Characterisation and Improvement of Whiskey Yeast

by

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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.

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SUMMARY

Scotch whiskey is of two main types, namely Scotch malt whiskey, made from malted barley alone, or Scotch grain whiskey, made from cereals, such as wheat or maize, together with malted barley. In both processes, the enzymes from the barley are responsible for starch conversion and should always be derived entirely from the malted barley. No exogenous enzymes are allowed to be added to any mashing. The enzymes involved in the conversion process to fermentable sugars, are the α- and β-amylases, limit dextrinase and β-glucosidase.

Maize, on the other hand, contains no enzyme activity, therefore enzymes need to be added when producing whiskey from maize alone. In other whiskey-producing countries where maize is freely available and cheaper than barley, the use of exogenous enzymes are allowed in the mashing process and is crucial for the formation of fermentable sugars from complex carbohydrates. The cost of the enzymes, however, can push the production cost of whiskey to higher levels.

Saccharomyces cerevisiae does not have any amylolytic activity, but is an excellent fermenter and produces favourable organoleptic notes, which makes it very suitable for producing potable spirit. Efforts have been made to genetically improve industrial strains, relying on classical genetic techniques followed by the selection of broad traits, such as ethanol tolerance, absence of off-flavours and carbohydrate/starch utilisation. No strain has thus far been selected for total starch degradation during the fermentation of whiskey mash.

Over the last decade, considerable progress has been made in the development of genetically improved strains for the distilling, wine, brewing and baking industries. The expression of heterologous genes introduced a new dimension in approaches to the genetic improvement of industrial strains. It would therefore be cost-effective to use a yeast strain that can produce active and sufficient enzymes to ferment raw starch efficiently to alcohol without lowering the quality of the end product. No such strain has been developed to date, but the continuous improvement of starch-utilising strains has made this goal more achievable.

Two α-amylase genes, namely *LKA1* and *LKA2,* were previously isolated from *Lipomyces kononenkoae*. In this study, we selected 4 strains on the basis of criteria that are important for whiskey-specific strains. The selected strains were transformed with *LKA1,* as well as with a combination of *LKA1 and LKA2* genes. The wine yeast VIN13 was included in the transformation of *LKA1* and *LKA2* because of its rapid fermentation rate. The genes were integrated into the genomes of the yeast strains and were stable after many generations. Assays showed that a significant increase in enzyme activity was induced in the whiskey strains, compared to the untransformed strains. The strains also showed good fermentation ability in whiskey fermentations, although optimum alcohol production was still not achieved.

OPSOMMING

Skotse whiskey bestaan uit 2 tipes, nl. mout whiskey, gemaak slegs van mout d.w.s. gars wat die mout proses ondergaan het, en graan whiskey wat gemaak word van gewasse soos mielies of koring, waarby mout gevoeg word. Die ensieme afkomstig van die mout is verantwoordelik vir die omsetting van stysel na fermenteerbare suikers en geen eksogene ensieme mag by die gars- of graanmengsel gevoeg word nie. Die ensieme wat betrokke is by die omsetting van stysel, is die α- en β-amilases, limiet dekstrinase en β-glukosidase.

Mielies bevat geen ensiemaktiwiteit nie, dus moet ensieme by die proses gevoeg word indien slegs mielies vir die vervaardiging van whiskey gebruik word. In whiskey produserende lande waar mielies vryelik beskikbaar is en goedkoper is as gars, word eksogene ensieme by die graanmengsel gevoeg vir die vrystelling van fermenteerbare suikers vanaf komplekse koolhidrate. Die hoë koste van die ensieme kan egter die produksiekoste van whiskey verhoog.

Saccharomyces cerevisiae besit geen amilolitiese aktiwiteit nie, maar is 'n uitstekende fermenteerder en produseer gewensde organoleptiese geure. Om hierdie redes is *S. cerevisiae* baie geskik vir die produksie van drinkbare etanol. Navorsingspogings om industriële rasse geneties m.b.v. klassieke genetiese metodes te verbeter, kom wydverspreid in die literatuur voor. Dit sluit in die seleksie van rasse met 'n verskeidenheid van eienskappe soos etanol toleransie, die afwesigheid van afgeur produksie en koolhidraat/stysel benutting. Geen ras is egter tot op hede geselekteer vir totale stysel afbraak gedurende fermentasie nie.

Groot vordering is gedurende die laaste dekade gemaak in die ontwikkeling van genetiese verbeterde rasse vir die wyn- stokery- en brouers industrieë. Die uitdruk van heterogene gene in gisrasse gee 'n nuwe dimensie aan die genetiese verbetering van industriële rasse. Die gebruik van 'n gisras wat aktiewe en genoegsame ensieme produseer om rou stysel te fermenteer, sonder om die kwalitiet van die eindproduk nadelig te beïnvloed, kan die produksiekoste van whiskey aansienlik verminder. Geen gisras met hierdie eienskap is tot op hede ontwikkel nie, maar die voortdurende verbetering van rasse om stysel af te breek maak hierdie doel meer bereikbaar.

Twee α-amilase gene, nl. *LKA1* en *LKA2* is voorheen uit *Lipomyces kononenkoae* geïsoleer. In hierdie studie is 4 gisrasse geselekteer op grond van die kriteria wat nodig is vir whiskey giste. Die geselekteerde rasse is getransformeer met *LKA1* sowel as 'n kombinasie van *LKA1* en *LKA2* gene. Die wyngis VIN13 is ingesluit by die transformasie met die *LKA1* en *LKA2* gene, omrede VIN13 bekend is as 'n vinnige fermenteerder. Die gene is geïntegreer in die genoom van die verskillende gisrasse en is stabiel na vele generasies. Die getransformeerde rasse het 'n betekenisvolle verhoging in ensiemaktiwiteit teenoor die nie-getransformeerde

rasse getoon. Al die transformante het ook goeie fermentasie vermoë getoon in whiskey fermentasie proewe. Optimum alkoholproduksie is egter nie verkry nie.

This thesis is dedicated to my family, but most of all to my husband, for his loving support, encouragement and interest in my progress.
Hierdie tesis is aan my familie opgedra, veral aan my eggenoot, vir sy liefdevolle ondersteuning, aanmoediging en belangstelling in my vordering.

BIOGRAPHICAL SKETCH

Karin la Grange-Nel was born in Bellville, South Africa on 3 December 1971. She attended Simonsberg Primary School in Kraaifontein and matriculated from Eben Dönges High School in 1989. Karin enrolled at Stellenbosch University in 1990 and graduated with the degree BSc in Food Science in 1993. In 1996, she started working at the former Stellenbosch Farmers' Winery as a microbiologist. She enrolled at the Institute of Wine Biotechnology for her MSc degree in 1999 and is still employed at Distell in the Quality Management and Research Department: Microbiology.

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PREFACE

This thesis is presented as a compilation of four chapters and each chapter is introduced separately. Chapter 3 will be prepared and submitted for possible publication in Annuals of Microbiology.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Development of specialised yeast strains for use in whiskey production : A Review

Chapter 3 **Research Results**
Characterisation of whiskey yeast and the expression of different amylolytic genes of *Lipomyces kononenkoae* in distiller's yeast

Chapter 4 **General Discussion and Conclusions**

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Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

CHAPTER 1

1.1 GENERAL INTRODUCTION

The earliest reports on the history of whiskey production indicate that it was produced in Ireland in the 12th century, but it was in Scotland that its qualities came to be extensively appreciated (Berry, 1984). Whiskey is the potable spirit obtained by distillation of an aqueous extract of an infusion of malted barley and other cereals that has been fermented with strains of *Saccharomyces cerevisiae* yeast. Various types of whiskey are produced in a number of different countries in the world. Principally, they differ in the nature and proportion of the cereals used as raw materials, as well as in the type of still used in distillation.

