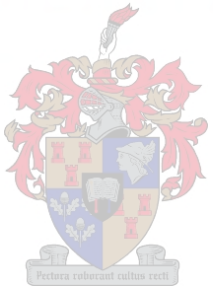
Construction of recombinant *Saccharomyces cerevisiae* strains for starch utilisation

by

Jeremy Michael Eksteen



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DECLARATION

I, the undersigned, hereby declare that the work contained 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.

Jeremy M Eksteen

SUMMARY

Starch-containing agricultural crops are widely available as feedstocks for the production of fuel ethanol, potable spirits or beer, single-cell protein (animal feed) and high-fructose corn syrups (sweeteners). Starch-rich crops, such as maize, rye, barley and wheat, are usually used for the production of whisky. One of the first steps in the production of whisky is to boil the raw starch at temperatures exceeding 100°C. This gelatinisation step is performed to disrupt and solubilise the starch granules to make them more accessible for enzymatic hydrolysis. After this cooking process, the starch is liquefied by α-amylase and then saccharified by glucoamylase and a debranching enzyme.

Lipomyces kononenkoae and *Saccharomycopsis fibuligera* secrete highly effective α-amylases and glucoamylases, making them two of the most efficient raw-starch-degrading yeasts known. However, *L. kononenkoae* and *S. fibuligera* cannot be used in existing industrial fermentations because of their low ethanol tolerance, slow growth rate, catabolite repression, poorly characterised genetics and lack of GRAS (**G**enerally **R**egarded **A**s **S**afe) status.

This study is divided into two sections. The aim of the first section was to clone a gene (*LKA2*) encoding a novel starch-degrading enzyme, a second α-amylase (Lka2p) from *L. kononenkoae*. *LKA2* was cloned into a multicopy plasmid, the yeast episomal plasmid, YEp352, under the control of the phosphoglycerate kinase promoter ($PGK1_P$) and terminator ($PGK1_T$) expression cassette. This recombinant plasmid was designated pJUL3 and transformed into a laboratory strain of *S. cerevisiae*, ∑1278b. Plate and liquid assays revealed that the recombinant yeast secreted active α-amylase into the medium. The optimum pH for Lka2p was pH 3.5 and the optimum temperature 60°C.

The aim of the second part of the study was to construct recombinant strains of *S. cerevisiae* secreting α-amylase and/or glucoamylase. The individual genes were cloned into a yeast-integrating plasmid, YIp5, under the control of the $PGK1_P$-$PGK1_T$-expression cassette. Two indigenous yeasts were selected on the basis of their ability to utilise raw starch, *L. kononenkoae* and *S. fibuligera*, as gene donors. Eight constructs containing the *L. kononenkoae* α-amylase genes, *LKA1* and *LKA2*, and the *S. fibuligera* α-amylase (*SFA1*) and glucoamylase (*SFG1*) genes were prepared: four single-cassette plasmids expressing the individual coding sequences under the control of the $PGK1_P$-$PGK1_T$-expression cassette, resulting in pIPLKA1, pIPLKA2, pIPSFA1 and pIPSFG1, respectively; two double-cassette plasmids (expressing both *LKA1* and *LKA2* under the control of the $PGK1_P$-$PGK1_T$-expression cassette, and *SFA1* and *SFG1* under their respective native promoters and terminators), resulting in pIPLKA1/2 and pIPSFAG, respectively, and two single-cassette plasmids expressing *SFA1* and *SFG1* with their native promoters and

terminators, resulting in pSFA1 and pSFG1, respectively. The respective constructs were transformed into a laboratory strain of *S. cerevisiae*, Σ1278b. By homologous recombination, each plasmid was integrated into the yeast genome at the *ura3* locus. *S. cerevisiae* ∑1278b that had been transformed with pIPLKA1/2, *LKA1* and *LKA2* under the control of the $PGK1_P$-$PGK1_T$-expression cassette resulted in the highest levels of α-amylase activity when assayed for amylolytic activity in a liquid medium. This recombinant strain resulted in the most efficient starch utilisation in batch fermentations, consuming 80% of starch and producing 6 g/L of ethanol after 156 hours of fermentation. The strain expressing *SFG1* under the control of the $PGK1_P$-$PGK1_T$-expression cassette gave the highest levels of glucoamylase activity. These results confirmed that co-expression of α-amylase and/or glucoamylase synergistically enhance starch degradation.

This study paves the way for the development of efficient starch-degrading strains of *S. cerevisiae* for the production of whisky, beer and biofuel ethanol.

OPSOMMING

Styselbevattende landbougewasse kom wydverspreid voor as die substraat vir die produksie van brandstofetanol, drinkbare spiritualië of bier, enkelselproteïen en hoë-fruktose graanstroop. Styselbevattende gewasse, soos mielies, rog, gars en koring, word gewoonlik vir die produksie van whisky gebruik. Die eerste stap in die produksie van whisky is om die stysel by temperature bo 100°C te kook. Hierdie jelatinisasie stap word uitgevoer om die styselkorrels te versteur en vloeibaar te maak sodat hulle meer toeganklik vir ensimatiese hidrolise is. Na dié kookproses word die stysel deur α-amilases vervloei en dan deur glukoamilases en 'n vertakkingsensiem versuiker.

Lipomyces kononenkoae en *Saccharomycopsis filuligera* skei hoogs effektiewe α-amilases en glukoamilases uit, wat dit twee van die effektiefste rou-stysel-afbrekende giste bekend, maak. *L. kononenkoae* en *S. fibuligera* kan egter nie in reeds bestaande industriële fermentasies gebruik word nie, as gevolg van hulle lae etanoltoleransie, stadige groeitempo, katabolietonderdrukking, swak gekarakteriseerde genetika en gebrek aan ABAV (**A**lgemeen **B**eskou **A**s **V**eilig) status.

Hierdie tesis is in twee afdelings verdeel. Die doel van die eerste deel was om 'n geen (*LKA2*) wat vir 'n nuwe, unieke styselafbrekende ensiem kodeer, te kloneer, 'n tweede α-amilase (Lka2p) van *L. kononenkoae*. *LKA2* is in 'n multikopie plasmied, die gis episomale plasmied, YEp352, onder beheer van die fosfogliseraatkinasepromotor- en termineerder-kasset ($PGK1_P$-$PGK1_T$), gekloneer. Hierdie rekombinante plasmied is pJUL3 genoem en in 'n laboratoriumras van *Saccharomyces cerevisiae*, Σ1278b, getransformeer. Plaat- en vloeibare-ensiem toetse het getoon dat die rekombinante gis aktiewe α-amilase in die medium uitskei. Die optimum pH vir Lka2p is 3.5 is en die optimum temperatuur 60°C.

Die doel van die tweede deel van die studie was om rekombinante rasse van *S. cerevisiae* te konstrueer wat α-amilases en/of glukoamilases uitskei. Die individuele gene is toe in 'n gis-integreringsplasmied, YIp5, onder beheer van die $PGK1_P$-$PGK1_T$-ekspressiekasset, gekloneer. Twee inheemse giste is op grond van hulle vermoë om stysel te benut geselekteer, *L. kononenkoae* en *S. filuligera*, as geen donors. Agt konstrukte bevattende die *L. kononenkoae* se α-amilasegene, *LKA1* en *LKA2*, en *S. filuligera* se α-amilasegeen (*SFA1*) en glukoamilasegeen (*SFG1*), moes gekonstrueer word: vier enkel-kasset plasmiede wat die individuele koderende sekwense onder beheer van die $PGK1_P$-$PGK1_T$-ekspressiekasset uitdruk, wat onderskeidelik pIPLKA1, pIPLKA2, pIPSFA1 en pIPSFG1 lewer; twee dubbel-kasset plasmiede (wat beide *LKA1* en *LKA2* onder beheer van die $PGK1_P$-$PGK1_T$-ekspressiekasset, en *SFA1* en *SFG1* met hulle onderskeie inheemse

promotors en termineerders) uitdruk, wat onderskeidelik pIPLKA1/2 en pIPSFAG lewer, en twee enkel-kasset plasmiede wat *SFA1* and *SFG1* met hul onderskeie inheemse promotors en termineerders, en wat onderskeidelik pSFA1 en pSFG1 lewer. Die onderskeie konstrukte is in 'n laboratoriumras van *S. cerevisiae*, Σ1278b, getransformeer. Deur middel van homoloë rekombinasie, is die onderskeie plasmiede in die *ura3*-lokus van die gisgenoom geïntegreer. *S. cerevisiae* Σ1278b, getransformeer met pIPLKA1/2, *LKA1* en *LKA2* onder die beheer van die $PGK1_P$-$PGK1_T$-ekspressiekasset, het die hoogste vlakke van α-amilase aktiwiteit gelewer toe dit vir amilolitiese aktiwiteit in vloeibare medium getoets is. Hierdie rekombinante ras het stysel die effektiefste benut, nl. 80% van die stysel en 'n opbrengs van 6 g/L etanol na 156 ure in lotfermentasies. Die ras wat *SFG1* onder beheer van die $PGK1_P$-$PGK1_T$-ekspressiekasset uitdruk, het die hoogste vlakke van glukoamilase-aktiwiteit gelewer. Hierdie resultate bevestig dat die gesamentlike uitdrukking van α-amilase- en/of glukoamilase-ensieme styselafbreking sinergisties bevorder.

Hierdie studie baan die weg vir die ontwikkeling van 'n effektiewe styselfermenterende ras van *S. cerevisiae* wat moontlik gebruik kan word vir die produksie van whisky en biobrandstofalkohol.

This thesis is dedicated to my family and Ruwaida Gelderbloem.
Hierdie tesis is opgedra aan my familie en Ruwaida Gelderbloem.

BIOGRAPHICAL SKETCH

Jeremy Eksteen was born in George, South Africa on the 4th of July 1976. He attended George Primary School and matriculated at George High School, George in 1994. Jeremy enrolled at Stellenbosch University in 1995 and obtained a BSc degree, majoring in Microbiology and Genetics, in 1997. In 1998 he completed a HonsBSc degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University.

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PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately. Chapter 3 is written according to the style of the journal *Yeast* and has been accepted for publication in this journal. Chapter 4 is written according to the style of the journal *Applied and Environmental Microbiology*, to which Chapter 4 is submitted for publication.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Whisky production and amylolytic yeasts

Chapter 3 **Research results**
Cloning and characterisation of a cDNA and genomic copy of a second α-amylase gene (*LKA2*) from *Lipomyces kononenkoae* IGC4052B and its expression in *Saccharomyces cerevisiae*

Chapter 4 **Research results**
The evaluation and comparison of recombinant *Saccharomyces cerevisiae* strains expressing α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*

Chapter 5 **General discussion and conclusions**

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

After cellulose, starch is the most widely distributed naturally occurring organic compound. The main sources of starchy material are higher plants, in which starch may account for 20 to 70% of the weight (Solomon, 1978). Starch is produced commercially from the seeds, tubers and roots of plants. Starch-containing agricultural crops are widely available as feedstocks for the production of fuel ethanol, potable spirits or beer, single-cell protein and high-fructose corn syrups. The conversion of starch, consisting of linear (amylose) and branched glucose polymers (amylopectin) (Nigam and Singh, 1995), to commercially important commodities by *Saccharomyces cerevisiae* employs a three-step process: (i) liquefaction by α-amylase, (ii) saccharification by glucoamylases and debranching enzymes, and (iii) fermentation of the sugars (De Mot *et al.*, 1985; Kim *et al.*, 1988). A one-step starch fermentation process would obviously be advantageous. Therefore, considerable attention is being paid to the evaluation and construction of amylolytic yeasts (Tubb, 1986).

Grain whisky is usually prepared from a starch-rich crop, such as maize (Piggott and Conner, 1995). One of the first steps in the production of whisky is to boil the starch at temperatures exceeding 100°C. The aim of this expensive, energy-intensive gelatinisation step is to make the starch more accessible for enzymatic hydrolysis. Thereafter, amylolytic enzyme preparations are added to the starch to break it down to fermentable sugars. *S. cerevisiae* then converts these sugars to alcohol and CO_2 (Korhola *et al.*, 1989), after which distillation takes place. Both the boiling process and the addition of enzymes are expensive and make a considerable contribution to the cost of the final product.

S. cerevisiae has a fast growth rate, a high ethanol tolerance, is an efficient ethanol producer, consist of 48% high quality protein and has been associated with food and beverage production for centuries (De Mot *et al.*, 1985). Furthermore, *S. cerevisiae* has become one of the premier organisms for basic research and the ideal experimental model for unravelling molecular mechanisms, such as gene regulation and secretion. However, *S. cerevisiae* is unable to utilise starch.

Several yeasts, such as *Lipomyces* spp. (Kelly *et al.*, 1985; Spencer-Martins and Van Uden, 1979; Steyn *et al.*, 1995), *Saccharomycopsis* spp. (Gašperík *et al.*, 1991) and *Schwanniomyces* spp. (Abarca *et al.*, 1991; Claros *et al.*, 1993; Dohmen *et al.*, 1989; Dohmen *et al.*, 1990; Strasser *et al.*, 1989; Yáñez *et al.*, 1998), secrete amylolytic

enzymes that are capable of degrading starch. Several studies have been done on the cloning and characterisation of amylolytic enzymes, e.g. α-amylases, glucoamylases and debranching enzymes, produced by these organisms (McCann and Barnett, 1986). These amylolytic yeasts, however, do not have the so-called GRAS (**G**enerally **R**egarded **A**s **S**afe) status, and thus cannot be used in the fermentation process. A logical step would be to introduce genes encoding amylolytic enzymes into laboratory and industrial strains of *S. cerevisiae*. For this reason, heterologous amylase genes derived from various organisms have been expressed in *S. cerevisiae*.

1.2 PROJECT AIMS

The present study forms an integral part of a research programme designed to broaden the substrate range of *S. cerevisiae*. α-Amylases and/or glucoamylases from the indigenous species, *Lipomyces* and *Saccharomycopsis*, have been cloned and expressed in *S. cerevisiae* previously. Here we express and compare these genes under the control of the phosphoglycerate kinase 1 promoter and terminator when cloned into yeast-integrating plasmids and transformed into *S. cerevisiae*.

The specific aims and approaches of this study were the following:

(i) the cloning and characterisation of a second α-amylase gene, (*LKA2*) from *L. kononenkoae*;

(ii) the expression of multiple copies of *LKA2* in *S. cerevisiae* and determination of the substrate range of the secreted enzyme;

(iii) the cloning of the *L. kononenkoae* α-amylase genes (*LKA1* and *LKA2*) and the *S. fibuligera* α-amylase (*SFA1*) and glucoamylase (*SFG1*) genes under the control of an expression cassette consisting of the yeast phosphoglycerate kinase gene promoter ($PGK1_P$) and terminator ($PGK1_T$);

(iv) the insertion of the $PGK1_p$-*LKA1*-$PGK1_T$, $PGK1_p$-*LKA2*-$PGK1_T$, $PGK1_p$-*SFA1*-$PGK1_T$ and $PGK1_p$-*SFG1*-$PGK1_T$ gene cassettes and the *SFA1* and *SFG1* genes under the control of their native promoter and terminator sequences (separately and jointly) into a set of yeast-integrating plasmids;

(v) the transformation of the full set of the constructed yeast-integrating plasmids into a laboratory strain of *S. cerevisiae* and the selection of amylolytic *S. cerevisiae* transformants;

(vi) the quantification of the amylolytic activities secreted by the *S. cerevisiae* transformants, as well as of starch utilisation and hydrolysis;

(vii) the set-up of small-scale fermentations to determine the production of ethanol produced by the recombinant *S. cerevisiae* strains.

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CHAPTER 2

LITERATURE REVIEW

Whisky production and amylolytic yeasts

LITERATURE REVIEW: WHISKY PRODUCTION AND AMYLOLYTIC YEASTS

2.1 WHISKY PRODUCTION

Whiskies are distilled alcoholic beverages, which are prepared from fermented cereals and normally matured in oak barrels. There are many possible ways of producing whiskies within the limitations set by the materials and processes available, and details vary depending on the customs and regulations in the producing countries.

Many fermentable materials that can be used to produce alcoholic beverages are found around the world, such as corn (maize), rye, barley, wheat, sugar, molasses, honey, fruit, oats and potatoes, to name but a few. Corn (maize), rye, barley and wheat are the major cereals used for whisky production (Bronsky and Schumann, 1989). These grains, which are all from the family of grasses (Graminaceae), have the prerequisite characteristic for alcohol production, namely a high concentration of starch. The composition of these four grains is shown in Table 1. Grains for the distilling industry are obtained either directly from a supplier (which could be a farmer or a grain company) or, in the case of some large distilleries, through their own grain-handling facilities. In either case, it is the responsibility of the supplier and receiver to monitor incoming materials carefully, so that defects in the grain do not enter the grain supply system to contaminate the existing grain and affect the outcome of the distillate (Bronsky and Schumann, 1989).

For the production of Scotch whisky, the processing of cereals is controlled by legal requirements, which state that it may only be produced from water and malted barley (to which only whole cereal grain may be added). All materials for the production of whisky should be produced into a mash in a distillery, converted by endogenous enzyme systems, fermented only by the addition of yeast and then distilled to below an alcoholic (ethanol) strength by volume of less than 94.8% so that the distillate has an aroma and taste derived from the materials used (Palmer, 1998).

2.2 MATERIALS USED FOR THE PRODUCTION OF WHISKY

Corn (*Zea mays*), with its high starch content, is normally the cheapest cereal grain available in the USA, due to the size of the crop and its availability. Corn is also the predominant grain used for whisky production in the USA (Bronsky and Schumann, 1989). In Scotland, it was the prime cereal grain used for Scotch grain whisky. However it has

been displaced partially by European wheat, owing to the price effects of EU agricultural policies (Piggott and Conner, 1995).

Table 1. Composition of the major cereals used for the production of whiskies (Bronsky and Schumann, 1989).

	Composition (% of total)			
	Corn	Rye	Barley	Wheat
Endosperm	82	87	84	85
Germ	12	3	3	3
Bran	6	10	13	12
Chemical composition (dry basis)				
Nitrogen-free extract	69.2	70.9	66.6	69.9
Starch	72	68	63-65	69
Sugars	2.6	0	2-3	0
Protein	8	12.6	12	13.2
Soluble N (% of total)	4.7	0	11	0
Crude fibre	2	2.4	5.4	2.6
Fat	3.9	1.7	1.9	1.9
Ash	1.2	1.1	2	1.9

Rye (*Secale montanum*) is used in whiskies for its flavour contribution, since it contains less starch than corn and wheat (Piggott and Conner, 1995) (**Table 1**). Rye malt is sometimes used alone or in addition to other grain to improve a special flavour and aroma, normally spicy, in whisky. When rye is the predominant grain in the mash, it commonly causes foaming problems. The amount of α-amylase in rye malt is lower than in distiller's barley malt (Bronsky and Schumann, 1989)

Barley (*Hordeum polystichum*) is used predominantly for malting and the resultant important enzyme content of malted barley, but also for the flavour characteristics it provides to the spirit (Bronsky and Schumann, 1989; Piggott and Conner, 1995; Sim and Berry, 1996). It is the fourth most important cereal in the world in terms of production (12% of total cereal production) after wheat, rice and corn (Jadhav *et al.*, 1998). The enzyme content, irrespective of the starch content, is the major quality criterion. The starch content of barley is about the same as that of rye and less than that of corn (Bronsky and Schumann, 1989) (**Table 1**). For distilling, barley varieties are selected on the basis of diastatic power (DP, largely a measure of β-amylase), α-amylase and fermentable extract (Piggott and Conner, 1995).

Wheat (*Triticum vulgare*) is a major EU, USA and CIS crop, with a total production being approximately equal to that of maize (Bronsky and Schumann, 1989). Wheat contains more or less the same amount of starch as other grains, but the alcohol yield is lower (Piggott and Conner, 1995).

2.2.1 FOOD RESERVES: SUBSTRATES FOR WHISKY PRODUCTION

The major substrates (**Table 1**) in malting barley are located in the starchy endosperm (Palmer, 1998) (**Figure 1**). In unmalted barley, the endosperm contains thousands of intact cells, such as those shown in **Figure 2**. Each cell has a wall, which encloses large and small starch granules embedded in a matrix of protein. The large starch granules (25 μm in diameter) constitute about 90% of the weight of the total starch of the grain and are therefore responsible for most of the ethanol produced during whisky production. The chemical composition of the large and small granules appears to be similar, with each containing about 25% amylose and 75% amylopectin. Despite this similar composition as regards amylose and amylopectin, the large granules gelatinise at 62°C, whereas the small starch granules gelatinise at 80°C. Unknown differences in physico-chemical properties are responsible for these differences in gelatinisation (Palmer, 1998).

2.3 PRODUCTION OF MALT

Malting is the treatment of a cereal grain (barley in Scotland, elsewhere on occasion rye or, very rarely, wheat or corn) to render the starches contained within its seed more soluble. Malt production is an expensive and a time-consuming process. Considerable work has been done with the aim of reducing malting times and losses without negative effects on the quality of the malt (Dolan, 1976; Dolan, 1981; Peterson, 1994). Grujić (1998) worked on the use of a commercial enzyme preparation "CELLUCLAST" (a multiactive carbohydrase for the degradation of cellulose, cellobiose and higher polymers of glucose) in the production of brewer's malt. Malt wort consists of maltose, maltotriose, glucose and dextrins (Klaassen *et al.*, 1996). These will subsequently be converted into sugars and then alcohol. The malting activates enzyme systems within the grain. The procedure is to steep the grain in water to awaken the embryo within the seed. The dampened grain is then allowed to germinate partially. This is halted by drying and slight cooking over hot air in a kiln. The kilning also develops the flavour and the colour of the malt. In Scotland, kilns were originally stoked with peat, which overlaid a smoky flavour. Although peat is no longer the principal fuel, a portion is usually burned in the kiln because of its contribution to the traditional Scotch whisky palate.

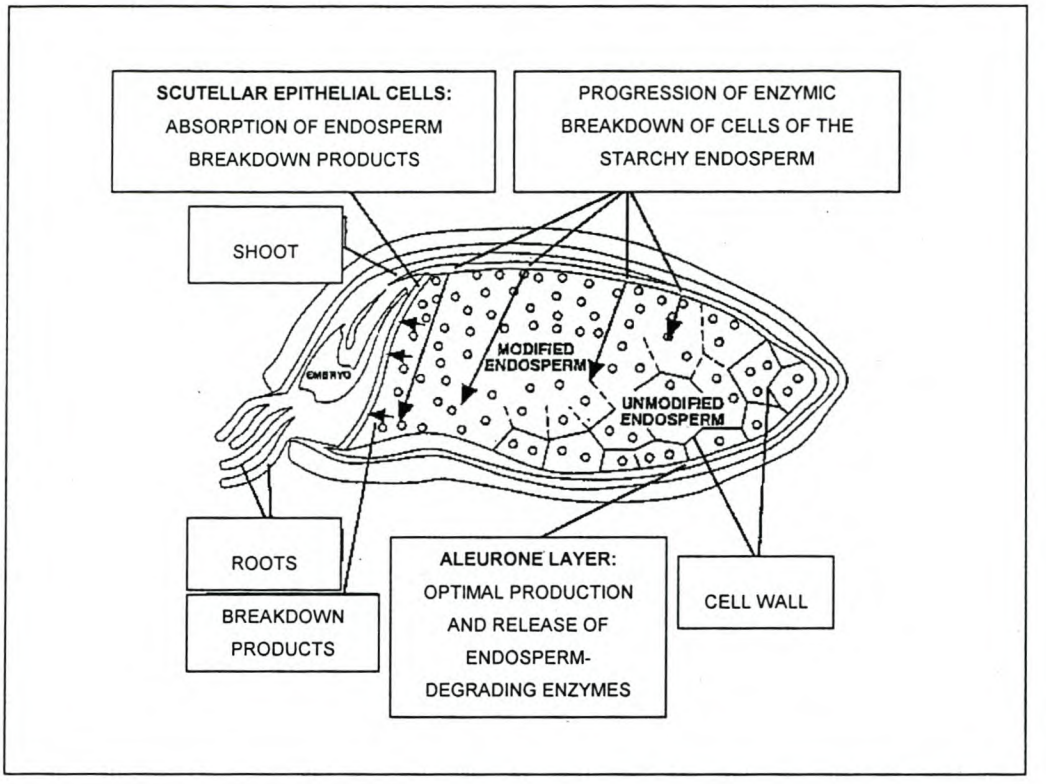

Figure 1. The enzymatic breakdown (modification) of the starchy endosperm of malting barley (Palmer, 1998).

