground, and particle size of spent ground. The results were presented in a confidential report. Two different mannanase enzymes were tested. The Shin Nihon Mannanase (SNM) enzyme and Genencor Mannanase (GM) enzyme were applied to spent ground at different enzyme dosages which included 0.2, 0.5, 1.0, and 2.0 nkat mg^{-1} spent ground. The original intent was to dose at 50 nkat per mg dry spent ground, which would render an 18% increase in yield. However, at the given cost and activity of the enzymes, such a dose rate proved uneconomical, and the dosage with optimum economic contribution was found to be 0.5 nkat per mg dry spent ground. The SNM enzyme performed the best and was also the most competitive when regarding the cost of the enzyme.

With regards to contact time, samples were taken periodically during enzyme hydrolysis, which lasted 16 hours. The sugars released as a result of enzyme hydrolysis were determined as an indication of how long the spent ground needed to be in contact with enzymes to ensure maximum yield. The release of total soluble sugars reached a plateau after 12 hours of incubation of spent ground with enzyme.

Different particle sizes of coffee spent ground were subjected to enzyme hydrolysis. Both spent ground in its original form, as well as finely milled spent ground, was treated with enzyme. More sugars were released as a result of hydrolysis from finely milled spent ground than sugars released from coffee spent ground in it original form.

Discussion

The increase in soluble solids extracted from roasted coffee beans for instant coffee processing without unnecessary changing the extraction process is in high demand. This calls for innovative methods to modify roasted coffee beans to release more solids during extraction, or acquiring solids from the coffee spent ground before the waste product is discarded. Galactose, mannose and xylose

are the major monosaccharides present in roasted beans (Oosterveld et al., 2003b). During the extraction process to produce soluble solids used for instant coffee production, arabinogalactans and xylan are easily degraded. The cellulose and galactomannan are more recalcitrant components that need to be broken down more effectively. Pure mannan is capable of forming a hard, insoluble crystalline structure, much like cellulose (McCleary et al., 1981). Therefore the galactomannan in the beans is more difficult to solubilise by hot water, chemical extraction or enzyme treatment when the beans reach maturity on the coffee plant (Bradbury, 2001). The use of hydrolytic enzymes at this stage of the process (after roasting of beans and before water extraction) would therefore not be very effective.

Heat administered during the water extraction for instant coffee production, however, breaks some of the interchain hydrogen bonding by opening the cell-wall matrix (Leloup and Liardon, 1993; Oosterveld et al., 2003b). This has proven to be the case where the extractability of galactomannans from green beans using water was very low (Fischer et al., 2001; Oosterveld et al., 2003a) and improved dramatically after roasting (Nunes and Coimbra, 2001; Oosterveld et al., 2003b). It can therefore be argued that the polysaccharides remaining in spent ground should be more readily hydrolysable by hydrolytic enzymes.

Hydrolytic enzyme hydrolysis of residual polysaccharides in the spent ground should increase the soluble solids that can be extracted from the material. It was therefore necessary to determine which polysaccharides or monosaccharides were still present in the spent ground after the roasting and extraction process to identify which enzymes would be beneficial to the process. Monosaccharide analysis confirmed our speculation that the residual polysaccharides in the spent ground were mannan (42%) and cellulose (51%). For this reason, mannanases and cellulolytic enzyme cocktails were sourced to enable soluble solid extraction. Xylanases and pectinases were also included in the analysis as small amounts of xylan and pectin were still present in the coffee spent ground.

Recombinant and native enzymes were characterised with regards to optimum temperature and temperature stability to confirm that they will perform well during the selected enzyme hydrolysis conditions. The optimum temperatures for Man1, Eg2 and the mannanase from ABO503 (75-80°C) were higher than for the Eg1 and Xyn2 (55-60°C) enzymes. The mannanase and endo-glucanase enzymes were, however, not stable at these high temperatures, but were more stable at 60°C. The Xyn2 did not perform well at 60°C and stability was ensured by doing enzyme hydrolysis experiments at 40°C for this enzyme.

It is necessary to denature the enzymes after hydrolysis to ensure that functional enzymes do not end up in the final product (Nicolas et al., 1998). To determine if the thermal extraction after enzyme hydrolysis would result in activity loss and therefore denaturing of the enzymes, the stability of the Man1 enzyme was determined at high temperatures. All Man1 activity was

abolished after 1 min. at 90°C and 2 min. at 80°C. It would therefore be safe to say that the Man1 enzyme will denature during the thermal extraction process at 121°C for 30 min.

Soluble solids or sugars were generated from coffee spent ground. The highest increases in soluble solid yield were recorded when spent ground was treated with Mannanase-L, recombinant Man1, the mannanase from ABO503, TP668L (cocktail containing mainly mannanase), Cellulosin GM5 (galactomannanase) and the maceration enzyme M263L (cocktail of mannanase, endo-galacturonase and pectinase). It was therefore evident that the highest soluble solid yields were a result of the hydrolysis action of β-mannanase.

The recombinant Xyn2 performed better at generating soluble solids than the Man1 when low concentrations of enzyme were used. This was due to the fact that the Xyn2 enzyme has very high specific activity. Monosaccharide analysis (Fig. 1) however suggests that the soluble solids generated by Xyn2 will reach a plateau at medium concentrations of enzyme, as only small amounts of residual xylan (3%) are present in the spent ground.

Smaller quantities of soluble solids were generated when cellulase and pectinase recombinant and commercial enzyme cocktails were applied to spent ground. This result was not surprising, as cellulose is a recalcitrant substrate that is very difficult to hydrolyse (Lynd et al., 1999). It is furthermore possible that pectinase enzymes did not perform well because the residual amount of pectin in spent ground is limited, as pectin is susceptible to degradation during roasting.

Higher protein concentrations were tested for selected commercial enzymes. Very high yields (44%) of soluble solids were obtained when using a large amount of Pectinex Ultra SP-L (0.233 g g^{-1} spent ground). Although this yield (44%) would be advantageous for the industry, it has to be noted that as enzyme load increases, so does the cost factor. Furthermore, when a larger quantity of enzyme is used, more protein and/or medium components present in the enzyme cocktail are also added to the end product, and hence contributing to the soluble solids content.

To determine whether soluble solid generation would reach a plateau when treated with a mannanase enzyme, increasing amounts of a mannanase preparation (Mannanase-L) was added to spent ground for hydrolysis. As the amount of enzyme added was increased, more broth or growth medium components were also added, which would negatively impact the soluble solid weight measured when weighing the freeze-dried samples by skewing the results. Total soluble sugars were therefore determined using the phenol-sulphuric method. The sugars and respective soluble solid yield increases generated as a result of hydrolysis started reaching a plateau when enzyme activities larger than 50 nkat mg^{-1} spent ground were added. As expected, saturation was evident. This could be due to end-product inhibition or the maximum possible yield being reached from the portion of mannan left in the spent ground which is hydrolysable under these conditions.

One way of increasing the soluble solids yield even further when applying hydrolytic enzymes, is by applying an enzyme cocktail which contains components which work synergistically (Abe et al., 1988; Ueda, 1981). The synergy between Man1 and other enzymes was therefore explored as a synergistic reaction would ensure an enzyme cost reduction in a hydrolysis approach. Man1 was tested in combination with Eg1, the mannanase from ABO503, Pectinex Ultra SP-L, Pectinase 062L (P062L), Pectinase 444L (P444L), Cellulase complex (NS50013), Enzyme complex (NS50012), and β-glucanase (NS50029) in different enzyme ratios. However none of the enzymes tested however reacted synergistically with Man1, as all reactions showed an additive percentage yield increase in soluble solids. Mannanase alone was therefore the most viable economic option for the hydrolysis of spent ground.

The efficacy of using additives to stabilise and increase enzyme activity was furthermore explored as an additional avenue of increasing soluble solid yield generated. BSA increased activity and stability of the enzymes during enzyme characterisation. BSA however did not significantly increase the percentage yield obtained when added to hydrolysis reactions, and this protein will only add to end-product cost over and above enzyme purchase costs.

In preparation for up-scaled experiments, an industrial environment was created for enzyme hydrolysis of spent ground by determining enzyme stability in un-buffered waste water drained from spent ground instead of the citrate buffer used during hydrolysis experiments. The enzymes tested proved to be stable in the un-buffered water, which indicated that the enzymes will also function well in the industry, and generated spent ground, could be treated as is.

During the synergistic experiments it was observed that different yields were being obtained for different batches of spent ground. This was confirmed by doing hydrolysis with a single Man1 enzyme preparation tested on different batches of spent ground. It is therefore essential for the industry to ensure that their process of coffee extraction and treatment of the waste product is standardised, as the ability of the enzyme to hydrolyse residual polysaccharides is adversely affected. The variation could be due to particle size distribution, therefore surface area of the spent ground particles.

Various parameters such as contact temperature, enzyme dosage, contact time, spent ground to water ratio and spent ground particle size had an affect on soluble solid yield generated. Optimisation of these parameters was performed at laboratory, semi-commercial and pilot-plant scale by River Biotech to ensure the best parameters would be chosen for the industry. An enzyme hydrolysis reaction performed at 60°C yielded the highest increase in soluble solids when spent ground was treated with a mannanase enzyme. This was a result of the high stability of the enzyme at this high temperature.

The original intent was to dose at 50 nkat per mg dry spent, which rendered an 18% increase in yield. However, at the given cost and activity of the enzyme, such a dose rate proved uneconomical, and the dosage with optimum economic contribution was found to be 0.5 nkat per mg dry spent ground. The Shin Nihon Mannanase enzyme performed the best and was also the most competitive when considering the cost of the enzyme.

With regards to contact time, the hydrolysis and release of soluble total sugars reached a plateau after 12 hours of incubation. This time could probably be shortened if a higher hydrolysis temperature was used, although an optimum temperature and contact time balance would have to be determined if the full-scale plant is run closer to the denaturing limit of the enzyme.

All laboratory-scale experiments were performed with a spent ground to water ratio of 30:100. During optimisation experiments, a ratio of 45:100 yielded the highest increase in generated soluble solids. This was as a result of an increase in hydrolysable material while in an environment where mixing of the spent ground and enzyme preparation was not hindered.

It is commonly agreed that the smaller the particle size of the spent ground, the larger the surface area for enzyme attack. This was also the finding in the optimisation experiments, and the River Biotech group indicated that it would be advisable to mill spent ground before treating it with enzyme to increase surface area of the particles. As very fine material will impede the passage of the coffee liquor in an industry set-up, this finding will have to be tested in an up-scaled test scenario.

In conclusion, monosaccharide analysis of coffee spent ground indicated that mannanases and cellulases should increase soluble solids extracted from spent ground in a hydrolysis reaction. This was proven to be true, as treatment with the mannanase (Man1) enzyme reached yield increases of up to 16.6%. This figure was increased to 23% when the enzyme hydrolysis procedure was optimised using the Shin Nihon mannanase enzyme. Cellulases did not perform as well as the mannanase enzymes due to the recalcitrant nature of the polysaccharide. Other enzyme activities such as xylanase (Xyn2) and pectinase cocktails also increased soluble solids, but did not result in yields as high as with the mannanase. Synergism was not detected between any of the enzyme combinations tested. Variation in hydrolysability between different spent ground batches had a significant effect on yield, highlighting the importance of setting up a standardised procedure for soluble solid extractions from roasted coffee beans, as well as treatment of the spent ground waste product.

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CHAPTER 6: GENERAL CONCLUSION AND REMARKS

Costs associated with the current starch conversion technologies remain high. The engineering of microbial systems to convert starch to bio-ethanol in a one-step process and therefore applying the CBP concept may yield large cost reductions. These reductions will be due to decreased heating energy requirements, as well as a decrease in money spent on the purchase of commercial enzymes for liquefaction and saccharification. The recombinant organism will be capable of utilising the raw starch as sole carbon source. The organism will produce its own raw starch hydrolysing enzymes to convert the starch to fermentable sugars, which in turn is utilised for ethanol production.

In this study a raw starch utilising yeast was engineered. The *GA I* and *amyl III* genes from *A. awamori* and *glaA* gene from *A. oryzae* that encode for raw starch hydrolysis were selected and heterologously expressed in *S. cerevisiae*. The presence of functional genes was demonstrated through raw-starch hydrolysing zones appearing around colonies spotted onto raw starch agar. Enzyme activities were confirmed in liquid assays. The recombinant *S. cerevisiae* strain expressing the wild-type *A. awamori* glucoamylase and α-amylase separately was able to utilise raw corn starch as carbon source, and displayed a maximum specific growth rate of 0.003 h^{-1}. The growth rates on equivalent amounts of soluble starch and glucose were 0.085 h^{-1} and 0.248 h^{-1} respectively. The strain converted raw corn starch into bio-ethanol at a specific volumetric productivity of 0.037 g (g DW cells)$^{-1}$ h^{-1}. The ethanol yield was 0.40 gram ethanol per gram available sugar from starch, which translates to 71% of the theoretical maximum. The ethanol yield and specific ethanol productivity of the generated strain compared well to previously generated strains for raw starch conversion, although the volumetric productivity of 0.018 g l^{-1} h^{-1} was very low.

In an attempt to improve microbe performance, codon optimised synthetic genes were heterologously expressed in *S. cerevisiae* in a similar way as the wild-type genes. The strain secreting the two optimised enzymes, however, did not perform better than the strain secreting the wild-type enzymes. The reason for this result was assigned to the lower expression of the optimised α-amylase which counteracts the higher expression levels of the optimised glucoamylase. Molecular techniques which should still be performed to possibly improve the microbe's performance include generating a yeast strain with the codon optimised glucoamylase and wild-type α-amylase. Nonetheless, the preliminary serum bottle fermentations indicate that the generated strain is a promising raw starch converter. Up-scaled anaerobic fermentations in batch culture or fed-batch will surely confirm these preliminary results.

Coffee is the second most important product in world trade. To increase soluble solids extracted from roasted coffee beans for instant coffee processing without complicating the extraction process, calls for innovative methods to acquire solids from the coffee spent ground before the waste product is discarded. Heat administered during the water extraction for instant coffee production breaks some of the interchain hydrogen bonding by opening the cell-wall matrix, and therefore it can be argued that the polysaccharides remaining in spent grain should be more hydrolysable by hydrolytic enzymes.