In Scotland, a Scotch malt whiskey is produced using only malted barley, with the fermented mash being distilled in small pot stills. These products are produced in small distilleries, of which there are over 100 in Scotland. Scotch grain whiskey is produced using malted barley together with wheat or maize (up to 90%) and it is distilled continuously in Coffey-type patent stills.

The tremendous popularity of whiskies manufactured in Scotland, Ireland, the US and Canada has prompted several other countries to start manufacturing whiskies, usually using Scotch whiskey as the benchmark. Today, there are over a dozen countries with minor but significant whiskey-distilling industries including Australia, Japan, Spain, the Netherlands and South Africa (Lyons, 1995).

Most discussion of the quality of malt whiskey focuses on the quality of peat, malt, water and air, the effect of still design and maturation, but rarely emphasises the role of the main organism active during fermentation, the yeast (Berry, 1979). The production of ethanol by microorganisms as a result of the fermentation of substrates, such as sugars or starch, is a process that predates recorded history. More than 90% of the fermentation ethanol produced today employs the yeast *S. cerevisiae* (Stewart, 1985). The main requirements for distiller's yeast strains include efficient substrate utilisation, a fast rate of fermentation, high ethanol yield and tolerance, high viability of cells, tolerance to low pH and high temperature, good flavour production and genetic stability (Korhola, 1987).

The most utilised raw material for grain whiskey production is maize, which consists of starch, that is not fermentable by *S. cerevisiae* because it lacks enzymes with amylolytic activity (Hinrichs and Stahl, 1993). The production of grain whiskey depends on the addition of enzymes (α-amylases and glucoamylases) for the conversion of starch to fermentable sugars. The group of α-amylase enzymes cleaves α(1-4) linkages in the starch molecule, whereas glucoamylases cleave α(1-6) linked branch points, in addition to α(1-4) linkages, to produce glucose, maltose and maltotriose (sugars which are readily taken up and fermented to ethanol by strains of *S. cerevisiae*) (Tubb, 1986). It therefore is of great commercial interest to create new

yeast strains to convert the polysaccharides of plant biomass directly into fermentable sugars (Hollenberg and Strasser, 1990).

With the advent of DNA transformation systems for yeast, it is now possible to modify the genetic composition of distiller's yeast strains by introducing genes from other sources (Stewart, 1997). Several laboratories are involved in the construction of amylolytic active strains, but there have been no reports on industrial strains that can fully utilise raw starch. Of all the yeast species able to grow on starch, only very few degrade starch with high efficiency, as a result of the combined action of α-amylase and debranching activity. One such strain is *Lipomyces kononenkoae* (Spencer-Martins and Van Uden, 1979). However, owing to its low ethanol tolerance, slow growth rate, poorly characterised genetics and lack of GRAS (**G**enerally **R**egarded **A**s **S**afe) status, it cannot be used as a fermenter. In contrast, a genetically engineered strain of *S. cerevisiae* secreting heterologous raw starch-degrading enzymes could be beneficial for the beverage industries (Steyn *et al.*, 1996). We have tried to accomplish the engineering of such a strain in this study through the transformation of whiskey strains with two α-amylase genes from *L. kononenkoae.*

1.2 PROJECT AIMS

The aims of the present study were the following:

(i) Characterisation of whiskey yeast strains by comparing chromosome profiles.

(ii) Selection of whiskey yeast strains for low/no H_2S production, high fermentation rate and high alcohol production.

(iii) Construction of two yeast integrating plasmids, one containing the *LKA1* gene and the other one containing both *LKA1* and *LKA2* genes, with the *SMR1* gene as marker, and introduction of these cassettes into selected whiskey strains.

(iv) Performance of enzyme assays and testing the ability of the yeast to grow in a medium with starch as sole carbon source.

(v) Evaluation of transformants in whiskey fermentations, in comparison to the control strains.

1.3 LITERATURE CITED

Berry DR. 1979. The physiology of whisky fermentation. *Brewers' Guardian* **6**:25-29.

Berry DR. 1984. The physiology and microbiology of Scotch whisky fermentations. *Prog. Ind. Microbiol.* **19**:199-243.

Hinrichs J and Stahl U. 1993. Genetic engineering in the brewing industry. *Brauwelt International* **4**:293-298.

Hollenberg CP and Strasser AWM. 1990. Improvement of baker's and brewer's yeast by gene technology. *Food Biotechnology* **4**:527-534.

Korhola M. 1987. Yeast in the Alcohol Industry. *Proceedings of the 21st Congress of the European Brewery Convention, Madrid*. pp 105-121.

Lyons TP. 1995. The Production of Scotch and Irish Whiskies. In: *The Alcohol Textbook*. Edited by: Lyons TP, Kelsall DR and Muragh JE. pp 127-156. Altech Inc., UK.

Spencer-Martins I and Van Uden N. 1979. Extracellular amylolytic system of the yeast *Lipomyces kononenkoae*. *Appl. Environ. Microbiol.* **44**:1253-1257.

Stewart GG. 1985. New developments in ethanol fermentation. *Am. Soc. Brew. Chem.* **43**: 61-65.

Stewart GG. 1997. Genetic manipulation of brewer's and distiller's yeast strains. *Food Science and Technology Today* **11**:181-182.

Steyn AJC, Marmur J and Pretorius IS. 1996. Cloning, Mapping and Characterization of a Genomic Copy of the *Lipomyces kononenkoae* α-Amylase-Encoding Gene (*LKA1*). *Yeast* **12**:925-937.

Tubb RS. 1986. Amylolytic yeasts for commercial applications. *Tibtech* **4**: 98-104.

Chapter 2

LITERATURE REVIEW

Development of specialised yeast strains for use in whiskey production: A Review

CHAPTER 2

2.1 INTRODUCTION

The art of making wine and beer can be traced back to 5000 to 6000 B.C., but the history of distillation is much shorter (Reed and Nagodawithana, 1991). The origin of distilled beverages is lost in antiquity, but it is generally assumed that the Chinese were the first to use the process of distillation about 3000 years ago. However, it is unclear how this knowledge was passed on to other civilisations.

During the Christian era, the Egyptians were the first of the highly developed civilisations to produce distilled beverages. The first distillation apparatus was described by Arab alchemists and this is quite similar to a pot that some distillers still use today (Nagodawithana, 1986). It is likely that the first whiskey was distilled in Ireland, and not in Scotland. The spirit was already known in 1170, when Ireland was invaded by the English, and it is therefore most likely that Irish missionary monks imported the art of distillation into Scotland (Lyons, 1995). The first historical record of whiskey production was by Friar John Corr in Scotland in 1494 (Berry, 1984). The word "whiskey" is derived from the Gaelic term, "visge beatha", meaning water of life (Nagodawithana, 1986). Whiskey is the name given to the distilled product of the fermented substrates obtained from cereal grains that have been matured in wooden casks. Scotch whiskey can only be produced in Scotland and, at present, the Scottish brands are the most well known whiskies in the world. Today, many other countries also produce this "water of life" and whiskies from Canada, Ireland and America (the so-called Bourbons) need no introduction. Many changes have occurred in whiskey production over the past two to three hundred years, making today's product different from that produced by John Corr. Through ongoing research, the process today is much more refined and will continue to improve as new technologies become available.

Whiskey production is an enzyme-dependent process and the enzymes for the production of Scotch whiskey are derived entirely from malted barley (Walker *et al.*, 2001). For the production of whiskey from maize alone, as in South Africa, commercial enzymes are commonly used. In order to produce ethanol, the complex carbohydrates must be reduced to sugars that are utilisable by yeast. Not only is the yeast responsible for the production of alcohol, but also for the formation of minute quantities of flavour compounds, which together make up a large part of the final perceived flavour and aroma. Distilling yeast strains have a number of limitations (Hammond, 1994). The conversion of starch to ethanol is limited by the inability of the yeast to ferment all the substrate present, the control over flavour production by the yeast is limited and, despite high standards of hygiene, contamination by other microorganisms is always a possibility. The range of improvements possible is still

restricted, but these problems can be addressed with increasing knowledge of yeast genetics and physiology.