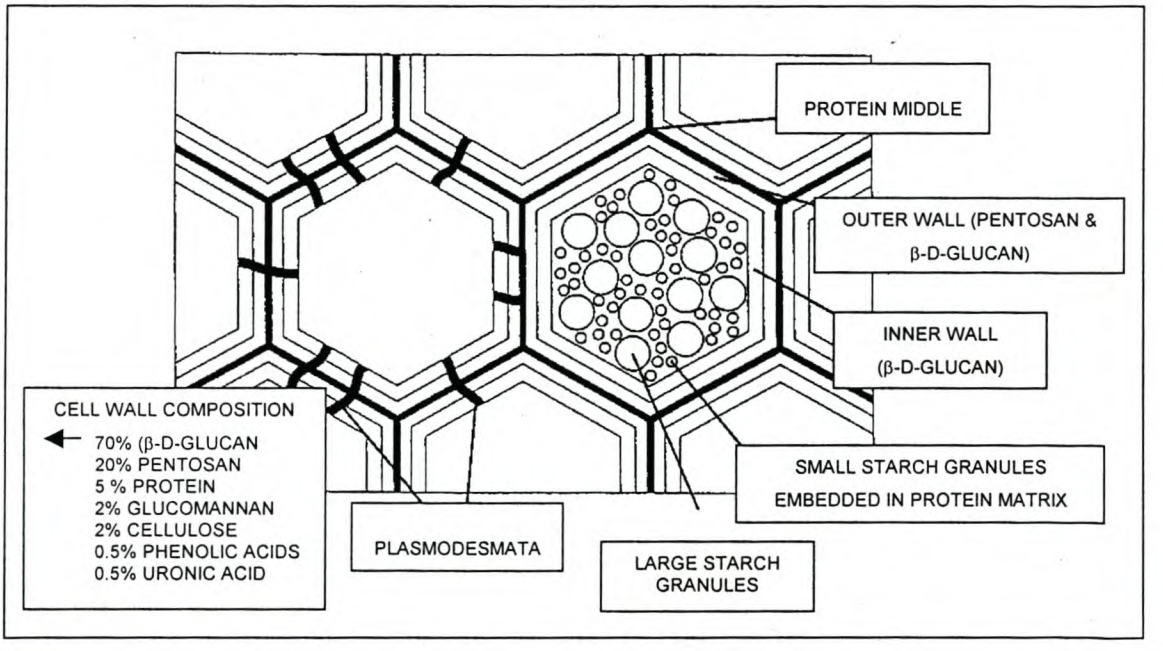

Figure 2. The structure of the cells of the starchy endosperm of barley (Palmer, 1998).

2.3.1 BIOCHEMICAL PRINCIPLES

The conversion of barley into malt is a biochemical process (**Figures 1** and **3**). The grain is hydrated and allowed to germinate. During the germination and subsequent stages of seedling growth, the embryo develops the plant hormone gibberellic acid (GA_3) (Palmer, 1998). The exogenous application of even very small amounts of gibberelic acid (<0.25 ppm) is not permitted as a malting aid in the production of malt. Therefore the natural potential of the germinated embryo to produce gibberelic acid and of the endosperm layer to respond to it by producing endosperm-degrading enzymes is critical. Dormancy caused by the genetics of the barley, low temperature storage (<8°C) of undried (>12% moisture) barleys, or low temperature steeping of ungerminated barley is likely to damage embryo vigour and the potential of the grain to malt optimally. Physical damage to the embryo (or aleurone layer) will also render grains unsuitable for malt production. This natural plant hormone is transported naturally to the aleurone layer, where it induces the production of a mixture of hydrolytic enzymes that are released into the starchy endosperm (**Figure 1**). These enzymes, together with activated enzymes already present in the starchy endosperm, break down or modify the starchy endosperm, so that its contents, previously hard as barley but now friable as malt, can be extracted in hot water and allowed to digest further in the mashing process (**Figure 4**) (Palmer, 1998).

Hydrolytic enzymes that are produced in the aleurone layer (**Figure 1** and **2**) are: endo-β-1,3-1,4-glucanase and pentosanases for breaking down cell wall β-glucans and pentosans, respectively, thereby exposing starch and proteins; α-amylase for breaking down starch granules; and endoproteases for breaking down matrix proteins. Phytase develops to hydrolyse phytic acid to phosphoric acid and inositol, and lipases are formed to break down lipids to glycerol and fatty acids. There is no evidence that phytase, lipase or endo-β-1,3-glucanase require stimulation by gibberellic acid to develop (Palmer, 1998).

Enzymes such as β-amylase and carboxypeptidase develop in the starchy endosperm, possibly as a result of reducing activity. They complement the hydrolytic activities of the enzymes formed in the aleurone layer. Carboxypeptidases continue the hydrolysis of solubilised proteins to amino acids, and β-amylase, which has no action during the germination process, will cause extensive production of maltose sugars during the extraction (mashing) stage of the distilling process (**Figure 4**). The proper functioning of the physiological, biochemical and chemical processes described ensures that the commercial production of distiller's malt is efficient. As is the case for brewing malts, the inadequate breakdown (modification) of the starchy endosperm of distilling malts will cause more processing problems than variations in enzyme levels. The *raison d`etre* of the entire malting process is the production of malted grain of which the hydrolysed endosperm contains sufficient quantities of the enzymes (i) to release all the starch and soluble proteins from the grains (**Figure 1**) without excessive milling and (ii) to hydrolyse

all the extracted starch (during mashing) into the special profile of sugars characteristic for whisky production. Unlike starch, where only 5-10% is broken down during the growth process, about 70% of the protein breakdown occurs during the extraction (mashing) stage. In commercial practice, the main role of the maltster is to ensure that sufficient enzyme hydrolysis has occurred during the growth (malting) process to ensure optimal starch and protein release into the mash tun (**Figure 4**), where the enzyme of the malted grain will hydrolyse the released starch and proteins into sugars and amino acids. Optimal levels of starch and amino acids (and small peptides) will result in the optimal production of ethanol and flavour compounds (i.e. whisky spirit) (Palmer, 1998).

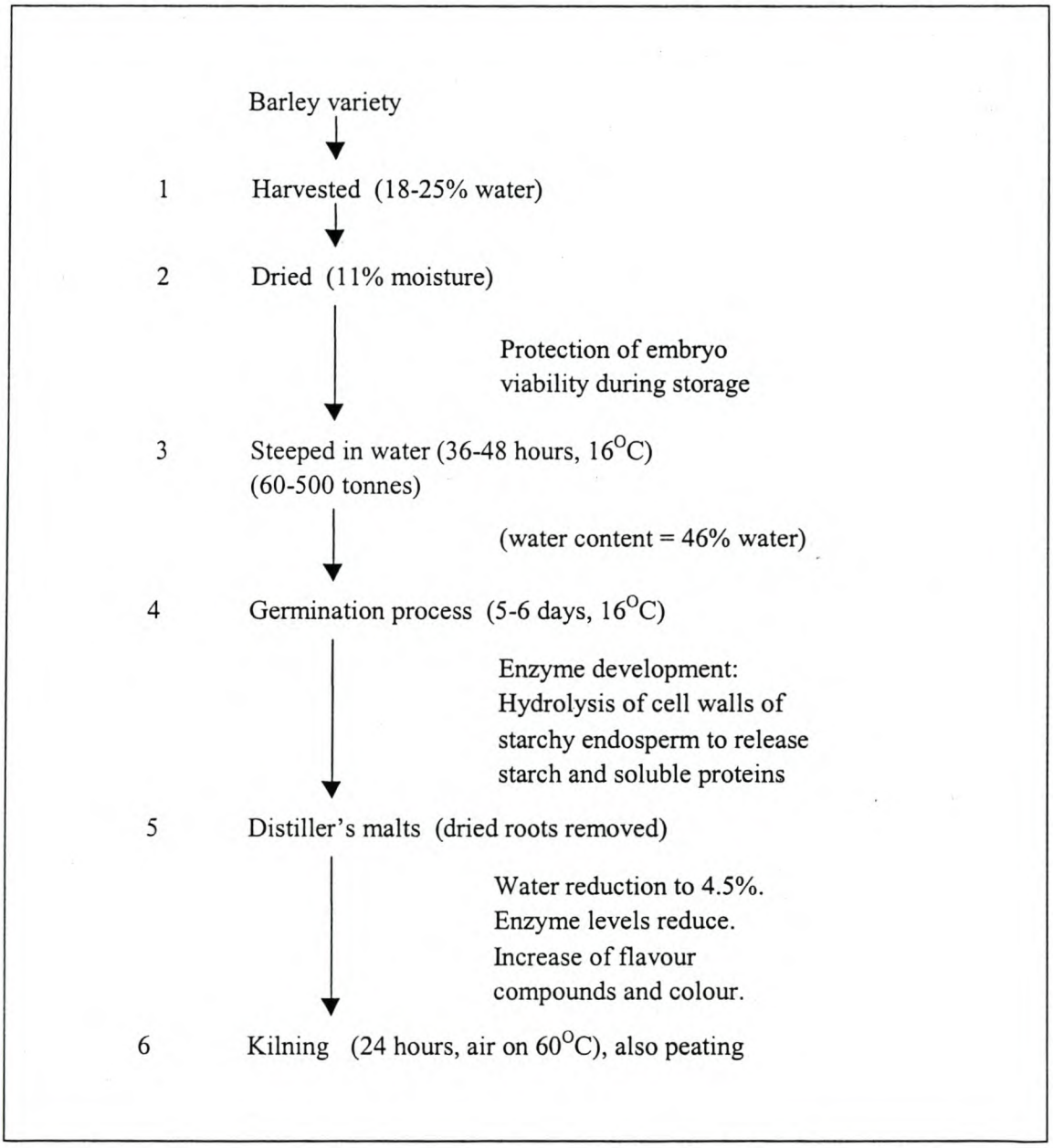

Figure 3. Schematic representation of the production of malt (Palmer, 1998).

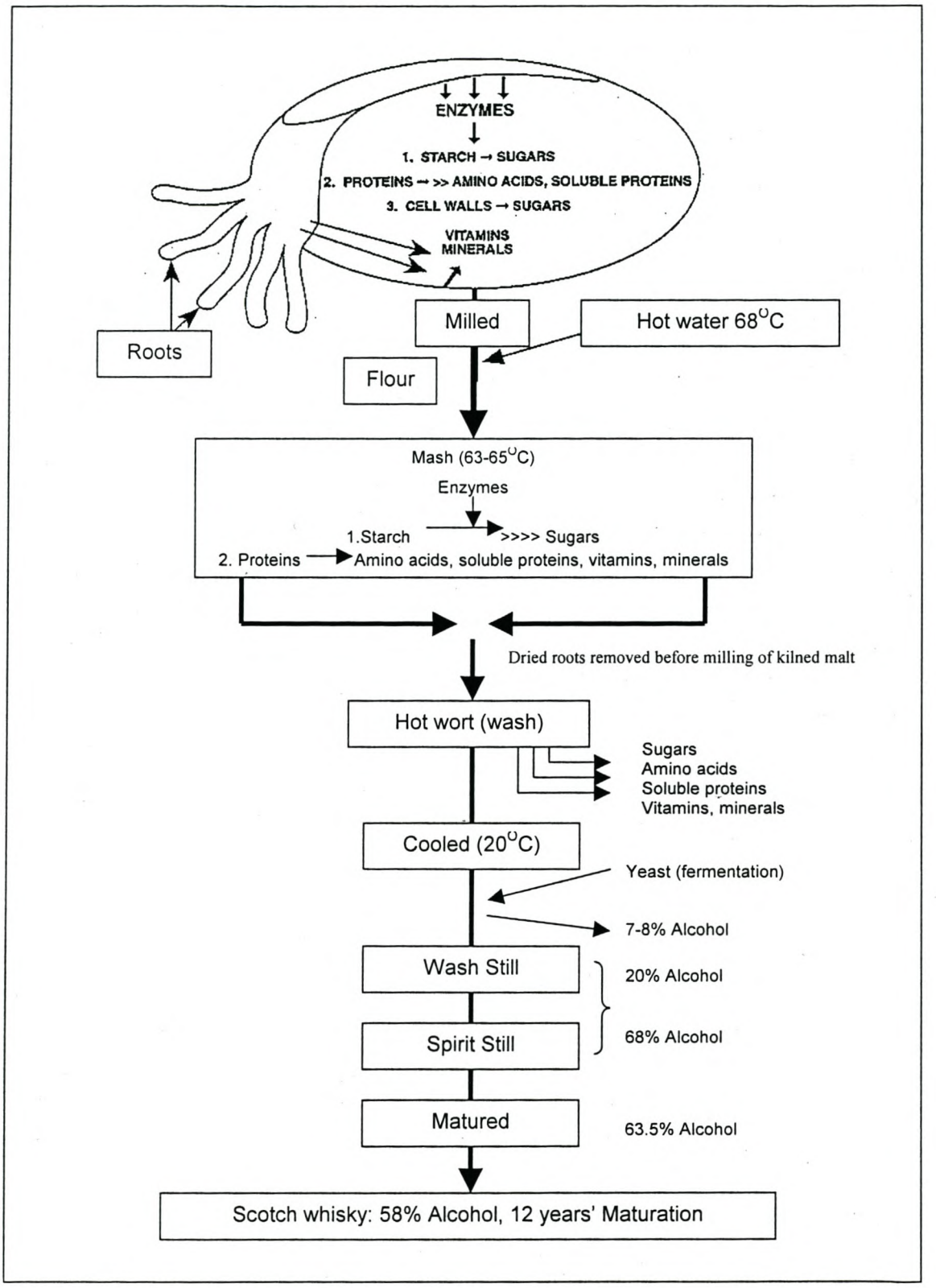

Figure 4. Barley malt to Scotch malt whisky production (Palmer, 1998).

2.3.2 STEEPING

Steeping is the first stage of the malting process (**Figure 3**). Basic steeping requirements are the provision of water, a suitable temperature and a supply of air as a means of removing excess heat, metabolic waste products (such as carbon dioxide) and endogenous germination inhibitors (Bathgate and Cook, 1989). The purpose of the steeping process is to initiate uniform germination and to hydrate the endosperm to a level, that is suitable for modification. Steeping conditions must subsequently allow the satisfactory development of enzymes without encouraging undue malting losses from respiration and growth (Bathgate and Cook, 1989). After harvest, malting barley is dried to 11-12% water to protect grain viability during storage. About 60-500 tonnes of grain are steeped (covered with water) at 16°C for 36-48 hours. Aeration is usually employed during steeping, but short periods of air-resting are allowed after the water has been drained to encourage uniform germination (Bathgate and Cook, 1989; Palmer, 1998; Piggott and Conner, 1995). The steeped grain contains about 46% water. At the end of the steeping stage, the grains should have started to germinate (protrusion of coleorhiza). The disposal of the steeping water and distilling effluents is expensive because of their high biological oxygen demand (BOD). Where necessary, maltsters and distillers have installed effluent treatment plants to meet disposal requirements.

2.3.3 GERMINATION

For malt spirit production, the purpose of germination is to maximise the fermentable extract by promoting both endosperm modification and the development of amylolytic enzymes (Bathgate and Cook, 1989). For grain spirit, there is less of a requirement for full modification, but a demand for relatively high enzyme levels. This type of malt must also supply sufficient amino nitrogen for yeast activity during fermentation (Bathgate and Cook, 1989). The germinated grain is allowed to grow in a germination chamber or box for about five days. During this process, water-saturated air is blown through the growing grain to maintain a relative humidity of nearly 100% and to keep the temperature at 16°C. Mechanical turners are used twice a day to prevent the growing roots from matting and to ensure that the airflow is not impeded. It is during this five to six day period of growth that the biochemical changes outlined in **Figure 1** and **Table 2** take place (Palmer, 1998).

2.3.4 KILNING

Green malt is kilned to produce a dry, storable product and to arrest the further development of biological activity when the required enzyme content and degree of modification have been reached (Uriyo and Eigel, 2000). In addition: (i) kilning brings about a degree of sterilisation; (ii) it is easier to remove rootlets from kilned malt; and (iii)

the dried product can be milled in a way that suits the mashing requirements. Unwanted flavour components are removed by kilning, whilst other desirable ones are introduced, either from existing precursors or from extraneous sources, such as peat smoke (Bathgate and Cook, 1989).

Kilning is carried out with an applied temperature of 60°C to dry the 'green' malt from about 44% water to 4.5% water over a 24-hour period. During the first ten hours of kilning, the enzymes continue to degrade the starchy endosperm, because the evaporation rate of the water is high and the temperature of the drying malt is lower than the applied temperature (60°C), at 30°C. Although one of the aims of kilning is to ensure that enzyme activity is preserved for the hot water extraction stage (mashing), the activities of heat labile enzymes, such as β-amylase and endo-β-1,3-1,4-glucanase, are reduced. After 10 hours of kilning, the drying grain reaches its 'breakpoint', at which the temperature of the malt begins to rise because of the slower rate of water evaporation. During this second phase of kilning, colour and flavour compounds develop in the malted grain (Palmer, 1998).

Table 2. The increased enzyme levels, increased hydrolysis (modification) and solubilisation of endosperm substrates when barley is transformed by germination process (of malting) into malt (Palmer, 1998).

	Barley	**Malted barley**
Starch %	65	60
β-D-glucan %	3.5	0.3
Lipids %	3.5	3.1
Total nitrogen %	1.6	1.5
Total soluble nitrogen %	0.3	0.7
α-Amino nitrogen %	0.05	0.17
Total sugars %	2	4
Sucrose %	1	2
Hot water extract % (Dry)	30	81
Friability %	1	92
Homogeneity %	-*	98
Whole grains %	99	2
Diastatic power (β-amylase ^{O}L)	20	75
Dextrinising unit (α-amylase)	< 5.0	30
Endo-β-glucanase (IRV units)	< 80,0	500
Colour ^{O}EBC	< 1.0	2
Moisture	11	4.3
Fermentation extract %	-*	67
Fermentability %	-*	86
Peatiness (phenol units) ppm	-*	1-55
Nitrosodimethylamine (ppb)	-*	< 2.0
Sulphur dioxide (ppm)	-*	< 15.0
Spirit yield (l/al/tonne) **	-*	406

* Not determined because values too low and/or analytically meaningless.

** Litres of alcohol per tonne of malt.

2.3.5 PEATING

Some characteristic flavour notes of Scotch whisky are derived from the smoke released when peat (plant material) is burned in the kiln. During the early phase of kilning, when the malt moisture is high, phenols such as isomeric cresols, guaiacol and xylenols are deposited on the drying malt in small but significant quantities. Although only about 20% of the phenolic flavours of the peated malt add distinct smoky, medicinal flavours to the whisky, the peatiness of a whisky is related to the intensity of peating the malt received during the kilning process (**Table 2**). Peating (i.e. the burning of mineralized plant residues) can, like the burning of other fossil fuels, release compounds such as benzo(a)pyrene, which are regarded as possible carconogens (Palmer, 1998). After the malt is dried (kilned) to about 4.5% moisture, the roots are removed and the remaining grain is regarded as distillers' malt. As a result of the chemical changes brought about by the enzymatic action during malting (**Table 2**), the value of the finished malt is much higher than the price of the starting barley material (Palmer, 1998).

2.4 MASHING

Mashing is the process of forming a fermentable extract. Two major routes may be followed, depending on whether a malted or unmalted cereal is used. In the former case, the process is essentially similar to the production of wort for brewing beer, a clear or filtered extract being required to prevent 'burning' in batch (pot) stills (Piggott and Conner, 1995). In the latter case, in modern continuous processes (**Figure 6**) in preparation for column distillation, the separation stage has become redundant and the fermentation (and distillation) are commonly carried out in the presence of the total grain solids.

2.4.1 MALT WHISKY

The initial step in batch mashing (most common in small distilleries typical of the Scotch industry) is a coarse milling of the grain. In malt whisky production, particularly in the Scottish tradition, it is important that the barley has been well modified during malting, as no additives can be used in the mashing procedure to help out a poor quality malt (Wilkin, 1989). Under these circumstances, the objective of milling is to obtain efficient recovery of the fermentable extract contained in the malt. If milling is too coarse, there will be a loss of extract, and if it is too fine, problems can occur with fine filtration (Piggott and Conner, 1995). A conventional malt distillery mashing process involves a multi-stage extraction of the malt (Wilkin, 1989). A batch of malt is loaded into the mash-tun, followed by the first water (4-4.5 tonnes/tonne of malt) at 64-68°C. The water is drained and water at a slightly higher temperature is added (1.5-2 tonnes/tonne of malt). This is repeated for a third and possibly a fourth time at a higher temperature, up to 95°C, the quantity of the water being adjusted to give the intended starting specific gravity for fermentation. Alternatively, later

extracts may be stored for recycling into the next mash. The temperature of the first water especially must be controlled to minimise enzyme damage; a proportion of the conversion of starch occurs in the fermenter during and after mashing and the temperature therefore must be limited. This 'secondary conversion' is obviously inhibited by the destruction of residual malt enzymes, particularly α-amylase. The time required for this process is typically 8-12 hours, and in an effort to reduce these cycle times, many distilleries use lauter tuns. In order to achieve the faster drainage rates required, a lauter tun uses a shallower bed and a careful design of rakes to stir the bed. Other systems may also be used, all aimed at producing a clear wort with maximum extract recovery in the minimum time (Wilkin, 1983, 1989). Milling becomes more critical as filtration speeds increase, and a higher degree of milling generally is required (Piggott and Conner, 1995).

2.4.2 GRAIN WHISKY

Producing spirit from a mash of mainly unmalted cereal requires a substantially different worts preparation procedure from that described above. The starch in the unmalted cereal has to be released from its matrix so that the enzymes, introduced by the addition of malt, can function effectively. This is traditionally achieved by a high-temperature cooking process. For the production of grain whisky, the batch process is applied (traditionally used in Scotch grain distilleries). The process is straightforward and can apply equally to maize or wheat (**Figure 5**). The grain may be milled or used whole; the cost of milling must be set against the energy saving achieved by the quicker cooking of the milled grain. The grain is loaded into batch pressure cookers with water (2.5 tonnes/tonne of grain) and steam is injected to raise the pressure to typically ≈200 kPa for 2 hours (Wilkin, 1989). The cooked cereal is then transferred to the mash tun, cooled to 62.5°C, and ground malt is added to achieve the necessary conversion of starch. The malt may be dried or may be used as 'green' malt, thus saving the energy cost of drying. The most important characteristic of the malt is that it has a high enzyme activity, which minimises the quantity required (typically 10-15% dry weight).

In a continuous process (**Figure 6**), the milled cereal slurry is mixed with a premalt (a proportion of the malt to be used for conversion) and preheated. Some conversion occurs at this stage, and the viscosity of the mix is reduced to ease future processing. The main cooking stage follows, at about 165°C, followed by cooling to 60°C, the addition of malt and conversion. The wort is finally cooled again for transfer to the fermentation stage.

The Scotch whisky industry developed the use of maize as the main non-barley cereal. The change to the use of wheat (Palmer, 1985) introduced some problems, requiring the removal of pentosans, small starch granules and proteins. The total economics of the process may then be affected by the income obtained from the disposal of gluten.

The energy demand of the high-temperature cooking stage has prompted an investigation into 'cold cooking' processes (Macher, 1982), but such methods require fine grinding of the cereal grain and, therefore, some of the energy saved is required for grinding.