In this study, soluble solid yields were increased by hydrolysing spent ground with hydrolytic enzymes. It was speculated that the residual polysaccharides remaining in spent ground after heat extraction were mannan and cellulose. This was later confirmed as the spent ground used in this study contained 42% mannan and 51% cellulose. The residual 7% of spent ground constituted xylose, arabinogalactan and minor sugars. For this reason, hydrolytic enzymes with appropriate action were selected to hydrolyse the residual material. Enzymes included recombinant enzymes secreted from engineered *Aspergillus* strains (Man1, Eg1, Eg2, and Xyn2), enzymes secreted from wild-type organisms (mannanases), and commercial enzyme cocktails displaying the necessary activities (mannanase, cellulase, and pectinase). Enzymes were characterised by determining their optimum temperatures and stability at specific temperatures. Hydrolysis experiments indicated that an enzyme cocktail containing mainly mannanase activity increased soluble solids extracted dramatically (16.6%). Synergism between mannanase and endoglucanases, xylanases, and pectinases was not detected. Variation between spent ground batches highlighted the importance of standardising current manufacturing processes, as the ability of enzymes to hydrolyse the substrate may be adversely affected. In conclusion, this study has proved that soluble solid yields extracted from coffee spent ground are increased when applying a mannanase in a hydrolysis extraction. A 23% yield increase in soluble solids was determined when using an optimised enzyme hydrolysis procedure. It is therefore recommended that the process be integrated in the current instant coffee production partner company.

APPENDIX A

Görgens JF, van Zyl WH, Rose S, Setati ME, de Villiers T (2006) Method for producing hemicellulase-containing enzyme compositions and the use thereof. South African Patent 2006/03771.

SPOOR & FISHER

PRETORIA

COMPLETE PATENT SPECIFICATION

COUNTRY	:	SOUTH AFRICA
APPLICATION NUMBER	:	2006/03771
DATE OF FILING	:	11 MAY 2006
NAME OF APPLICANT	:	UNIVERSITY OF STELLENBOSCH
NAMES OF INVENTORS	:	GÖRGENS, JOHANN FERDINAND; VAN ZYL, WILLEM HEBER; ROSE, SHUANITA HELLOUISE; MATHABATHA, EVODIA SETATI; DE VILLIERS, TANIA
TITLE OF INVENTION	:	METHOD OF PRODUCING HEMICELLULASE-CONTAINING ENZYME COMPOSITIONS AND USE THEREOF

FILE REF : PA141307/ZA
DATE : 11 July 2006

SPOOR & FISHER

FORM P.1

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT

AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) – Regulation 22)

SPOOR P

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

OFFICIAL APPLICATION NO.		S & F REFERENCE
21 01	2006/03771	PA141307/ZA

FULL NAME(S) OF APPLICANT(S)

71	UNIVERSITY OF STELLENBOSCH

ADDRESS(ES) OF APPLICANT(S)

	VICTORIA STREET, STELLENBOSCH, 7600, WESTERN CAPE, SOUTH AFRICA

TITLE OF INVENTION

54	METHOD OF PRODUCING HEMICELLULASE-CONTAINING ENZYME COMPOSITIONS AND USE THEREOF

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. THE EARLIEST PRIORITY CLAIM IS:

COUNTRY:	NUMBER:	DATE:
ZA	2005/01283	11 FEB 2005

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.~~

21	01	

~~THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND IS BASED ON APPLICATION NO.~~

21	01	

THIS APPLICATION IS ACCOMPANIED BY:

- ☒ 1. Two copies of a complete specification of 36 pages.
- ☒ 2. Drawings of 12 sheets.
- ☒ 3. Publication particulars and abstract (Form P.8 in duplicate).
- ☒ 4. A copy of Figure 12 of the drawings for the abstract.
- ☐ 5. Assignment of invention.
- ☐ 6. Certified priority document.
- ☐ 7. Translation of the priority document.
- ☐ 8. Assignment of priority rights.
- ☐ 9. A copy of the Form P.2 and the specification of S.A. Patent Application No .
- ☐ 10. Declaration and power of attorney on Form P.3.
- ☐ 11. Request for ante-dating on Form P.4.
- ☐ 12. Request for classification on Form P.9.
- ☒ 13. Form P.2 in duplicate.
- ☐ 14. Other.

74	ADDRESS FOR SERVICE: SPOOR & FISHER

Dated: 11 May 2006

D. G. GILSON

SPOOR & FISHER
PATENT ATTORNEYS FOR THE APPLICANT(S)

RECEIVED

REGISTRAR OF PATENTS

SPOOR & FISHER

FORM P.7
(To be lodged in duplicate)

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

COMPLETE SPECIFICATION

(Section 30(1) – Regulation 28)

OFFICIAL APPLICATION NO.		LODGING DATE	
21	01	22	11 MAY 2006

INTERNATIONAL CLASSIFICATION	
51	A23F; C12N

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TITLE OF INVENTION	
54	METHOD OF PRODUCING HEMICELLULASE-CONTAINING ENZYME COMPOSITIONS AND USE THEREOF

BACKGROUND OF THE INVENTION

The present invention relates to a method for the production of hemicellulolytic-, cellulolytic- and pectolytic-containing enzyme compositions and to their use in coffee production.

More particularly, the present invention relates to a method for providing yeast and fungal strains adapted to produce enzyme compositions containing hemicellulolytic, cellulolytic and pectolytic activities, and to the use of these compositions in the production of coffee extracts.

Polysaccharides such as arabinogalactan, mannan, and cellulose constitute nearly 50 % of the green coffee bean weight (Nunes *et al.*, 2001; Sachslehner *et al.*, 2000). The major polysaccharide of this fraction is a water-insoluble, crystalline mannan that forms approximately 20-30% of the dry weight of *Arabica* and *Robusta* beans (Sachslehner *et al.*, 2000). These carbohydrates play an important role in the

retention of volatile substances due to their capacity to bind aromatic compounds at the adsorptive sites (Nunes *et al.*, 2001; Trugo, 1985).

Modern instant coffee production entails cleaning, roasting and grinding of coffee beans, followed by a split extraction and concentration to achieve a high solids concentrate for a low-energy spray-drying operation (Stoltze and Masters, 1979). During the roasting procedure of the coffee beans, the physical and chemical properties of carbohydrates change drastically (Sachslehner *et al.*, 2000). For instance, green coffees contain 62 % arabinogalactan, 24 % galactomannan, and glucans in the high-molecular weight material extracted with water, whereas roasted coffees contain 28 % arabinogalactan and 69 % galactomannan (Nunes *et al.*, 2001). After roasting and grinding, extraction is the key operation in the large-scale manufacture of instant coffee in which both soluble solids and volatile aroma/flavour compounds are extracted (Clarke, 1987). Technically produced extracts from roasted *Arabica* and *Robusta* coffee contain 20-36 % carbohydrates depending on the degree of extraction. They are predominantly composed of mannan and galactan in about the same proportions, with glucan and araban making up only 1-3 % of the extracts (Thaler, 1979).

Coffee extraction techniques lead to extracts of around 25 % w/w soluble solids concentration (Clarke, 1987). Spray-drying of these concentrations can provide coffee of the required physical form, although there will be substantial loss of volatile compounds. Consequently, pre-concentration methods for coffee extracts prior to drying have been introduced to circumvent extensive losses (Clarke, 1987). The extracts can be concentrated to 45 % w/w soluble solids concentration in vacuum evaporators, resulting in lower costs of removing water during drying (Stoltze and Masters, 1979). However, concentrations above 42 % w/w solids are difficult to reach, due to the high viscosity of coffee extracts. Also highly viscous extracts will require longer pre-concentration times (Clarke, 1987). The viscosity of coffee extracts can be reduced by hydrolysing the mannan, xylan, cellulose and pectin to short oligosaccharides (Sachslehner *et al.*, 2000; Wong and Saddler, 1993). This would make it possible to concentrate the extracts to concentrations higher than 42 % w/w soluble solids.

SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided a DNA expression cassette for use in transforming a yeast or fungus so as to provide it with a capability of producing β-mannanase, the expression cassette including:

a gene encoding a β-mannanase enzyme; and

a suitable promoter for promoting transcription of the gene in the transformed yeast or fungus.

The expression cassette may include a suitable terminator sequence for promoting efficient expression of the β-mannanase gene.

The gene encoding β-mannanase may be the *man1* gene from a fungus such as *Aspergillus aculeatus* MRC11624.

The yeast or fungus strain may be a *Saccharomyces cerevisiae* yeast strain (especially *Saccharomyces cerevisiae* Y294(pMES1)), *Yarrowia lipolytica, Pichia pastoris, Hansenula polymorpha, Aspergillus awamori or Aspergillus niger* (especially *Aspergillus niger* D15(pGT-man1)).

The yeast promoter and terminator sequences may be the *PGK* promoter and terminator DNA sequence, respectively.

The fungus promoter and terminator sequences may be the *gpd* promoter and *glaA* terminator DNA sequence, respectively.

According to a second embodiment of the invention, there is provided a DNA vector including the gene encoding the β-mannanase gene. The DNA vector containing the gene for β-mannanase, as well as promoter and terminator sequences, may be the yeast/*Escherichia coli* shuttle vector YEp352 or the *Aspergillus*/*Escherichia coli* shuttle vector pGT.

According to a third embodiment of the invention, there is provided a DNA expression cassette for use in transforming a yeast or fungus so as to provide it with a capability

of producing β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, or β-cellobiohydrolase 1-4, the expression cassette including:

one or more genes encoding one or more of a β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, or β-cellobiohydrolase 1-4 enzyme;

a suitable promoter for promoting transcription of the gene(s) in the transformed fungus or yeast strain.

The expression cassette may include a suitable terminator DNA sequence for promoting efficient expression of the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, or β-cellobiohydrolase 1-4 genes.

The recombinant β-endoglucanases 1, 2, and 3 may be produced from *Aspergillus niger* D15(pGT-eg1), *Aspergillus niger* D15(pGT-eg2), and *Aspergillus niger* D15(pGT-eg3).

The recombinant β-xylanase may be produced from *Aspergillus niger* D15(pGT-xyn2).

The recombinant β-cellobiohydrolase may be produced from *Aspergillus niger* D15(pGT-cbh1-4).

The genes encoding β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, and β-xylanase 2 may be the *eg1, eg2, eg3 or xyn2* genes, respectively, from a fungus such as *Trichoderma reesei* QM6a (see Table 1 for list of genes, donor organisms and Genbank accession numbers for the DNA sequences and deduced protein sequences).

The gene encoding β-cellobiohydrolase 1-4 may be the *cbh1-4* gene from a fungus such as *Phanerochaete chrysosporium* ATCC 24725.

The fungus promoter and terminator sequences may be the *gpd* promoter and *glaA* terminator DNA sequence (Rose and van Zyl, 2002).

According to a further embodiment of the invention, there is provided a DNA vector including the gene encoding one or more of the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, and β-xylanase 2 genes. The DNA vector may be the *Aspergillus*/*Escherichia coli* shuttle vector pGT.

According to a further embodiment of the invention, there is provided a method of producing a yeast strain which is capable of expressing β-mannanase, the method including the step of:

transforming a yeast strain with a nucleotide sequence including a gene encoding a β-mannanase enzyme and a suitable promoter for promoting transcription of the gene in the transformed yeast.

The transformation of the fungus strain may be effected by:

(a) constructing the yeast expression vector containing the *ADH2* promoter and terminator DNA regions, called plasmid pDLG1 (La Grange *et al.*, 1996);

(b) amplifying the *man1* gene from a *Aspergillus aculeatus* MRC11624 culture by the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloning as a 1180-bp *Eco*RI/*Xho*1 DNA fragment into plasmid pDLG1 to generate plasmid pMES1 (Figure 12A) (Setati *et al.*, 2001);

(c) isolating total RNA from an *Aspergillus aculeatus* MRC11624 culture prepared on locust bean gum as carbon source;

(d) purifying poly-A mRNA from the total RNA and preparing first strand complementary DNA (cDNA) for the poly-A mRNA; and

(e) designing oligodeoxyribonucleotide DNA primers by conventional techniques.

The yeast strains may be selected from *Saccharomyces cerevisiae, Yarrowia lipolytica, Pichia pastoris,* and *Hansenula polymorpha*.

According to a further embodiment of the invention, there is provided a method of producing a fungus strain which is capable of expressing β-mannanase, the method including the step of:

transforming a fungus strain with a nucleotide sequence including a gene encoding a β-mannanase enzyme and a suitable promoter for promoting transcription of the gene in the transformed fungus.

The transformation may be effected by:

(a) constructing the fungus expression vector containing the *gpd* promoter and *glaA* terminator DNA sequences, called plasmid pGT (Rose and Van Zyl, 2002);

(b) amplifying the *man1* gene from a *Aspergillus aculeatus* MRC11624 culture with the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques and cloned as a 1180-bp *Eco*RI/*Xho*1 DNA fragment into plasmid pGT to generate plasmid pGT-man1 (Figure 12B).

According to a further embodiment of the invention, there is provided a method of producing a fungus strain which is capable of expressing one or more of β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and β-cellobiohydrolase 1-4, the method including the step of:

transforming a fungus strain with a nucleotide sequence including one or more genes encoding β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and β-cellobiohydrolase 1-4 and a suitable promoter for promoting transcription of the gene in the transformed fungus.

The transformation may be effected by:

(a) constructing the fungus expression vector containing the *gpd* promoter and *glaA* terminator DNA sequences, called plasmid pGT (Rose and Van Zyl, 2002); and

(b) cloning the selected genes into plasmid pGT to generate plasmids pGT-eg1, pGT-eg2, pGT-eg3, pGT-xyn2, pGT-cbh1-4 using similar methods described above for construction of pGT-man1.

The fungus strain may be an *Aspergillus niger* fungus strain. The recombinant β-endoglucanases 1, 2, and 3 may be produced from *Aspergillus niger* D15(pGT-eg1), *Aspergillus niger* D15(pGT-eg2), and *Aspergillus niger* D15(pGT-eg3). The recombinant β-xylanase may be produced from *Aspergillus niger* D15(pGT-xyn2).

The recombinant β-cellobiohydrolase may be produced from *Aspergillus niger* D15(pGT-cbh1-4).

According to a further embodiment of the invention, there is provided a host cell which has been transformed as described above. The host cell may be a yeast or fungus, such as *Saccharomyces cerevisiae, Yarrowia lipolytica, Pichia pastoris, Hansenula polymorpha, Aspergillus awamori or Aspergillus niger.*

According to a further embodiment of the invention, there is provided β-mannanase enzyme produced by:

causing a transformed host cell to express the β-mannanase gene; and

recovering the β-mannanase enzyme.

According to a further embodiment of the invention, there is provided one or more of β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and β-cellobiohydrolase 1-4 enzymes produced by:

causing a host cell to express the gene(s) encoding the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and/or β-cellobiohydrolase 1-4; and

recovering the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and/or β-cellobiohydrolase 1-4 enzyme.

According to a further embodiment of the invention, there is provided a method of producing a coffee extract, the method including the step of subjecting roasted, ground coffee beans or leftovers thereof to an enzymatic hydrolysis extraction.

The extraction yield and/or quality of coffee extracts, soluble solids and/or volatile compounds obtained from the roasted, ground coffee beans, or leftovers thereof, may be improved by the enzymatic hydrolysis.

The coffee extract may be used to produce coffee by percolation.

The hydrolytic enzyme extraction procedure may occur at a temperature of from about 30 to about 100°C. More particularly, the extraction may occur at a temperature of from about 60 to about 80°C.