The aim of this literature study is to investigate the possibilities of improving distiller's yeast with regard to the utilisation of starch-containing raw material. An overview of the Scotch whiskey production is necessary for a full understanding of the strategies that are going to be discussed.

2.2 STARCH AS A FUNCTIONAL INGREDIENT FOR ALCOHOL PRODUCTION

Starch contains two components that contribute to its molecular structure, namely amylose and amylopectin (Hough, 1985). Amylose is a linear-chain molecule in which all the D-glucose units are bound in α(1-4) linkages, whereas, in amylopectin, the α(1-4)-linked chains are highly branched through a number of α(1-6) linkages. A cooking-and-conversion process is necessary to produce fermentable sugars before fermentation can take place. Seeds store energy in the form of starch granules. In this form, the starch is poorly accessible to hydrolytic enzymes. The pretreatment of starch consists of three steps, namely gelatinisation, liquefaction and saccharification. Heat and water are needed for gelatinisation, and liquefaction needs the use of heat and amylolytic enzymes, such as heat stable α-amylases. Starch molecules are only partially hydrolysed during liquefaction and are then further converted to sugars, such as glucose and maltose, through the process of saccharification, which is accomplished by making use of glucoamylases (Stewart and Russell, 1986).

Starch-hydrolysing enzymes fall into three basic categories, namely α-amylase, β-amylase and glucoamylase. An α-amylase is known as an endo-enzyme, meaning that it acts from within the starch molecule, catalysing the hydrolysis of α(1-4) linkages. Once the starch is liquefied, it must be converted to fermentable sugars. A glucoamylase is used to accomplish this. This is an exo-enzyme, as it acts from outside the molecule, removing glucose units from the non-reducing end of the starch molecule. This enzyme can also catalyse the hydrolysis of α(1-6) bonds, in addition to α(1-4) bonds. Figure 1 shows the action of these two enzymes in amylose and amylopectin.

α-Amylase is obtained from malted barley and catalyses the hydrolysis of every second α(1-4) glycosidic bond from the non-reducing end of the starch molecule to produce maltose units. α-Amylase is noted for its extreme thermostability during the cooking process and is stable up to 95°C. It is important to note that calcium acts as a cofactor for α-amylase stability. α-Amylase contains calcium at a vulnerable position in the molecule and, if it is lost, it can cause inactivation of the enzyme (Sears, 1995).

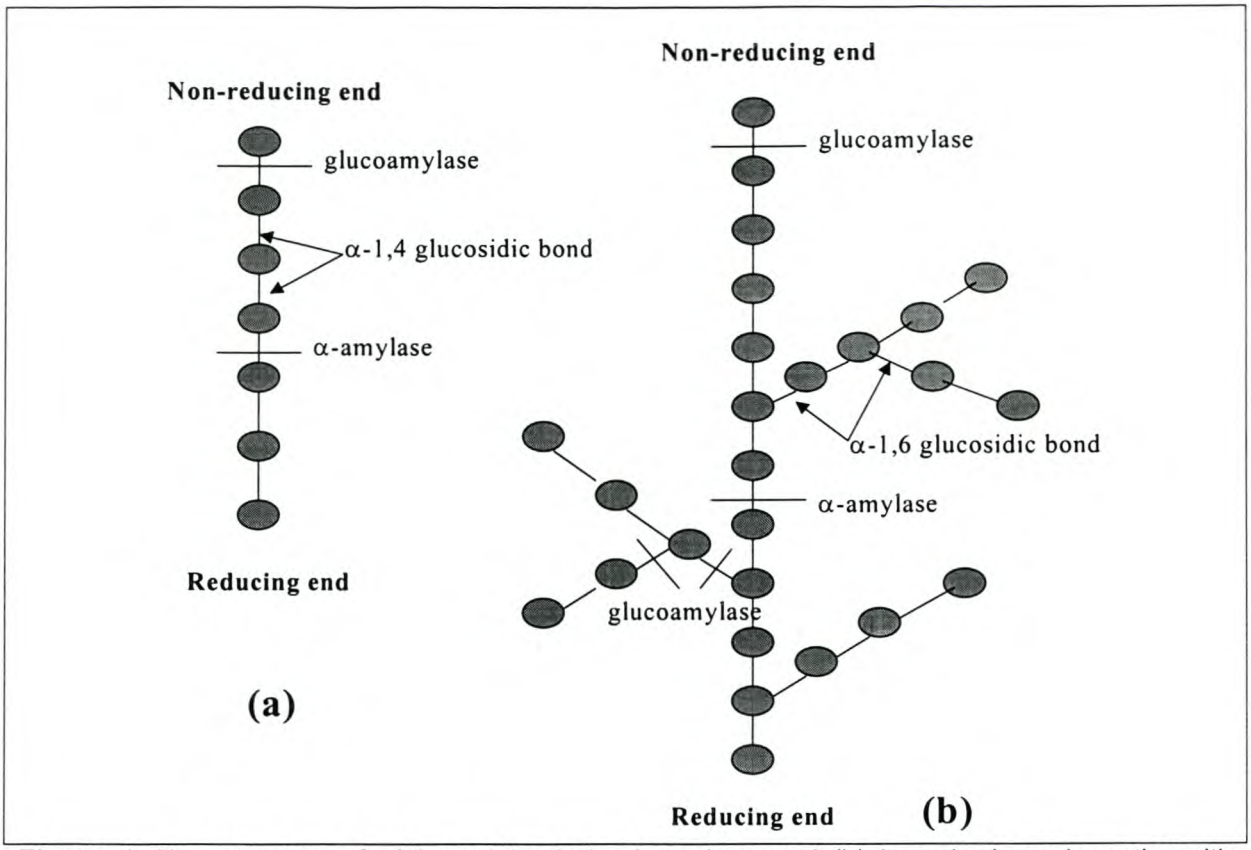

Figure 1. The structure of (a) straight-chained amylose and (b) branched amylopectin, with possible points of attack for α-amylase and glucoamylase. Each circle represents a glucose unit (Keim, 1995; Hough, 1985).

2.3 MALT WHISKEY VERSUS GRAIN WHISKEY

Scotch whiskey is an alcoholic beverage that can only be produced in Scotland. There are two main types. If the whiskey is distilled from the fermented extract of malted barley alone, it is called Scotch malt whiskey. If the fermented extract is made from cereals such as wheat or maize, in conjunction with malted barley, however, the distilled product is called Scotch grain whiskey. The distilled product from either fermented extract is matured in oak barrels for at least three years. Blended Scotch is made from as many as 40 malt and grain whiskies, ranging in age from three to over 30 years (Palmer, 1997).

2.3.1 SCOTCH MALT WHISKEY PRODUCTION

2.3.1.1 Raw material: Barley

Barley is used to make malted barley and is rarely used in the unmalted state in Scotch whiskey production. Unmalted barley is high in husk content, which decreases the amount of starch present and the associated yields of ethanol. Barley used for the production of distiller's malt should comply with certain criteria: it should be of a specified variety, must germinate to 98%, should be free from visual signs of fungal and insect attack, must have a starch content of at least 64% and a low

protein content (ca. 10%), and should also be free from pre-germination and husk damage (Palmer, 1997). After the harvest, the barley is dried to a water content of 11-12% to protect the viability of the grain during storage (Palmer, 1997).