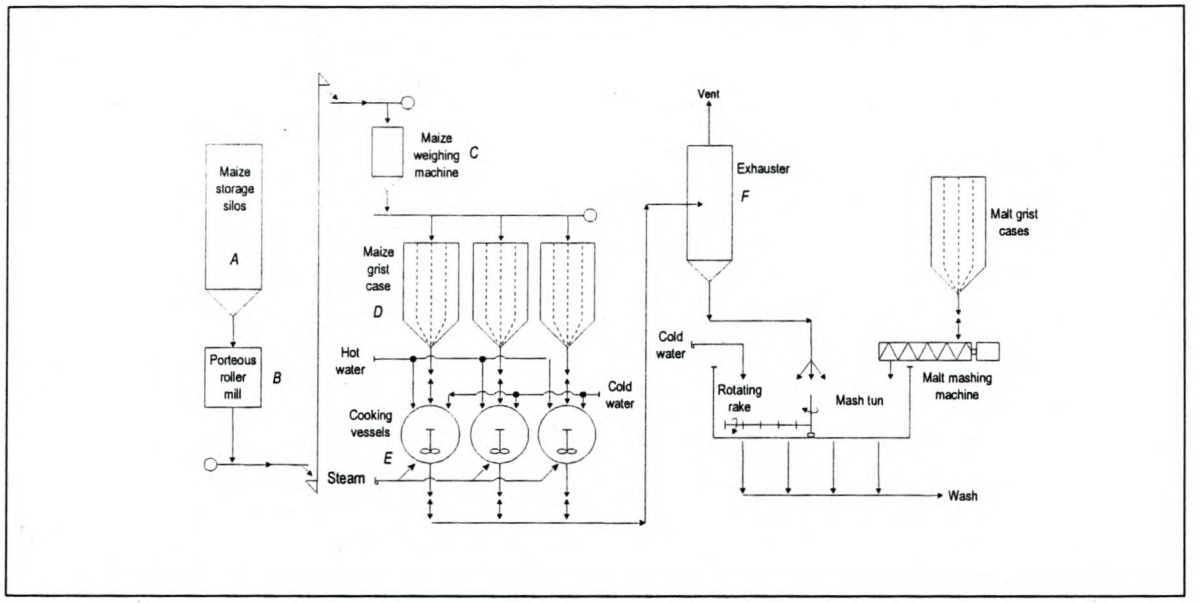

Figure 5. Cereal batch cooking equipment for Scotch whisky (Wilkin, 1989).

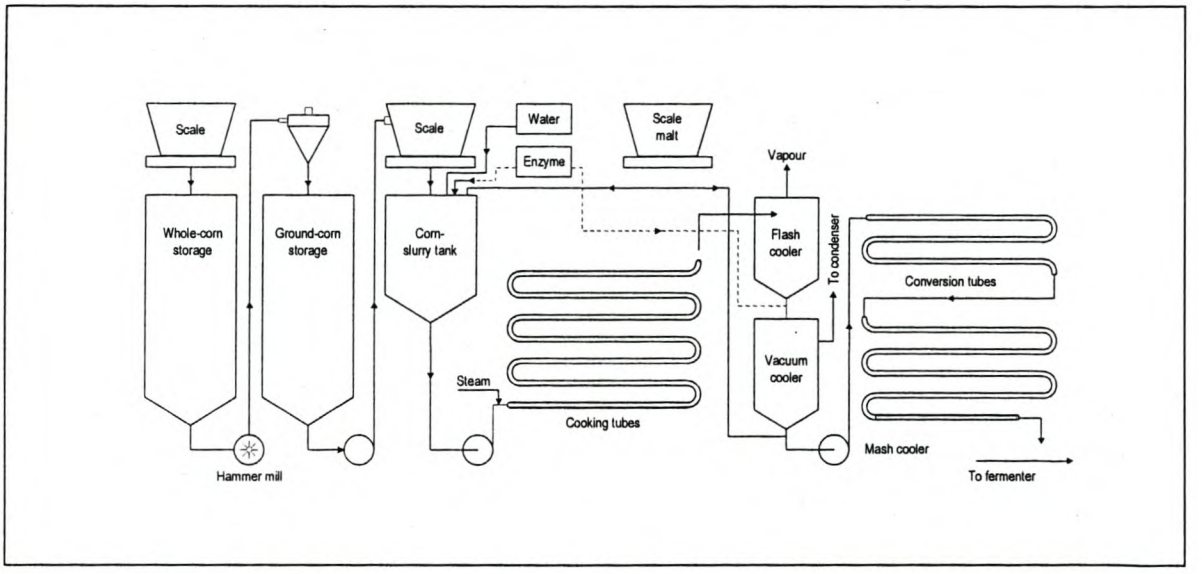

Figure 6. Continuous maize process for whisky (Piggott and Conner, 1995).

2.5 FERMENTATION

The fermentation stage is similar to that used for many other alcoholic beverages, during which sugars hydrolysed from grain starch are metabolised by yeast into ethanol, carbon dioxide, byproducts and cell material. In most regulations, yeast (*S. cerevisiae*) is specified as the only organism allowed to be used (Piggott and Conner, 1995). While malt and other cereals may be contaminated with a wide variety of organisms, such as yeast and bacteria, whisky fermentations are started by pitching the wort with a known yeast culture, normally a specific strain of high-performance distilling yeast. In some cases, typically in Scotch malt production, a brewer's yeast may be used also because it is believed that it produces a distillate with a more desirable flavour (Korhola *et al.*, 1989). In small-scale production, fermenters are closed vessels, traditionally of wooden construction, with no means of temperature control. It is uneconomical to collect carbon dioxide, so it is simply vented. In large-scale production (e.g. continuous grain distilleries), the fermenters are stainless steel, with facilities for cooling and, in some cases, the ability to collect carbon dioxide. A typical fermentation will run for 40-48 hours (Piggott and Conner, 1995).

For the production of Scotch whisky a yeast mixture of distiller's yeast and a brewer's ale strain is added (20 million cells per ml) to the wort. Three main phases of yeast activity are identified: starting at pH 5.4, an active yeast growth phase at pH 4.5, a stationary phase and a final yeast decline phase at pH 4.5. During fermentation the temperature increases from 20°C to about 30°C as the yeast multiplies tenfold. At the end of fermentation about 8% ethanol and large volumes of carbon dioxide have been produced from an original sugar concentration of about 15% (Palmer, 1998).

2.6 DISTILLATION

The fermented malt wort extract now contains 8% ethanol, a wide range of flavour compounds, such as higher alcohols, esters, carbonyls, organic acids and phenols, in addition to a large quantity of yeast (Piggott *et al.*, 1989). This complex alcoholic mix of flavours is transferred from the fermenters to copper pot stills (**Figure 8** and **9**), which can vary in capacity and can be as large as 25 000 litres to distil 15 000 litres of fermented worts (Whitby, 1992).

Two distinct distillation systems have been used for the production of whiskies: the batch or pot still, normally a double (occasionally triple) still, to produce highly-flavoured spirit (**Figure 7**), and the continuous column still, to produce lighter-flavoured spirits that normally are used as the base for blending.

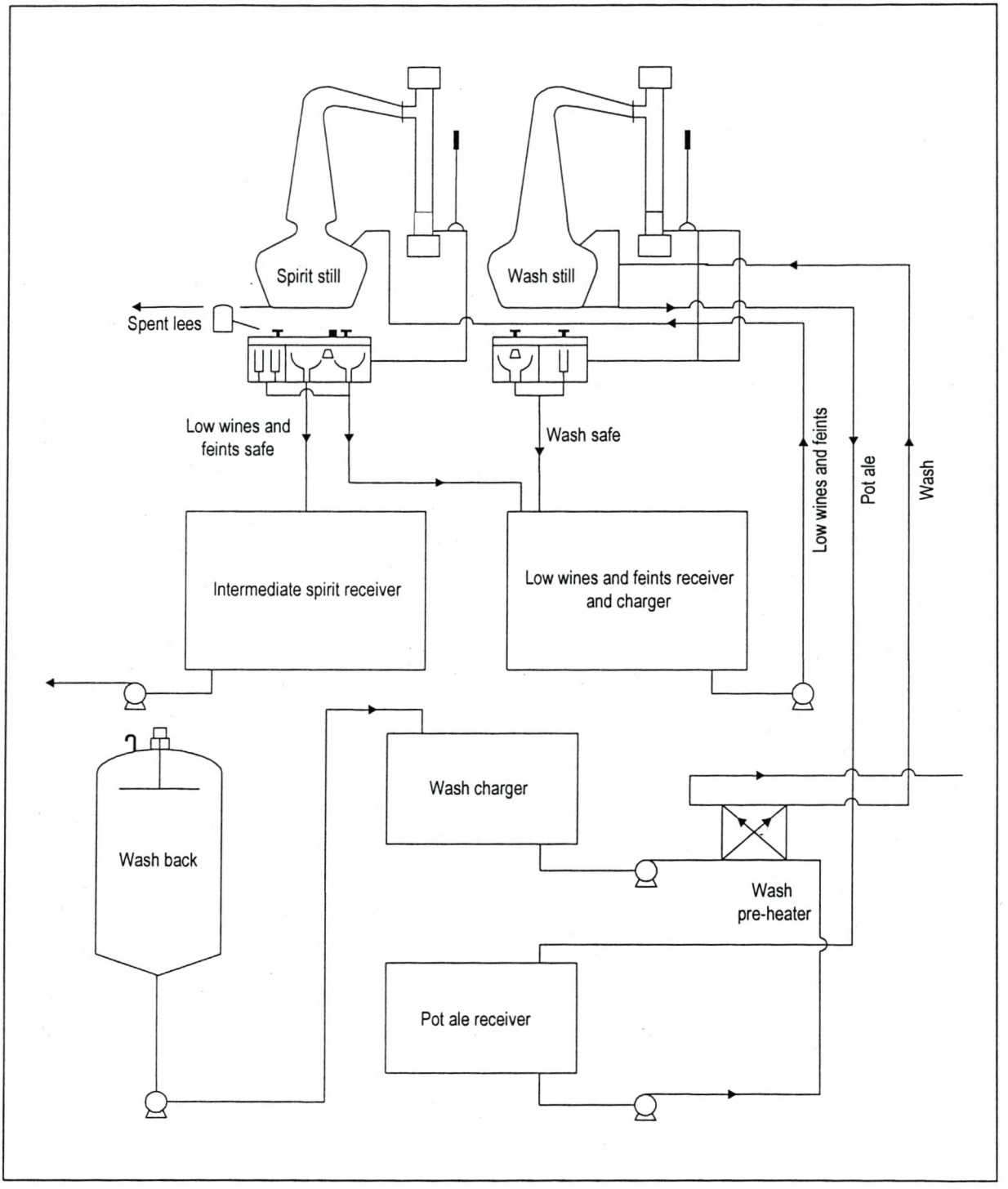

Figure 7. Typical malt distillery flow diagram (Nicol, 1989).

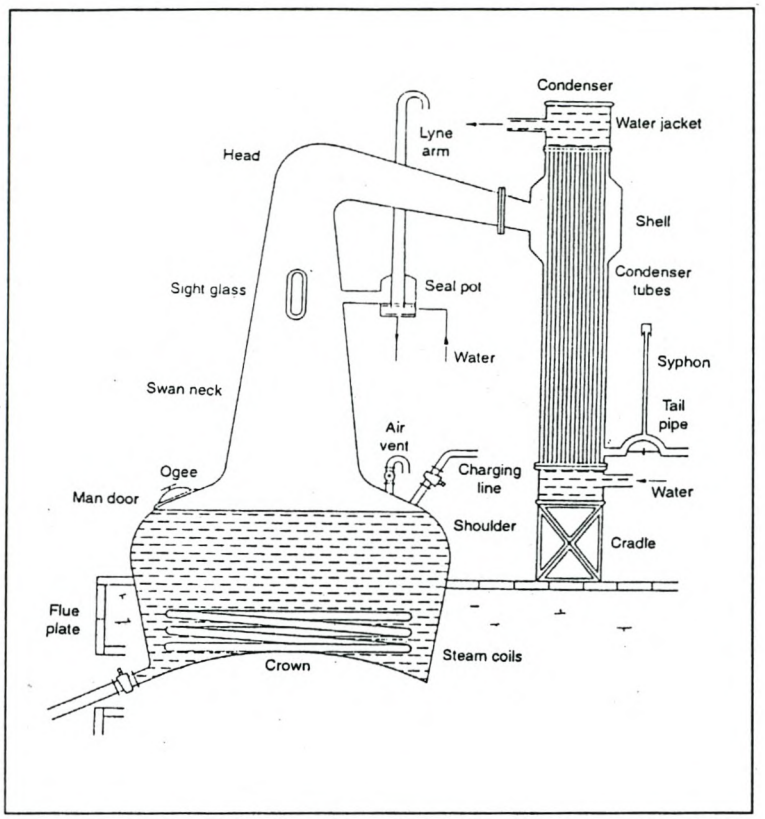

Figure 8. Plain wash still (Piggott and Conner, 1995).

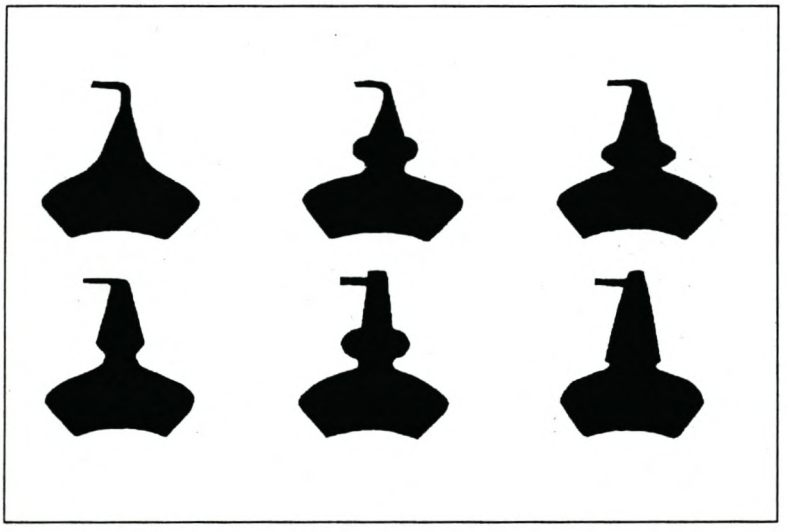

Figure 9. A selection of different pot still shapes (Whitby, 1992).

2.6.1 BATCH DISTILLATION

The batch still is normally constructed from copper. This has traditionally been the chosen material, as it provides ease of working, good heat conduction and resistance to wear; more recently, its importance in influencing flavour, by aiding the removal of sulphur compounds, has been appreciated (Whitby, 1992). A still consists of three major parts, the pot containing the liquid to be distilled, the swan neck and the lyne arm, and the condenser (**Figure 8**). There are many minor variations on this basic pattern (**Figure 9**), which have largely unknown effects on the distillate. It is clear, however, that variations in the swan neck and lyne arm, by allowing differing extents of reflux, may effect flavour, and malt distillers are most reluctant to change the shape of a still when it has to be replaced in order to avoid unexpected effects.

Heating of the pot may take place by direct flame heating or indirectly by steam coil or jack. The first (wash) still may be fitted with a stirrer or rummager to prevent burning in the case of directly heated stills (Nicol, 1989). The wash typically contains about 8% v/v ethanol, and efficient distillation should result in the collection of virtually all the ethanol, at about 21-23% v/v, as low wines. The second (spirit) distillation essentially proceeds in the same way, except that a portion of the distillate (middle cut) must be selected as new spirit. The selection of the cut points at the start and the end of spirit collection is critical for the quality of the product and depends on the particular distillery. After the collection of spirit has ceased, distillation is continued until the ethanol is recovered, though it may be uneconomical to continue distillation to collect all the ethanol. The initial and final fractions of the distillate, the foreshots and feints, contain the undesirable highly volatile and less-volatile congeners, together with a large amount of ethanol, and are recycled for distillation with the low wines. The charge to the still is, therefore, a mixture of foreshots, feints and low wines, at 25-30% v/v ethanol. The middle cut collected as spirits is typically at 65-75% v/v, depending on the distillery. The recycling of foreshots and feints onto the distillation has substantial implications for spirit quality, and it is essential that a consistent and uniform procedure is followed and that a consistent charge to the spirit still is maintained (Nicol, 1989; Whitby, 1992). The entire distillery system constitutes a complex balance, and if the balance is disturbed by changes in equipment or operating procedures, there may be undesirable effects on product quality (Piggott and Conner, 1995).

2.6.2 CONTINUOUS DISTILLATION

Column stills are used to produce the lighter grain spirits for blending (or occasionally for consumption as such) (Piggott and Conner, 1995). The still is constructed from two columns situated side-by-side (**Figure 10**), though functionally it can be regarded as a single column mounted in sections to reduce the overall height (Panek and Boucher, 1989). Stills are normally constructed of copper or stainless steel, although in this case

copper must be present in the system to remove undesirable flavour compounds. The wash or beer is preheated by passing it through a tube winding through the second column (rectifier) and is then fed into the first column (analyser) towards the top. Steam is sparged into the base of the column and, as the wash falls, the volatiles are stripped out and removed from the top of the column. The vapour then passes to the base of the rectifier, and the separation into alcohol and water occurs. The spirit product is removed at a level towards the top of the column. Fusel oil (largely isoamyl alcohol) is taken off from near the top of the column (Piggott and Conner, 1995). Single and multi-column systems are available. The single distillation column is normally used in North America for producing bourbon and other relatively highly flavoured grain spirits, and multi-column systems are used for the production of less strongly-flavoured spirits (Piggott and Conner, 1995).

2.6.3 BYPRODUCTS

The two major byproducts of whisky production are the residues of the cereals used as the source of carbohydrate (spent grains), and the residues of the distillation (pot ale). These may arise separately, as in the case of Scotch malt whisky production, or they may arise together, as in the case of a whole mash grain distillation. In any case they have some value as animal feed and are normally dried as 'dark grains'. The economics of the process is finely balanced and depends on the cost of energy for drying, the value as feed and the cost of alternative disposal (Alsaker, 1989). As mentioned above, carbon dioxide may be vented or collected and compressed. Other minor products include fusel oil from column distillations, for which there may be a market, spent lees from the second pot distillation and the heads from column distillation. These are normally disposed of and may require further treatment on site (Piggott and Conner, 1995).

2.7 MATURATION

Maturation is an important step in the development of whisky flavour. Freshly distilled whiskies generally have unacceptable sensory characteristics and are matured in oak casks to produce an acceptable product. During the maturation period, the new distillate becomes highly modified as a result of its contact with the cask. Although many reactions have been identified as occurring during maturation, there is no reliable chemical or physical index available for measuring the progress of maturation (Nishimura and Matsuyama, 1989). Three varieties of oak are important in the maturation of Scotch whisky. They are: *Quercus ruber* (English), *Quercus petraea* (Spanish and French) and *Quercus alba* (American) (Palmer, 1998).

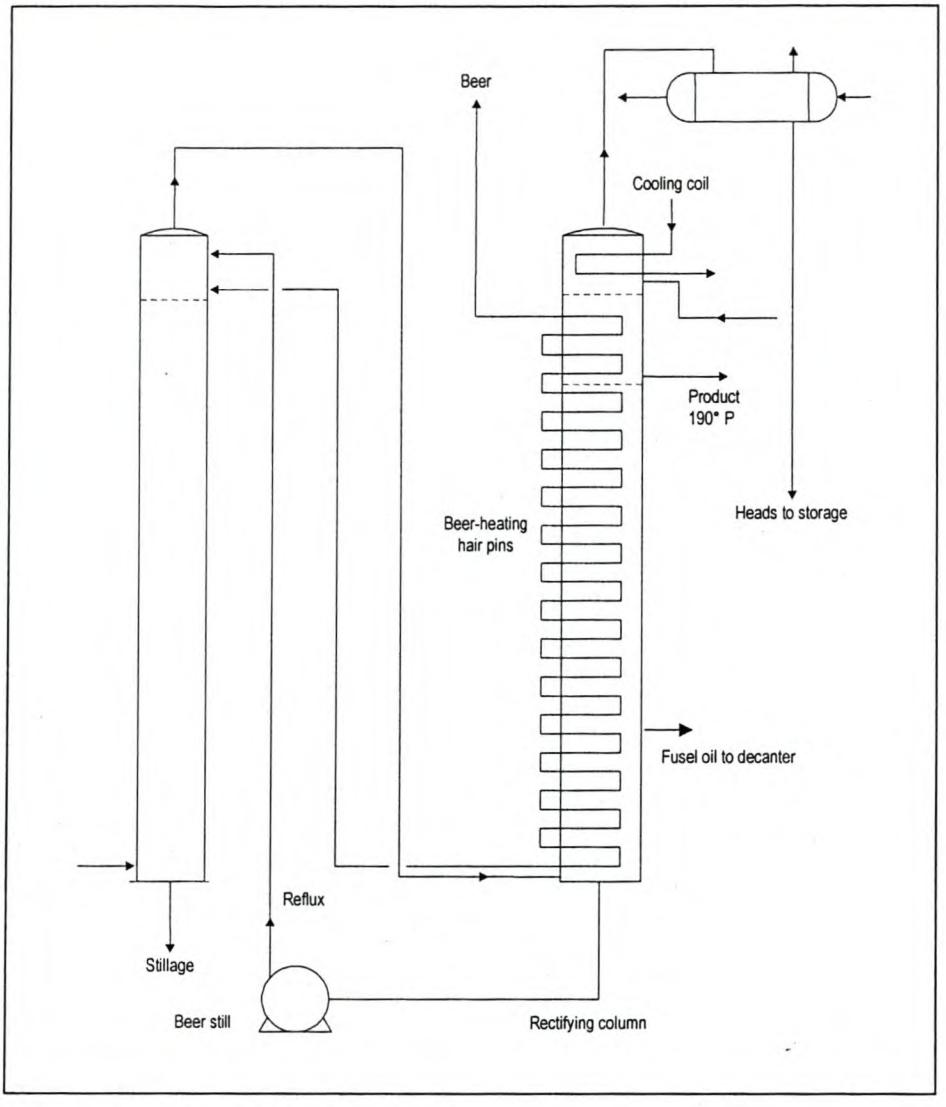

Figure 10. Coffey still (Panek and Boucher, 1989).

The legal minimum maturation time in most countries is three years, although if an age is stated on the bottle, this is the minimum age of all whiskies used in production, including grain whiskies used in blends (Piggott and Conner, 1995). A number of factors will influence the maturation process and have an impact on the quality and flavour characteristics of the whisky. The microclimate of the maturation warehouse, notably the temperature, relative humidity and air flow, has a large effect on maturation, and distillers will go to great lengths to reduce atmospheric variables to help maintain product consistency. Distillers will take advantage of other factors to impart specific maturation characteristics to the whisky. The maturation time, the style of the barrel, the depth of char, the age of the barrel and the strength of the spirit entering the barrel all have their own influences on maturation, as well as on the finished whisky (Wright, 1999).

Charring the inner surface of the barrel produces carbon, which absorbs the unpleasant flavours of sulphur compounds from the whisky. These include dimethylsulphide,

dimethyldisulphide, methional and methionol acetate, but not dimethyltrisulphide, which can be detected organoleptically even at the very low concentration of 4 ppb (10^{-9}) (Nishimura and Matsuyama, 1989). Important complexes of substances are released into the whisky from the scorched sub-surface (**Figure 11**), thereby adjusting and mellowing its flavour and increasing its colour (Palmer, 1998).