The enzymatic hydrolysis step may be performed for a period of up to about 24 hours.

Up to about 800 kg of one or more enzymes per ton dry spent ground or dry coffee beans may be used to perform the extraction step. However, an amount of as little as about from 1 to about 7 kg of the enzymes per ton dry spent ground or dry coffee beans may be used to perform the extraction.

The method may include the step of drying the coffee extract.

The coffee extract may comprise between 8 % and 40 % w/v concentration.

The coffee beans may be *Arabica* and/or *Robusta* beans.

The coffee beans may contain 20-36% w/v carbohydrates, predominantly in the form of β-1,4-mannan and β-1,3-galactan.

β-1,4-mannan and β-1,3-galactan may be hydrolysed by the enzyme.

The coffee extract may have a concentration higher than 42% w/v.

One or more of galactomannan, cellulose, xylan and pectin present in the coffee beans or leftovers may be hydrolysed by the enzyme(s) during the enzymatic hydrolysis extraction step.

The enzyme(s) may be recombinant or native, and may be β-mannanase, β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, and/or β-cellobiohydrolase 1-4.

Recombinant β-mannanase may be produced from *Saccharomyces cerevisiae* (especially *Saccharomyces cerevisiae* Y294(pMES1)), *Yarrowia lipolytica, Pichia pastoris, Hansenula polymorpha, Aspergillus awamori or Aspergillus niger* (especially *Aspergillus niger* D15(pGT-man1)).

The recombinant β-endoglucanases 1, 2, and 3 may be produced from *Aspergillus niger* D15(pGT-eg1), *Aspergillus niger* D15(pGT-eg2), and *Aspergillus niger* D15(pGT-eg3).

The recombinant β-xylanase may be produced from *Aspergillus niger* D15(pGT-xyn2).

The recombinant β-cellobiohydrolase may be produced from *Aspergillus niger* D15(pGT-cbh1-4).

Natural fungal strains may be isolated from green or roasted coffee beans, or leftovers thereof. Natural fungal strains may also be isolated from nature or from decaying plant material.

β-mannanase, β-endoglucanase, β-xylanase, β-cellobiohydrolase, polygalacturonase, pectin lyase, and pectin esterase enzyme activities may also be complimented by additional β-mannanase, β-endoglucanase, β-xylanase, β-cellobiohydrolase, polygalacturonase, pectin lyase, and pectin esterase enzyme activities.

According to a further embodiment of the invention, there is provided a method for determining the fraction of monosaccharide represented in the total measurable neutral sugars for roasted ground coffee beans and spent ground.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Pie charts indicating the fraction of minor sugars (1), arabinogalactan (2), xylan (3), mannan (4), and cellulose (5) in (A) roasted ground coffee, and (B) spent ground.

Figure 2: Total soluble solid yield increases measured in spent ground (Freeze-dry method) treated with recombinant enzymes followed by a thermal extraction process. The spent ground was treated with β-mannanase (Man1) (♦), β-endoglucanase 1 (Eg1) (■), β-endoglucanase 2 (Eg2) (●), and β-xylanase (Xyn2) (▲) at concentrations of 0.2 nkat/mg, 0.8 nkat/mg, 1.6 nkat/mg, 2 nkat/mg and 10 nkat/mg spent ground.

Figure 3: Total soluble solid yield increases measured in spent ground (Freeze-dry method) treated with recombinant enzymes followed by a thermal extraction process. The spent ground was treated with β-mannanase (Man1) (♦), β-endoglucanase 1 (Eg1) (■), β-endoglucanase 2 (Eg2) (●), and β-xylanase (Xyn2) (▲) at different protein concentrations.

Figure 4: Total soluble solid yield increases measured in spent ground (Freeze-dry method) treated with enzyme cocktails from natural fungal strains followed by a thermal extraction process. The spent ground was treated with cocktails from ABO 503 (●) and ABO 500 (■) at 0.2 nkat/mg, 0.6 nkat/mg, 1 nkat/mg, 2 nkat/mg or 8.8 nkat/mg spent ground.

Figure 5: Total soluble solid yield increases measured in spent ground (Freeze-dry method) treated with enzyme cocktails from natural fungal strains followed by a thermal extraction process. The spent ground was treated with cocktails from ABO 503 (●) and ABO 500 (■) at different protein concentrations.

Figure 6: Soluble solid yield increases measured in spent ground treated with commercial enzyme cocktails. The amount of enzyme added was

based on the amount of β-mannanase activity of the enzymes. An equivalent of 2 nkat/mg spent ground was added. The enzymes used are as follows; [1] Man1, [2] Cellulosin GM5 (Anchor Biotechnologies and Hankyu Bioindustry Co., LTD., Japan), [3] Pectinex Ultra SP-L (Novozymes SA, Sandton, SA), [4] Gamanase (Novozymes SA, Sandton, SA), [5] TP668L (Biocatalysts, Whales, UK), [6] D040L (Biocatalysts, Whales, UK), [7] D112L (Biocatalysts, Whales, UK), [8] M263L (Biocatalysts, Whales, UK), [9] D670L (Biocatalysts, Whales, UK), [10] G015L (Biocatalysts, Whales, UK), [11] C013L (Biocatalysts, Whales, UK), [12] M282L (Biocatalysts, Whales, UK), [13] TP692L (Biocatalysts, Whales, UK), and [13] D690L (Biocatalysts, Whales, UK).

Figure 7: Soluble solid yield increases measured in spent ground (Freeze-dry method) treated with commercial enzyme cocktails. The spent ground was treated with a total of 0.004 g total protein per 30 g spent ground. The enzymes used are as follows; [1] Man1, [2] Cellulosin GM5 (Anchor Biotechnologies and Hankyu Bioindustry Co., LTD., Japan), [3] Pectinex Ultra SP-L (Novozymes SA, Sandton, SA), [4] Gamanase (Novozymes SA, Sandton, SA), [5] TP668L (Biocatalysts, Whales, UK), [6] D040L (Biocatalysts, Whales, UK), [7] D112L (Biocatalysts, Whales, UK), [8] M263L (Biocatalysts, Whales, UK), [9] D670L (Biocatalysts, Whales, UK), [10] G015L (Biocatalysts, Whales, UK), [11] C013L (Biocatalysts, Whales, UK), [12] M282L (Biocatalysts, Whales, UK), [13] TP692L (Biocatalysts, Whales, UK), and [13] D690L (Biocatalysts, Whales, UK).

Figure 8: Saturation curve showing the soluble solid yield increases measured in spent ground (Total sugar method) after treatment with Mannanase-L (River Biotech (Pty) Ltd, Milnerton, SA). The spent ground was treated with the enzyme cocktail at concentrations of 10 nkat/mg, 50 nkat/mg, 100 nkat/mg, and 200 nkat/mg spent ground.

Figure 9: Saturation curve showing the soluble solid yield increases measured in spent ground (Total sugar method) after treatment with β-mannanase (Man1) (♦), and total soluble solid yield increases after and additional thermal extraction process (■). The spent ground was treated at concentrations of 10 nkat/mg, and 50 nkat/mg spent ground.

Figure 10: GC profiles of coffee samples. C-3 is the control sample, A-2 is the autoclaved sample, and E-1 is the enzyme treated sample.

Figure 11 Amino acid sequence of β-mannanase of *Aspergillus aculeatus* MRC11624. The neutral Ser→Thr substitution in the β-mannanase of *A. aculeatus* MRC11624 versus the published sequence (GenBank accession number L35487) of the β-mannanase of *A. aculeatus* KSM510 is encircled.

Figure 12 diagrams of a *man1* β-mannanase yeast expression cassette in (A) plasmid pMES1 and (B) plasmid pGT-man1.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a new method for producing yeast and fungal strains adapted to produce one or more enzymes containing hemicellulolytic, cellulolytic and/or pectolytic activities, and also provides for the use of hemicellulolytic-, cellulolytic- and/or pectolytic-containing enzymes in coffee production.

The invention also relates to a method for producing soluble coffee extracts, consisting of dissolved solids, from roasted coffee beans, or leftovers thereof. The purpose may be the production of an instant coffee product in an industrial process, or improving the extraction of soluble solids during percolation from roasted, ground coffee beans, sold as a final retail product. The method is based on the use of enzymes having hemicellulolytic, cellulolytic and/or pectolytic activity that may be used to improve either the yield and/or the quality of soluble solids and/or volatile compounds obtained from the roasted ground beans, or leftovers thereof.

The coffee extracts are typically obtained from *Arabica* and/or *Robusta* coffee beans.

The yeast strain or fungus strain (typically an *Aspergillus* fungus strain) is transformed to have the capability of producing endo-1,4-β-mannanase (β-mannanases) (Man1), endo-1,4-β-endoglucanase (β-endoglucanases) (Eg1), endo-1,4-β-endoglucanase (β-endoglucanases) (Eg2), endo-1,4-β-endoglucanase (β-endoglucanases) (Eg3), endo-1,4-β-xylanase (β-xylanases) (Xyn2), and/or exo-1,4-β-glucan cellobiohydrolase (β-cellobiohydrolases) (Cbh1-4).

More particularly, the yeast strain has the capability of producing a β-mannanase (Man1).

The fungus strain should have the capability of producing a β-mannanase (Man1), β-endoglucanase (Eg1, Eg2 or Eg3), β-xylanase (Xyn2) or β-cellobiohydrolase (Cbh1-4).

DNA expression cassettes for use in transforming the yeast or fungus strains are also part of the invention.

Apart from transforming yeast or fungus strains to produce the enzymes, the enzymes may also be obtained by cultivating natural fungal strains. Any natural fungal strains, capable of producing β-mannanase, β-endoglucanase, β-xylanase, β-cellobiohydrolase, polygalacturonase, pectin lyase, and/or pectin esterase, can be utilised for the production of the hydrolytic enzyme compositions. Such natural fungal strains may be isolated from nature, decaying plant material, green coffee beans, roasted coffee beans or the leftovers of roasted, ground coffee beans after water extraction.

The enzymes may also be obtained commercially.

Other enzymes containing hemicellulolytic, cellulolytic and/or pectolytic activities may be added to the recombinant, natural fungal strain compositions or commercial compositions containing β-mannanase to improve the efficiency thereof. For

example, β-endoglucanase, β-xylanase, β-cellobiohydrolase, polygalacturonase, pectin lyase, and/or pectin esterase may be added to the composition containing β-mannanase to improve the efficiency thereof.

Other enzymes, such as those having hemicellulolytic, cellulolytic and pectolytic activities, may be added to the compositions containing β-mannanase, where the addition of the other enzymes provides a synergistic effect to improve the efficiency thereof.

The viscosity of soluble solid coffee extracts may be reduced by the addition of enzymes containing hemicellulolytic, cellulolytic and pectolytic activities, such as those described above.

The present invention also relates to a method for determining the fraction of monosaccharide represented in the total measurable neutral sugars for roasted ground coffee beans and spent ground.

The term "a mannanase yeast expression cassette", as used herein, denotes a recombinant DNA molecule according to the invention which includes the *man1* gene, preferably the *man1* gene from *Aspergillus aculeatus* MRC11624 (Setati *et al.*, 2001), and the yeast *ADH2* promoter and terminator DNA sequences, preferably the *ADH2* promoter and terminator DNA sequences resident on the yeast/*Escherichia coli* shuttle vector pMES1 (Setati *et al.*, 2001).

The term "a mannanase fungus expression cassette", as used herein, denotes a recombinant DNA molecule according to the invention which includes the *man1* gene, preferably the *man1* gene from *Aspergillus aculeatus* MRC11624 (Setati *et al.*, 2001), and the fungus *gpd* promoter and *glaA* terminator DNA sequences, preferably the *gpd* promoter and terminator DNA sequences resident on the fungus/*Escherichia coli* shuttle vector pGT-man1 (Figure 12B).

β-Mannanases hydrolyse linear mannan polysaccharides and complex substituted mannan polysaccharides such as glucomannan, galactomannan and galactoglucomannan into oligosaccharides of various chain lengths (Sabini *et al.*,

2000; Setati *et al.*, 2001). Hydrolysis of substituted mannans is greatly affected by the degree and pattern of substitution so that as the galactose content increases, the rate of hydrolysis (V_{max}) by β-mannanase decreases and the K_m increases (McCleary, 1983). β-mannanases can therefore be used to perform hydrolysis experiments in which the intention is only to modify the properties of polysaccharides without complete degradation. The viscosity of polysaccharides is proportional to chain length, branching, and entanglement, and reduction of viscosity can be effected through partial hydrolysis.

Considering the high galactomannan content (69%) in roasted coffee beans, β-mannanases can also be considered for enzymatic pre-treatment of ground coffee beans before extraction (percolation) to (i) improve coffee solid yields and (ii) improve extraction of volatile substances. The leftovers of coffee beans after the first extraction of soluble solids (percolation), often referred to as "spent ground" or "spent grain," may similarly be treated with enzyme cocktails to improve the extraction of soluble coffee solids and/or volatile substances.

Table 1: List of recombinant DNA sequences used for the production of recombinant enzymes by yeast and *Aspergillus* strains

Gene description	Gene name	Donor organism	Accession number
Cellobiohydrolase 1-4	cbh1-4	*Phanerochaete chrysosporium* ATCC 24725	L22656
Endoglucanase 1	*eg1*	*Trichoderma reesei* QM6a	M15665
Endoglucanase 2	*eg2*	*Trichoderma reesei* QM6a	M19373
Endoglucanase 3	*eg3*	*Trichoderma reesei* QM6a	AB003694
Mannanase 1	*man1*	*Aspergillus aculeatus* MRC11624	L35487
Xylanase 2	*xyn2*	*Trichoderma reesei* QM6a	U24191

Examples:

The invention will now be described by way of example with reference to the accompanying schematic Figures.

(a) Method for isolation of natural fungal strains

Fungi able to produce β-mannanases were isolated from green coffee beans (Indonesia and Vietnam Robusta beans), as well as from coffee and chicory spent grain. A substrate consisting of coffee beans or spent grain, or chicory spent-grain, was incubated in a moisture chamber at 22°C for a period of two weeks. During this time fungal growth was periodically transferred to malt extract agar (MEA). The fungal isolates growing on MEA were purified by preparing single-spore cultures, followed by successive cultivation on MEA at 22°C. The pure cultures were deposited in the fungal culture collection of the Department of Microbiology at the University of Stellenbosch. The isolates were maintained in this culture collection until they were tested for production of β-mannanase. Other fungal strains in the culture collection mentioned above, which were originally selectively isolated from the natural environment using complex plant materials (e.g. lignocellulose, mannan or xylan) as carbon source in selective media, were also screened for efficiency of growth and β-mannanase on roasted, ground coffee beans, of leftovers thereof.

ABO500, ABO503, PPRI 5471 and PPRI 5469 represent four natural fungal strains that produce high levels of β-mannanase, and were isolated using the methods described above.