2.3.1.2 Malting barley

2.3.1.2.1 Steeping

Steeping is the first stage of the malting process and involves the immersion of barley in water (Palmer, 1997). The purpose of steeping is to ensure a uniform germination. The uptake of water during steeping is generally regarded as a three-stage process. The initial absorption of water continues rapidly until a moisture level of 32% is reached. A lag phase follows, which can last for a further 10 hours. A third phase of active water uptake follows to produce a maximum moisture content after a total steeping time of about 50 hours. Aeration is usually employed during steeping (Bathgate and Cook, 1989), not only to increase the rate of water uptake by the embryo and consequently the rate of development of the germ, but also to remove excess CO_2 and ethanol, which can have an inhibitory effect on germination. The steeped grain contains about 46% water and, at the end of the steeping stage, the grains should have started to germinate (Palmer, 1997).

2.3.1.2.2 Germination

The purpose of germination for malt spirit production is two-fold: firstly, to generate amylolytic enzymes and, secondly, to release starch grains from the cells of the barley endosperm, thereby initiating the breakdown of the starch grains (Berry *et al.*, 1987). Hydrolytic enzymes, such as endo-β-(1-3), (1-4)-glucanase and pentosanases, are released for breaking down cell wall β-glucans and pentosans respectively to expose starch and proteins (Palmer, 1997).

The enzymes involved in the conversion of starch to fermentable sugars are the α- and β-amylases, limit dextrinase and β-glucosidase (Bathgate and Cook, 1989). The germinated grain is allowed to grow in a germination chamber for about five days. A relative humidity of nearly 100% is maintained by blowing water-saturated air through the grain and the temperature is kept at 16°C. The temperature is then increased to 63-64°C to complete the conversion of all the gelatinised starch to a complex of glucose-related sugars (Palmer, 1997).

2.3.1.2.3 Kilning

The "green" malt, as it is now called, undergoes a drying stage (Bathgate and Cook, 1989). The malt should be dried at the lowest temperature possible, with high airflow to preserve the enzyme activity. This is also an important step for the development of flavour compounds in the malted grain. For characteristic flavour notes, the malt can be peated by burning plant material (peat) during the drying stage, thereby releasing smoke, and phenols are deposited onto the drying malt in small but

significant quantities. Only about 20% of the phenolic flavours of the peated malt is distilled over into the spirit, giving a distinct smoky, medicinal flavour to whiskey (Palmer, 1997). The malt is then milled and ready for use.

2.3.1.2.4 Mashing

Hot water is added to the malt to give a final mash temperature of 64-65.5°C (Berry *et al.*, 1987). The extract is drained off after about two hours and kept separate. A further extraction then follows with water of increasing temperature, at 68-74°C, to ensure that minimal sugar is lost. The extractions are pooled together in a fermentation tank and cooled to ±20°C. About 95% of grain starch hydrolysis takes place during mash extraction (Palmer, 1997). Seventy percent of protein hydrolysis occurs during malting and 30% during mash extraction.

The mashing procedure is biochemically complex (Berry *et al.*, 1987). A major proportion of the large starch granules is partially degraded during malting, which means that these more modified parts are hydrolysed rapidly. The bulk of the starch is made soluble after the gelatinisation temperature (63-64°C) is reached. The major products are maltose, glucose, maltotriose and dextrins. These dextrins can be linear or branched. Limit dextrinase in malted barley can survive the temperature conditions of both kilning and mashing, and has the capability to hydrolyse α-(1-6) linkages in α- and β-limit dextrins. It has recently been shown that the majority of limit dextrinase is protected as the bound form during mashing and it appears that the release of active limit dextrinase from its inhibitor during fermentation is highly regulated, so that constant levels of free limit dextrinase are available throughout the fermentation (Walker *et al*, 2001). The larger dextrins are liberated by the action of α-amylase, which is known as an endo-enzyme because it acts from within the molecule (Berry *et al.*, 1987). The dextrins then become susceptible to β-amylase, which is known as an exo-enzyme, producing maltose units from the non-reducing end of the dextrin or starch molecule. A final wash at 90-100°C is applied and this extraction water or sparging water, as it is also known, is used for the first mash water of the next mash. The only viable carbohydrase activity up to the last wash at 90°C will be that of α-amylase. At this stage, any remaining large starch granules will begin to dissolve into the wort or are partially broken down into larger dextrins.

2.3.2 GRAIN WHISKEY PRODUCTION

2.3.2.1 Raw material: maize

Since the last quarter of the 1800s, maize was commonly employed as the main raw material for Scotch grain whiskey. No enzyme additives are allowed, so the only source of enzymes for the conversion of starch to fermentable sugars is malted barley (Pyke, 1965). The starch in the cereal first has to be released from its matrix by a high temperature cooking process (Wilkin, 1989).

2.3.2.2 Cooking

The maize can be cooked in batches or continuous cooking can be employed. In batch cooking, the grist is pumped into pressure cookers fitted with a stirring gear to prevent sticking and burning of the grain. Water is added and steam is injected to bring the pressure up to 1.5×10^5 Pa and the temperature to 120°C, which is then maintained for about two hours or until the maize starch is completely gelatinised (Varnam and Sutherland, 1999). During the cooking process, the starch granules start to absorb water, swell and gradually lose their crystalline structure. These large gel-filled sacs then break with agitation and the starch becomes gelatinised (Keim, 1995). Conversion malt may be added to aid the liquefaction of the starch (Berry *et al.*, 1987). The enzymes of this so-called "pre-malt" do not survive the cooking process (Reed and Nagodawithana, 1991). Insufficient treatment leaves a proportion of the starch granules intact; excessive heating, however, appears to cause a degree of caramelisation of the starch. Production of pentose sugars from the maize husk is also possible and all of this can lead to a reduction in the amount of alcohol produced (Pyke, 1965).

2.3.2.3 Mashing

After the cooking stage, the hot, cooked maize is pumped into the mash tun. It is cooled down and mixed with the ground, malted barley at the most suitable conversion temperature, usually about 63-65°C (Berry *et al.*, 1987). It is essential for the cooked maize to be brought into contact with the malt enzymes immediately, because the entire mash will solidify if the gelatinised starch is allowed to cool. The diastiatic enzymes are released from the malted barley and allowed to degrade the gelatinised starch to fermentable sugars. At one time, the conversion mixture was washed out of the solid residues and pumped to a separate mash-tun (Pyke, 1965). Now, the majority of grain distillers ferment the unfiltered, converted grain mixture, which results in higher alcohol yields. The mashing process continues in the fermentation vessel since the residual malt enzymes maintain their activity for part of the fermentation (Berry *et al.*, 1987).

2.4 FERMENTATION

2.4.1 YEAST STRAINS

Fermentation in the production of whiskey is basically similar to any other alcohol fermentation, during which sugars are hydrolysed from grain or maize starch and then metabolised by yeast into ethanol, carbon dioxide, byproducts and cell material (Korhola *et al.*, 1989). The use of specific raw materials, yeast strains and fermentation conditions, as well as bacterial contaminations, will lead to uniquely different whiskey fermentations and, subsequently, a different product.

Most traditional Scottish malt distilleries use two types of yeast in their whiskey fermentations, namely a primary and a secondary strain (Sharp and Watson, 1979). Primary strains are used as the main fermenting yeast and are mostly strains of *Saccharomyces cerevisiae*. This yeast is a fast fermenter and tends to outcompete other microorganisms that may be present in the fermentation. It also produces a high alcohol yield. Secondary strains are used in conjunction with the primary strain, mainly for adding complexity and for their cost-effectiveness. The main source of these strains is the excess yeast produced by the brewing industry and, because it is a waste product, it is sold at a much lower price than the primary yeast. Grain distilleries use only a primary strain.

In the production of Scotch malt whiskey, the wort, which is the fermentation medium, is very complex (Berry, 1979). Since it has not been boiled, it contains active malt enzymes as well as many microorganisms, such as lactic acid bacteria and wild yeasts. These wild yeasts can contribute to the aroma during the early stages of fermentation. They are outcompeted by the primary strain and do not survive the high alcohol levels produced in the final stages of the fermentation (Korhola *et al.*, 1989).