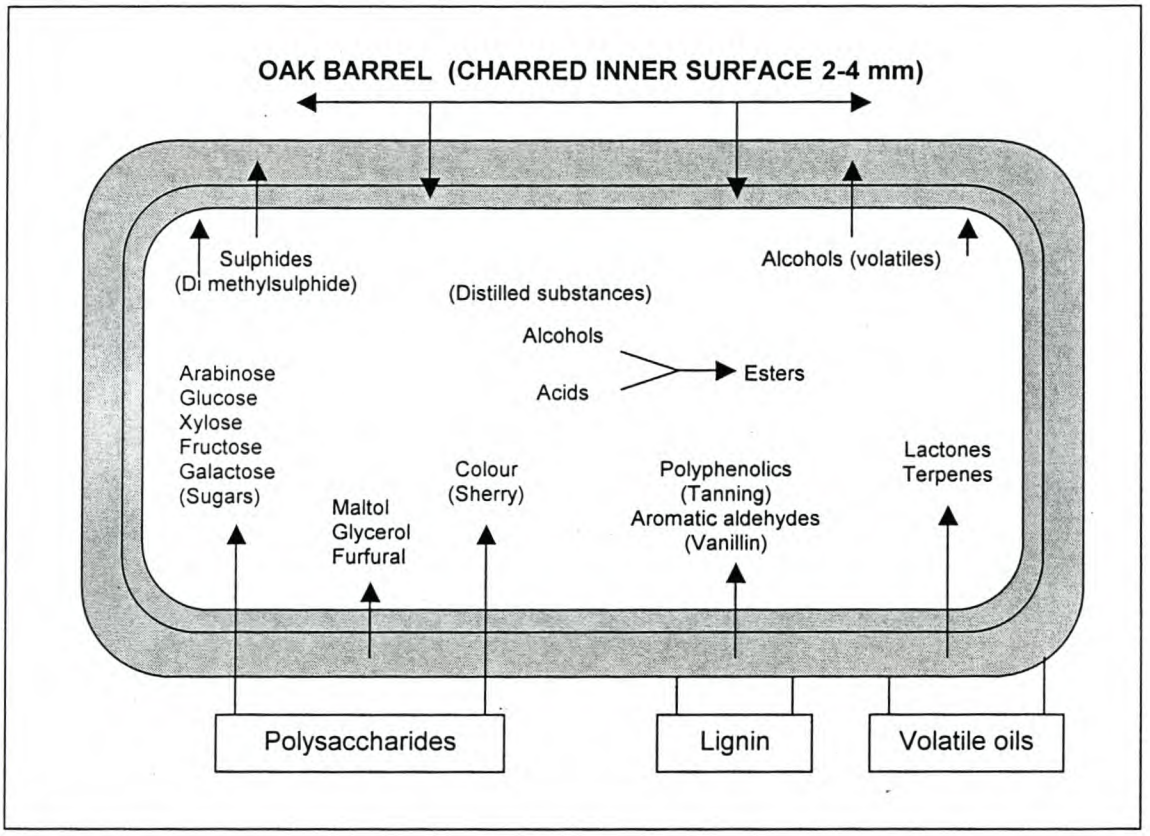

Figure 11. Some changes in Scotch malt whisky during maturation (Palmer, 1998).

2.7.1 SENSORY CHANGES DURING MATURATION

Maturation should produce a significant improvement in flavour quality. This results from the development of mellow or mature characteristics from the wood and a loss of the harsh or immature characteristics of the new distillate. Mature flavours that develop during maturation include vanilla, spicy, floral, woody and smooth. The harsh or immature characteristics have been described as sour, grassy, oily and sulphury, although there is considerable variation in the vocabularies used (Canaway, 1983). Both the magnitude and the rate of change during maturation are dependent on the type of cask used. Charring has been shown to increase the intensity of mature characteristics, such as smooth, vanilla and sweet, and to decrease the intensity of immature characteristics (pungent, sour and oily). Cask reuse conversely decreases the intensity of mature characteristics and increases that of immature characteristics (Piggott *et al.*, 1993).

2.7.2 CHEMICAL CHANGES DURING MATURATION

A number of chemical changes have been identified as important during the maturation and they have been divided into additive, subtractive and interactive reactions (Piggott and Conner, 1995).

2.7.2.1 Additive reactions

Additive reactions refer to the extraction of components from the cask wood during maturation. Many wood components are not present in the barrel wood and are the result of the decomposition of the macromolecules forming the framework of the wood, such as lignin, cellulose and hemicellulose. Polymetric material is also extracted from the wood and this, in turn, may be broken down by the spirit solution (Nishimura *et al.*, 1983).

2.7.2.2 Subtractive reactions

Subtractive reactions are typified by the evaporation of low-boiling-point compounds through the wood of the cask, but may also involve either absorption of compounds onto the surface of the cask or chemical reactions, which result in a less volatile product. The cask is a porous container and the evaporation of volatile compounds through the cask surface occurs during the course of maturation (Piggott and Conner, 1995). For a model whisky, the rate of evaporation ranged from 32% of the total acetaldehyde present in the spirit to 5% for isoamyl alcohols and 1% for ethyl hexanoate and acetic acid. Evaporation is thought to be the main route for the loss of dimethylsulphide and dihydro-2-mathyl (2H)-thiophene (Nishimura and Matsuyama, 1989). The rate of evaporation may be affected by cask stave thickness, the airflow around the cask, humidity and temperature (Piggott and Conner, 1995).

2.7.2.3 Interactive reactions

Interactive reactions may occur between components of the original distillate or between wood components and components already present in the original distillate. Such reactions are typified by esterification, though they could theoretically include oxidation and acetyl formation. During maturation, the concentration of esters increases as a result of the esterification of free acids by ethanol. A large fraction of this is the formation of ethyl acetate from acetic acid, either extracted from the wood or the product of ethanol oxidation (Reazin, 1981). Trans-esterification reactions are also thought to occur, which favour the formation of ethyl esters in the presence of the large excess of ethanol. Aromatic acids extracted from the cask wood, such as syringic and vanillic acids, are also known to form ethyl esters during maturation (Nishimura *et al.*, 1983).

2.8 BLENDING

The aim of blending is to produce a consistent product that has a distinctive flavour (Lang, 1983). Generally, blends consist of a light-bodied spirit mixed with a number of heavier bodied spirits in a wide range of proportions. 'Light-bodied' spirits are those distilled to high ethanol concentrations using continuous stills and include Scotch grain whisky and American light whiskies, grain spirits and grain neutral spirits. 'Heavier bodied' whiskies are either batch-still products or column-still products distilled to lower ethanol concentrations (Booth *et al.*, 1989). The traditions and regulations of the country of origin determine the nature and components of the blends. The actual process of blending is, however, very similar. Approved whiskies are delivered to the blending house and drained from the casks, in correct proportions, into passivated steel troughs. The troughs convey the whiskies to a blending vat, where they are thoroughly mixed with mechanical agitators and compressed air. When the blend is correct, deproofing water is added to the blend to reduce the strength for bottling. Minor variations do occur. In Scotland, blending may be followed by a further period of maturation, and in Canada, distillates may be mixed prior to any maturation (preblending) (Piggott and Conner, 1995).

2.9 FILTRATION

Whisky is filtered in order to present a clear, bright product to the consumer and to reduce the risk of haze formation (Piggott and Conner, 1995; Wright, 1999). Spirits are traditionally matured at 50 to 70% alcohol by volume, but are bottled at 40 to 45% alcohol by volume. For heavier-bodied older whiskies and whiskies matured at higher strengths, this can result in haze formation caused by high-molecular-weight lipids and esters and ethanol-soluble lignins being less soluble in water than ethanol (Piggott and Conner, 1995). This is controlled by chill filtration, during which the whisky is cooled to between –0°C and 10°C and held for a specified period of time before the problem compounds are removed by physical separation and absorption by a filter (Booth *et al.*, 1989).

The conventional filter used is a plate and frame variety using preformed pads made of cellulose, or cellulose impregnated or precoated with diatomaceous earth. Typical particle retentions are of the order of 5 to 7μm. The operational parameters depend on the batch size, the nature of the product and the filtration rate required. In general, higher filling strengths and new wood require more filter area per unit volume (Piggott and Conner, 1995).

2.10 THE IMPROVEMENT OF WHISKY PRODUCTION BY GENETIC ENGINEERING OF YEAST FOR EFFICIENT EXPRESSION AND SECRETION OF RAW-STARCH-DEGRADING ENZYMES

In the whisky production process, the fermentation is carried out by yeast on a wort substrate. The wort is prepared by mashing malt grist. Malt consists of maltose, maltotriose, glucose and dextrins. The utilisation of wort sugars is one of most important factors that controls the fermentation efficiency during the whisky production process (Klaassen *et al.*, 1996). Yeast ferments sucrose, glucose, fructose and maltose, leaving the larger dextrins unfermented (Klaassen *et al.*, 1996). In addition, maltotriose is fermented incompletely. Whisky producers have to use commercial enzyme preparations, such as α-amylase and glucoamylase, to break down larger dextrins, such as maltotriose, to make them more susceptible for fermentation. This is an unnessecary loss of money if one takes into account the existence of amylolytic yeasts. The use of yeast capable of fermenting these larger dextrins, such as maltotriose, would offer a great advantage to the whisky producer, as it would save money and give rise to a higher ethanol yield. A logical step would be to introduce amylolytic genes into *S. cerevisiae*, as this yeast is unable to degrade starch.

Metabolic engineering has become a very important approach to strain improvement in parallel with classical strain development. *S. cerevisiae* has several industrial applications in the production of beer, wine, alcohol, baker's yeast and heterologous proteins. *S. cerevisiae* is a domesticated microorganism, and thousands of years of use has resulted in the development of several strains that are optimised for these specific applications (Olsson and Nielsen, 2000).

2.10.1 OCCURRENCE AND COMPOSITION OF STARCH

After cellulose, starch is the most widely distributed naturally occurring organic compound. The main sources of starchy material are higher plants, in which starch may account for 20 to 70% of the weight (Solomon, 1978). Starch is produced commercially from the seeds, tubers and roots of plants. The major source in the Western Hemisphere is maize (corn) while in the developing countries cassava, tubers and the stern of the sago plant are important sources. Starch is composed of two fractions of high molecular weight, amylose and amylopectin (Nigam and Singh, 1995). These two polymers differ significantly in molecular size, in their solubility in water, their iodine staining and their susceptibility to enzymatic hydrolysis (Zobel, 1988).

2.10.1.1 Amylose

Amylose, the minor component (20-30%) of starch, is a flexible, mainly linear polysaccharide that is formed by α-1,4-linked α-D-glucose residues and some α-1,6-branching points (Zobel, 1988). The double helical crystalline structure, which contains six D-glucose molecules per turn, is responsible for the incomplete degradation of amylose by α-amylase (about 70% conversion into maltose) (Nigam and Singh, 1995). The average chain length of amylose is approximately 500-1000 glucose units (Zobel, 1988). The chains are capable of twisting and coiling in three-dimensional space and the majority are interlinked to form a very lightly branched structure. The degree of branching in amylose is much less than in amylopectin (Manners, 1989). The molecular size varies with the source of the starch, as is evident from the degree of polymerisation (DP). Amylose dissolves in water only to a limited extent. The α-1,4 bond enables the molecule to attain various conformations in solution, which are responsible for the 'retrogradation' and 'gelatinisation' of a concentrated amylose solution. Retrogradation describes the process whereby starch in the dissolved or hydrated state reverts to a water-soluble form (Solomon, 1978). The complex of amylose and iodine produces a blue colour and is the basis for the quantitative determination of amylose. Dextrins containing less than six D-glucose residues do not produce the characteristic coloured complex (Solomon, 1978).

2.10.1.2 Amylopectin

Amylopectin, in contrast to amylose, is a highly branched structure with 4-6% α-1,6 bonds of branch points. It represents the major fraction (70-80%) and is undoubtedly more complex than amylose, particularly because of its highly branched structure. Amylopectin is also composed of α-1,4-linked α-D-glucose residues, but is connected at the branch points by α-1,6 linkages (Solomon, 1978). The average length of the branch chains are 20-25 glucose units (Nigam and Singh, 1995). Amylopectin may have a molecular weight (Mr) in excess of 10^8, making it the largest molecule in nature (Nigam and Singh, 1995). Amylopectin is reasonably soluble in water and, with iodine, forms a red colour, in contrast with the blue colour obtained with amylose (Solomon, 1978; Zobel, 1988).

2.10.2 AMYLOLYTIC YEASTS

Starch-containing raw materials (e.g. maize, barley, other grains, potatoes and cassava) are widely available as feedstock for fermentation processes and are used, in particular, for the large-scale production of industrial or fuel ethanol, potable spirits or beer (Steyn and Pretorius, 1990). A developing trend is to produce ethanol (particularly for addition to unleaded gasoline) from low-grade starch fractions derived in the processing of grain starches for the enormous, and still expanding, soft drinks business. Produced in this

way, ethanol becomes a byproduct of the manufacture of glucose and high-fructose syrups (Tubb, 1986).

Amylolytic yeasts are potentially valuable in the use of starchy substrates for direct conversion into single-cell protein (Nigam and Singh, 1995). Studies have also been done on the use of amylolytic strains for starch utilisation in alcoholic fermentation (Amin *et al.*, 1985; De Mot and Verachtert, 1987). Strains of about 150 species of yeast have been reported to be able to utilise starch as the sole carbon source for aerobic growth (McCann and Barnett, 1986). However, only a few secrete a combination of enzymes, consisting of α-amylase, glucoamylase and debranching enzymes, which is necessary for the complete hydrolysation of starch (De Mot *et al.*, 1985). Commercial amylase preparations, both bacterial and fungal, are commonly used for the liquefaction and saccharification of starch in the production of industrial ethanol by the fermentation of starchy substances. Considerable cost savings could be realised by the use of yeast that produces its own α-amylase and glucoamylase for starch conversion, and that is also capable of ethanol fermentation (Wilson and Ingledew, 1982).

Several studies have been done on yeast that could produce extracellular α-amylase and glucoamylase. These yeasts include *Lipomyces kononenkoae* (Spencer-Martins and Van Uden, 1979), *Lipomyces starkeyi* (Kelly *et al.*, 1985), *Saccharomycopsis* (Gašperík *et al.*, 1991), *Cryptococcus* (Iefuji *et al.*, 1996) and *Schwanniomyces* (Nigam and Singh, 1995). Spencer-Martins and Van Uden (1977) reported that, among 59 species examined, the highest biomass production on starch was obtained with strains of *L. kononenkoae* and *L. starkeyi*, which converted 84 and 99% of the starch, respectively, and thus appear to be promising candidates for industrial exploitation. *Lipomyces* strains were also compared with various *Schwanniomyces* strains and it was found that *L. kononenkoae* was the most efficient starch-degrading yeast among those screened (Horn *et al.*, 1988). *L. kononenkoae* secretes a group of enzymes including an α-amylase, glucoamylase, isoamylase and cyclodextrin glucanotransferase (Spencer-Martins and Van Uden, 1979). *L. starkeyi* secretes a novel heat stable α-amylase (Kelly *et al.*, 1985). *Saccharomycopsis fibuligera* has been used to ferment premelted 20% wheat starch (Nigam and Singh, 1995). The ability of yeast species belonging to the genus *Schwanniomyces* to synthesise the extracellular amylolytic enzymes, α-amylase and glucoamylase, has made them useful in biomass production and in the direct fermentation of starch to as much as 3% (v/v) ethanol (Abarca *et al.*, 1991; Calleja *et al.*, 1982; Claros *et al.*, 1993; Clementi *et al.*, 1980; Dohmen *et al.*, 1989, 1990; Strasser *et al.*, 1989; Wilson *et al.*, 1982)

2.10.2.1 Application of amylolytic yeasts

In recent years, several laboratories around the world have been involved in the construction and/or evaluation of amylolytic yeast strains. A major thrust of this work has been towards generating some amylolytic capability *in situ* during the fermentation stages of the processes outlined in **Figure 12**. The advantages are those relating to:

(i) a reduced need to purchase (or produce in separate process) amylolytic enzymes or barley malt;
(ii) simplification of the processing stages which precede fermentation;
(iii) improved efficiencies of starch conversion;
(iv) the possibility of producing amylolytic enzymes for use in syrup manufacture, as byproducts of alcohol fermentations (Tubb, 1986).

S. cerevisiae var. diastaticus can produce large amounts of ethanol from glucose, but it is not attractive as a starch-degrading yeast, since it has only α-1,4-glucosidase activity and lacks the α-1,6-glucosidic debranching and α-amylase activities (De Mot and Verachtert, 1984). The use of other yeasts with stronger amylolytic activities such as *Schwanniomyces* species (Calleja *et al.*, 1982; Horn *et al.*, 1988; Wilson and Ingledew, 1982; Wilson *et al.*, 1982) and *Lipomyces* species (Horn *et al.*, 1988; Spencer-Martins and Van Uden, 1977), are well documented. These yeasts, however, are subject to catabolic repression and can only ferment starch to ethanol in low polysaccharide concentrations because of their low ethanol tolerance (<5%) (De Mot *et al.*, 1985). Unlike *S. cerevisiae* they also lack GRAS (**G**enerally **R**egarded **A**s **S**afe) status. For these reasons, an efficient amylolytic yeast strain with a fast growth rate, high ethanol tolerance (for ethanol production) and a high content of quality protein (for SCP production) would be ideal. That is why several studies have been done to introduce amylolytic enzymes, such as α-amylases and glucoamylases, into *S. cerevisiae* (Benkel *et al.*, 1997; Compagno *et al.*, 1995; De Moraes *et al.*, 1995; Ho *et al.*, 1998; Janse and Pretorius, 1995; Kim *et al.*, 1988; Shibuya *et al.*, 1992a, 1992b; Steyn *et al.*, 1995; Tokunaga *et al.*, 1997; Yáñez *et al.*, 1998).

2.10.3 HYDROLYSIS OF STARCH AND AMYLOLYTIC YEASTS

Starch is the universal major storage carbohydrate in most plants and consists of linear amylose and branched amylopectin structures (Nigam and Singh, 1995). One major group of starch-hydrolysing enzymes, the amylases, catalyses the endolytic cleavage of α-1,4-glucosidic linkages in starch and related substrates, releasing short oligosaccharides and α-limit dextrins (Pandey *et al.*, 2000). A wide range of organisms, including bacteria, yeasts, fungi, plants, insects and mammals, produce α-amylase, which play an important role in their metabolism (Vihinen and Mäntsälä, 1989).

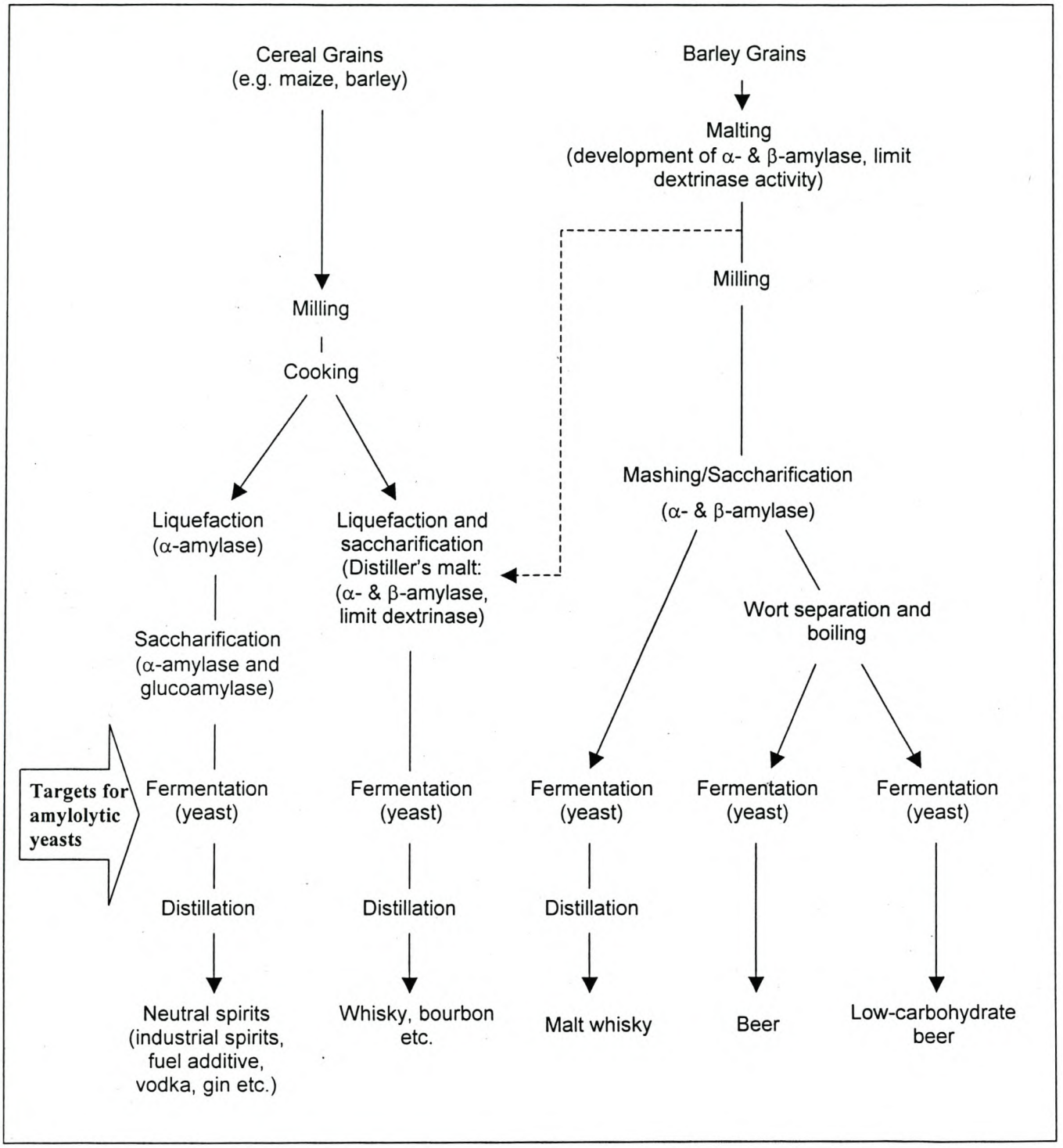

Figure 12. Schematic representation of the major pathways for converting starch-containing materials to ethanol or alcoholic beverages (Tubb, 1986).

2.10.3.1 Enzymes involved in starch degradation

Traditionally, starch was, and still is, hydrolysed to low-molecular weight dextrins and glucose using acid, but enzymes have several advantages. Firstly, the specificity of enzymes allows the production of sugar syrups with well-defined physical and chemical properties. Secondly, the milder enzymatic hydrolysis results in fewer side reactions (Nigam and Singh, 1995).

Indeed, enzymatic hydrolysis is essential for the production of glucose syrups from starch. A summary of starch-degrading enzymes is shown in **Figure 13** (Nigam and Singh, 1995). What follows are the main industrial enzymes used in starch degradation:

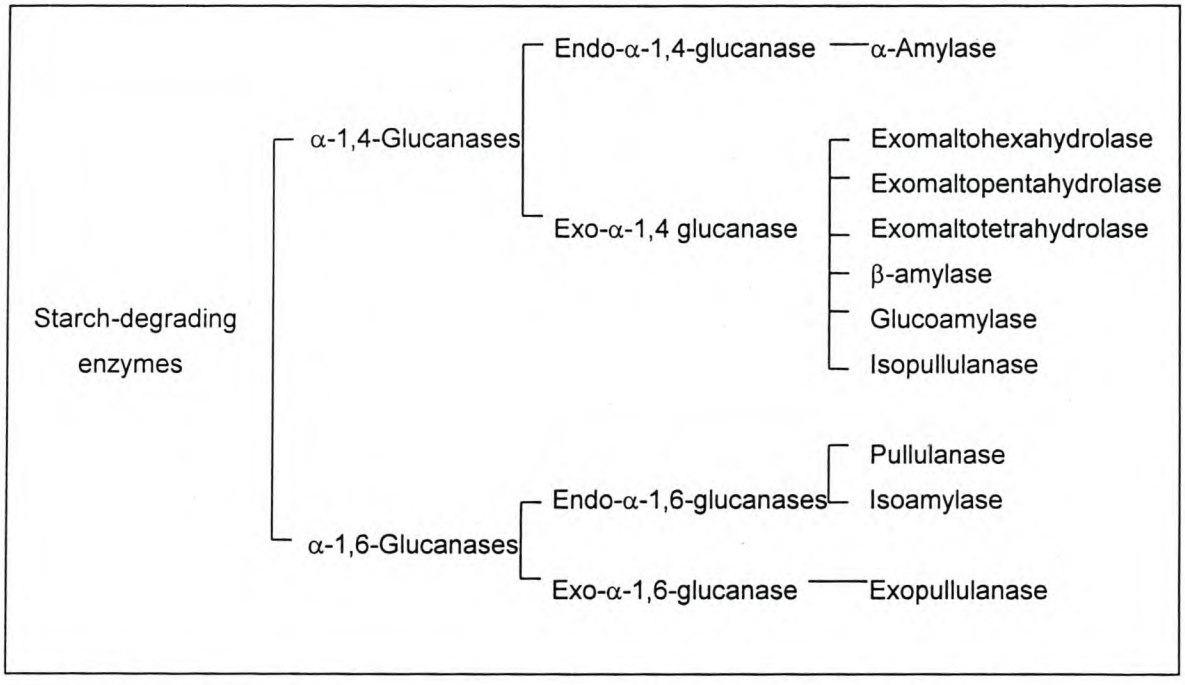

Figure 13. A summary of starch-degrading enzymes (Nigam and Singh, 1995).