(b) Enzyme production by recombinant *Saccharomyces cerevisiae*

The recombinant β-mannanase producing yeast strain *Saccharomyces cerevisiae* Y294(pMES1) was cultivated in minimal synthetic complete medium supplemented with 4 % glucose, 0,17 % yeast nitrogen base without ammonium sulphate, and an amino acid drop-out mix without methionine. The cultures were incubated at 30°C on a rotary shaker for 4 days, after which the cells were removed by centrifugation. The supernatant (enzyme source) was concentrated through the Minitan cross-flow ultra filtration device (Millipore Corporation, Bedford, Massachusetts, USA). The filtrate was freeze-dried and used for hydrolysis experiments.

(c) Enzyme production by recombinant *Aspergillus niger*

The recombinant producing fungal strains of *Aspergillus niger* (Rose and van Zyl, 2002) were cultivated in double strength liquid minimal medium containing 10 % (w/v) glucose supplemented with 0.4 % (w/v) casamino acids, 0.08 % (w/v) $MgSO_4.7H_20$, 1.2 % (w/v) $NaNO_3$, 0.3 % (w/v) KH_2PO_4, 0.1 % (w/v) KCl additives and trace elements. The cultures were incubated at 30°C on a rotary shaker for 6-9 days, after which the cells were removed by filtering cultures through Miracloth (Calbiochem biosciences inc., La Jolla, CA, USA). The supernatant (enzyme source) was concentrated through either the Minitan cross-flow ultra filtration device (Millipore Corporation, Bedford, Massachusetts, USA), or the Pellicon device (Millipore Corporation, Bedford, Massachusetts, USA).

(d) Enzyme production by natural fungal strains

The natural fungal strains were cultivated in liquid minimal medium containing 5 % (w/v) spent ground supplemented with 0.5 % (w/v) glucose, 0.2 % (w/v) casamino acids, 0.04 % (w/v) $MgSO_4.7H_20$, 0.6 % (w/v) $NaNO_3$, 0.15 % (w/v) KH_2PO_4, 0.05 % (w/v) KCl additives and trace elements. The cultures were incubated at 30°C on a rotary shaker after which the cells were removed by filtering cultures through Miracloth (Calbiochem biosciences inc., La Jolla, CA, USA). The supernatant (enzyme source) was concentrated through either the Minitan cross-flow ultra filtration device (Millipore Corporation, Bedford, Massachusetts, USA) or the Pellicon device (Millipore Corporation, Bedford, Massachusetts, USA).

(e) Determination of monosaccharide fraction

Polysaccharides were hydrolysed by subjecting 0.25 g of sample to two-stage sulphuric acid hydrolysis (Moore and Johnson 1967). After neutralization with $CaCO_3$ to pH ~5.5, samples were amended with 20 mg of myo-inositol (internal standard), then centrifuged (1,500 x g, 15 min.), and 10 ml of supernatant was lyophilised. The lyophilised samples were resuspended in 1000 µl deionised water, then centrifuged at 12,000 x g for 5 min. Supernatants were dried under an air stream and then

subjected to reduction with Na borodeuteride, and acetylation with acetic anhydride, as described by Blakeney et al. (1983).

Gas-liquid chromatography of alditol acetates was performed using a Hewlett-Packard 6890 Plus GC fitted with a flame ionisation detector and a Supelco SPB-225 capillary column (30 m x 0.25 mm, with 0.25 µm film thickness). Helium was used as carrier gas. The temperature program was as follows: 215°C for 2 min., then increased at 4°C per min. for 3.75 min., and then held at 230°C for 11.25 min.

(f) Hydrolysis of locust bean gum (galactomannan)

A 1 % (w/v) locust bean gum solution was prepared in 50 mM citrate buffer pH 5 and used as a substrate for viscosity analysis. The viscosity analyses were performed using a Brookfield viscometer model (Brookfield Engineering Laboratories, Inc., Stoughton, Mass. USA). The initial viscosity was determined at 40°C, after which the β-mannanase enzyme was added to the final concentration of 2 nkat/mg substrate. The viscosity of the reaction mixture was measured at different time points and samples were collected concurrently and analysed for reducing sugars. The reaction was terminated by boiling for 5 min. The reducing sugars were determined using the modified DNS method as previously described (Stålbrand *et al.*, 1993).

(g) The flow dynamics of coffee extracts

Ground *Arabica* coffee (Boveldt, pure South African) was supplied by SAPEKOE (Pty) Ltd. (Tzaneen, SA). Coffee extracts were prepared by pre-wetting 400 g of coffee in 5 mM citrate buffer at pH 5 for 5 hrs. Extraction was carried out overnight at 80°C with constant stirring. The soluble extract was collected by filtration through a Miracloth (CALBIOCHEM), and freeze-dried. The dried extract was used to prepare different extract concentrations (20 %, 40 % and 60 % w/v) for viscosity analysis. The flow dynamics of the extracts were studied at 30°C on a HAAKE RV12 Viscometer (HAAKE Mess-Technik GmbH & Co., Germany). The effect of β-mannanase on the viscosity, and flow dynamics of the coffee extract was evaluated. The enzyme was added to a final concentration of 2 nkat/mg extract and 100 µg/ml

BSA was added to stabilize the enzyme. The reaction was carried out at pH 5, 50°C for 3 h followed enzyme inactivation at 100°C for 5 min.

(h) Extraction process on roasted coffee beans

Thirty grams of ground *Arabica* coffee was pre-wet to 50°C in 100 ml of sodium citrate buffer (50 mM, pH 4.5 – 5.0) for 5 hours, followed by hydrolysis with enzyme for 18 hours with constant stirring. An extraction was performed afterwards consisting of two autoclave cycles at 121°C for 20 min per cycle at 100 kPa. The soluble extracts were freeze-dried and volatile compounds were analysed by gas chromatography.

(i) Extraction process on spent ground: Freeze-dry method

Thirty grams of spent ground was pre-wet at 50°C with agitation using 100 ml sodium citrate buffer (50 mM, pH 4.5 – 5.0). Enzyme was added for hydrolysis at different enzyme activities and protein concentrations. BSA (0.1 mg/ml) was added to certain extractions to stabilise the recombinant enzymes. Spent ground was hydrolysed by incubation with agitation at temperatures of 40°C-70°C for 18 hours. Supernatant was filtered through 150 mm diameter filter papers (Whatman, International Ltd., Maidstone, England) to remove spent ground and 50 ml supernatant was freeze-dried for soluble solid determination. The spent ground was re-suspended to a final volume of 100 ml with sodium citrate buffer (50 mM, pH 4.5 – 5.0) and subjected to 2 liquid autoclave cycles for 15 minutes per cycle at 100 kPa (thermal extraction). Supernatant was filtered and soluble solids determined after freeze-dry process.

(j) Extraction process on spent ground: Total sugar method

Thirty grams of spent ground was pre-wet at 50°C with agitation using 100 ml sodium citrate buffer (50 mM, pH 4.5 – 5.0). Enzyme was added for hydrolysis at different enzyme activities and protein concentrations. BSA (0.1 mg/ml) was added to certain extractions to stabilise the recombinant enzymes. Spent ground was hydrolysed by incubation with agitation at temperatures of 40°C-70°C for 18 hours. Supernatant was filtered through 150 mm diameter filter papers (Whatman, International Ltd.,

Maidstone, England) to remove spent ground. Total sugars in the supernatant were determined using the phenol-sulphuric acid method. Shortly, samples were mixed with 5% phenol and mixed. Concentrated sulphuric acid was added and the optical density measured at 490 nm. Total sugars were then determined from a standard curve. Soluble solids were calculated from a standard curve taking total sugars and soluble solids produced from control experiments (no enzymes added) into account. The spent ground was re-suspended to a final volume of 100 ml with deionised water and subjected to 2 liquid autoclave cycles for 15 minutes per cycle at 100 kPa (thermal extraction). Supernatant was filtered and soluble solids calculated after total sugar determination.

(k) Analytical methods

Gas chromatography analysis was performed on 3.57 g of coffee samples. SPME fibre (100 μm Polydimethylsiloxane) was used for sampling. Extraction was carried out for 120 min at 65°C (head space), and desorption at 230°C for 5 min. Compounds were separated on PS089 (0.25 μM film), 40 m x 0.25 mm GC column using 38 cm/s He as a carrier gas. The GC program was 40°C (0 min) to 230°C at 4°C/min.

(l) Monosaccharide fraction of total measurable neutral sugars

A large portion of the arabinogalactan and almost all of the xylan present in the roasted beans are hydrolysed during the coffee extraction. The residual monosaccharides present in the spent ground are largely mannan and cellulose (Figure 1A and 1B).

(m) The effect of recombinant enzymes, commercial enzymes and natural fungal enzyme cocktails on soluble solid yield

An increase in soluble solids was measured after spent ground was treated with the different enzymes. Figure 2 indicates that β-mannanase showed the highest increase in soluble solids for the different recombinant enzyme activities used to treat spent ground. The β-xylanase enzyme however performs better when the experiment was

based on the amount of protein added (Figure 3). The enzyme cocktail obtained by cultivation of ABO500 showed a higher increase in soluble solids than ABO503 at the lower levels of enzyme treatment based on β-mannanase activity (Figures 4), whereas the ABO503 performed better when yields are observed for protein concentration added (Figures 5). The Pectinex Ultra SP-L, M263L and M282L commercial enzymes showed the highest increase in soluble solid yields after treatment based on β-mannanase activity (Figure 6), but the recombinant β-mannanase enzyme performed the best at protein level (Figure 7). The TP668L and M263L also compared very well with the recombinant β-mannanase enzyme (Figure 7). Overall, the recombinant β-mannanase induced the highest soluble solids yield when performing experiments based on enzyme activity added, and was most effective for the intended purpose. Theoretical yields per gram total protein of enzyme added to treatments however indicate that the recombinant β-xylanase enzyme will induce the highest increase in soluble solid yield. Enzyme saturation is prevalent when using concentrations larger than 50 nkat/mg (100 nkat/mg and 200 nkat/mg) for Mannanase-L (Figure 8). An additional thermal extraction process further increased soluble solid yields after enzyme treatment (Figure 9).

(n) The effect of enzyme-aided extraction on volatile compounds

It has previously been shown that hydrolysis of coffee galactomannan with β-mannanase results in reduction of viscosity, and that the viscosity remained constant after a few hours of hydrolysis (Sachslehner *et al.*, 2000). Therefore, it was expected in the experiment that hydrolysis of coffee extracts at 50°C for 5 h would generate high concentrations of oligosaccharides which together with high molecular weight arabinogalactan have the capacity to bind volatile compounds (Nunes *et al.*, 2001; Trugo, 1985). Due to the mildness of the treatment, retention of higher concentrations of volatile compounds would be achieved. Figure 10, shows the chromatograms obtained from coffee extract samples that were extracted by enzyme-aided treatment (E-1), and through autoclaving at 121°C (A-2). The profiles of volatile compounds were compared to those detected in ground coffee. The three samples displayed similar profiles, which, indicated that they contained the same type of volatile compounds. However, the differences in peak sizes indicate that the autoclaved sample had a significant loss of volatile compounds, relative to the

control. Enzyme treatment does also seem to affect the amount of compounds retained, resulting in improved retention of some compounds and slight losses of others.

The preliminary results indicate that β-mannanase is capable of hydrolysing coffee galactomannan, leading to substantial reduction in viscosity. In addition the results seem to indicate that enzyme-aided extraction might be a valuable technique for instant coffee production since this could allow extractions at lower temperatures, and therefore, better retention of volatile compounds. In addition, lower extract viscosity would allow recovery of concentrates higher than 42 % after pre-concentration, and also improve the drying process due to the lower water content of the extracts.

Construction of recombinant yeast and fungal strains

(a) Isolation of β-mannanase (man1) gene

For the purpose of isolating the *man1* gene, the fungus *Aspergillus aculeatus* MRC11624 was cultivated in minimal medium containing: 0,3% locust been gum [Sigma], 0.1% bacto tryptone; 0.5% yeast extract; 0.1% NaNO3; 0.001% FeSO4.7H2O; pH 5.5 in shaking flasks for 48 hours at 30°C. Total RNA was isolated essentially according to Crous *et al (1995)*: The poly $(A)^+$mRNA was purified from total RNA using the Oligotex™ mRNA isolation kit (Qiagen, Hilden, Germany). First strand cDNA was synthesised from 116 ng of mRNA using a first strand cDNA synthesis kit (Roche Molecular Biochemicals, Ottweiler, Germany), and used as template for amplification of the β-mannanase encoding gene *man1* by PCR on a Biometra Trio Thermoblock TB1 (Biometra Biomedizinische Analytik, Göttingen, Germany). The primers used were designed based on the sequence of the *man1* gene of *A. aculeatus* strain KSM510 (Accession. No. L35487) (Christgau *et al.*, 1994).

1. MANR (28-mer, the *Eco*RI restriction site is underlined)
(5'-GATCGAATTCCACCACCACACAACCAAG-3')

2. MANL (28-mer; the *Xho*I restriction site is underlined) (5′-CTAGCTCGAGCGCCAACAGTCTACTTCG-3').

The cDNA amplified *man1* gene was ligated to pBLUESCRIPT and sequenced, and the nucleotide sequence showed 99.7% identity with the sequence of the *A. aculeatus* KSM510 as reported by Christgau *et al.* in 1994 (GenBank accession number L35487). Three base-pair discrepancies were detected on the DNA level and one resulted in an amino acid sequence difference (Ser → Thr) at position 225 (Figure 16). This region is variable according to sequence alignment by Hilge *et al.* in 1998 and is not crucial for its structure and function. The neutral Ser→Thr substitution is thus not likely to affect the enzyme activity.

(b) Construction of recombinant yeast and fungal strains

For the purpose of constructing a yeast strain capable of producing β-mannanase, plasmid pMES1 was engineered. The PCR product was pre-digested with the restriction enzymes *Eco*RI and *Xho*I, the DNA purified through agarose gel electrophoresis and ligated as a 1180-bp *Eco*RI/*Xho*I DNA fragment into the *Eco*RI and *Xho*I sites between the *ADH2* promoter and terminator in plasmid pDLG1, thereby generating plasmid pMES1, which constitutes a β-mannanase yeast expression cassette according to the invention (Figure 12A). Plasmid pMES1 was transformed into *Saccharomyces cerevisiae* strain Y294 following the DMSO-lithium acetate method described by Hill *et al.* in 1991. Auto-selective *Saccharomyces cerevisiae* strains contain this plasmid was constructed by replacing the *FUR1* gene on the chromosome with a *fur1::LEU2* disruptive allele, as described by La Grange *et al.* in 1996.