If commercial enzymes are permitted to be used for starch conversion, a high temperature cooking process is involved. This process sterilises the mash and therefore minimises the presence of other microorganisms in the fermentation.

2.4.2 SUGAR UPTAKE BY YEAST

The mash in whiskey fermentation is a rich source of sugars, nitrogenous compounds, vitamins, salts, such as sulphate and phosphate and minerals, such as potassium, magnesium, calcium and zinc (Korhola *et al.*, 1989). The sugars present in a whiskey mash are mainly maltose, maltotriose, maltotetraose and possibly higher oligosaccharides, plus glucose, fructose and sucrose. Three methods of entry into the yeast cell are possible, namely simple diffusion, catalysed diffusion and active transport, which requires ATP for energy. The disaccharide maltose, which is an important product of amylolytic activity during whiskey production, is dependent on ATP for active transport into the cell.

Glucose, fructose and sucrose are consumed rapidly, maltose is consumed more slowly, followed by maltotriose (Hough, 1985). Sucrose is first hydrolysed to glucose and fructose at the cell wall by invertase. Amino acids are absorbed in sequence. One group, containing glutamate, asparagine and serine, is used before a second group, containing histidine and leucine. Finally, a group containing glycine and tryptophan is absorbed.

2.4.3 YEAST CARBOHYDRATE METABOLISM

Mash is fermented under anaerobic conditions, except for the air that remains in the headspace until it is replaced by carbon dioxide.

Yeasts use the glycolytic pathway to convert sugars into energy, intermediates and major end products, such as ethanol and carbon dioxide. All these reactions are catalysed by enzymes (Korhola *et al.*, 1989). Figure 2 shows the glycolytic pathway for the breakdown of glucose.

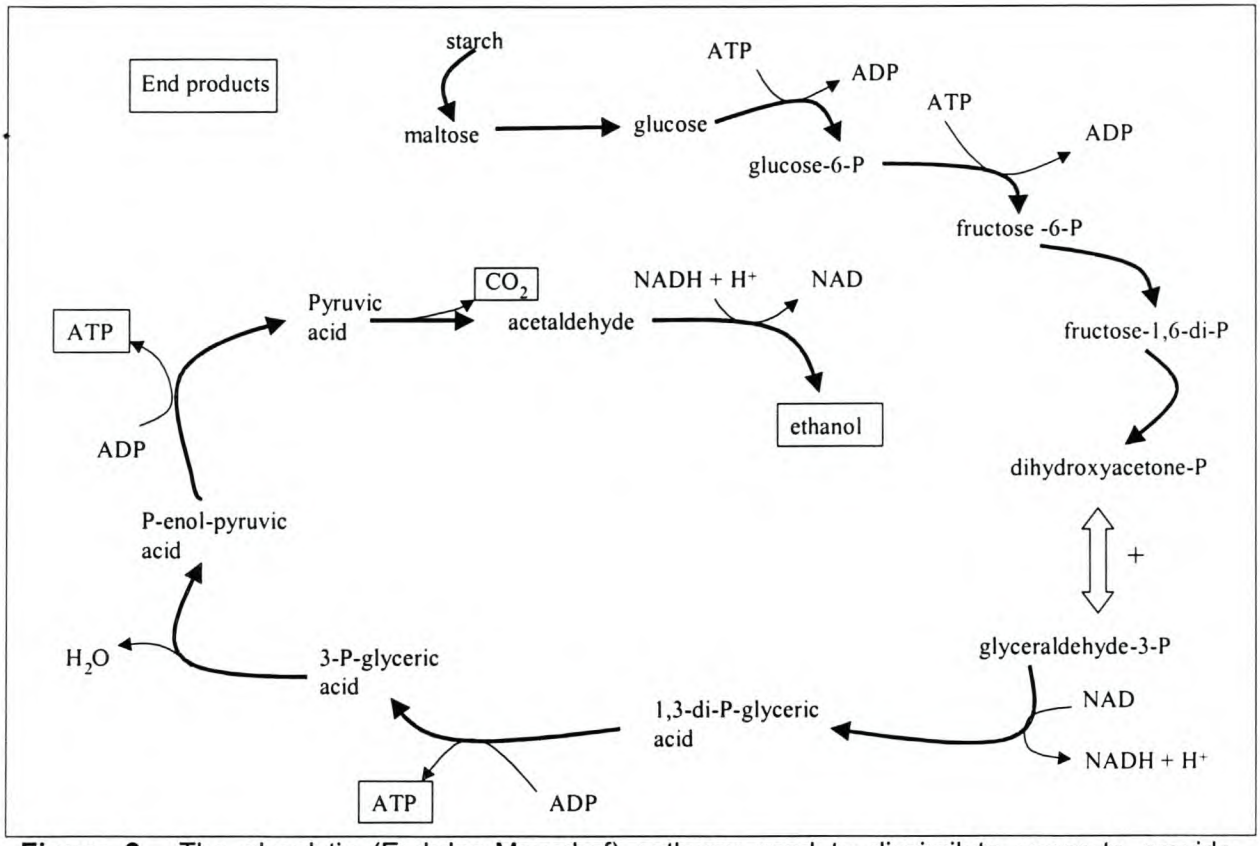

Figure 2. The glycolytic (Embden-Meyerhof) pathway used to dissimilate sugar to provide energy and carbon to the cell. The end product, pyruvate, is converted to ethanol and carbon dioxide by yeast. Note that, due to the interconversion of dihydroxyacetone phosphate to glyceraldehyde 3-P, two molecules of each of the subsequent intermediates and end products result for every glucose molecule degraded (Ingledew, 1995).

The fermentation process can be divided into three stages, namely a lag phase, an exponential or growth phase and a stationary phase, when the process slows down and the yeast starts to die and autolyse (Watson, 1981). Berry (1979) studied the whiskey fermentation process and found that the maximum yeast growth occurred in the first eight hours and was associated with a rapid utilisation of α-amino nitrogen and a drop in pH. He compared a laboratory-scale fermentation with a commercial-scale fermentation and found that the assimilable sugars had been utilised after only 12 hours in the industrial fermentation, whereas it took 28-30 hours to level off in the laboratory-scale fermentations. This was explained by the lack of a lag phase, as well as the high inoculum levels used in the Scotch whiskey industry. An increase in bacterial count was also observed during the final stages of the industrial fermentation, followed by a decrease in pH. Yeast viability dropped markedly after 24 hours, although no reduction was observed in the rate of ethanol production.

2.4.4 FERMENTATION PARAMETERS

Whiskey fermentation is started at a temperature of 20-23°C, which can rise to 33°C during the initial growth period as a result of the heat released by the metabolism of sugars (Watson, 1981). Some distilleries have temperature control devices fitted onto the fermenters that keep the temperature constant at 30°C, but most traditional distilleries do not control the fermentation temperature (Korhola *et al.*, 1989). This increase in temperature does not have an inhibitory effect on the yeast, as *S. cerevisiae* can ferment at temperatures of up to 46°C (Paterson and Piggott, 1989), but it can have an effect on the formation of organoleptic compounds. The inoculation level is in the order of 1-2 x 10^7 cells/ml (Ramsay and Berry, 1984).

An industrial-scale fermentation in which a high gravity wort was inoculated at a level of 2 x 10^7 cells/ml during the first runnings from the mash tun is described by Watson (1981). Four hours later, when the fermenter was filled, fermentation had already started. The initial growth phase was relatively short and was completed within 12 hours. The yeast concentration had risen to 2 x 10^8 cells/ml. During this time, the temperature had risen to 33°C from an ambient temperature of 20-23°C. The temperature remained stable during the stationary phase and gradually dropped towards the end of the fermentation. During the first 16 hours of the fermentation, sucrose, fructose, glucose and maltose were utilised, while alcohol production was completed after about 32 hours. A common description of the characteristics required for a distiller's yeast is as follows: a yeast with high alcohol tolerance that gives a good yield; a yeast that ferments rapidly and hence minimises the risk of contamination; and, finally, a yeast that produces the best concentration and balance of congeners desired in the distilled beverage (Reed and Nagodawithana, 1991).