2.10.3.1.1 α-Amylase

The α-amylases are used in starch hydrolysis for sugar syrups and brewing, and are obtained from *Aspergillus oryzae*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* (Nigam and Singh, 1995). Mitsui and Itoh (1997) did a study on the α-amylase multigene family. There are two varieties of α-amylases: thermostable and thermolabile (Guzmán-Maldonado and Paredes-López, 1995). The thermostable α-amylases are bacterial in origin and is derived from the *Bacillus* species. The other group of endoamylases, thermolabile α-amylases, are derived from fungal sources, usually *A. oryzae*. In the starch

industry, their utilisation is confined mostly to the saccharification reaction. Products from this reaction are mainly maltose and maltotriose. α-Amylases (endo-1,4-α-D-glucan glucohydrolase, EC 3.2.1.1) are extracellular enzymes that randomly cleave the α-D-1,4-glucosidic linkages between adjacent glucose units in the linear amylose chain. These are endoenzymes that split the substrate randomly in the interior of the molecules (Pandey *et al.*, 2000).

2.10.3.1.2 Glucoamylase

Glucoamylase is an exo-splitting enzyme that releases glucose units successively from the non-reducing end of the substrate. Glucoamylase is generally extracellular and is produced by many fungi (Yoshizumi and Ashikari, 1987), yeasts (McCann and Barnett, 1986) and only a few bacteria (Vihinen and Mäntsälä, 1989). Glucoamylase is active at the relatively low pH that is characteristic of fungal growth conditions and is glycosylated, while the optimum temperature varies from 50 to 60°C (McCann and Barnett, 1986). The glucoamylases are used in syrup production and are usually obtained from *Aspergillus niger* and *Rhizopus* species (Nigam and Singh, 1995). Chiba (1997) did a study on the molecular mechanism on glucoamylase.

2.10.3.1.3 Pullulanase and isoamylase

Pullulanase and isoamylase are defined as debranching enzymes that are capable of cleaving α-1,6-glucosidic linkages in a variety of oligo- and polysaccharide substrates. These enzymes are used in debranching starch in sugar syrup manufacturing, and are prepared from *Klebsiella pneumoniae* and *Bacillus acidopullulyticus* (Nigam and Singh, 1995).

2.10.3.2 Commercially available amylolytic enzymes

Bacterial α-amylases are produced by *Bacillus amyloliquefaciens*, in a fermentation at 30-40°C, pH 7.0, using soybean meal with lactose in the medium. The production of α-amylases starts when the bacterial count reaches 10^9-10^{10} cells ml^{-1}, usually after 10-20 h, and continues for another 100-150 h. Bacterial amylases usually are marketed as liquid preparations, which are preserved with 20% sodium chloride. The most active preparation contains 2% active α-amylase protein, and the most active solid preparations contain 5% active α-amylase protein (Nigam and Singh, 1995).

2.10.3.2.1 Fungal α-amylases

These amylases are produced by *A. oryzae* and *A. niger*, which are grown on wheat bran. *Aspergillus oryzae* amylase can be produced in a submerged culture employing media similar to those used for bacterial amylase. Because the viscosity is very high due to mycelia, aeration and agitation present problems. *Aspergillus* amylase produces more sugar than bacterial amylase (Nigam and Singh, 1995).

2.10.3.2.2 Glucoamylase

Glucoamylase is produced industrially by *Aspergillus*, *Rhizopus* and *Endomycopcis* species. The optimum temperature for fermentation is 30-35°C. *The Aspergillus* species are cultivated in a submerged culture using a medium with a high concentration of starch. A typical composition is 20% corn and 2.5% corn-steep liquor. The starch is liquefied with a heat-stable bacterial α-amylase prior to sterilisation. Glucose does not repress the formation of the enzyme, but the pH is not allowed to drop below 4.5; otherwise, the α-amylase is denatured. Fermentation continues for 4-5 days, during which the pH drops to 3-4. The glucose formed by the hydrolysis of starch is also used by *Endomycopsis*. Glucoamylase is marketed in liquid form. The active enzyme protein in commercial preparations is about 5%. The products contain small amounts of other enzymes, such as α-amylase, protease and cellulase (Nigam and Singh, 1995).

2.10.4 HETEROLOGOUS EXPRESSION OF α-AMYLASE GENES IN YEAST

A number of strategies have been adopted for the construction of starch-utilising systems, including the addition of amylolytic enzymes in culture broth and the introduction of heterologous genes encoding amylases into yeast cells for the secretive production of the enzymes. A bifunctional enzyme, having both α-amylase and glucoamylase activities may be extremely usefull because it could eliminate the process and period of liquefaction and saccharification, and reduce the overall consumption of amylases. Shibuya *et al.* (1992) constructed an α-amylase/glucoamylase fusion gene by joining cDNAs encoding α-amylase and glucoamylase from *Aspergillus shirousamii* and then expressed it in *S. cerevisiae* under the control of the *ADH1* promoter. The fusion gene had higher raw-starch-digesting activity than that of the original α-amylase and glucoamylase, and absorbed onto starch like the glucoamylase (Shibuya *et al.*, 1992). A novel way of overproducing amylolytic enzymes is by targeting α-amylase and glucoamylase to the cell wall of *S. cerevisiae* (Murai *et al.*, 1997, 1999). Genetic engineering techniques employing a yeast adhesion molecule, α-agglutin, which has a glycosylphosphatidylinositol (GPI) anchor as a transient membrane on the cell surface, anchored the *Rhizopus oryzae* glucoamylase and the *Bacillus stearothermophilus* α-amylase to the *S. cerevisiae* cell wall. In this system, the cell surface of the yeast was used as a carrier for immobilising the

enzymes that were expressed, and the living whole cells were remade as "cell biocatalists". Targeting recombinant proteins to the cell surface of *S. cerevisiae* offers several advantages: as *S. cerevisiae* is widely used in the industrial production of proteins and chemicals, enzyme-coated yeast surface cells could be used as novel whole-cell biocatalists, because surface-expressed proteins are covalently linked to glucan in the cell wall, rendering them resistant to extraction; and as *S. cerevisiae* is safe for oral use, it can be used in food and pharmaceutical products and for oral vaccines.

2.11 CONCLUSION

Whisky is a very complex, traditional alcoholic beverage derived from the processing of selected natural materials (Palmer, 1998). The chemical composition of each whisky is determined by the use of specified natural raw materials and processing parameters that do not display observable changes. Therefore, Scotch whisky is produced by the traditional processing of natural materials rather than by the chemistry of manufacturing (Palmer, 1998). In the malt whisky process, the fermentation is carried out by yeast on a malt wort substrate. Malt wort consists of maltose, maltotriose, glucose and dextrins (Klaassen *et al.*, 1996). In a malt fermentation, yeast ferments sucrose, glucose, fructose and maltose, leaving the larger dextrins unfermented. In addition, maltotriose is fermented incompletely. Research has been done on the construction of amylolytic yeasts for better utilisation of the starch-rich substrates used for whisky production. Klaassen *et al.* (1996) did a study on malt whisky fermentations, using yeast strains expressing glucoamylase. They found better utilisation of maltotriose with the strain expressing the glucoamylase gene than with the control strains. These results showed that the use of recombinant strains of *S. cerevisiae*, expressing a amylolytic enzyme will be of a tremendous advantage for the whisky industry

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CHAPTER 3

RESEARCH RESULTS

Cloning and characterisation of a cDNA and genomic copy of a second α-amylase gene (*LKA2*) from *Lipomyces kononenkoae* IGC40552B and its expression in *Saccharomyces cerevisiae*

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RESEARCH RESULTS

Cloning and characterisation of a second α-amylase gene (*LKA2*) from *Lipomyces kononenkoae* IGC4052B and its expression in *Saccharomyces cerevisiae*

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ABSTRACT

Lipomyces kononenkoae secretes a battery of highly effective amylases (*i.e.* α-amylase, glucoamylase, isoamylase and cyclomaltodextrin glucanotransferase activities) and is therefore considered as one of the most efficient raw-starch-degrading yeasts known. Previously, we have cloned and characterised genomic and cDNA copies of the *LKA1* α-amylase gene from *L. kononenkoae* IGC4052B (CBS5608T) and expressed them in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Here we report on the cloning and characterisation of the genomic and cDNA copies of a second α-amylase gene (*LKA2*) from the same strain of *L. kononenkoae*. *LKA2* was cloned initially as a 1663-bp cDNA harbouring an open reading frame (ORF) of 1496 nucleotides. Sequence analysis of *LKA2* revealed that this ORF encodes a protein (Lka2p) of 499 amino acids, with a predicted molecular weight of 55307 Da. The *LKA2*–encoded α-amylase showed significant homology to several bacterial cyclomaltodextrin glucanotransferases and also to the α-amylases of *Aspergillus nidulans*, *Debaryomyces occidentalis*, *Saccharomycopsis fibuligera* and *S. pombe*. When *LKA2* was expressed under the control of the phosphoglycerate kinase gene promoter ($PGK1_p$) in *S. cerevisiae*, it was found that the genomic copy contained a 55-bp intron that impaired the production of biologically active Lka2p in the heterologous host. In contrast to the genomic copy, the expression of the cDNA construct of $PGK1_p$-*LKA2* in *S. cerevisiae* resulted in the production of biologically active α-amylase. The *LKA2*-encoded α-amylase produced by *S. cerevisiae* exhibited a high specificity towards substrates containing α-1,4 glucosidic linkages. The optimum pH of Lka2p was found to be 3.5 and the optimum temperature was 60°C. Besides *LKA1*, *LKA2* is only the second *L. kononenkoae* gene ever cloned and expressed in *S. cerevisiae*. The successful expression of these two genes encoding these highly

efficient α-amylases paves the way for the development of amylolytic strains of *S. cerevisiae* for the efficient bioconversion of starch into commercially important commodities.

3.1 INTRODUCTION

Lipomyces is one of the most effective starch-degrading yeasts known (Horn *et al.*, 1988). This lipid-forming yeast hydrolyses starch completely due to the secretion of α-amylase, glucoamylase (Spencer-Martins and Van Uden, 1979), isoamylase (Spencer-Martins, 1982) and cyclodextrin glucanotransferase (Spencer-Martins, 1984) activities. Furthermore, of 81 starch-assimilating yeasts evaluated, the highest biomass production on starch medium was obtained with strains of *Lipomyces kononenkoae* and *Lipomyces starkeyi* (Spencer-Martins and Van Uden, 1977). When the efficiency of starch degradation was compared between *Lipomyces* strains and various *Schwanniomyces* strains, it was found that *L. kononenkoae* IGC4052B (CBS5608T) was the most efficient starch-degrading yeast among those screened (Horn *et al.*, 1988). In this mutant strain, amylase production is not repressed by glucose (Van Uden *et al.*, 1980). There is conflicting biochemical evidence for some of the properties of the purified α-amylase from *L. kononenkoae* IGC4052B. For example, three independent groups have purified an α-amylase, reporting molecular weights of 76 kD (Steyn and Pretorius, 1995; Prieto *et al.*, 1995) and 38 kD (Spencer-Martins and Van Uden, 1977). Differences in purification protocols and matrixes may partially explain some of the differences in molecular weight. However, the optimum temperature of 70°C for the α-amylase purified by Prieto *et al.* (1995) is in stark contrast with the optimum temperature of 40°C reported by Steyn and Pretorius (1995) and Spencer-Martins and Van Uden (1979). NH_2-terminal sequencing of the α-amylase purified by Steyn and Pretorius (1995) and the translation of a cloned α-amylase gene (*LKA1*) demonstrated that Lka1p is indeed the gene product of *LKA1*. The amino acid composition of the α-amylase purified by Prieto *et al.* (1995) differs significantly from that of Lka1p, ruling out the possibility that this α-amylase is Lka1p. Additional differences between α-amylases purified from *L. kononenkoae* IGC4052B are the optimum pH (Steyn *et al.* 1995: pH 4.0; Prieto *et al.*, 1995: pH 4.5-5; Spencer-Martins and Van Uden, 1979: pH 5.5) and pI (Steyn and Pretorius, 1995: pI 4.17; Prieto *et al.*, 1995: pI 3.5; Spencer-Martins and Van Uden, 1979: pI 7.1). Dissimilarities in amylase enzyme properties are not limited to the α-amylase produced by this yeast. For example, during purification of the α-amylase, Steyn and Pretorius (1995) observed two glucoamylase bands of M_r 100-200 kD. Subsequently, Chun *et al.* (1995) reported the purification of two glycosylated glucoamylases, with a M_r of 150 kD (GI) and 128 kD (GII) respectively, from *L. kononenkoae* IGC4052B. These data are in contrast to the predicted M_r of 81.5 for the glucoamylase of *L. kononenkoae* IGC4052, reported by Spencer-Martins

and Van Uden (1979). Taken together, these discrepancies highlight the need for a thorough molecular investigation of the properties of the purified amylases produced by *Lipomyces* spp.

Based on the conflicting data reviewed above, the purpose of this study was to screen a *L. kononenkoae* IGC4052B cDNA expression library in *S. cerevisiae* for other potential α-amylase-encoding genes. In this study, we describe the cloning and characterisation of a cDNA and genomic copy of a second *L. kononenkoae* α-amylase-encoding gene (*LKA2*). The *LKA2* gene was also expressed in *S. cerevisiae*. A comparison of the deduced primary sequences of amylase proteins is also presented.

3.2 MATERIALS AND METHODS

3.2.1 MICROBIAL STRAINS, PLASMIDS, MEDIA AND ENZYME ASSAYS

The microbial strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* was cultured on synthetic dextrose (SD), synthetic complete (SC) and YPD media (Rose *et al.*, 1990). YNBS medium (0.67% yeast nitrogen base without amino acids, 1.5% starch, 1% $NaH_2PO_4H_2O$, 0.4% $Na_2HPO_42H_2O$, 0.3% peptone, 0.1% yeast extract) was used to grow *L. kononenkoae* routinely. *S. cerevisiae* transformants were selected on $PHSC^{-Ura}$ or $PHSS^{-Ura}$ (PHSC refers to SC containing 0.4% Phadebas starch and lacking uracil, whereas PHSS contained 1% Phadebas starch instead of 2% glucose as a carbon source). Plates were incubated at 30°C for 2-6 days and the digestion of starch was indicated by the appearance of blue halos surrounding the colonies (Steyn and Pretorius, 1995). α-Amylase activity was quantitatively measured using the Phadebas Amylase Test (Pharmacia Diagnostics, Uppsala, Sweden) assay (Steyn and Pretorius, 1995). The saccharolytic activity of α-amylase was assayed by the dinitrosalicylic acid (DNS) method (Miller *et al.*, 1960). The standard reaction mixture, containing 700 µl of substrate, 100 µl of enzyme solution and 200 µl of buffer, was incubated at 30°C for 60 min. The reaction was stopped with 1.5 ml of DNS reagent and boiled for 15 min. The contents of the assay tubes were diluted with 5 ml of distilled water and absorbancy readings (A_{540}) were taken at 540 nm in a Beckman DU-5 spectrofotometer. Various substrates were assayed: soluble starch (Lintner), dextrin, amylose, potato starch, amylopectin, glycogen (oyster) and pullulan. Polysaccharide substrates (0.5%) were heated on a magnetic stirrer prior to use in enzyme assays to facilitate their dissolution. For the determination of substrate specificity, the assay was carried out at optimum pH and temperature. The effect of pH on enzyme activity was determined by the DNS assay using various buffers. Citrate phosphate was used for the pH range 3.0-5.0, while Tris-HCl was used for the pH range 7.0-8.0. The DNS assay was also used to determine the effect of temperature on the enzyme activity. The reaction mixtures were incubated at

temperatures ranging between 20 and 80°C. These incubations were buffered at the pH of the highest enzyme activity determined as described above. For the determination of the optimum pH and temperature, 0.5% of soluble starch (Lintner) was used as a substrate in the reaction mixture. *Escherichia coli* strains were routinely cultured on LB with 50 μg/ml ampicilin. Plasmid DNA was isolated using the Qiagen plasmid kit (Qiagen).

3.2.2 DNA SEQUENCING, AMPLIFICATION AND TRANSFORMATION

Bi-directional sequencing was performed using the Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and analysed on an Applied Biosystems Model 373A DNA sequencing system. Oligodeoxyribonucleotides (20-30 nucleotides) were synthesised by the phosphoramidate method on an Applied Biosystems Model 394 DNA/RNA apparatus. DNA was amplified using Expand™ High Fidelity mix (Boehringer Mannheim, Indianapolis, USA) according to the manufacturer's instructions. PCR products were purified by phenol/chloroform extraction and ethanol precipitation. Restriction enzyme digestion of the PCR products was performed at 37°C for 14 h. The following oligodeoxyribonucleotides were used as primers: LKA2-F 5'-GC*GAATTC*ATGCGGTTAAATCTCAAAC-3' and LKA2-R 5'-GCCTCGAGTTAAGAACAAAATTTCCCAG-3', for PCR amplification of the genomic copy of *LKA2* (*LKA2*g). Both primers contained restriction endonuclease sites for *Eco*RI (italicised) and *Xho*I (underlined). *S. cerevisiae* was transformed using the method described by Gietz *et al.* (1992). *E. coli* DH5α was prepared according to Dower *et al.* (1988) and electrotransformed using a Bio-Rad Gene Pulsar Apparatus (Bio-Rad, Richmond, CA, USA). The isolation of plasmid DNA from the yeast transformants was done according to Adam and Polaina (1991). The nucleotide sequence of the *LKA2* gene was deposited in Gene Bank AF443872.

3.2.3 CONSTRUCTION OF LIBRARY AND EXPRESSION VECTORS

A genomic library of *L. kononenkoae* IGC4052B was constructed in cosmid pYC1 (Hohn and Collins, 1980), as described before (Steyn *et al.*, 1996). High molecular weight DNA was isolated from *L. kononenkoae* IGC4052B according to Steyn and Pretorius (1995). Plasmid pJUL3 was constructed by cloning the *Eco*RI-*Xho*I fragment of pJUL1 into *Eco*RI-*Xho*I-digested pJC1, thereby positioning the *LKA2* cDNA fragment between the promoter and terminator elements of the *PGK1* gene. Oligonucleotides LKA2-F and LKA2-R were used to amplify *LKA2*g from genomic cosmid pools (Steyn and Pretorius, 1995). The digestion of the genomic PCR products with *Eco*RI and *Xho*I and the subsequent cloning into plasmid pJC1 resulted in the plasmid pJUL2.

Table 1.
Microbial strains and plasmids used in this study

Strains and plasmids	Relevant genotype/description	Source/References
E. coli		
DH5αF'	F'/endA1 hsdR17($r_k^-m_k^+$)supE44 thi-1 recA1 gyrA *(Nal')*relA1 Δ(lacIZYA-argF)U169 deoR *(80*dlacΔ(lacZ)M15)	BRL
S. cerevisiae		
Σ1278b	*MATa/MATα URA3*	Liu *et al.*, 1993
Σ1278b-pJUL3	Σ1278b transformed by pJUL3 episomal plasmid	Eksteen *et al.*, 2002
YPH259	*MATα ura3 lys2 ade2 his3 leu2*	Sikorski and Hieter, 1989
Plasmids		
pBluescript KS$^+$	Ap^R	Stratagene
pJC1	Ap^R $PGK1_{P-T}$ *URA3* 2μ	Crous *et al.*, 1995
pJUL1	pBluescript KS$^+$ with *Eco*RI-*Xho*I containing *LKA2c*	This work
pJUL2	pJC1 containing *LKA2g*	This work
pJUL3	pJC1 containing *LKA2c* gene	This work

3.3 RESULTS AND DISCUSSION

3.3.1 CLONING OF A cDNA COPY OF *LKA2*

The production of α-amylase, glucoamylase and isoamylase by *L. kononnenkoae* IGC4052 is subject to carbon catabolite repression (Van Uden *et al.*, 1980). Currently, our research efforts are focused on the glucose derepressed mutant strain IGC4052T, which produces large quantities of α-amylase in media containing glucose or starch as the carbon source (Van Uden *et al.*, 1980). Previously, our laboratory reported the purification and characterisation of an α-amylase from *L. kononenkoae* IGC4052B (Steyn and Pretorius, 1995), identified the α-amylase-encoding gene *LKA1* (Steyn *et al.*, 1995), and studied the expression of a cDNA and genomic copy of *LKA1* in *S. cerevisiae* and *Schizosaccharomyces pombe* (Steyn *et al.*, 1996). To investigate the presence of a second putative amylase, we transformed plasmid cDNA library pools into *S. cerevisiae* YPH259 and screened them on PHSC^{-Ura} plates for halo-producing colonies. The use of

Phadebas tablets in minimal medium enabled us to easily distinguish between glucoamylase- and α-amylase-secreting cells (Steyn and Pretorius, 1995). We obtained a single *S. cerevisiae* clone (*LKA2*) showing a distinctly small blue halo on PHSC^{-Ura} medium, which is in contrast to the other large, halo-producing clones. Free plasmid DNA was isolated from these transformants and transformed into *E. coli* DH5α. Plasmids isolated from the bacterial clones were reintroduced into strain YPH259 to validate the secretion of α-amylase on PHSC^{-Ura} and starch plates. Plasmids isolated from halo-producing yeast clones were digested with *Xho*I and *Eco*RI and the inserts were cloned into pBluescript KS (Stratagene) for sequence analysis. The sequence results showed that the translated cDNA insert is not identical to *LKA1* and that it showed homology to a large number of α–amylases present in bacteria, yeast and fungi (see below). Sequence analysis of the inserts obtained from two large halo-producing clones identified cDNA fragments containing the *LKA1* open reading frames (ORFs).

We conclude that we identified a second novel *L. kononenkoae* α-amylase-encoding gene, *LKA2*, which can be expressed and produced in *S. cerevisiae* in a biologically active form.

3.3.2 NUCLEOTIDE SEQUENCE OF *LKA2*

Restriction analysis of a plasmid pUS1 obtained from an α-amylase-producing *S. cerevisiae* transformant showed the presence of a cDNA fragment with an approximate size of 1.7 kb. The nucleotide sequence of the 1663-bp cDNA contained a single ORF of 1497 nucleotides and the translated amino acid sequence showed homology to α-amylases from a variety of organisms. We designated this α-amylase-encoding gene *LKA2*. The nucleotide sequence of the 1.66-kb *Eco*RI-*Xho*I fragment is presented in Fig. 1. The *LKA2* ORF starts with an ATG start codon at position 1 and ends with a TAA stop codon at position 500, followed by a poly(A) region. We assumed that translation initiation occurs at the first in-frame AUG at the 5' end of the mRNA encoded by the cDNA fragment, as proposed by Kozak (2001). The nucleotide sequence context of the *LKA2* translational start signal, GCTCTGATGCGG, does not resemble the yeast consensus sequence, WAMAMAAUGUCY, for highly-expressed genes (Hamilton *et al.*, 1987). However, it does resemble the eukaryote consensus sequence, TCATCATGCG (Kozak, 2001). The TAA termination signal at position 1497 of the *LKA2* ORF is followed by several stop codons that are both in and out of frame. The *LKA2* 3' non-coding region contains sequences similar to the TAG and TA(T)GT sequences that are believed to play a role in the termination and polyadenylation of yeast genes (Zaret and Sherman, 1982). These include the TAG, TAGT and TACG sequences that are present 9 to 55 nucleotides downstream from the stop codon (Fig. 1).