For the purpose of constructing a fungus strain capable of producing endo-1,4-β-mannanase, plasmid pGT-man1 was engineered. The *manI* gene was retrieved as an *Eco*RI-*Xho*I DNA fragment from plasmid pMES1 (Setati *et al.*, 2001) and cloned into the corresponding sites of *Escherichia coli* plasmid pBLUESCRIPT. The *man1* gene was retrieved from pBLUESCRIPT-man1 as a *Not*I-*Xho*I DNA fragment and cloned into the *Not*I and *Sal*I sites of plasmid pGT (Rose and Van Zyl, 2002), generating plasmid pGT-man1 (Figure 12B). Plasmid pGT-man1 was transformed

into *Aspergillus niger* strain D15 by the spheroplasting method of Ballance *et al.* of 1983. Plasmid pGT-man1 was transformed, together with plasmid p32R2 and selection for successful transformants was performed on medium containing acetamide as nitrogen source (Rose and Van Zyl, 2002).

(c) Enzymatic assays

Mannanase, endoglucanase, xylanase and polygalacturonase activities were measured using the modified DNS method (Stålbrand *et al.*, 1993). The substrates used for liquid assays were 0.25% Locust bean gum (Sigma-Aldrich Co., Missouri, USA), 1% CMC (Sigma-Aldrich Co., Missouri, USA), 1% Birchwood xylan (Carl Roth GmbH, Karlsruhe, Germany), and 0.05% Polygalacturonic acid (Sigma-Aldrich Co., Missouri, USA). Cellobiohydrolase activity was quantified using the chromophoric substrate PNPC, essentially as described for β-glucosidase activity determinations with the chromophoric substrate PNPX (La Grange *et al.*, 1997).

Total protein concentration of enzyme cocktails was determined using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, München, Germany).

(d) Production levels of recombinant β-mannanase with *S. cerevisiae*

The maximum β-mannanase activity obtained from the recombinant *fur1::LEU2* *Saccharomyces cerevisiae* Y294(pMES1) strain (521 nkat/ml at 50°C) and recombinant *Aspergillus niger* D15(pGT-man1) strain (6 000 nkat/ml at 50°C) compares very well with that of *Aspergillus aculeatus* MRC11624 (about 500 nkat/ml) and other recombinant strains reported in literature (Table 2), if one takes into account that the *Aspergillus aculeatus* MRC11624 culture supernatant contains the auxiliary enzymes involved in mannan degradation.

Table 2: β-Mannanase activity levels measured from expression in different hosts.

Expression Host	Enzyme Activity (nkat/ml)	Reference
S. lividans IAF10-164	1450	Arcand *et al.*, 1993
S. lividans 1326	1917	Marga *et al.*, 1996

E. coli JM109	81.7	Mendoza *et al.* 1995
E. coli RR28	1.33	Gibbs *et al.*, 1992
S. cerevisiae DBY746	0.22	Stålbrand *et al.*, 1995
S. cerevisiae Y294(pMES1)	521	This patent application
S. cerevisiae Y294	6000	This patent application

(e) Production levels of recombinant β-mannanase with *A. niger*

An average activity of 1700 nkat/ml was reached after 6 days of cultivation.

(f) Production levels of β-mannanase in cocktails from natural fungal strains

Natural strains ABO 500 and ABO 503 showed a maximum β-mannanase activity of 120 nkat/ml and 2000 nkat/ml after 9 days of cultivation, respectively.

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CLAIMS:

1. A DNA expression cassette for use in transforming a yeast or fungus so as to provide it with a capability of producing β-mannanase, the expression cassette including:

a gene encoding a β-mannanase enzyme; and

a suitable promoter for promoting transcription of the gene in the transformed yeast or fungus.

2. An expression cassette according to claim 1, which includes a terminator sequence for promoting efficient expression of the β-mannanase gene.

3. An expression cassette according to either of claims 1 or 2, wherein the gene encoding β-mannanase is the *man1* gene from a fungus.

4. An expression cassette according to claim 3, wherein the fungus is *Aspergillus aculeatus* MRC11624.

5. An expression cassette according to any one of claims 1 to 4, wherein the yeast or fungus strain is *Saccharomyces cerevisiae*, *Yarrowia lipolytica, Pichia pastoris, Hansenula polymorpha, Aspergillus awamori* or *Aspergillus niger*.

6. An expression cassette according to claim 5, wherein the yeast strain is *Saccharomyces cerevisiae* Y294(pMES1))

7. An expression cassette according to claim 5, wherein the fungus strain is *Aspergillus niger* D15(pGT-man1).

8. An expression cassette according to any one of claims 1 to 6, wherein the yeast promoter and terminator sequences are the *PGK* promoter and terminator DNA sequences, respectively.

9. An expression cassette according to any one of claims 1 to 5 and 7, wherein the fungus promoter and terminator sequences are the *gpd* promoter and *glaA* terminator DNA sequences, respectively.

10. A DNA vector including the expression cassette of any one of claims 1 to 9.

11. A vector according to claim 10, which is the yeast/*Escherichia coli* shuttle vector YEp352.

12. A vector according to claim 10, which is the *Aspergillus*/*Escherichia coli* shuttle vector pGT.

13. A DNA expression cassette for use in transforming a yeast or fungus so as to provide it with a capability of producing β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, and/or β-cellobiohydrolase 1-4, the expression cassette including:

one or more genes encoding one or more of a β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, or β-cellobiohydrolase 1-4 enzyme;

a suitable promoter for promoting transcription of the gene(s) in the transformed fungus or yeast strain.

14. An expression cassette according to claim 13, which includes a terminator DNA sequence for promoting efficient expression of the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, and/or β-cellobiohydrolase 1-4 genes.

15. An expression cassette according to either of claims 13 or 14, wherein the fungus strain for producing the recombinant β-endoglucanases 1, 2, and 3 is any one of *Aspergillus niger* D15(pGT-eg1), *Aspergillus niger* D15(pGT-eg2), and *Aspergillus niger* D15(pGT-eg3).

16. An expression cassette according to either of claims 13 or 14, wherein the recombinant β-xylanase is produced from *Aspergillus niger* D15(pGT-xyn2).

17. An expression cassette according to either of claims 13 or 14, wherein the recombinant β-cellobiohydrolase is produced from *Aspergillus niger* D15(pGT-cbh1-4).

18. An expression cassette according to any one of claims 13 to 17, wherein the genes encoding β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, and/or β-xylanase 2 are the *eg1, eg2, eg3 or xyn2* genes, respectively, from a fungus.

19. An expression cassette according to claim 18, wherein the fungus is *Trichoderma reesei* QM6a.

20. An expression cassette according to any one of claims 13 to 19, wherein the gene encoding β-cellobiohydrolase 1-4 is the *cbh1-4* gene from a fungus.

21. An expression cassette according to claim 20, wherein the fungus is *Phanerochaete chrysosporium* ATCC 24725.

22. An expression cassette according to any one of claims 13 to 21, wherein the fungus promoter and terminator sequences are the *gpd* promoter and *glaA* terminator DNA sequences.

23. A DNA vector including an expression cassette of any one of claims 17 to 22.

24. A DNA vector according to claim 23, which is the *Aspergillus/Escherichia coli* shuttle vector pGT.

25. A method of producing a yeast strain which is capable of expressing β-mannanase, the method including the step of:

transforming a yeast strain with a nucleotide sequence including a gene encoding a β-mannanase enzyme and a suitable promoter for promoting transcription of the gene in the transformed yeast.

26. A method according to claim 25, wherein the yeast strains are selected from *Saccharomyces cerevisiae, Yarrowia lipolytica, Pichia pastoris,* and *Hansenula polymorpha*.

27. A method of producing a fungus strain which is capable of expressing β-mannanase, the method including the step of:

transforming a fungus strain with a nucleotide sequence including a gene encoding a β-mannanase enzyme and a suitable promoter for promoting transcription of the gene in the transformed fungus.

28. A method according to claim 27, wherein the fungus strain is an Aspergillus strain.

29. A method of producing a fungus strain which is capable of expressing one or more of β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and β-cellobiohydrolase 1-4, the method including the step of:

transforming a fungus strain with a nucleotide sequence including one or more genes encoding β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and β-cellobiohydrolase 1-4 and a suitable promoter for promoting transcription of the gene in the transformed fungus.

30. A method according to claim 29, wherein the fungus strain is an *Aspergillus niger* fungus strain.

31. A method according to either of claims 29 or 30, wherein the recombinant β-endoglucanases 1, 2, and 3 are produced from *Aspergillus niger* D15(pGT-eg1), *Aspergillus niger* D15(pGT-eg2), and/or *Aspergillus niger* D15(pGT-eg3).

32. A method according to either of claims 29 or 30, wherein the recombinant β-xylanase is produced from *Aspergillus niger* D15(pGT-xyn2).

33. A method according to either of claims 29 or 30, wherein the recombinant β-cellobiohydrolase is produced from *Aspergillus niger* D15(pGT-cbh1-4).

34. A host cell which has been transformed according to any one of claims 25 to 33.

35. A host cell according to claim 34, which is a yeast or fungus.

36. A host cell according to claim 34, which is a *Saccharomyces cerevisiae, Yarrowia lipolytica, Pichia pastoris, Hansenula polymorpha, Aspergillus awamori or Aspergillus niger.*

37. A β-mannanase enzyme produced by:

causing a transformed host cell to express the β-mannanase gene; and

recovering the β-mannanase enzyme.

38. An enzyme which is β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 or β-cellobiohydrolase 1-4 produced by:

causing a host cell to express the gene(s) encoding the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and/or β-cellobiohydrolase 1-4; and

recovering the β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2 and/or β-cellobiohydrolase 1-4 enzyme.

39. A method of producing a coffee extract, the method including the step of subjecting roasted, ground coffee beans or leftovers thereof to an enzymatic hydrolysis extraction.

40. A method according to claim 39, wherein the extraction yield and/or quality of coffee extracts, soluble solids and/or volatile compounds obtained from the roasted, ground coffee beans, or leftovers thereof, is improved by the enzymatic hydrolysis.

41. A method according to either of claims 39 or 40, wherein the coffee extract is used to produce coffee by percolation.

42. A method according to any one of claims 39 to 41, wherein the hydrolytic enzyme extraction step is performed at a temperature of from about 30 to about 100°C.

43. A method according to claim 42, wherein the extraction is performed at a temperature of from about 60 to about 80°C.

44. A method according to any one of claims 39 to 43, wherein the enzymatic hydrolysis step is performed for a period of up to about 24 hours.

45. A method according to any one of claims 39 to 44, wherein from about 1 to about 800 kg of one or more enzymes per ton dry spent ground or dry coffee beans is used to perform the extraction step.

46. A method according to any one of claims 39 to 45, which includes the step of drying the coffee extract.

47. A method according to any one of claims 39 to 46, wherein the coffee extract comprises between 8 % and 40 % w/v concentration.

48. A method according to any one of claims 39 to 47, wherein the coffee beans are *Arabica* and/or *Robusta* beans.

49. A method according to any one of claims 39 to 48, wherein the coffee beans contain 20-36% w/v carbohydrates, predominantly in the form of β-1,4-mannan and β-1,3-galactan.

50. A method according to any one of claims 39 to 49, wherein β-1,4-mannan and β-1,3-galactan are hydrolysed by the enzyme during the enzymatic hydrolysis extraction step.

51. A method according to any one of claims 39 to 50, wherein the coffee extract has a concentration higher than 42% w/v.

52. A method according to any one of claims 39 to 51, wherein one or more of galactomannan, cellulose, xylan and pectin present in the coffee beans or leftovers is hydrolysed by the enzyme(s) during the enzymatic hydrolysis extraction step.

53. A method according to any one of claims 39 to 52, wherein the enzymes are recombinant or native.

54. A method according to any one of claims 39 to 53, wherein the enzyme or enzymes are selected from one or more of β-mannanase, β-endoglucanase 1, β-endoglucanase 2, β-endoglucanase 3, β-xylanase 2, and/or β-cellobiohydrolase 1-4.

55. A method according to claim 54, wherein the recombinant β-mannanase is produced from *Saccharomyces cerevisiae*, *Yarrowia lipolytica, Pichia pastoris, Hansenula polymorpha, Aspergillus awamori or Aspergillus niger.*

56. A method according to any one of claims 54 to 55, wherein the recombinant β-endoglucanases 1, 2, and 3 are produced from *Aspergillus niger* D15(pGT-eg1), *Aspergillus niger* D15(pGT-eg2), and *Aspergillus niger* D15(pGT-eg3).

57. A method according to any one of claims 54 to 56, wherein the recombinant β-xylanase is produced from *Aspergillus niger* D15(pGT-xyn2).

58. A method according to any one of claims 54 to 57, wherein the recombinant β-cellobiohydrolase is produced from *Aspergillus niger* D15(pGT-cbh1-4).

59. A coffee extract or coffee product produced by the method of any one of claims 39 to 58.

60. A method for determining the fraction of monosaccharide represented in the total measurable neutral sugars for roasted ground coffee beans and spent ground.

61. An expression cassette according to either one of claims 1 or 13, substantially as herein described with reference to any one of the illustrative examples.

62. A vector according to either one of claims 10 or 23, substantially as herein described with reference to any one of the illustrative examples.

63. A method according to any one of claims 25, 27 or 29, substantially as herein described with reference to any one of the illustrative examples.

64. A host cell according to claim 34, substantially as herein described with reference to any one of the illustrative examples.

65. An enzyme according to either one of claims 37 or 38, substantially as herein described with reference to any one of the illustrative examples.

66. A method according to claim 39, substantially as herein described with reference to any one of the illustrative examples.

67. A coffee extract of coffee product according to claim 59, substantially as herein described with reference to any one of the illustrative examples.

68. A method according to claim 60, substantially as herein described with reference to any one of the illustrative examples.