2.5 FLAVOUR/AROMA COMPOUNDS PRODUCED DURING FERMENTATION

2.5.1 YEAST

Compounds contributing to the unique aroma of whiskey have different origins, namely compounds originating from the raw material and its processing; compounds formed during fermentation; compounds extracted from the wooden barrels during ageing, and compounds formed by chemical reactions between constituents present in the beverage during ageing (Ter Heide, 1986). These compounds are present in very small concentrations and their major source is the metabolism of wort or grain nutrients by yeast during the fermentation.

Over the years, much work has been devoted to the investigation of biochemical mechanisms, which today enables us to describe routes by which the flavour compounds are formed in alcoholic beverages. It was revealed that there are no major differences in the chemical composition of different beverages, although they readily can be distinguished from each other organoleptically. The most important differences appear in the quantitative contents of aroma compounds in the different

beverages (Suomalainen and Lehtonen, 1979). The major groups of organoleptic compounds produced by the yeast are the higher alcohols, fatty acids, esters and carbonyl compounds. A general scheme for the formation of these compounds is outlined in Figure 3 (Watson, 1983).

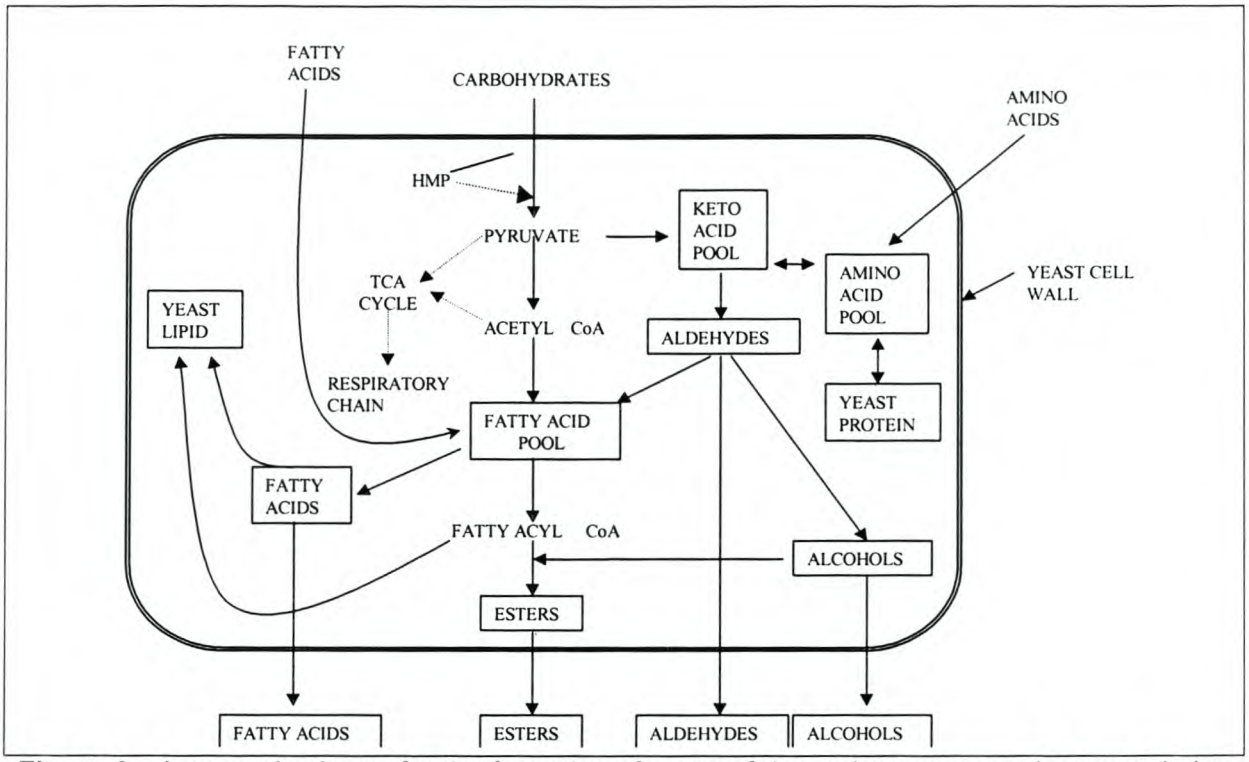

Figure 3. A general scheme for the formation of some of the major congeners by yeast during the malt whiskey fermentation (Watson, 1983).

2.5.2 LACTIC ACID BACTERIA

Flavour compounds are produced not only by the yeast, but also by lactic acid bacteria, which are always present in a malt whiskey fermentation. There is no wort boiling stage in malt whiskey fermentations, since the mash extract is transferred directly to the fermentation vessels (Barbour and Priest, 1988). Some degree of bacterial contamination therefore is normal in fermentations. However, these bacteria may proliferate in the mash, compete with the yeast and lead to a reduced ethanol yield. Although lactic acid bacteria are the main spoilage organisms in whiskey fermentations, other bacteria can also cause problems, as shown in Figure 4 (Ingledew, 1995). This figure illustrates the byproducts formed by various groups of microorganisms that are a problem in distilleries and which might, at times, develop in fermentation tanks or in spent grains. Glucose is converted to pyruvic acid by the homofermentative lactic acid bacteria, which are immediately converted to lactic acid through the enzyme lactic dehydrogenase. This will result in a drop in pH, and if the mash reaches a pH of less than 3.8, it is a sign of gross infection. A normal flora of about 10^4 cells/ml is considered desirable, and cell counts of 10^8 cells/ml are undesirable (Barbour and Priest, 1988). The lactic acid produced reacts with the

ethanol during distillation to form ethyl lactate. This flavour compound is desirable and makes a positive contribution to the sensory qualities of the final product.

Makanjuola and Springham (1990) studied the effect of added lactic acid, as well as bacteria, on malt whiskey fermentations, since lactic acid is known to have an inhibitory effect on yeast fermentations. A reduction in ethanol production of 16% and 12% was observed respectively when the bacteria were added prior to fermentation and when the bacteria were added at the start of fermentation. When added 24 hours after the yeast, no significant effect was observed. The viable yeast count and final pH were lowest in the case where bacteria were added prior to fermentation. The effect of added lactic acid was much the same, with the lowest ethanol yield occurring when it was added at the start of fermentation. However, the addition of lactic acid had a totally different effect on final viable yeast counts. When added 0 hours and 8 hours after the start of fermentation, the yeast counts were reduced to about 60% of the control values, but, when added later in the fermentation, at 20 and 30 hours, they were reduced to about 12% of the control values.