The coding region of *LKA2* has a G+C content of 47.47%, which is similar to that of the total DNA (48.3%) from *Lipomyces* spp. (Kurtzman, 1984). The codon usage of the *LKA2* gene indicates that the most abundant amino acids are Leu (8.82%), Val (8.02%),

Ala (8.02%), and Ser (7.41%). The amino acid composition of the α-amylase purified from *L. kononenkoae* by Prieto *et al.* (1995), namely 25.3% (w/w) of Thr, 14.3% (w/w) of Ser, 0.2% (w/w) of Lys and 0.8% (w/w) of Tyr, differs significantly from that of *LKA2*, which strongly suggests that these α-amylases are dissimilar.

To identify a putative signal peptide, we used the programs SIGNALP (web) and PSORT (web). Both programmes identified cleavage of a presumed signal peptide between A^{23} and K^{24} of Lka2p. The calculated M_r of the unmodified Lka2p (499 amino acids) precursor is 55.3 kDa. Processing of the first 23 amino acids of Lka2p during secretion gives rise to a 52.7-kDa (476 amino acids) mature protein with a theoretical pI of 4.71. In contrast, the calculated M_r of mature Lka1p is 65.7 kDa (596 amino acids), with an experimentally determined pI of 4.17 (Steyn *et al.*, 1995; Steyn and Pretorius 1995). It is interesting to note that Lka2p is approximately 100 amino acids shorter than Lka1p, which might suggest a different biological role. Lka2p contains seven putative glycosylation sites (N^{46}-L-T, N^{92}-T-T, N^{162}-T-S, N^{177}-Q-S, N^{254}-Q-S, N^{378}-T-S, N^{473}-G-T), whereas Lka1p contains only two, which are similar to that of the *Schwanniomyces occidentalis amylases SWA2* (Claros *et al.*, 1993) and *AMY1* (Strasser *et al.*, 1989). The α-amylase from *Saccharomycopsis fibuligera* (ALP) contains only one potential glycosylation site (Itoh *et al.*, 1987). Intriguingly, Lka2p contains six Cisteine residues, which potentially could form disulphide bonds when secreted or when translocated to the cell wall (Fig.1). A sulphated tyrosine signature is present at position 291 (Fig.1).

We conclude that the translated amino acid sequence of Lka2p differs significantly from that of Lka1p and agrees with previously conflicting data (Chun *et al.*, 1995; Steyn and Pretorius, 1995), thus providing clear evidence for the presence of a second α-amylase in *L. kononenkoae*.

3.3.3 CLONING OF A GENOMIC COPY OF *LKA2*

The transformation of *S. cerevisiae* YPH259 with pJUL2 containing the genomic copy of *LKA2* (*LKA2*g) did not result in halo formation on $PHSC^{-Ura}$ plates, suggesting that biologically active α-amylase was not synthesised in *S. cerevisiae*. We therefore concluded that *LKA2* might contain an intron, as does *LKA1* (Steyn and Pretorius, 1995). PCR amplification using primers designed from the cDNA sequence of *LKA2* (LKA2-F, -R), and cosmid DNA prepared from genomic DNA (Steyn and Pretorius, 1995), revealed the presence of the expected size band of ca. 1.7 kb in several cosmid pools (results not shown). Subsequent cloning and sequencing of *LKA2g* confirmed that the amplified PCR product was indeed the genomic copy of *LKA2* and revealed the presence of a 55-bp intron between nucleotide positions 304 and 305 of *LKA2*: 5'-*GTTAGTATT*GATACATATTCATTTGACTACCGTCTGGCTGACAAAAACT**TAATAG**-3'. The position of the intron and the donor, acceptor and splicing sites of *LKA2* differ from those of *LKA1*. The *LKA2* intron is 8 bp shorter than the *LKA1* intron of 63 bp and

contains a donor consensus sequence (italicised) that is similar to that of *S. pombe* (GTAA/TGT) (Prabhala *et al.*, 1992). However, the branch site (underlined) is identical to the *LKA1* sequence and correlates with the common branch motif shared by mammals and *S. cerevisiae* (T/AAYTRAY) (Zhang and Marr, 1994). The 3'-splicing site of the *LKA2* intron (bold) resembles the *S. pombe* splicing sequence (T/ATAG).

According to our knowledge, this is the second report of a yeast α-amylase containing an intron and, more specifically, its occurrence in the Lipomycetaceae family could contribute significantly to our understanding of the mechanisms of gene regulation in this family.

3.3.4 COMPARISON

Sequence analyses were performed using the GCG programme package (version 8) of the Genetics Computer Group, Madison, WI, USA (Devereux *et al.*, 1984). Using GAP and BESTFIT to compare *LKA1* with *LKA2*, we observed an identity and similarity of 35 and 39% respectively. A Gap-BLAST search revealed that mature *LKA2* shows homology to fungal (*Aspergillus nidulans*: 36 % identity), yeast (*Debaryomyces occidentalis*, *S. fibuligera*, *S. pombe, Cryptococcus* sp. S-2: 34 %, 34 %, 33 % and 38 % identity, respectively) and bacterial (*Thermoactinomyces vulgaris*: 31 % identity) α-amylases. In addition, we found significant similarities to bacterial cyclodextrin glycosyltransferases (*Thermoanaerobacterium thermosulfurigenes* and *Bacillus licheniformis*: 30 % and 33 % identity, respectively), which hydrolyse non-reducing cyclic oligodextrins (Fig. 2). With homologous amylases, residues and conserved regions involved in substrate and Ca^{2+} binding are indicated (Buisson *et al.* 1987, Itoh *et al.* 1987, Claros *et al.*, 1993). Janse *et al.* (1993) and Chiba (1997) provided an extensive list of four conserved regions, A, B, B' and C that are present in the catalytic domain of α-amylases, cyclodextrin glucanotransferases, maltases, pullulanases, isoamylases, oligo-1,6-α-glucosidases, and branching enzymes. Lka2p contains the conserved residues ^{137}DVVINH^{142}, ^{223}GIRLDTARH^{231}, ^{251}EALN^{254} and ^{312}FLDNQD^{317} in regions A, B, B' and C, respectively. However, two residues in these regions of Lka2p differ from the conserved residues present in a wide spectrum of α-amylases (Fig. 2). The most significant differences are the presence of L^{226} (region B), A^{252} (region B'), D^{314} and Q^{316} (region C), which potentially could influence catalytic activity and substrate binding and/or specificity. Holm *et al.* (1990) reported that mutating K^{237} (corresponding to R^{230} of *LKA2*) to R in region B of the *B. stearothermophilus* α-amylase had no effect on enzyme activity. However, this conclusion was based solely on estimations of halos on starch plates and did not directly address the catalytic effect or substrate affinity as a result of the changes. Matsui *et al.* (1992) altered K^{210} (corresponding to R^{230} in region B of *LKA2*) to R or N in the active site of the *S. fibuligera* α-amylase and reported a three-fold enhancement of short substrate-specific (maltose) catalytic activity of the mutant enzyme ($K^{210}\rightarrow$R). Based upon this observation and the homology (38%) between Lka2p and the *S. fibuligera* Alp1p, it is

tempting to speculate that Lka2p might have a high affinity for short substrates. Holm *et al.* (1990) suggested that W^{266} (corresponding to L^{253} in region B' of Lka2p) is in contact with the docked substrate and corresponds to Trp^{206} in the barley α-amylase, which has been shown to be essential for enzymatic function (Svensson *et al.*, 1987). The uncharged residue Q is present in region C of both Lka1p (Q^{443}) and Lka2p (Q^{318}) in the place of the highly conserved, charged H, which is involved in substrate binding (Kuriki and Okada, 1995). Søgaard *et al.* (1993) have shown that the corresponding residue, H^{290}, in barley α-amylase I is involved in the stabilisation of the transition state.

We conclude that the amino acid alterations in the conserved regions of Lka2p most probably affect the catalytic activity and/or substrate binding of Lka2p and needs to be investigated in greater detail.

3.3.5 PROPERTIES OF α-AMYLASE EXPRESSED BY *S. CEREVISIAE* TRANSFORMANTS

We tested the ability of the non-amylolytic yeast *S. cerevisiae* Σ1278b to secrete active α-amylase when transformed with pJUL3. Σ1278b transformants were able to form blue halos on $PHSC^{-Ura}$ plates within 96 h, indicating the stable expression of the *L. kononenkoae* IGC 4052B *LKA2* gene under control of the *PGK1* promoter, and effective secretion of the encoded gene product by *S. cerevisiae* Σ1278b. The amylase activity of the transformed *S. cerevisiae* strain was primarily cell wall associated. Intracellular extracts of Σ1278b-pJUL3 were used in the determination of the Lka2p activity at different temperatures. Intracellular protein was isolated as described in Eksteen *et al.* (2002). *LKA2* activity using the DNS assay was measured between 20°C and 80°C (Fig. 3A). The optimum temperature for Lka2p was 60°C, indicating a specific activity (specific activity refers to 1.0 U/mg). At temperatures below 55°C, Lka2p activity decreased gradually, whereas incubation at temperatures over 65°C drastically decreased Lka2p activity. At 30°C and 40°C, the activity was 45% and 50% of the maximum, respectively. Fig. 3B shows that the optimum pH is 3.5. At pH 6, the activity was reduced by almost 20%, while the activity at pH 4 was reduced to 85% of the maximum. The substrate specificity of the amylolytic enzyme was determined using a number of different glucose polymers, containing either α-1,4 glucosidic linkages or a mixture of α-1,4 and α-1,6 glucosidic linkages. The enzyme showed high reactivity towards soluble starch (Lintner), dextrin and amylose (Table 2), but only small amounts of reducing sugars were liberated from amylopectin, glycogen and pullulan. The reaction specificity of Lka2p is very similar to that of an α-amylase with associated dextranase activity and several bacteria and filamentous fungi do produce such enzymes (Hamilton *et al.*, 2000).

We conclude that the amylolytic capability of *L. kononenkoae* depends on the reaction specificity of Lka1p and Lka2p. Lka2p, with poor side pullulanase and glucoamylase activity, have great dextranase activity, thus complementing Lka1p which have better side pullulanase and glucoamylase activity.

```
  1 EcoRI                  M  R  L  N  L  K  Q  Q  A  L  L  G  L
-22 GAATTCTGTT GAATCGGCTC TGATGCGGTT AAATCTCAAA CAACAGGCCC TGTTGGGTTT     38

 14  L  A  T  F  V  T  N  V  Q  A  K  T  A  A  E  W  K  E  L  S
     GCTTGCCACC TTCGTTACCA ACGTGCAGGC CAAGACCGCG GCAGAATGGA AAGAGCTTTC   98

 34     I  Y  Q  V  I  T  D  R  F  A  T  T [N  L  T] A  P  D  C  W
     CATTTATCAA GTCATAACCG ATCGTTTCGC TACAACCAAT CTCACTGCTC CCGATTGTTG  158

 54     I  R  A  Y  C  G  G  T  W  K  G  L  E  R  K  L  D  Y  I  Q
     GATCAGAGCC TACTGTGGTG GGACATGGAA AGGTTTGGAA AGGAAACTTG ACTACATCCA  218

 74     N  M  G  F  D  A  V  W  I  S  P  V  I  H  N  I  E  V [N  T]
     AAATATGGGA TTTGATGCGG TTTGGATCTC TCCGGTCATT CACAATATTG AGGTAAACAC  278

 94  [T] W  G  F  A  F  H  G  Y  W  G  D  D  P  Y  R  L  N  E  H
     GACTTGGGGA TTTGCCTTTC ATGGATATTG GGGAGATGAC CCTTACAGAC TCAACGAACA  338

114     F  G  T  A  A  D  L  K  S  L  S  D  S  L  H  A  R  G  M  S
     TTTCGGTACT GCAGCCGACC TGAAGAGTCT CTCGGATTCT CTTCACGCCC GCGGAATGTC  398

134     L  M  V  D  V  V  I  N  H  L  A  S  Y  T  L  P  Q  D  V  D
     TCTCATGGTG GATGTTGTAA TTAACCACCT TGCATCATAT ACGTTGCCGC AAGATGTGGA  458

154     Y  S  L  Y  P  A  P  F [N  T  S] S  A  F  H  Q  P  C  P  I
     TTACAGCTTA TATCCGGCTC CGTTCAATAC TTCTAGCGCT TTCCACCAGC CCTGTCCAAT  518

174     D  F  S [N  Q  S] S  I  E  D  C  W  L  V  T  E  P  A  P  A
     CGATTTCTCT AATCAGTCAT CTATCGAAGA TTGTTGGCTT GTAACCGAGC CTGCGCCCGC  578

194     L  V  D  L  K  N  E  D  Q  V  I  L  D  A  L  I  N  S  V  V
     ACTTGTGGAT CTGAAAAATG AAGATCAAGT CATTCTGGAT GCCTTGATTA ACAGCGTGGT  638

214     D  L  V  E  T  Y  D  I  D  G  I  R  L  D  T  A  R  H  V  P
     TGATCTTGTC GAAACATATG ATATTGATGG CATCCGTCTC GACACGGCCC GCCATGTTCC  698

234     K  P  S  L  A  K  F  Q  E  K  V  G  V  F  V  T  G  E  A  L
     GAAGCCGTCG CTTGCGAAGT TTCAGGAGAA GGTCGGGGTT TTCGTTACTG GTGAGGCACT  758

254 [N  Q  S] V  P  Y  V  A  Q  Y  Q  G  P  L  N  S  A  I  N  Y
     CAACCAGAGT GTTCCATACG TGGCCCAATA TCAGGGTCCG CTCAATAGTG CGATTAATTA  818

274     P  L  W  Y  A  L  V  D  S  F  M  G  R  T  T  F  D  Y  L  E
     TCCGCTTTGG TACGCCTTGG TGGACAGCTT TATGGGTCGG ACCACATTCG ACTATCTGGA  878

294     S  V  V  K  S  E  Q  A  T  F  S  D  A  H  A  L  T  N  F  L
     ATCTGTTGTC AAGAGCGAAC AGGCAACTTT TTCTGATGCC CACGCGCTAA CAAATTTCCT  938

314     D  N  Q  D  Q  P  R  F  A  S  Y  L  G  D  G  N  G  D  D  V
     CGACAATCAA GATCAGCCTC GATTTGCCAG CTATTTAGGA GATGGTAATG GTGACGATGT  998

334     L  R  D  E  N  A  A  T  F  L  F  F  V  S  G  I  P  V  I  Y
     CTTACGGGAT GAGAATGCTG CTACTTTTCT TTTCTTTGTG AGCGGAATCC CGGTTATTTA 1058

354     Y  G  F  E  Q  R  F  D  G  G  F  D  P  V  N  R  E  P  M  W
     CTACGGTTTT GAACAGCGGT TTGATGGCGG CTTTGATCCC GTAAACAGAG AGCCAATGTG 1118

374     T  S  G  Y [N  T  S] T  P  L  Y  N  Y  L  A  R  L  N  A  I
     GACATCTGGC TACAATACAA GTACTCCCTT GTACAACTAT CTTGCTCGGC TTAATGCAAT 1178

394     R  K  Y  A  A  S  I  T  G  T  Q  V  F  Y  S  D  D  T  V  F
     CCGAAAGTAT GCTGCTTCCA TCACTGGCAC GCAAGTATTC TACTCGGACG ATACAGTTTT 1238

414     L  G  S  G  V  S  H  M  A  M  Q  R  G  P  L  V  I  V  L  T
     CTTGGGCTCT GGAGTGAGTC ATATGGCTAT GCAACGCGGA CCTTTGGTTA TTGTACTTAC 1298

434     N  V  G  Q  H  I  I  D  N  T  G  Y  T  V  T  G  S  Q  F  S
     AAACGTGGGC CAGCACATTA TCGACAACAC TGGCTATACC GTGACCGGTT CCCAGTTTAG 1358

454     A  G  D  S  L  T  D  L  V  S  C  T  K  V  K  V  V  G  A [N]
     TGCCGGGGAT TCATTGACAG ACTTGGTCAG CTGCACCAAG GTCAAAGTAG TCGGCGCCAA 1418

474 [G  T] F  T  S  P  S  N  G  G  K  A  R  I  W  I  K  S  K  Y
     CGGCACATTT ACTAGCCCAA GTAATGGCGG CAAAGCAAGG ATATGGATTA AGTCCAAGTA 1478

494     A  G  K  F  C  S  *
     TGCTGGGAAA TTTTGTTCTT AAGGTAAAAT TAGAGATTAA CTTAGTTAAT TAACCTTCCT 1538
     GTTATTACTG TGTACGGGTA TCTCATGTCC ATGGGTATCT CATGTCCAAG CAAATATATG 1598
     CTCAGTCTTA TGCACTATAA AAAAAAAAAA AAAAAAACTC GAG                    1641
                                                 XhoI
```

Fig. 1. Complete sequence of the 1680-bp cDNA fragment containing the *LKA2* α-amylase gene from *Lipomyces kononenkoae*. The putative N terminus of the protein is indicated in italics. The underlined sequence indicates the potential termination signal. Cysteine residues are typed in bold. The predicted *N* glycosylation sites are boxed.

```
AMYA    1                                        MRSPLFLS FAATVLAATP   20
ALP1    1                          MQI SKAALLASLA ALVYAQPVTL FKR...ETNA  30
SWA2    1                        MKFAT ILSTTALALS SLVASKPIFL SKRDAGSSAA  35
LKA1  101  WELWTLSAIA AGAVEITGAS YVDSDTSVTY TTSLDLPLTT TSASVPTGTA  150
AMY2    1                              MKHNEVFG WTLKVLSFLL VVIPANALDK  28
LKA2    1                                MRLNLK QQALLGLLAT FVTNVQAKTA  26

AMYA   21  AEWRSQSIYF LLTDRFARTD NSTTAECDTS .AKYCGGTWQ GIINQLDYIQ   70
ALP1   31  DKWRSQSIYQ IVTDRFARTD GDTSASCNTE DRLYCGGSFQ GIIKKLDYIK   81
SWA2   36  AAWRSESIYQ LVTDRFARTD GSTSATCNTG DRVYCGGTFQ GIIDKLDYIQ   86
LKA1  151  ANWRGRSIYQ VVTDRFARTD GSITYSCDVT DRVYCGGSYR GIINMLDYIQ  200
AMY2   29  HGWRKQSIYS LLTDRFASTN ...PKPCNPE DREYCGGNWR GIIDKLDYIQ   76
LKA2   27  AEWKELSIYQ VITDRFATTN ...LTAPDCW IRAYCGGTWK GLERKLDYIQ   74

AMYA   71  GMGFTAIWIT PVTANLEDGQ .HGEAYHGYW QQDIYALNPH FGTQDDLRAL  120
ALP1   82  DMGFTAIWIS PVVENIPDNT AYGYAYHGYW MKNIYKINEN FGTADDLKSL  132
SWA2   87  GMGFTAIWIS PVVEQIPDDT GYGYAYHGYW MKDIYAINSN FGTADDLKNL  137
LKA1  201  GMGFTAIWIS PIVENIPDDT GYGYAYHGYW MKDIFALNTN FGGADDLIAL  251
AMY2   77  GMGFTAIWIS PIIKNIEGRT KYGEAYHGYW PQDLYTLNPH FGTEQDLIDL  127
LKA2   75  NMGFDAVWIS PVIHNIEVNT TWGFAFHGYW GDDPYRLNEH FGTAADLKSL  125
                                  ** **
                          A
AMYA  121  SDALHDRGMY LMVDVVANHF GYDAPAASVD YSAFNP.FNS ADYFHTPCDI  170
ALP1  133  AQELHDRDML LMVDIVTNHY GSDGSGDSID YSEYTP.FND QKYFHNYCLI  182
SWA2  138  SNELHKRNMK LMVDIVTNHY AWNGAGSSVA YSNYNP.FNQ QSYFHDYCLI  187
LKA1  252  ATELHNRGMY LMVDIVVNHF AFSGNHADVD YSEYFP.YSS QDYFHSFCWI  301
AMY2  128  ADALHDRGMY LMVDTVVNHM GSSDPR.NID YGIYRP.FNQ SSHYHPMCPI  176
LKA2  126  SDSLHARGMS LMVDVVINHL ASYTLPQDVD YSLYPAPFNT SSAFHQPCPI  176
                     °   °*

AMYA  171  TDYDNQTQVE DCWLYTD.AV SLPDVDTTNE EVKEIWYDWV GDLVSDYSID  220
ALP1  183  SNYDDQAQVQ SCWEGDS.SV ALPDLRTEDS DVASVFNSWV KDFVGNYSID  232
SWA2  188  TNYDDQTNVE DCWEGDN.TV SLPDLRTEDS DVSSIFNLWV AELVSNYSID  237
LKA1  302  TDYSNQTNVE ECWLGDD.SV PLVDVNTQLD TVKSEYQSWV KQLIANYSID  351
AMY2  177  E.QDKPLSLE QCWIGTE.DM TLPDIDTENP QIIETLYNFI HDQVKQFKID  225
LKA2  177  D.FSNQSSIE DCWLVTEPAP ALVDLKNEDQ VILDALINSV VDLVETYDID  226
            *          °          * °
                B                    B'
AMYA  221  GLRIDTARHV QKDFWRDYND AAGVYCVGEV FQGDPDYTCG YQEVMDGVLN  271
ALP1  233  GLRIDSAKHV DQGFFPDFVS ASGVYSVGEV FQGDPAYTCP YQNYIPGVSN  283
SWA2  238  GLRIDSAKHV DESFYPSFQS AAGVYLLGEV YDGDPAYTCP YQNYMSGVTN  288
LKA1  352  GLRIDTVKHV QMDFWAPFQE AAGIYTVGEV FDGDPSYTCP YQENLDGVLN  402
AMY2  226  GLRVDATKHV RRTFWPGFCE SAGVYCQGEE WTGQADLFCE WQEYMDGLHN  276
LKA2  227  GIRLDTARHV PKPSLAKFQE KVGVFVTGEA LNQSVPYVAQ YQGPLNSAIN  277
              ▲  *°              *▲* *
                                                      C
AMYA  272  YPIYYPLLRA FSSTSG.SLS DLANMIETVK YTCSDATLLG NFIENHDNPR  321
ALP1  284  YPLYYPTTRF FKTTDS.SSS ELTQMISSVA SSCSDPTLLT NFVENHDNER  333
SWA2  289  YPLYYPMLRF FQGTSN.SVD ELNAMISSLE SDCKDITLLG NFIENHDQPR  338
LKA1  403  YPVYYPVVSA FQRVGG.SIS SLVDMIDTLK SECIDTTLLG SFLENQDNPR  452
AMY2  277  FPVQGVAAES VIPLNDRALR KTAIAMNLVA HHCKDSTLLG LFLESQDAPR  327
LKA2  278  YPLWYALVDS FMGRTT..FD YLESVVKSEQ ATFSDAHALT NFLDNQDQPR  326
           *                                          * *▲

AMYA  322  FASYTDDIS. ....LAKNVA AFVILSDGIP IIYAGQEQHY SGAGDPANRE  367
ALP1  334  FASMTSDQS. ....LISNAI AFVLLGDGIP VIYYGQEQGL SGKSDPNNRE  379
SWA2  339  LPSYTSDSA. ....LIKNAI AFNLMSDGIP IIYYGQEQGY SGSSDPNNRE  384
LKA1  453  FPSYTSDES. ....LIKNAI AFTILSDGIP IIYYGQEQGL NGGNDPYNRE  498
AMY2  328  LAALNNDYT. ....VLKNAM TLNLMSDGIP IVFYGQEQMF NGSHDPVNRP  373
LKA2  327  FASYLGDGNG DDVLRDENAA TFLFFVSGIP VIYYGFEQRF DGGFDPVNRE  377
                                                      *   *

AMYA  368  ATWLSGYDST SELYQFISKT NQIRNHAIWQ NET..YLSYK NYAIYNENNV  416
ALP1  380  ALWLSGYNKE SDYYKLIAKA NAARNAAVYQ DSS..YATSQ LSVIFSNDHV  428
SWA2  385  ALWLSGYSTS NGYYKLISSV NQIRNQAIYK DSK..YTTYW SDVLYASGHV  433
LKA1  499  ALWPTGYSTT STFYEYIASL NQIRNHAIYI DDT..YLTYQ NWVIYSDSTT  547
AMY2  374  ALWDQGYNTD GPLYQYTSKV NKIRRDLINS EDGEIYIRSI THAIMIGDHV  424
LKA2  378  PMWTSGYNTS TPLYNYLARL NAIRKYAASI TGTQVFYSDD TVFLGSGVSH  428

AMYA  417  LAMRKGFDGS QIITILTNAG ADAGS.STVS VPNTGFTAGA AVTEIYTCED  466
ALP1  429  IATKRGS... .VVSVFNNLG SSGSS..DVT ISNTGYSSGE DLVEVLTCST  474
SWA2  434  IALQRGADDQ RIVSVFNNLG SSGS...QTV TFSTKYSGGE KVVDVLTCQT  481
LKA1  548  IAMRKGFTGN QIITVLSNLG SSGSS.YTLT LSNTGYTASS VVYEILTCTA  597
AMY2  425  MVMYKGP... .VITFITNYG AVDK...EYL IK...MPGSE TMIDLLTCTL  465
LKA2  429  MAMQRGP... .LVIVLTNVG QHIIDNTGYT VTGSQFSAGD SLTDLVSCTK  475

AMYA  467  ITVSGSG.EV SVPMESGLPR VLYPKAKLEG SGICGL                 502
ALP1  475  VSGSSD...L QVSIQGGQPQ IFVPAKYASD ICS                    505
SWA2  482  SYANSDS.TL TVSISGGAPR IYAPASLIAN SGICNF                 517
LKA1  598  VTVDLSG.NL AVPMSGGLPR VFYPESQLVG SGICSM                 633
AMY2  466  IEVEGEV..M RTSIKKGEPK ILYPYQLAFR DGFCQEQITL QEIDDVFMGR  514
LKA2  476  VKVVGANGTF TSPSNGGKAR IWIKSKYAGK FCS                    509
```

Fig. 2. Alignment of the amino acid sequences of several amylases. The sequences of the mature proteins from *Aspergillus nidulans* (AMYA), *Saccharomycopsis fibuligera* (*ALP1*), *Debaromyces occidentalis* (*SWA2*), *Lipomyces kononenkoae* (*LKA1*), *Schizosaccharomyces pombe* (*AMY2*) and *Lipomyces kononenkoae* (*LKA2*) have been aligned by introducing gaps (.) to maximise the similarity. Bolds letters represent identical amino acids or conservative replacements. ▲, residues implicated in catalysis; *, residues for substrate binding; °, residues for Ca^{++} binding.