Dated this 11th day of May 2006

SPOOR & FISHER
APPLICANT'S PATENT ATTORNEYS

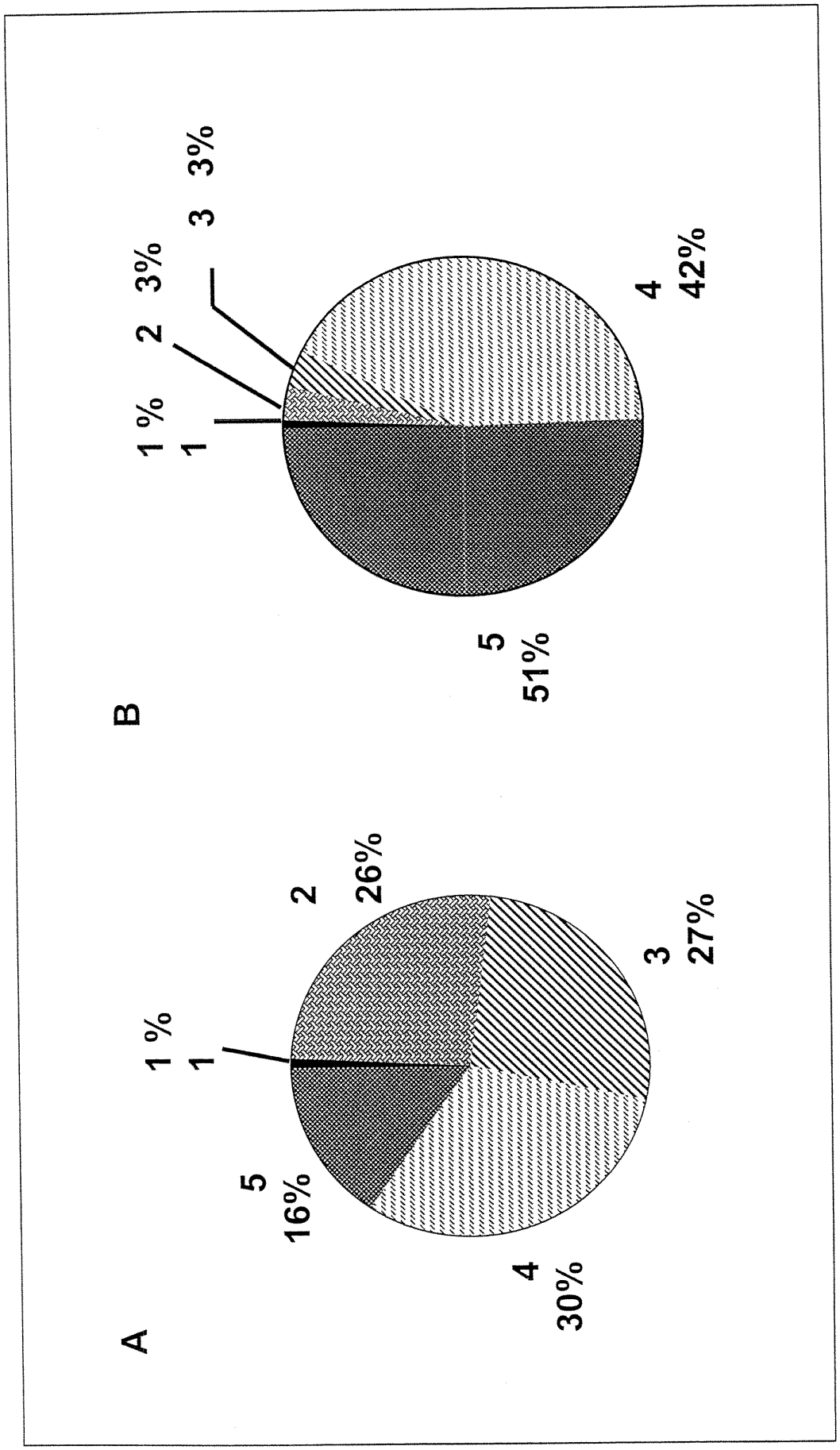


Fig. 1

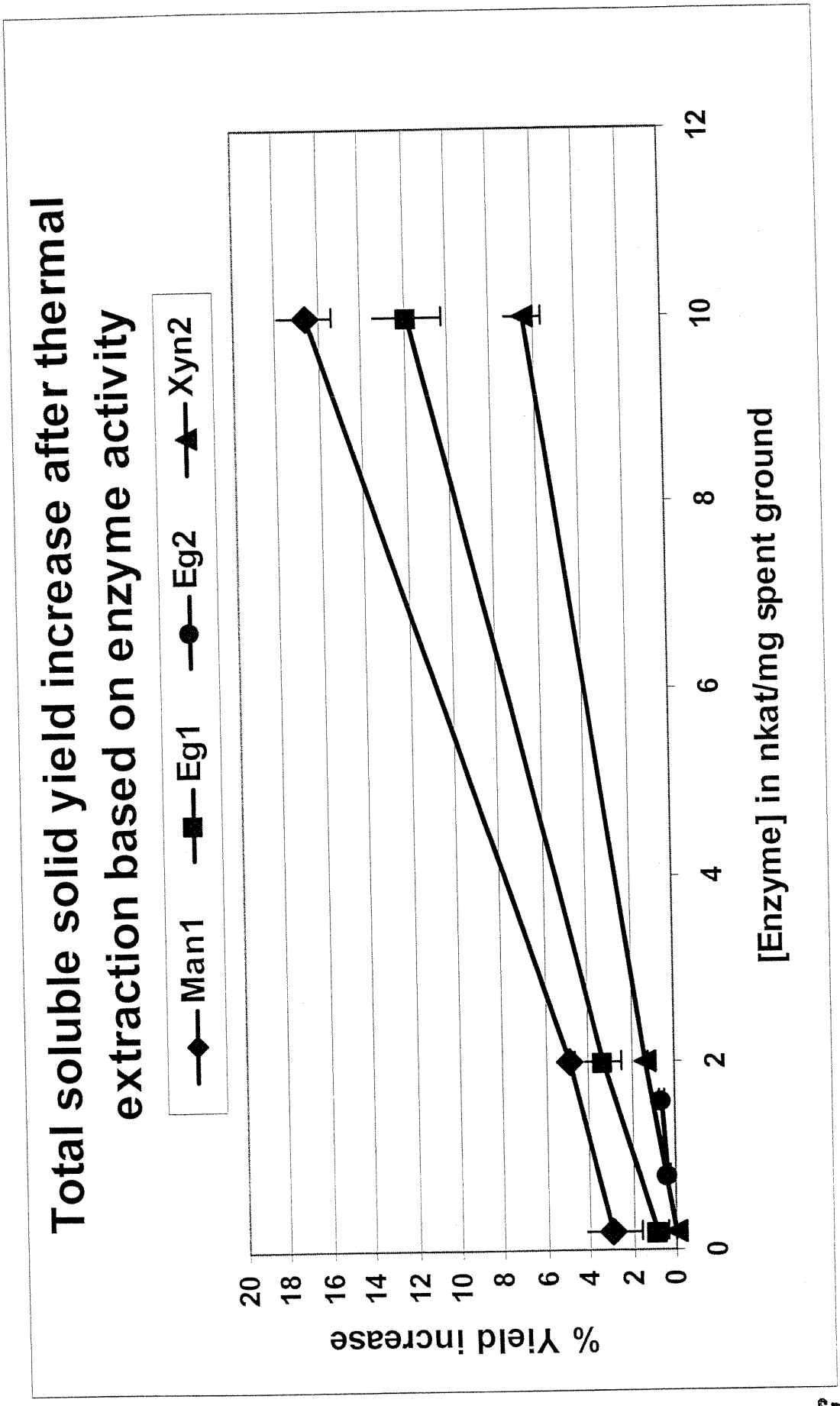


Fig. 2

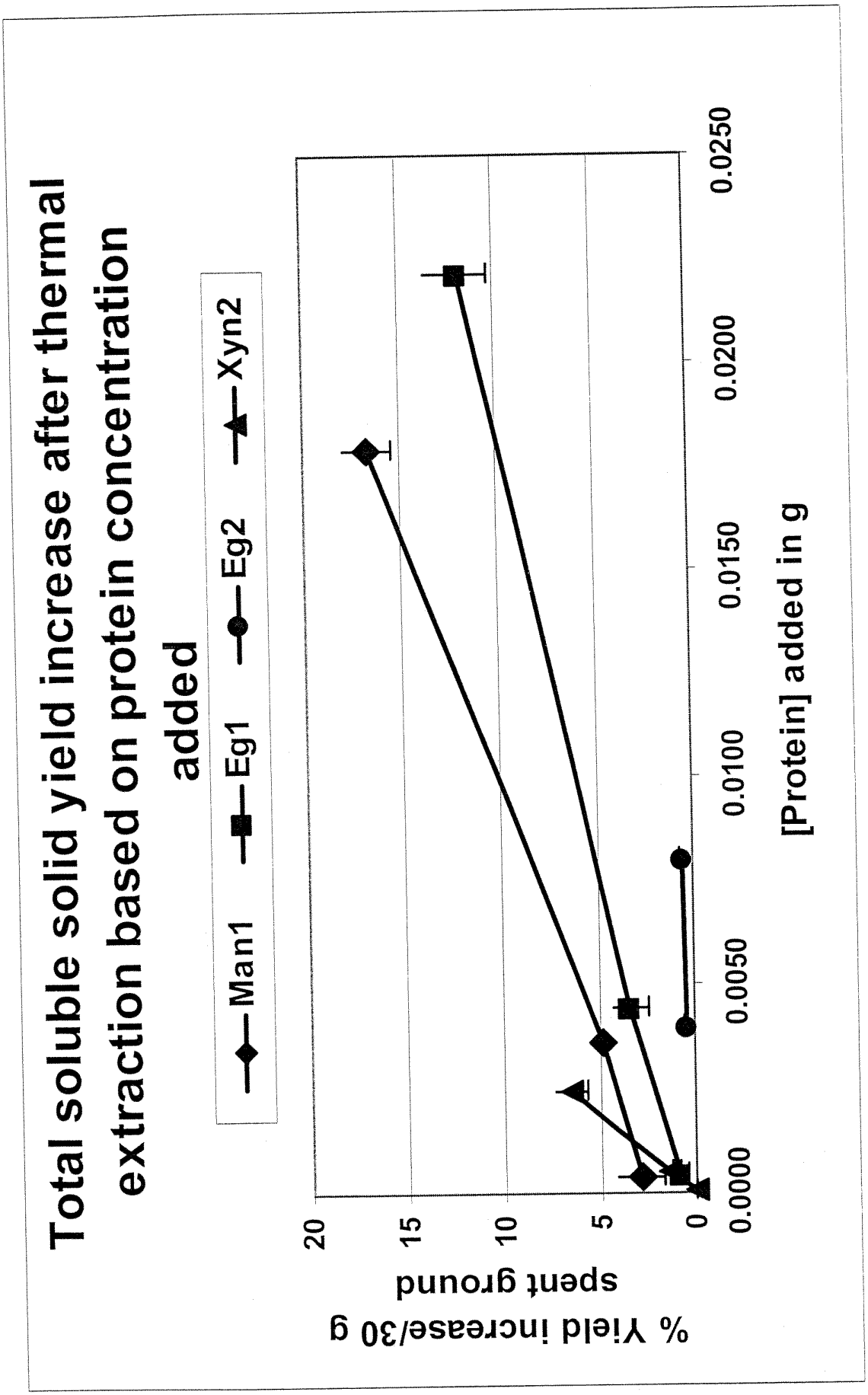


Fig. 3

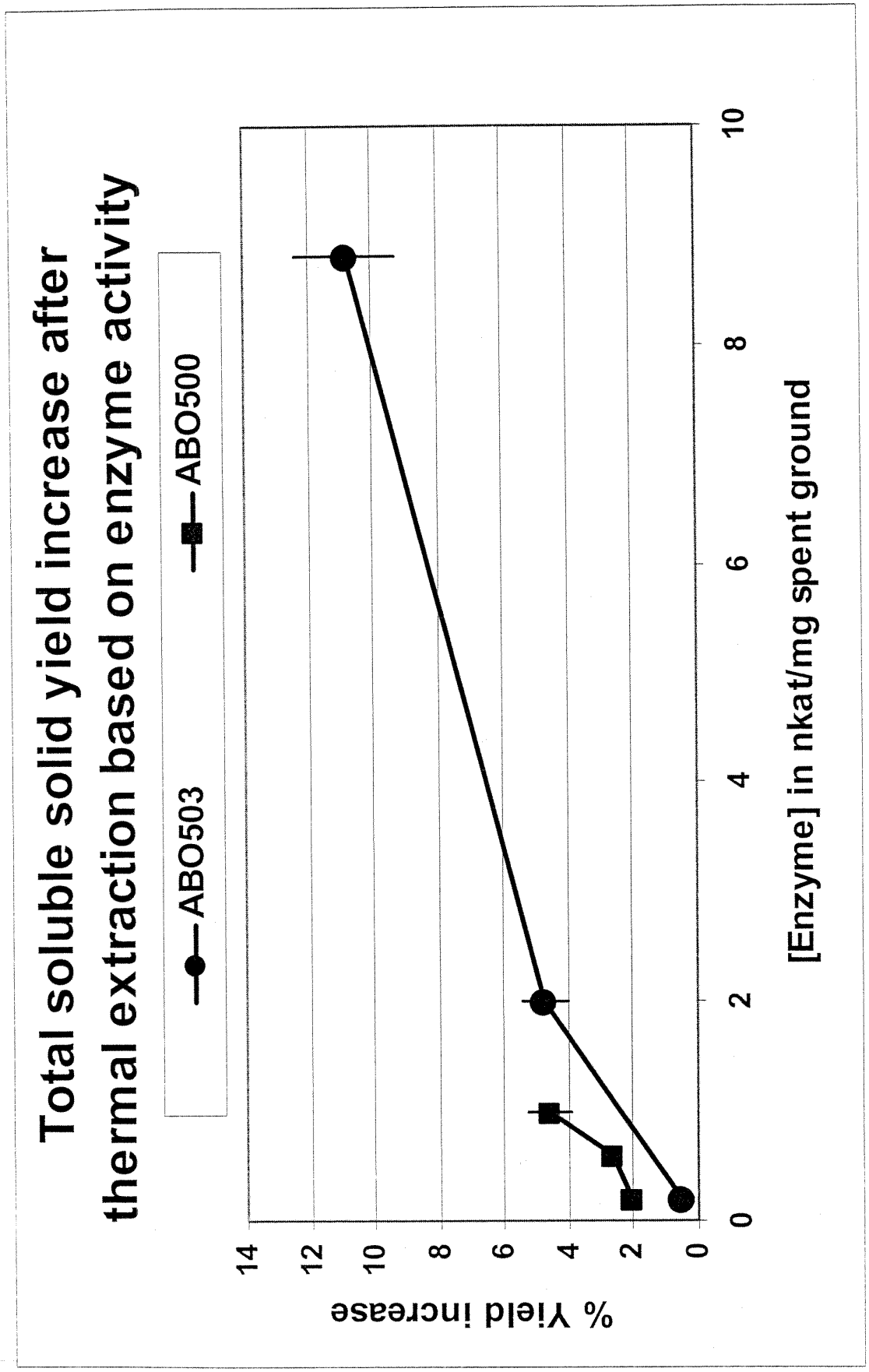


Fig. 4

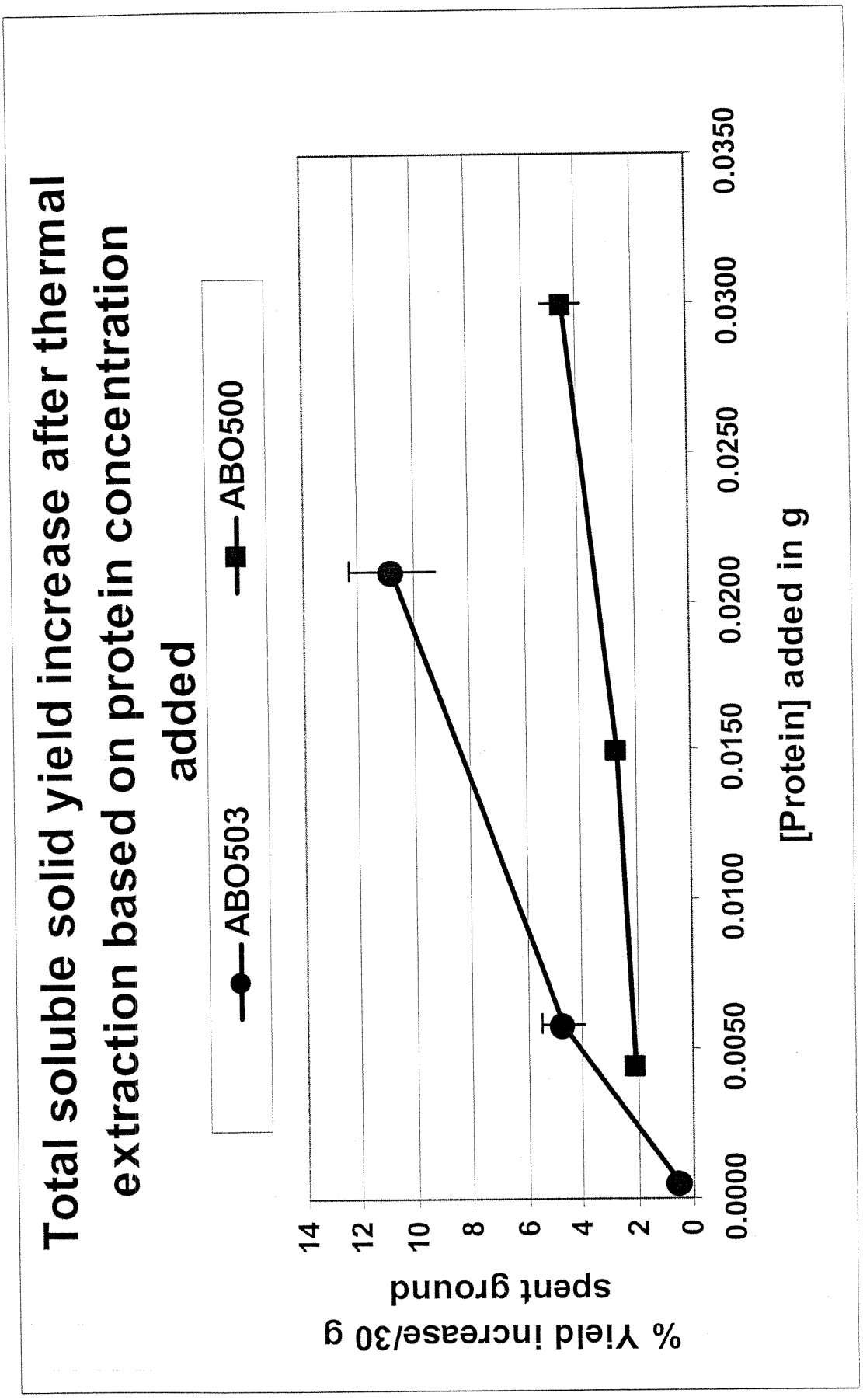
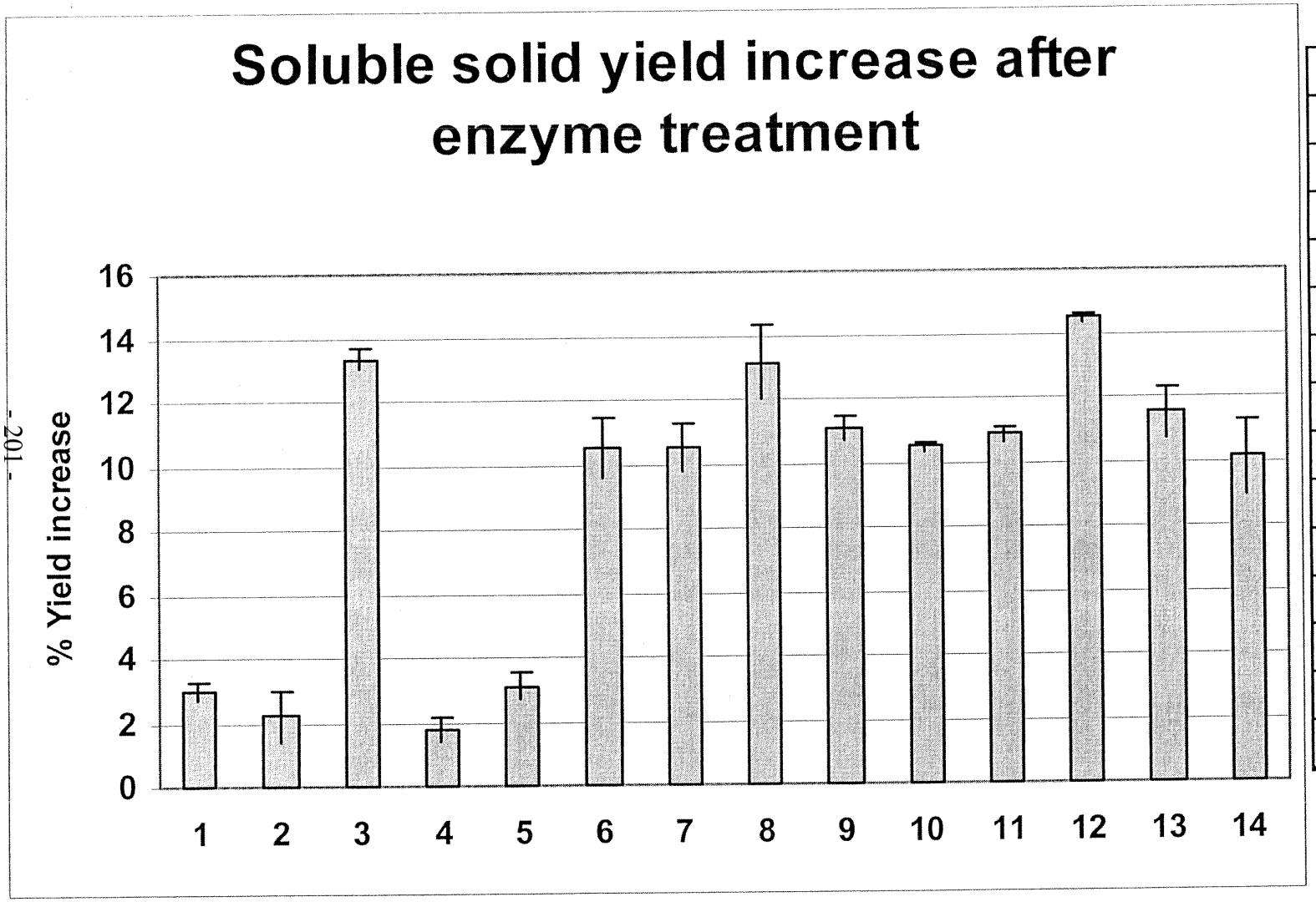
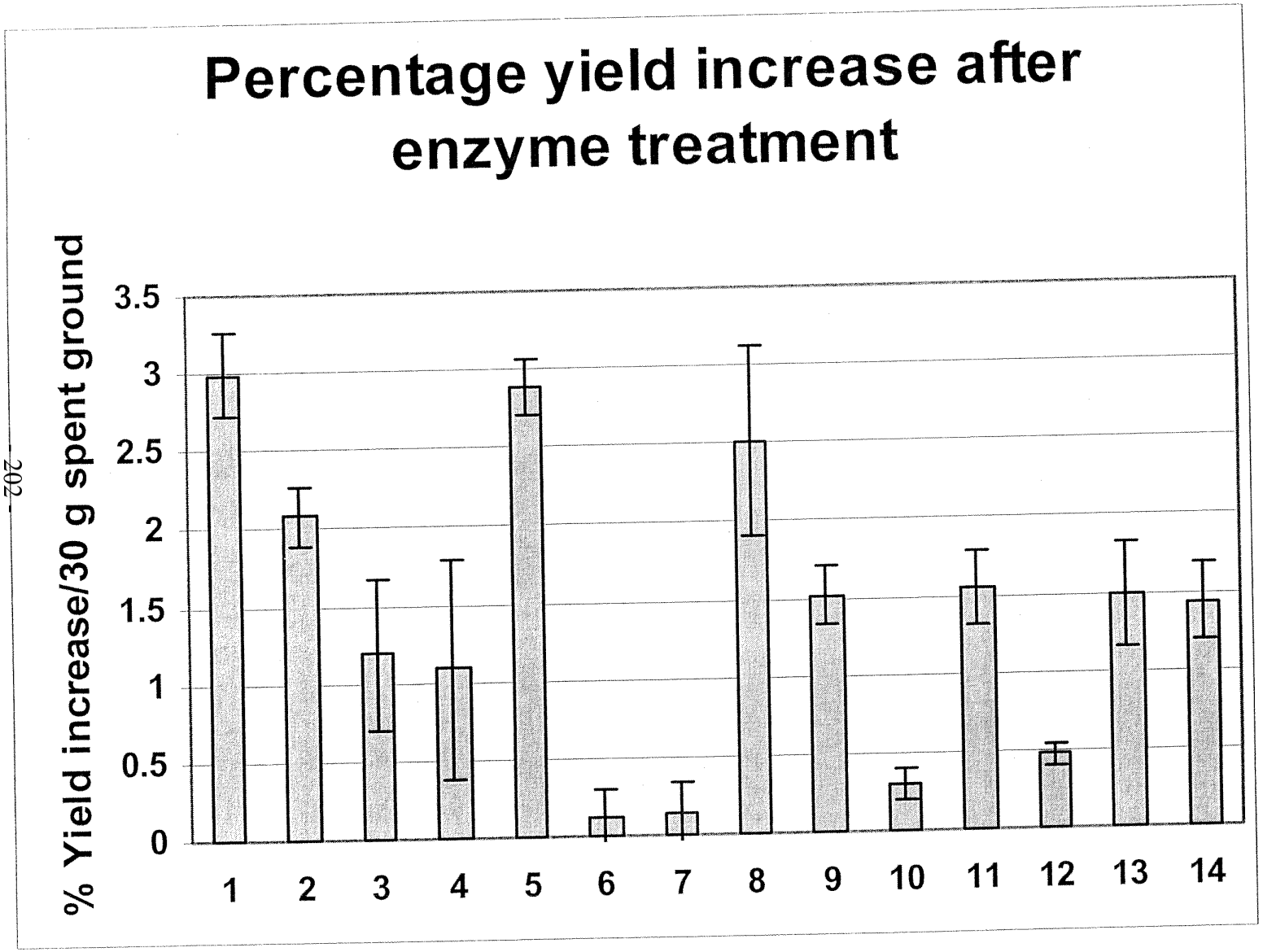