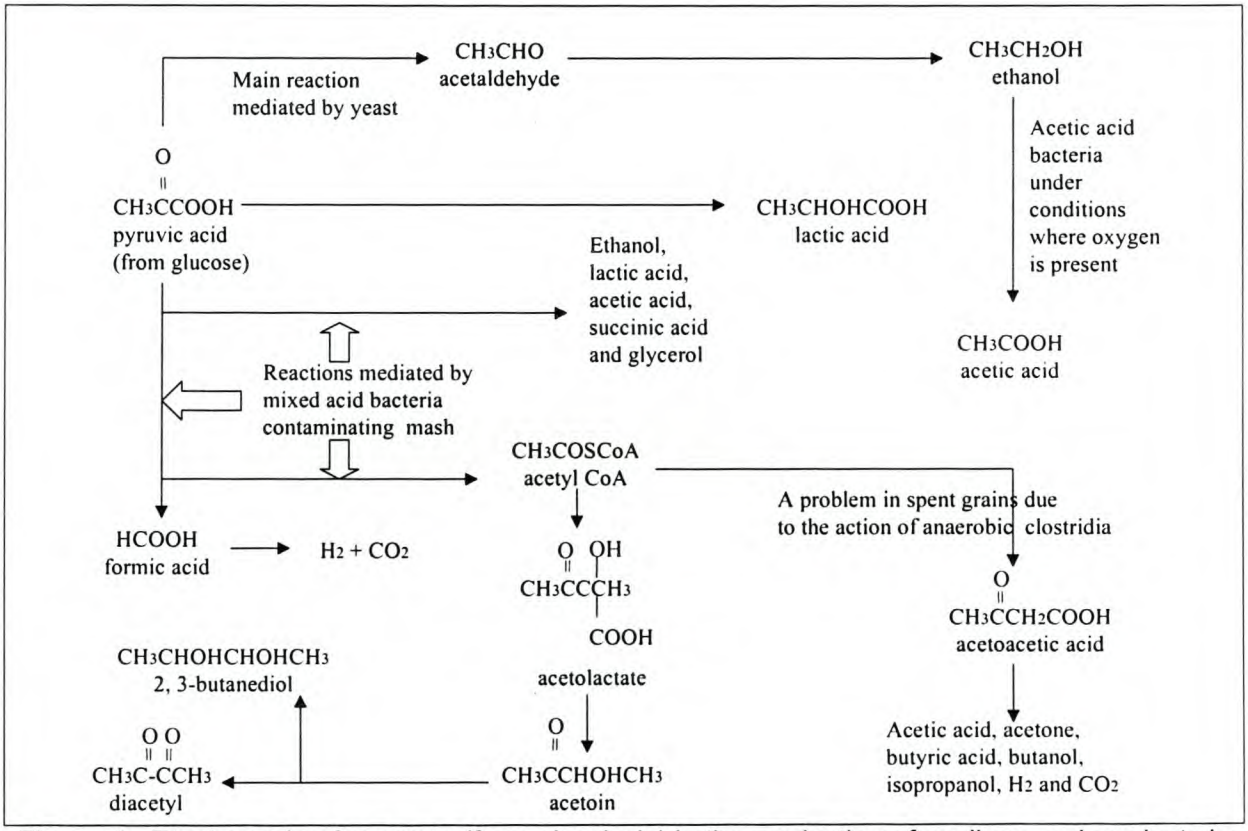

Figure 4. The key role of pyruvate (from glycolysis) in the production of spoilage end products in fermentations contaminated with organisms other than yeast (Ingledew, 1995).

It appears therefore that the inhibitory effect of the acid was dependent on the stage at which it was added, and not on the contact time between the acid or bacteria and the yeast cells. The amount of lactic acid produced by bacteria therefore will depend on the time of addition of the bacteria and how quickly bacterial numbers increase. Therefore, the bacteria added prior to the yeast were more inhibitory than

those added later. It is clear that early bacterial contamination during whiskey production can be quite deleterious when accompanied by a rapid and large production of lactic acid.

Lactobacilli can also metabolise glycerol to produce a flavour precursor, hydroxypropionaldehyde, which is converted again in the still to acrolein, giving unpleasant acrid notes to the whiskey (Barbour and Priest, 1988; Paterson and Piggott, 1989). Both beneficial and detrimental effects therefore can be attributed to contamination by lactic acid bacteria.

Barbour and Priest (1988) studied the effect of varying levels of *Lactobacillus* contamination on the whiskey fermentation using a laboratory system. In the control samples, in which no bacteria were inoculated, bacterial growth occurred at the end of the fermentation that results from the metabolism of the products of yeast autolysis. This tendency is found during a typical whiskey fermentation. When bacteria were inoculated at the start of fermentation, they grew during the initial stages of the fermentation and peaked at 17 hours. This is an atypical situation and will possibly occur in a distillery where the level of contamination is unacceptably high. It was also found that the higher the inoculum level, the lower the final pH and the higher the ethanol loss. The concentration of lactic acid also increased with bacterial numbers. Acetic acid, which will form ethyl acetate during distillation, was also detected in amounts proportional to cell numbers. It therefore is important to maintain a balanced mixed flora to produce unique organoleptic properties without causing a reduced ethanol yield.

Makanjuola and Springham (1984) did an extensive study of contamination levels of different stages of malt whiskey production and also identified the lactic acid bacteria isolated. From a total of 85 isolates, 36 strains were of the genus *Lactobacillus*, mainly *Lactobacillus plantarum*. Thirty-eight strains were from the *Leuconostoc* spp., the most frequent contaminant, but these tended to die off at later stages of the fermentation.

Priest and Pleasants (1988) further demonstrated that leuconostocs occur in both grain and malt whiskey distillery fermentations. The 73 test strains isolated were clustered according to the properties of reference strains. Some strains were difficult to identify, but *Leuconostoc mesenteroides* was found to be the most predominant strain occurring in malt and grain distillery fermentations. Various lactobacilli were also isolated. The stages at which the samples were taken were not specified, so these bacteria could have occurred at any time during the fermentation.

Geddes (1986) reported on the production of hydrogen sulphide by *Lactobacillus* spp. in fermenting wort. Sulphury off-notes are not always the result of yeast metabolism. Conditions of low pH, anaerobiosis and low carbohydrate concentration prevail during the final fermentation stages and, consequently, this is the stage when bacterial growth occurs. Lactobacilli were isolated from various sources in a malt distillery where sulphury aromas were detectable during fermentation. It was found that nearly 20% of the isolates were able to produce some H_2S. Three main types of

lactobacilli were predominant, namely *L. casei, L. plantarum* and an unidentified species.

Geddes and Riffkin (1989) studied the influence of lactic acid bacteria on the flavour of Scotch whiskey. They came to the conclusion that the presence of lactobacilli was essential for the contribution of organoleptic compounds in the final whiskey. Controlled levels of lactic acid bacteria led to reduced levels of acetaldehyde and higher levels of fatty acids and their ethyl esters. The levels of ethyl lactate were also elevated. The secondary lactobacillus fermentation had no apparent influence on higher alcohol formation.

It therefore is evident that the control of contaminants to minimum levels will result in efficient fermentations without undesirable flavours and with increased ethanol yields and, at the same time, add characteristic flavour compounds to the whiskey.

2.6 DISTILLATION

The purpose of the distillation process is to separate the ethanol produced during fermentation from the ethanol-water mixture. Most malt whiskey new spirit is double distilled in copper pot stills (Watson, 1983). Continuous distillation is also used for the production of grain whiskey and at least two columns are required, namely a beer still and a rectifying column. This type of distillation system is called a Coffee still. The Coffee still produces a spirit that has a high congener concentration and a very heavy flavour. The first Coffee still was built in Scotland in the early 1830s and, today, it is the traditional method for the production of grain whiskey. The beer still strips the alcohol from the supply stream and usually contains a few sieve plates that concentrate the alcohol to approximately 50-60% (w/w) in the overhead. This alcoholic product is called a high wine or primary distillate (Panek and Boucher, 1989). The feed to the rectifying column may be in the form of vapour, vapour-liquid or liquid. It further concentrates the ethanol fraction by removing water from the product distillate until an ethanol concentration of 95.6% (w/w) is reached (Nagodawithana, 1986).

In alcoholic products, the flavour is determined by minute quantities of volatile congeners that are formed as byproducts during the fermentation process and then concentrated into the final product by distillation (Panek and Boucher, 1989). These compounds, no matter how small in quantity, have a significant impact on the flavour and quality of the product. The congeners can be classified into three groups according to their behaviour in a distillation column. Most esters and aldehydes are more volatile relative to alcohol and they are the most abundant in the overhead product from a distillation column. They are commonly referred to as heads compounds. The organic acids and phenolic compounds are less volatile relative to alcohol and are most abundant in the base effluent of a distillation column. They are referred to as tails compounds. A third group of compounds includes amyl alcohols,

butyl alcohols, propyl alcohols and other higher alcohols. This group of compounds is more volatile than alcohol at low alcohol concentrations and less volatile than alcohol at high alcohol concentrations. They are collectively known as fusel oils. The region of the column in which the highest concentration of the fusel oils occurs, depends on the particular operating conditions of the column and, most importantly, on the alcohol concentration profile over the length of the distillation column. The effect of distillation on flavour and odour can be dramatic (Reed and Nagodawithana, 1991). By quantitative removal of the heads and tails fraction during distillation, it is possible to remove the perceptible odour almost completely.