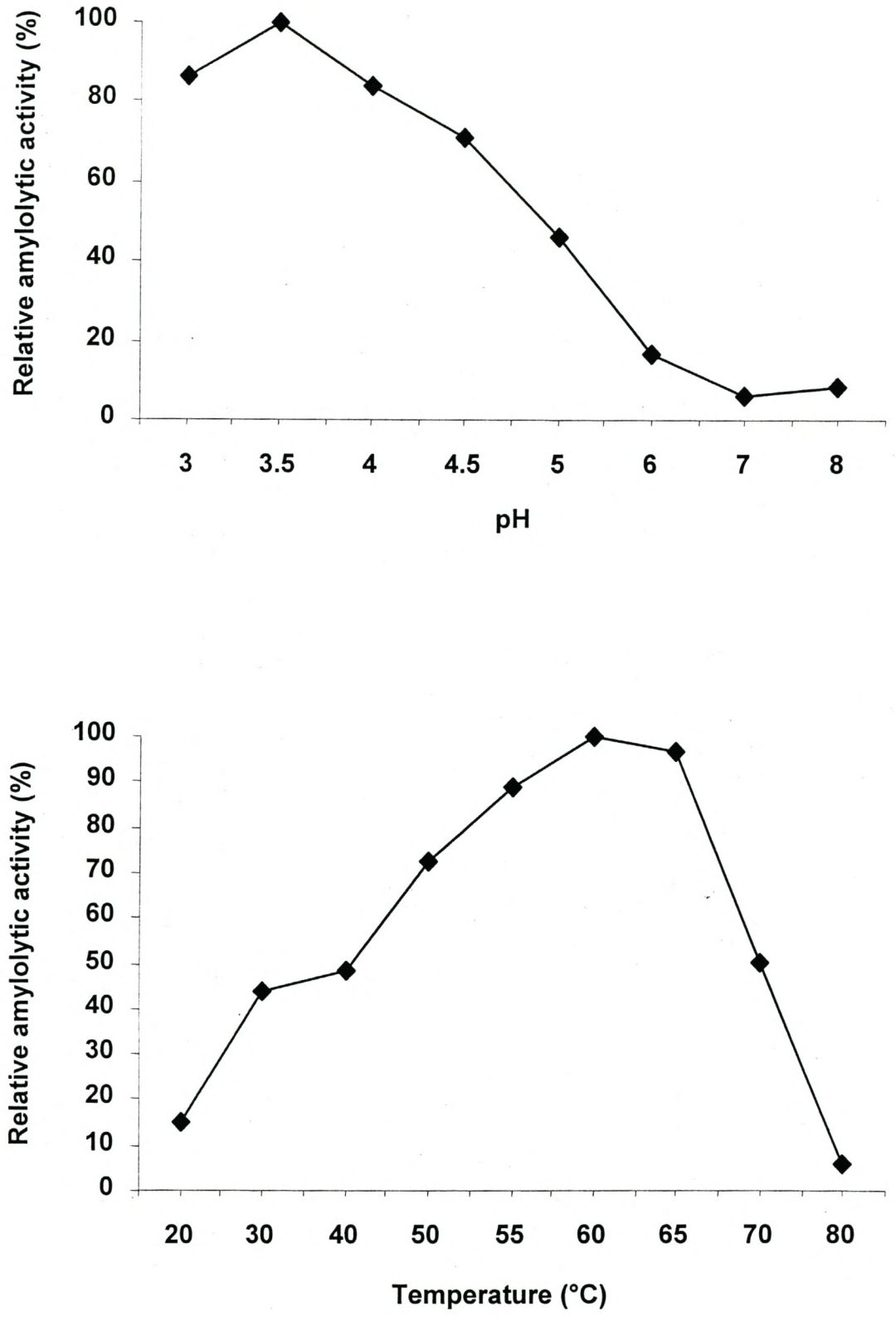

Fig. 3. Relative activities of *Lipomyces kononenkoae LKA2*-encoded α-amylase at different pH (A) and temperatures (B). Assays were done in triplicate with a standard deviation within 10%.

Table 2.

Substrate specificity of the *L. kononenkoae LKA2* – encoded α-amylase secreted by the *S. cerevisiae* transformant. Assays were done in triplicate with a standard deviation within 10%.

Substrate	Relative activity (%)
Starch (Lintner)	100
Dextrin	91
Amylose	90
Potato Starch	83
Amylopectin	17
Glycogen (oyster)	9
Pullulan	2

3.4 ACKNOWLEDGEMENTS

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CHAPTER 4

RESEARCH RESULTS

The evaluation and comparison of recombinant *Saccharomyces cerevisiae* strains expressing α–amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*

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The evaluation and comparison of recombinant *Saccharomyces cerevisiae* strains expressing the α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*

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ABSTRACT

Lipomyces kononenkoae and *Saccharomycopsis fibuligera* were selected on the basis of their ability to utilise raw starch. Both organisms possess α-amylase and/or glucoamylase activity. Eight constructs containing the *L. kononenkoae* α-amylase genes (*LKA1* and *LKA2*), the *S. fibuligera* α-amylase (*SFA1*) and glucoamylase (*SFG1*) genes were prepared: four single cassette plasmids expressing the individual coding sequences under the control of the phosphoglycerate kinase gene (*PGK1*) promoter and terminator, two double cassette plasmids (expressing both *LKA1* and *LKA2*) under the control of the *PGK1* promoter and terminator, and *SFA1* and *SFG1* under their respective native promoters and terminators, and two single cassette plasmids expressing the *SFA1* and *SFG1* with their native promoters and terminators, respectively. These constructs were transformed into Σ1278b, a laboratory strain of *Saccharomyces cerevisiae*. Southern-blot analysis confirmed the stable integration of the different gene constructs into the *S. cerevisiae* genome and plate assays revealed amylolytic activity. The strain expressing *LKA1* and *LKA2* resulted in the highest levels of α-amylase activity in liquid media. This strain was also the most efficient at starch utilisation in batch fermentations, utilising 80% of the available starch and producing 0.61 g/100 ml of ethanol after 156 h of fermentation. The strain expressing *SFG1* under the control of the *PGK1* expression cassette gave the highest levels of glucoamylase activity. These results show that there is a non-linear relationship between starch hydrolysis and the ability to produce ethanol. This study paves the way for the development of efficient starch-degrading *S. cerevisiae* strains that could be used in the brewing, whisky and biofuel industries.

4.1 INTRODUCTION

After cellulose, starch is the most widespread, naturally occurring organic compound and is derived mainly from higher plants. The potential of starch as a renewable biological resource has stimulated research into amylolytic enzymes and into the broadening of the substrate range of *S. cerevisiae* (Steyn and Pretorius, 1990). Starch is composed of two fractions of high molecular weight, amylose and amylopectin. Amylose, the minor component (20-30%) is a mainly linear polysaccharide formed by α-1,4-linked glucose residues and some α-1,6 branching points, while amylopectin represents the major fraction of starch (70-80%) and is highly branched. Gelatinisation, the first step in raw starch hydrolysis, entails the disruption and solubilisation of the starch granules. This is normally achieved by heating the starch to temperatures often in excess of 100°C for several minutes (Vihinen and Mantsala, 1989). After the gelatinisation, the starch slurry is liquefied by α-amylases and then saccharified by glucoamylases and debranching enzymes (e.g. isoamylase and pullulanase). During liquefaction, α-amylase (1,4-α-D-glucan maltohydrolase) degrades starch to oligosaccharides, reducing the viscosity of the starch solution. Finally, the oligosaccharides are hydrolysed by β-amylase (1,4-α-D-glucan maltohydrolase) and glucoamylase. These enzymes act in a stepwise and synergistic process and the major end product of concerted α-amylase, glucoamylase and isoamylase activity is glucose (Vihinen and Mantsala, 1989).

Of the more than 150 starch-assimilating yeast species, only a few secrete a combination of enzymes consisting of α-amylase, for endo-α-1,4-cleavage, and glucoamylase, for cleaving terminal α-1,4-glucosidic bonds and, in some cases, α-1,6-branched bonds. The ability of *L. kononenkoae* and *S. fibuligera* to degrade raw starch is well documented (Spencer-Martins and Van Uden, 1979; Wilson and Ingledew, 1982). Unfortunately, their low ethanol tolerance, slow growth rate, poorly characterised genetics and lack of GRAS (Generally Regarded As Safe) status limit the potential of these yeasts to be used in a one-step bioconversion process of starch-rich biomass to commercially important commodities. One of the main limitations of the industrially important yeast, *S. cerevisiae* (that has GRAS status), is its inability to convert relatively inexpensive polysaccharide-rich substrates (such as starch) into commercially important commodities. Microorganisms have not been described that possess the combination of substrate utilisation (e.g. high-level production of hydrolytic enzymes, and utilisation of resulting sugars) and product production (e.g. high yield and tolerance) properties required for consolidated bioprocessing (CBP). The development of such microorganisms can proceed via either of two strategies, namely (i) the improvement of desired production properties of a naturally hydrolytic organism having excellent substrate utilisation capabilities or, alternatively, (ii) the desired substrate utilisation (hydrolytic) properties could be conferred to an organism having optimal product production properties. We

have opted for the latter strategy, by attempting to extend the limited range of carbohydrates that the versatile yeast, *S. cerevisiae*, is capable of fermenting.

In this paper, we report the engineering and comparison / evaluation of recombinant yeast strains that express α-amylases and glucoamylases under the control of either the *PGK1* promoter or their native promoters. α-Amylases from *L. kononenkoae* (*LKA1* and *LKA2*), as well as the α-amylase and glucoamylase from *S. fibuligera* (*SFA1* and *SFG1*), were used. This study compared these different recombinant yeast strains with regard to the efficiency of expression of the heterologous genes, as well as their ability to produce ethanol from small-scale fermentations, using starch as the only carbon source. The aim of this study was to select the best combination of starch-degrading genes for future use in industrial yeast strains. The optimization of the synthesis and secretion of these amylase enzymes in genetically modified *S. cerevisiae* is thus central to the efficient one-step bioconversion of starch-rich materials to ethanol.

4.2 MATERIALS AND METHODS

4.2.1 MICROBIAL STRAINS AND PLASMIDS

The sources and relevant genotypes of bacterial and yeast strains, together with the plasmids used in this study, are listed in Table 1.

4.2.2 GROWTH MEDIA AND CULTURE CONDITIONS

Escherichia coli was grown in Luria Broth (LB) (Sambrook *et al.*, 1989). Bacterial transformants were cultured in LB medium containing ampicillin (100 μg/ml). Yeasts were cultured in YPD medium containing 1% yeast extract, 2% bacto-peptone and 2% glucose. The *S. cerevisiae* transformants were cultured and selected on synthetic complete (SC) medium containing 2% glucose and 0.67% yeast nitrogen base without amino acids. No growth factors were added and uracil was used as the selectable marker. Starch medium, containing soluble starch (Sigma-Aldrich, Steinheim, Germany) as the only carbon source, and SC^{-Ura}, containing Phadebas starch (Pharmacia Diagnostics, Uppsala, Sweden) $PHSC^{-Ura}$ medium (20 Phadebas tablets per liter), were used to screen for amylolytic activity. All solid media contained 2% agar. Selective plates were incubated at 30°C for 2-6 days and the appearance of halos around the colonies was taken to be indicative of starch utilisation (Steyn and Pretorius, 1995). Bacteria and yeasts were routinely cultured at 37°C and 30°C respectively.

Table 1. Microbial strains and plasmids used in this study

Strain or plasmid	Genotype	Source/reference
***Escherichia coli* strains:**		
DH5α	*F⁻ recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*argF-Lac-ZYA*) (φ80d*lacZ*(M15)λ	BRL
***S. cerevisiae* strains:**		
Y294	*MATa ura3 leu2 trp1*	This laboratory
Σ1278b	*MATa/Matα URA3*	Liu *et al.* (1993)
Σ1278b IPLKA1	*MATa/Matα URA3*::pIPLKA1	This study
Σ1278b IPLKA2	*MATa/Matα URA3*::pIPLKA2	This study
Σ1278b IPLKA1/2	*MATa/Matα URA3*::pIPLKA1/2	This study
Σ1278b IPSFA1	*MATa/Matα URA3*::pIPSFA1	This study
Σ1278b IPSFG1	*MATa/Matα URA3*::pIPSFG1	This study
Σ1278b ISFA15	*MATa/Matα URA3*::pISFA15	This study
Σ1278b ISFG15	*MATa/Matα URA3*::pISFG15	This study
Σ1278b ISFAG15	*MATa/Matα URA3*::ISFAG15	This study
Plasmids:		
Ylp5	Ap^R Tc^R *URA3*	Struhl *et al.* (1979)
pJC1	Ap^R $PGK1_{PT}$ *URA3 2μm*	Crous *et al.* (1995)
pPGK1	Ap^R Tc^R $PGK1_P$ $PGK1_T$ *URA3*	This study
pGEM	Ap^R *f1 ori LacZ* $T7_P$ $SP6_P$	Yanish-Perron *et al.* (1985)
pSport	Ap^R *f1 ori LacZ* $T7_P$ $SP6_P$	Stratagene
YEp13	Ap^R Tc^S *Leu2*	Broach *et al.* (1983)
pSport-LKA2	Ap^R *f1 ori LacZ* $T7_P$ $SP6_P$ $PGK1_P$ *LKA2* $PGK1_T$	Eksteen *et al.* (2001)
pJUL3	Ap^R Tc^R $PGK1_P$ *LKA2* $PGK1_T$ *URA3*	Eksteen *et al.* (2001)
pEPSFG1	Ap^R Tc^R $PGK1_P$ *SFG1* $PGK1_T$ *URA3*	This study
pIPLKA1	Ap^R Tc^R $PGK1_P$ *LKA1* $PGK1_T$ *URA3*	This study
pIPLKA2	Ap^R Tc^R $PGK1_P$ *LKA2* $PGK1_T$ *URA3*	This study
pIPLKA1/2	Ap^R Tc^R $PGK1_P$ *LKA1* $PGK1_T$ $PGK1_P$ *LKA1* $PGK1_T$ *URA3*	This study
pIPSFA1	Ap^R Tc^R $PGK1_P$ *SFA1* $PGK1_T$ *URA3*	This study
pIPSFG1	Ap^R Tc^R $PGK1_P$ *SFG1* $PGK1_T$ *URA3*	This study
pIPSFA15	Ap^R Tc^R $SFA1_P$ *SFA1* $SFA1_T$ *URA3*	This study
pIPSFG15	Ap^R Tc^R $SFG1_P$ *SFG1* $SFG1_T$ *URA3*	This study
pSFAG15	Ap^R Tc^R $SFA1_P$ *SFA1* $SFA1_T$ $SFG1_P$ *SFG1* $SFG1_T$ *URA3*	This study

4.2.3 CONSTRUCTION OF THE *SACCHAROMYCOPSIS FIBULIGERA* GENOMIC LIBRARY

The total genomic DNA from *S. fibuligera* (containing the *SFA1* and *SFG1* genes) was isolated and prepared as described by Yamashita *et al.* (1985). The genomic DNA was partially digested with *Sau*3A. DNA fragments larger than 5 kb were recovered and inserted into the *Bam*HI site of the *E. coli-S. cerevisiae* shuttle plasmid (YEp13) by ligation. The resulting constructs were used to transform *E. coli* for ampicillin resistance (Ap^R). Approximately 20 000 Ap^R clones were obtained, 90% of which were tetracycline sensitive (Tc^S). Selection for plasmids containing the *SFA1* and *SFG1* genes (pSFA15 and pSFG15) was carried out in *S. cerevisiae* strain Y294. pSFA15 and pSFG15 were characterised and the DNA inserts were sequenced.

4.2.4 DNA MANIPULATIONS, SEQUENCING AND AMPLIFICATION

Techniques employed for the manipulation and subcloning of DNA fragments, plasmid DNA isolation, the transformation of *E. coli*, agarose gel electrophoresis and the purification of DNA fragments were carried out as described by Sambrook *et al.* (1989). Restriction endonucleases, T4 DNA ligase, the Klenow (large) fragment of *E. coli* DNA polymerase I and Shrimp Alkaline Phosphatase (SAP) were purchased from Roche Biochemical Products (Randburg, SA) and used as recommended by the supplier.

DNA sequencing was carried out using an ABI PRISM® Big Dye™ Terminator cycle sequencing ready reaction kit with an ABI PRISM™ 377 DNA sequencer (PE/Applied Biosystems).

DNA was amplified by the polymerase chain reaction (PCR) technique. The PCR was conducted in a 50 µl volume containing 25 mM of $MgCl_2$, 200 µM of a nucleotide mixture (dNTPs), 200 nM of each primer, 2.6 U of Expand High Fidelity mix (Roche Biochemical Products) and 2-10 ng of template DNA. After an initial denaturation step at 94°C for 2 min, the PCR was conducted for 30 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 2 min. The PCR products were purified by ethanol precipitation. Restriction enzyme digestion of the PCR products was performed at 37°C for 14 h. Primers were obtained from Roche Biochemical Products (South Africa). The following oligodeoxyribonucleotides were used as primers:

SFA5' (5'-ATCGAGATCTTATGCAAATTTCAAAAGCTG-3'),
SFA3' (5'-ATCGAGATCTCCAGCCATCATGAACAA-3'),
$SFGLU_{EcoRI}$-F (5'-GATCGAATTCCGCTTCTATTTGCTATG-3'),
$SFGLU_{XhoI}$-R (5'-GATCCTCGAGTAAGCCAAAGCCTTGACCTT-3').

4.2.5 YEAST TRANSFORMATION

S. cerevisiae strain Σ1278b was transformed using the lithium acetate method, as described by Gietz and Schiestl (1991). The different constructs were first linearised by digesting them in the *URA3* gene of the vector, YIp5, with either *Stu*I, *Nco*I or *Apa*I, depending on the gene involved.

4.2.6 SOUTHERN-BLOT ANALYSIS

Southern hybridisation was performed using the DIG Labeling Kit from Roche Biochemical Products (South Africa), using the method as described in the Dig Application Manual.

4.2.7 ISOLATION OF INTRACELLULAR PROTEIN

50 ml of YPD broth was inoculated to an optical density at 600 nm (OD_{600}) of 0.1 from an overnight culture and grown at 30°C for 48 h. Cells were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 5 ml of 50 mM Tris (pH 7.5, containing 10 mM $NaCl_2$) buffer. Glass beads (0.1 g of 0.2-mm) were added and the cells were vortexed vigorously for 3 min. After centrifugation at 6000 rpm for 2 min, the supernatant, containing the intracellular protein extract, was carefully removed and used for the enzyme assays.

4.2.8 ENZYME ASSAYS

Intracellular and extracellular α-amylase activity was spectrophotometrically (OD_{620}) measured, using 200 µl of intracellular extract and culture supernatants respectively, by means of the Phadebas Amylase Test (Pharmacia Diagnostics, Uppsala, Sweden) assay (Steyn and Pretorius, 1995). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 nmole of reducing sugar/ml/min.

Intracellular and extracellular glucoamylase activity was measured by adding 600 µl of either intracellular extract or culture supernatant to 200 µl of 500 mM sodium acetate and 200 µl of 50 mM maltotriose, respectively. After mixing, the enzyme slurry was incubated at 37°C for 20 min, boiled for 3 min at 100°C and placed on ice for 5 min. The reaction mixture (800 µl) was mixed with 1 ml of Trinder solution (Sigma Glucose Trinder Kit) and incubated at room temperature for 25 min. Readings were taken at OD_{540}. One U of glucoamylase was defined as the amount of enzyme required to release 1 mmol of glucose/min from maltotriose.

4.2.9 ALCOHOLIC FERMENTATIONS AND SAMPLING

Recombinant *S. cerevisiae* strains were used for alcoholic fermentation using soluble starch as the only carbon source. Yeast cells were inoculated into 50 ml of YPD medium and grown at 30°C for 24 h. The 50 ml precultures were used to inoculate 500 ml of SC^{-Ura} medium in 1 liter sterile flasks and grown at 30°C for 48 h. The yeast cells were removed by centrifugation at 5000 rpm for 5 min and aseptically inoculated into the fermentation media at a concentration of 2 g/l. Fermentations were carried out anaerobically on a magnetic stirrer at 30°C for 156 h. A gastrap was installed in the fermentors for the release of CO_2 as well as a syringe to remove samples sterile. Samples were taken every 12 h to determine the reducing sugar. The fermentation media were prepared using 2% soluble starch, 0.67% Yeast Nitrogen Base without amino acids, 42 mg/100 ml tween 80, 1 mg/100 ml ergosterol and 100 mg/ml ampicillin, and adjusted to a volume of 150 ml in sterile 200 ml bottles. Soluble starch was dissolved in a microwave oven prior to use.