Fig. 5



No	Enzyme
1	ManI
2	Cellulosin
3	Pectinex
4	Gamanase
5	TP668L
6	D040L
7	D112L
8	M263L
9	D670L
10	G015L
11	C013L
12	M282L
13	TP692L
14	D690L

Fig. 6



No	Enzyme
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8	M263L
9	D670L
10	G015L
11	C013L
12	M282L
13	TP692L
14	D690L

202

Fig. 7

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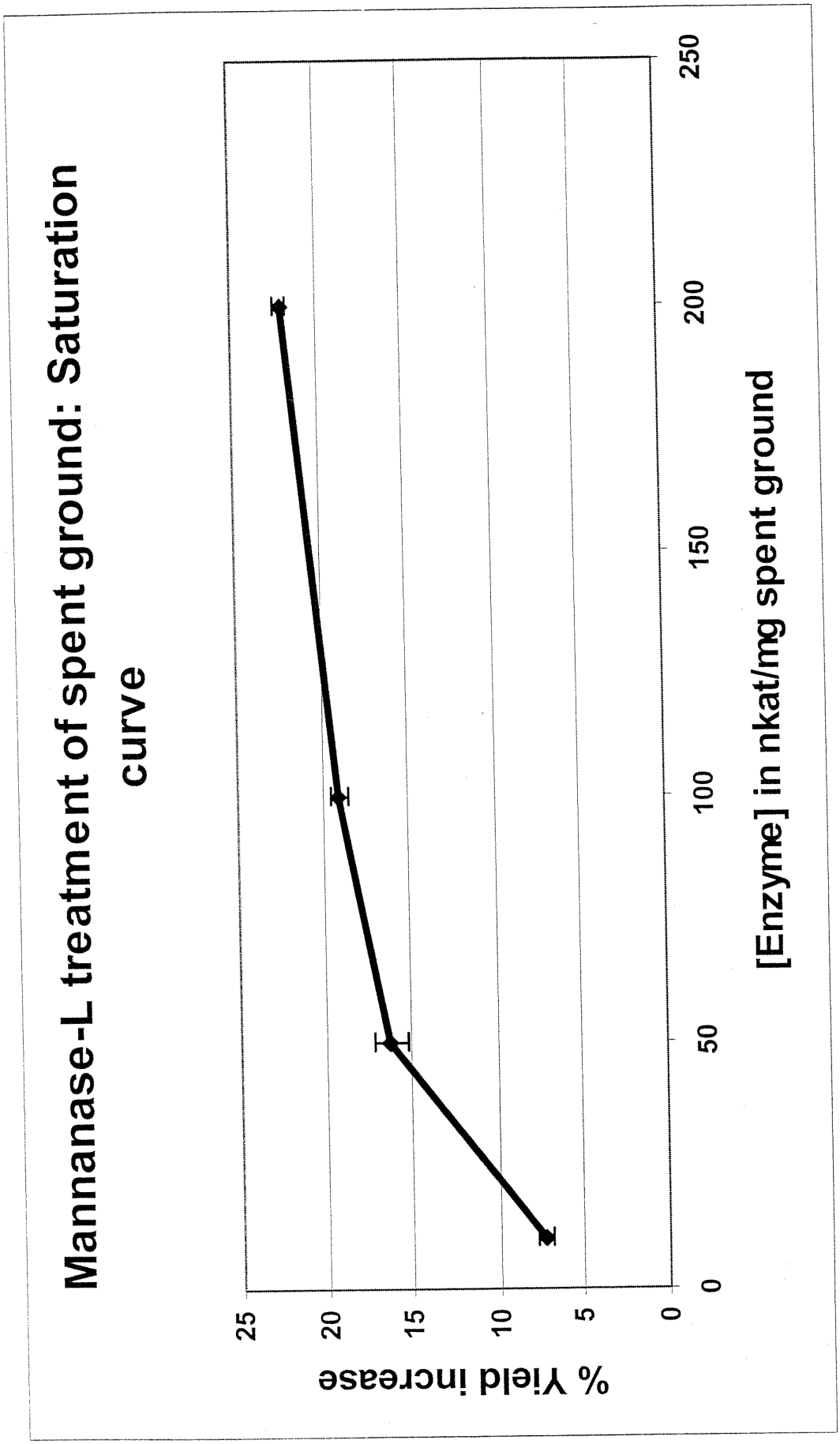