4.2.10 STARCH UTILISATION AND HYDROLYSIS

To analyse the residual starch in the culture medium, 200 µl of the supernatant of each of the different strains grown in the fermentation media was mixed with 1 ml of 2 M HCl and complete hydrolysis was accomplished by heating the mixture in a boiling water bath for 30 min. After neutrilisation of the hydrolysate with 1 ml of 2 M NaOH, the reducing sugars released from the starch were determined using the Glucose Trinder kit (Sigma).

4.2.11 GAS CHROMATOGRAPHY

Gas chromatography (GC) analysis was performed on sample supernatants from small-scale starch fermentations to screen the ethanol yield. A Hewlett Packard HP 6890 Series GC system, using an HP-INNOWAX capillary column, was used.

4.3 RESULTS AND DISCUSSION

4.3.1 CONSTRUCTION OF EXPRESSION CASSETTES

Eight plasmids were constructed: the *L. kononenkoae* α-amylase genes (*LKA1* and *LKA2*) were expressed under the control of the *PGK1* promoter and terminator cassette individually, as well as under the control of a double cassette plasmid expressing both the *LKA1* and *LKA2* under the *PGK1* expression cassette (Fig.1). The *S. fibuligera* α-amylase

gene (*SFA1*), as well as the *S. fibuligera* glucoamylase gene (*SFG1*), was expressed under the control of the *PGK1* promoter and terminator cassette, as well as under their respective native promoters and terminators.

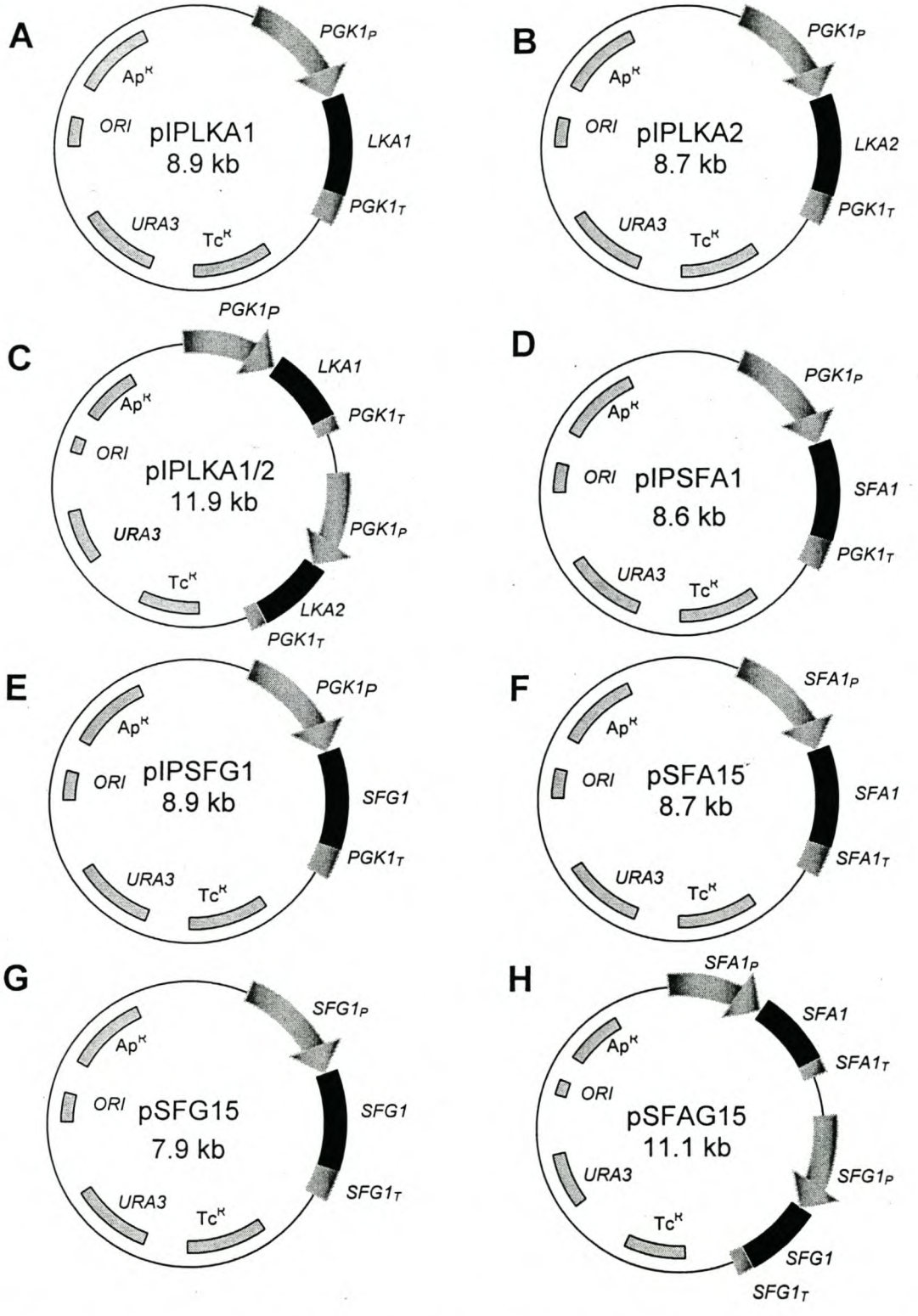

Fig. 1. Schematic representation of recombinant plasmids pIPLKA1 (A), pIPLKA2 (B), pIPLKA1/2 (C), pIPSFA1 (D), pIPSFG1 (E), pSFA15 (F), pSFG15 (G) and pSFAG15 (H).

The construction of the recombinant plasmids, pSFA15 and pSFG15, containing the *SFA1* and *SFG1* genes under their respective native promoters, is described in the materials and methods (Construction of Genomic Library, Fig.1). The PCR of the pSFA15 and pSFG15 plasmids yielded amplified products of 1481 bp and 1559 bp. The amplified *SFA1* product was cloned into the *Bgl*II site of pPGK1 (the *PGK1* promoter and terminator cassette, cloned into the *Hin*dIII site of YIp5), forming pIPSFA1 (Fig.1). The amplified *SFG1* product was digested with *Eco*RI and *Xho*I and cloned into the *Eco*RI/*Xho*I-digested pJC1, resulting in pEPSFG1. Plasmid pEPSFG1 was digested with *Pvu*II, resulting in a 3406-bp $PGK1_P$-*SFG1*-$PGK1_T$ fragment. This fragment was cloned into the *Pvu*II site of YIp5, generating pIPSFG1 (Fig.1). Steyn and Pretorius (1995) had previously cloned the cDNA copy of the *LKA1* gene from *L. kononenkoae* into the yeast episomal plasmid pJC1, containing the *PGK1* expression cassette, resulting in pAJC2. The $PGK1_P$-*LKA1*-$PGK1_T$ cassette was subcloned as a 3383-bp *Hin*dIII DNA fragment into the *Hin*dIII site of the yeast-integrating plasmid YIp5, generating recombinant plasmid pIPLKA1 (Fig.1). The *LKA2* gene was previously cloned into the yeast episomal plasmid, YEp352, resulting in pEPLKA2 (Eksteen *et al.*, 2002). Plasmid pEPLKA2 was digested with *Hin*dIII, resulting in a 3159-bp $PGK1_P$-*LKA2*-$PGK1_T$ fragment. This fragment was cloned into the *Hin*dIII site of both YIp5 (generating pIPLKA2) (Fig.1) and pSport (resulting in pSport-LKA2). pSport-LKA2 was digested with *Bam*HI and *Sph*I, resulting in a 3184-bp $PGK1_P$-*LKA2*-$PGK1_T$ fragment. This fragment was cloned into the *Bam*HI/*Sph*I site of pIPLKA1, resulting in pIPLKA12 (Fig.1).

4.3.2 EXPRESSION OF THE DIFFERENT GENE CONSTRUCTS IN *S. CEREVISIAE*

The eight constructs were transformed separately into the laboratory strain *S. cerevisiae* Σ1278b, resulting in Σ1278b IPLKA1, Σ1278b IPLKA2, Σ1278b IPLKA1/2, Σ1278b IPSFA1, Σ1278b IPSFG1, Σ1278b SFA15, Σ1278b SFG15 and Σ1278b SFAG15 (Table 1). Southern-blot hybridisations were performed to confirm integration into the yeast genome (results not shown). Once integration had been confirmed, plate assays were used to determine whether the transformants were capable of secreting functional amylolytic enzymes (results not shown).

4.3.3 AMYLOLYTIC ACTIVITY OF THE DIFFERENT TRANSFORMANTS

α-Amylase and glucoamylase activities were measured in cell extracts and culture supernatants from the different transformants (see Materials and Methods). Table 2 summarises the results. The transformant Σ1278b IPLKA1/2 (secreting both *LKA1* and *LKA2* under the control of the *PGK1* promoter) resulted in the highest activity: 134 U/l of α-amylase activity in the extracellular extract. Transformant Σ1278b IPLKA1, however,

resulted in higher α-amylase activity (165 U/l) in the intracellular extract. No α-amylase activity could be detected in the transformants Σ1278b IPSFG1 and Σ1278b SFG15. Strain Σ1278b IPSFG1 resulted in the highest levels of glucoamylase activity, reaching 36.5 U/l in the intracellular and 11.9 U/l in the extracellular extract after 48 h of growth. Transformants Σ1278b SFG15 and Σ1278b SFAG15 resulted in much lower values for both intracellular and extracellular extracts, reaching 22.8 and 30 U/l (intracellular), and 1.1 and 1.8 U/l (extracellular), respectively. It must be remembered that the secretion of all of these enzymes was directed by the native secretion signals and it should therefore be possible to obtain higher extracellular activity if *S. cerevisiae* secretion signals are used. The other strains (expressing only α-amylases) produced no glucoamylase activity. The control strain, Σ1278b, showed no amylolytic activity.

Table 2. Amylolytic activity of transformants. Assays were done in triplicate with a standard deviation within 10%.

Strain	α-Amylase activity (U/l)		Glucoamylase activity (U/l)	
	Intracellular	Extracellular	Intracellular	Extracellular
Σ1278b IPLKA1	165	124	0	0
Σ1278b IPLKA2	0	12	0	0
Σ1278b IPLKA1/2	144	134	0	0
Σ1278b IPSFA1	11	132	0	0
Σ1278b IPSFG1	0	0	36.5	11.9
Σ1278b SFA15	0	47	0	0
Σ1278b SFG15	0	0	22.8	1.1
Σ1278b SFAG15	0	25	30.6	1.8
Σ1278b	0	0	0	0

4.3.4 EVALUATION OF THE TRANSFORMANTS TO UTILISE STARCH IN SMALL-SCALE FERMENTATIONS

Amylolytic enzymes are continuously expressed and secreted during the growth of yeast cells - the sugar uptake and carbohydrate content of the culture broth at any given moment will therefore reflect both the types and activities of the amylolytic enzymes present, as well as the ability of the cells to assimilate various products of starch hydrolysis. The utilisation of starch by different transformants was compared in small-scale batch fermentations (150 ml), using soluble starch as the only carbon source (Fig. 2). Figure 2 summarises the ability of the different transformants to utilise starch as the only carbon source in small-scale fermentations over a period of 156 h. For the first 60 h of fermentation, the different transformants utilised more or less the same amount of starch with Σ1278b IPSFA1, utilising 26% of the available starch. After 156 h transformant Σ1278b IPLKA1/2 (expressing both *LKA1* and *LKA2* under the control of the *PGK1*

promoter) consumed 80% of the starch and produced 0.61 g/100 ml of ethanol (Fig. 2) (Table 3). Ethanol concentrations were determined by gas chromatography (GC) analysis. The native promoters of *LKA1* and *LKA2* were evaluated, but proved to be non-functional in *S. cerevisiae* (data not shown). Table 3 summarises the ability of the different transformants to produce ethanol from soluble starch in batch fermentations. These results illustrate the effectiveness of strain Σ1278b IPLKA1/2 to metabolically convert starch to ethanol. If one takes into account the individual α-amylase activities of this strain, it is not clear why this transformant was so effective. It may be that either *LKA1* or *LKA2* possesses additional minor amylolytic side activities and that these activities enhanced the synergistic effect on starch utilisation between Lka1p and Lka2p. Transformant Σ1278b SFAG15, expressing *SFA1* and *SFG1* under their respective native promoters, was the second best starch utiliser, consuming 70% of the starch. This strain, however, produced only 0.20 g/100 ml of ethanol. As expected, the control strain Σ1278b (with no amylolytic enzymes) degraded practically none of the starch. Transformants expressing only the α-amylase or the glucoamylase could utilise only a maximum of 50% of the starch, with transformant Σ1278b IPSFA1 utilising 52% of the starch. The results illustrate the effectiveness of expressing both an α-amylase and glucoamylase in the same strain for the utilisation of starch. Strain Σ1278b IPLKA1 produced 0.29 g/100 ml of ethanol, making it the second best producer of ethanol.

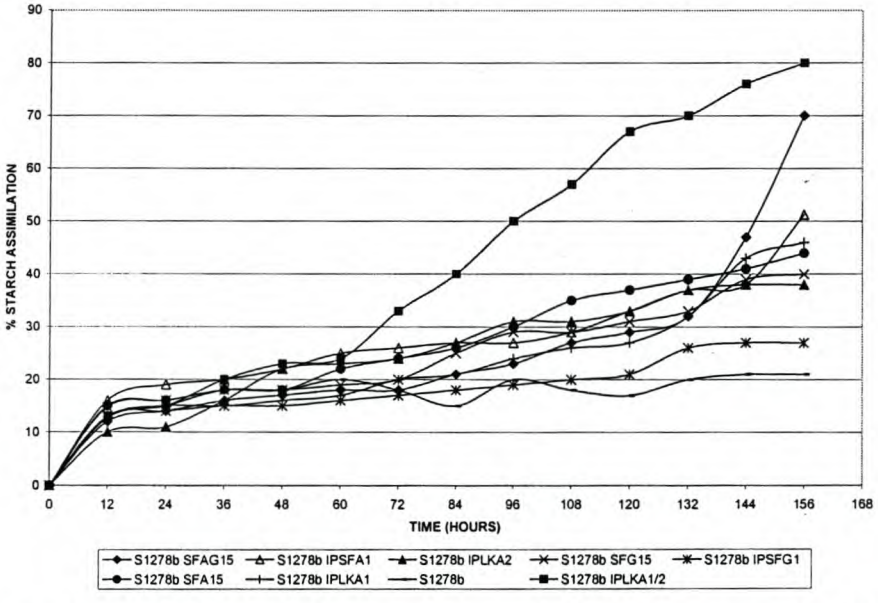

Fig 2. Starch utilisation and hydrolysis. Assays were done in triplicate with a standard deviation within 10%.

For the construction of an industrial recombinant yeast strain, the specific genetic background of the strain should be evaluated, as it can play a crucial role in the end result (Gundllapalli *et al.*, 2002). We only tested the constructs for starch utilisation in laboratory strains. Σ1278b IPLKA1/2 (expressing both *LKA1* and *LKA2* under the control of the *PGK1* promoter) can lead to interesting prospects when introduced into an industrial strain of

S. cerevisiae. This strain showed excellent amylolytic capabilities when compared to recombinant strains transformed with only a single α-amylase or glucoamylase. The introduction of a glucoamylase into the Σ1278b IPLKA1/2 strain could lead to even better utilisation of starch and ethanol production. Σ1278b SFAG15, expressing *SFA1* and *SFG1* under their respective native promoters, can also lead to interesting prospects when expressed under a different promoter. The phosphoglycerate kinase gene (*PGK1*) promoter would be a strong candidate. Under their respective native promoters, Σ1278b SFAG15 consumed 70% of starch but the ethanol production was much lower than that of Σ1278b IPLKA1/2, with the former producing 2 g/l of ethanol to the 6.1 g/l of ethanol produced by Σ1278b IPLKA1/2.

In conclusion, this study has attempted to demonstrate that, with the correct yeast development strategy, recombinant raw starch-degrading yeasts are a viable option for future one-step starch utilisation and subsequent ethanol production. We found that there is a non-linear relationship between starch hydrolysis and the ability to produce ethanol. In particular, efficient starch-utilising strains of *S. cerevisiae* would be valuable for consolidated bioprocessing (CBP) in which production of amylolytic enzymes, hydrolysis of starch-rich substrates, and fermentation of resulting sugars to a desired product (e.g. beer, whisky, biofuel ethanol, etc.) occurs in one step.

Table 3. Ethanol production by the different transformants after 156 h of fermentation

Strain	Ethanol Concentration (g/100ml)
Σ1278b IPLKA1	0.29
Σ1278b IPLKA2	0.16
Σ1278b IPLKA1/2	0.61
Σ1278b IPSFA1	0.22
Σ1278b IPSFG1	0.21
Σ1278b SFA15	0.19
Σ1278b SFG15	0.15
Σ1278b SFAG15	0.20
Σ1278b-controle	none

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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5.1 GENERAL DISCUSSION AND OTHER PERSPECTIVES

In general, yeast have not been described that possess the combination of starch utilisation (e.g., high-level production of effective amylases and efficient utilisation of resulting sugars) and product production (e.g., high selectivity and tolerance) properties required for the efficient utilisation of starch-rich substrates in the whisky industry. The development of such yeasts can proceed via either of two distinct strategies, namely (i) the improvement of desired production properties of a naturally amylolytic yeast (e.g. *Lipomyces kononenkoae*, *Saccharomycopsis fibuligera*) with excellent substrate utilisation capabilities or, alternatively, (ii) desired substrate (hydrolytic) properties can be conferred to a yeast (*Saccharomyces cerevisiae*) with optimal product production properties. In this study, we have opted for the latter strategy.

Chapter 1 of this thesis highlights the ever-increasing demand for a one-step starch fermentation process. The conversion of starch, consisting of linear (amylose) and branched glucose polymers (amylopectin) (Nigam and Singh, 1995), to commercially important commodities by *S. cerevisiae* employs a three-step process: (i) liquefaction by α-amylase, (ii) saccharification by glucoamylases and debranching enzymes, and (iii) fermentation of the sugars (De Mot *et al.*, 1985; Kim *et al.*, 1988). A one-step starch fermentation process would obviously be advantageous. Therefore, considerable attention is currently being paid to the evaluation and construction of amylolytic yeasts (Tubb, 1986). The specific aims and approaches to construct amylolytic strains of *S. cerevisiae* are outlined in this regard.

Chapter 2 reviews the process of whisky production. This overview concentrates on the materials used for the production of whisky (Bronsky and Schumann, 1989; Piggott and Conner, 1995), with particular emphasis on the starch content of the industrial crops used for whisky production as well as the α-amylase content of the crops (Bronsky and Schumann, 1989). The treatment of cereal crops for the production of malt is outlined. Considerable work has been done with the aim of reducing malting times and losses without negative effects on the quality of malt (Dolan, 1976, 1981; Peterson, 1994). Malt wort consists of maltose, maltotriose, glucose and dextrins (Klaassen *et al.*, 1996). These will subsequently be converted into sugars and then alcohol. The malting activates enzyme systems within the grain. The procedure is to steep the grain in water to awaken the embryo within the seed. The dampened grain is then allowed to germinate partially. This is halted by drying and slight cooking over hot air in a kiln. The kilning also develops the flavour and the colour of the malt. Mashing, the process of forming a fermentable

extract, is also discussed. Two major routes may be followed, depending on whether a malted or unmalted cereal is used. In the former case, the process is essentially similar to the production of wort for beer brewing, a clear or filtered extract being required to prevent 'burning' in batch (pot) stills (Piggott and Conner, 1995). In the latter case, in modern continuous processes in preparation for column distillation, the separation stage has become redundant and the fermentation (and distillation) are commonly carried out with the total grain solids present. Whisky fermentation is similar to that for many other alcoholic beverages, the only difference being that whisky fermentations are started by pitching the yeast culture, normally a specific strain of high-performance distilling yeast. The process of distillation is discussed, with emphasis on the two distinct distillation systems used for the production of whiskies: batch to produce highly flavoured and continuous to produce lighter spirits. The sensorial and different chemical changes during whisky maturation have been summarised and some comments were made on whisky filtration and blending were mentioned. Chapter 2 also focuses on starch as a renewable biological resource and on the structure and enzymatic hydrolysis of starch. The application of amylolytic yeasts for a reduced need to purchase (or produce in a separate process) amylolytic enzymes or barley malt; simplification of the processing stages that precede fermentation; and improved efficiencies of starch conversion and the possibility of producing amylolytic enzymes for use in syrup manufacture, as byproducts of alcohol fermentations are highlighted as the main advantages for the use of amylolytic yeasts (Tubb, 1986). *S. cerevisiae* is pointed out as a host for the expression of heterologous genes encoding amylolytic enzymes.

Chapter 3 contains the complete nucleotide sequence of a novel α-amylase, the *LKA2* gene. *LKA2*, cloned from *L. kononenkoae* IGC4052B (CBS5608T) in *S. cerevisiae,* revealed significant homology to the amino acid sequences of *Aspergillus nidulans*, *Debaromyces occidentalis*, *Saccharomycopsis fibuligera* and *Schizosaccharomycopsis pombe* α-amylases, as well as to various bacterial cyclomaltodextrin glucanotransferases. The amylolytic enzyme had a pH optimum of 3.5 and a temperature optimum of 60°C. The substrate specificity of this enzyme is very similar to an α-amylase combined with dextrinase activity. The substrate specificity of the amylolytic enzyme was determined using a number of different glucose polymers, containing either α-1,4 glucosidic linkages or a mixture of α-1,4 and α-1,6 glucosidic linkages. The enzyme showed high reactivity towards soluble starch, dextrin and amylose, but only small amounts of reducing sugars were liberated from amylopectin, glycogen and pullulan. The reaction specificity of this enzyme distinguishes it from other amylolytic enzymes already reported in other microorganisms and is very similar to that of an α–amylase with associated dextranase activity.

Chapter 4 describes the subcloning and expression of the *LKA1* and *LKA2* α-amylase genes from *L. kononenkoae,* and the α-amylase (*SFA1*) and glucoamylase (*SFG1*) genes

from *S. fibuligera*, in *S. cerevisiae*. The following constructs were expressed in *S. cerevisiae*: four single gene cassettes containing the individual amylase-coding sequences (*LKA1*, *LKA2*, *SFA1* and *SFG1*) under the control of the *PGK1* promoter and terminator; two double gene cassettes (containing both *LKA1* and *LKA2* under the control of the *PGK1* promoter and terminator, and *SFA1* and *SFG1* under their respective native regulatory sequences); and two single gene cassettes containing *SFA1* and *SFG1* with their native promoters and terminators. Southern-blot analyses confirmed the stable integration of these different gene constructs into the yeast genome and plate assays revealed amylolytic activity. When assayed for amylolytic activity in liquid medium, the strain bearing the *LKA1* and *LKA2* cassettes resulted in the highest levels of amylolytic activity.

In conclusion, the expression of the *LKA1* and *LKA2* double gene cassette (*LKA1* and *LKA2* under the control of the *PGK1* promoter and terminator) in *S. cerevisiae* could lead to interesting insights into starch metabolism in yeast and the potential role of amylolytic strains of *S. cerevisiae* in industrial fermentations. This construct resulted in the most efficient starch utilisation in batch fermentation, assimilating 80% of the starch and producing 6 g L^{-1} of ethanol. Transformant Σ1278b SFAG15, expressing *SFA1* and *SFG1* under their respective native promoters, was the second best starch utiliser, consuming 70% of the starch. This strain, however, produced only 0.20 g/100 ml of ethanol, showing a non-linear relationship between starch hydrolysis and the ability to produce ethanol. Taken together, the work implies that the engineering of a *S. cerevisiae* strain to effectively hydrolise starch is not the sole critical factor but the ability to effectively ferment the end products is equally (or perhaps more) important. In other words, good starch hydrolysis does not necessarily correlate with good ethanol production.

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