Fig. 8

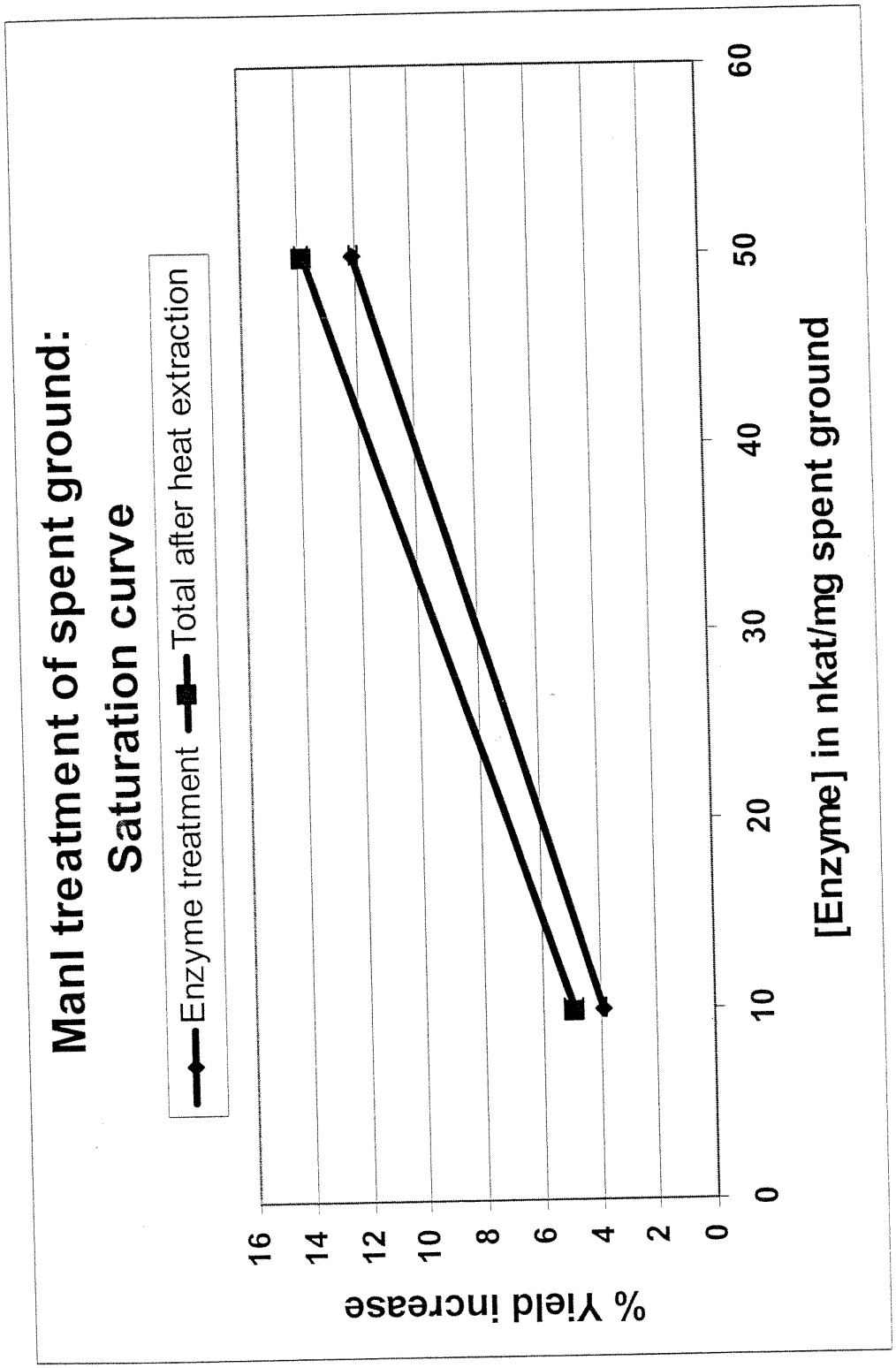
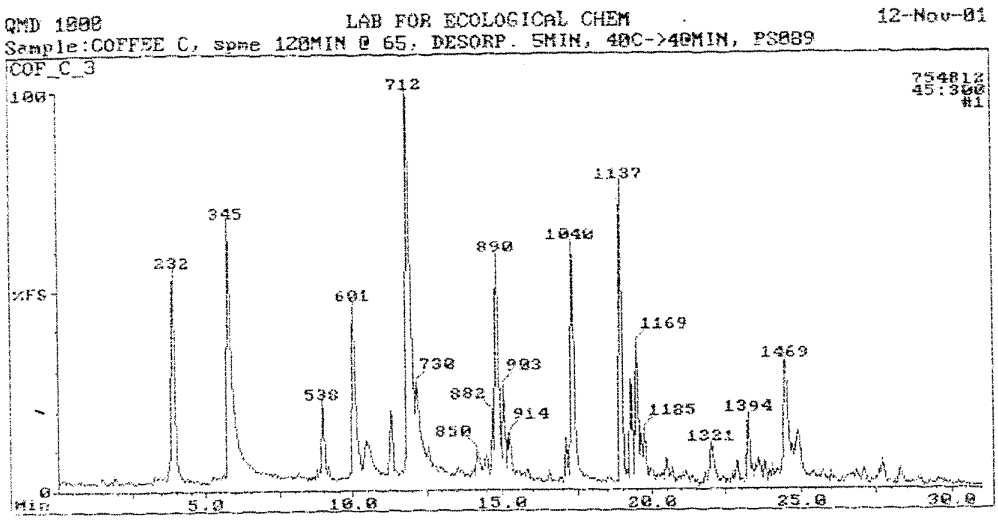
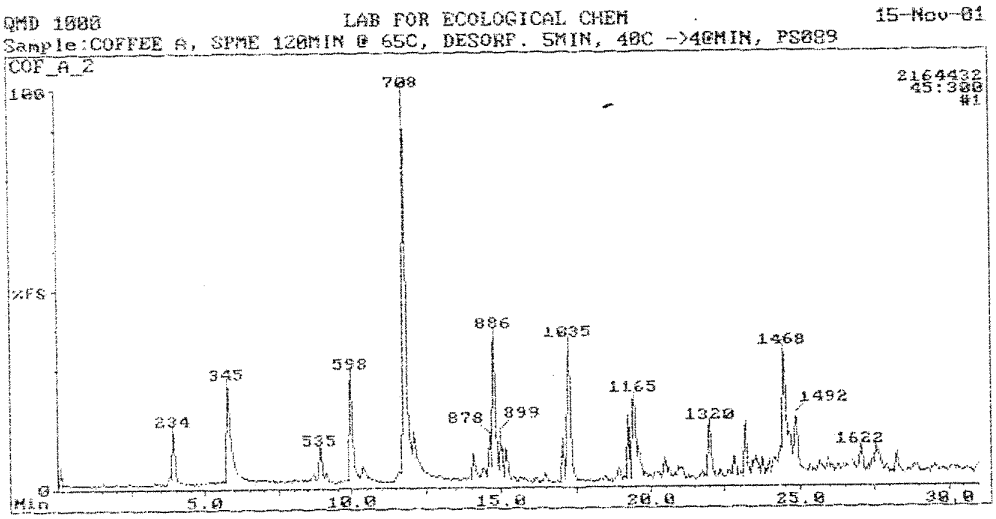


Fig. 9





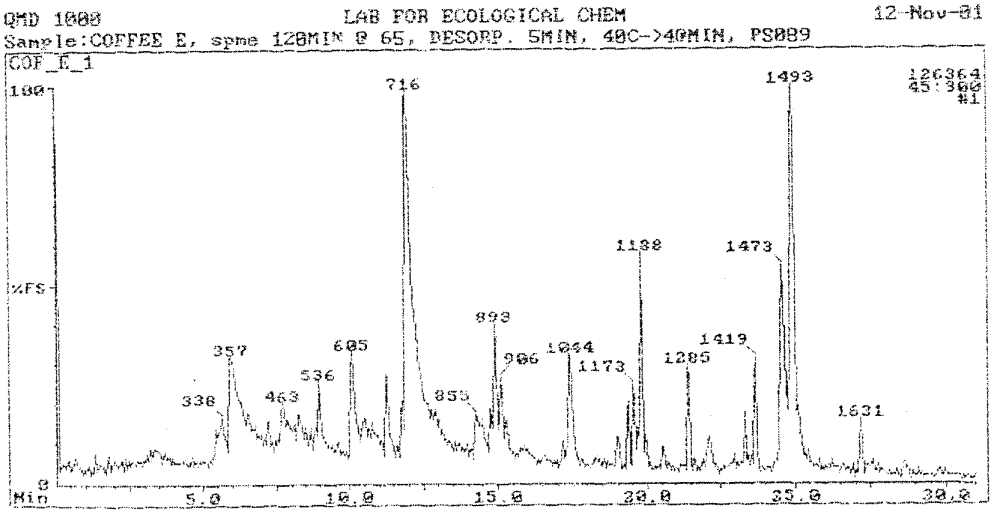


Fig. 10

```
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31                  41                  51                  60
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61                  71                  81                  90
TNNDDVDLVMSQLAASDLKILRVWGFNDVN

91                  101                 111                 120
TKPTDGTVWYQLHANGTSTINTGADGLQRL

121                 131                 141                 150
DYVVTSAEKYGVKLIINFVNEWTDYGGMQA

151                 161                 171                 180
YVTAYGAAAQTDFYTNTAIQAAYKNYIKAV

181                 191                 201                 210
VSRYSSSAAIFAWELANEPRCQGCDTSVLY

211                 221                 231                 240
NWISDTSKYIKSLDTKHLVTIGDEGFGLDV

241                 251                 261                 270
DSDGSYPYTYGEGLNFTKNLGISTIDFGTL

271                 281                 291                 300
HLYPDSWGTSYDWGNGWITAHAAACKAVGK

301                 311                 321                 330
PCLLEEYGVTSNHCAVESPWQQTAGNATGI

331                 341                 351                 360
SGDLYWQYGTTFSWGQSPNDGNTFYYNTSD

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Fig. 11

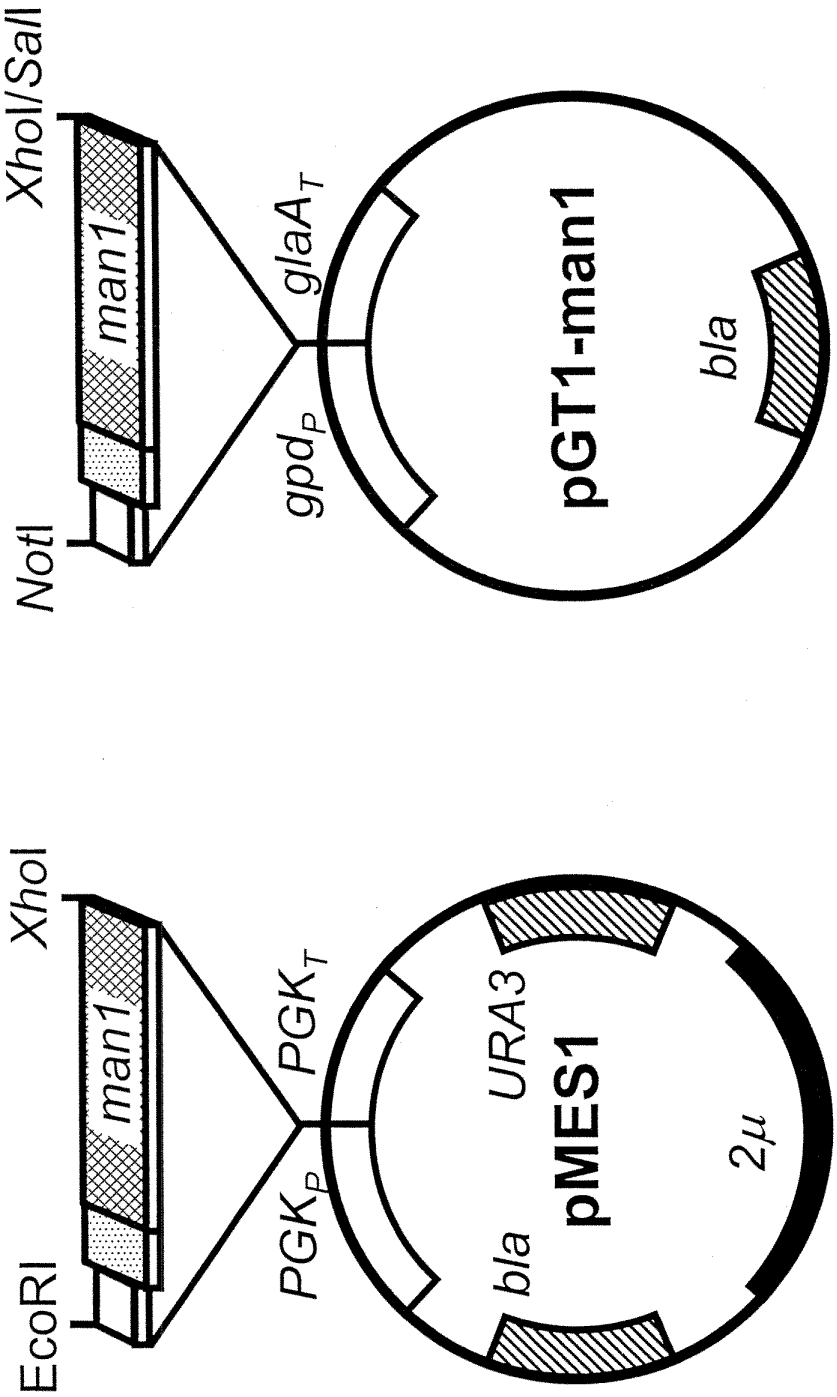

Fig. 12

ABSTRACT

The invention provides a method of transforming a yeast or fungus strain to produce one or more enzymes having hemicellulolytic, cellulolytic and/or pectolytic activities, and also provides for the use of these enzymes or the same or similar enzymes which have been produced by native yeast or fungus strains or which have been obtained commercially, in coffee production. Soluble coffee extracts, consisting of dissolved solids, are produced from roasted coffee beans, or leftovers thereof, by enzymatically hydrolysing the roasted coffee beans or leftovers in an extraction step. The method may be used to improve either the yield and/or the quality of soluble solids and/or volatile compounds obtained from the roasted ground beans or leftovers. An instant coffee product may be obtained by the method. Typical enzymes which are used in the method include β-mannanases (endo-1,4-β-mannanase) (Man1), endo-1,4-β-endoglucanase (β-endoglucanases) (Eg1), endo-1,4-β-endoglucanase (β-endoglucanases) (Eg2), endo-1,4-β-endoglucanase (β-endoglucanases) (Eg3), endo-1,4-β-xylanase (β-xylanases) (Xyn2), and/or exo-1,4-β-glucan cellobiohydrolase (β-cellobiohydrolases) (Cbh1-4).