Copper is used in the construction of most beer stills (Panek and Boucher, 1989). Fermented grain products contain volatile sulphur compounds that have a negative effect on distillate quality, giving it a cabbage-like odour. The copper acts as a catalyst, reacting with these compounds and removing them from the product vapour. A thick pad of fine copper mesh, called a demister, is also placed in the top of the column to remove sulphurous compounds, as well as any solid particles that have become entrained in the vapours.

Pot still distillation is a reflection of the unique product that emanates from such stills. Copper remains the only suitable material for distilling high quality malt whiskey and it surely is an essential ingredient in the malt whiskey recipe (Nicol, 1989). Distillation is not only an extraction step, but many interactions are continued, sometimes at a more significant level than during fermentation, e.g. the copper in the pot stills acts as a catalyst for ester production (Watson, 1983). The pot stills used for the production of malt whiskey produce a different product than that produced by the continuous Coffee stills (Nicol, 1989). The first distillation is carried out in a wash still, in which the fermented wort or wash, as it is called, is boiled. All of the ethanol is accumulated over a period of 5 to 6 hours and is called the low wine fraction, with an average ethanol concentration of 21% (v/v). The second stage is carried out in three phases. The foreshots, which are the first fractions, are responsible for cleansing out the heavy oils clinging to the internal surfaces from the previous distillation and are collected after 15 to 45 minutes, depending on the distillery. The second fraction, or middle cut, is the whiskey fraction and the strength of the distillate will be in the range of 70-75% ethanol. The strength and temperature of the distillate are monitored carefully. Depending on the distillery, the second cut point will be in the range of 62-64% (v/v) ethanol. The third fraction is called the feints and the distillation will continue until the distillate contains 1% (v/v) ethanol. The foreshots and feints are added back to the spirit still, together with a new batch of low wine. The timing of the cut between foreshots, whiskey and feints is of the utmost importance, since this will determine the final spirit quality.

There are so many factors that influence the congener composition of the new spirit that it is little wonder that each distillery produces a different product. For more consistent spirit quality, a full understanding of the parameters involved is necessary, and they also need to be controlled tightly.

2.7 MATURATION

Maturation is the important, final phase in whiskey manufacturing and it is needed to produce a well-balanced, drinkable product. Maturation chemistry is a very complex and complicated field and a lot of research has been done on spirit maturation, yet the chemistry is still not fully understood. Freshly distilled whiskies have pungent, unpleasant odours and a sharp taste, therefore storage in oak casks for a number of years is needed to modify their aroma (Nishimura and Matsuyama, 1989).

Before whiskey can be sold as Scotch whiskey, it must be matured in a warehouse in Scotland in oak barrels of not greater than 700 litres for 3 years (Palmer, 1997). For more than a hundred years, Scotch whiskey has been stored in used sherry casks, but due to the shortage of used sherry casks, American oak casks that have been used for maturing bourbon whiskey are now being used. American law requires that bourbon casks are used only once, but they can be used over and over again for Scotch whiskey (Nishimura and Matsuyama, 1989). About 2% of the content of the barrel is lost each year, thus the loss in a 12-year-old whiskey is about 24%. Some of the changes that take place in Scotch malt whiskey during maturation are illustrated in Figure 5.

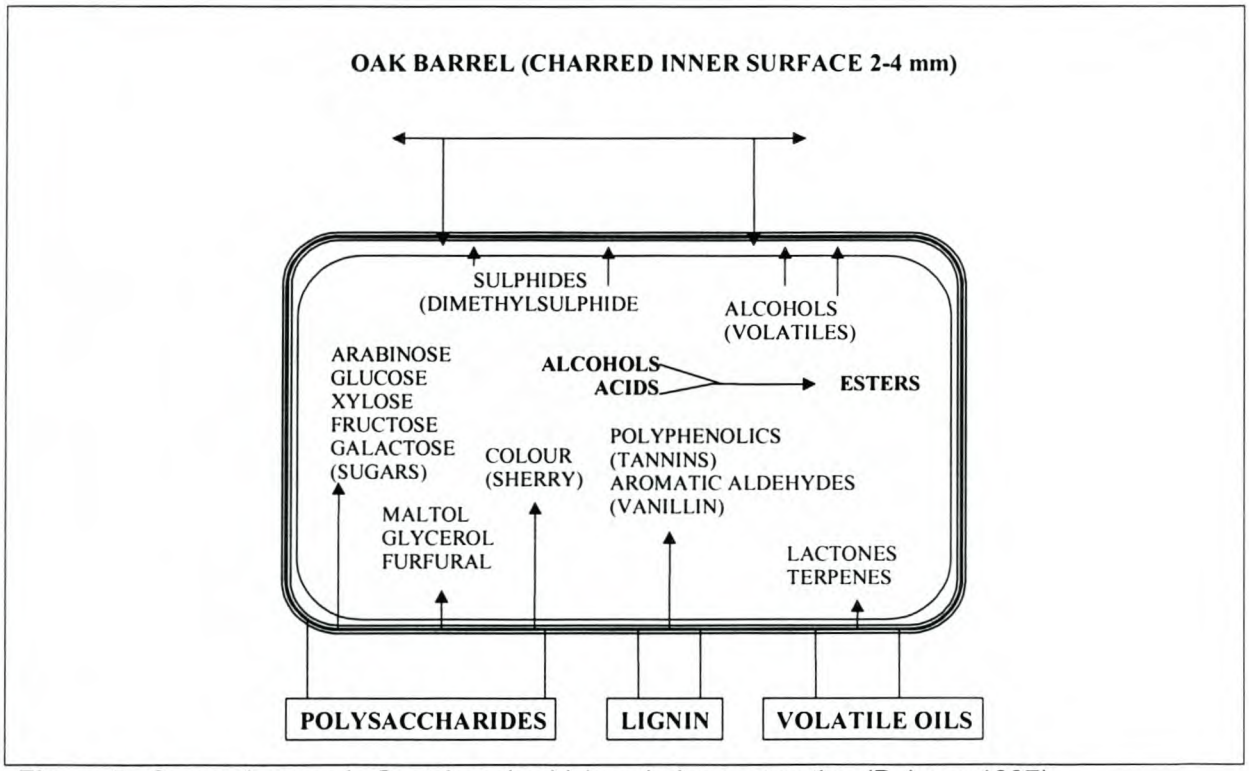

Figure 5. Some changes in Scotch malt whiskey during maturation (Palmer, 1997).

Nishimura and Matsuyama (1989) divided the changes that take place in the oak cask into seven types:

(i) Direct extraction of wood components.

(ii) Decomposition of wood macromolecules from the woody cell wall – lignin, cellulose and hemicellulose, followed by elution.

(iii) Reactions of wood components with spirit components.

(iv) Reactions involving only the extracted wood components

(v) Reactions involving only the distillate components.

(vi) Evaporation of low-boiling compounds through cask wood.

(vii) Physical rearrangement of components of the spirit to give structured liquid crystals.

Charring of the inner surface of the barrel is a common feature and the objectives of thermally degrading the wood in this manner are threefold. Firstly an active carbon layer is produced, which absorbs the unpleasant flavours of sulphur compounds from the whiskey. Secondly, oak lignin is anaerobically degraded in the layer immediately underneath the charcoal. Flavour compounds, such as vanillin, are released and dissolve into the spirit during maturation. Finally, the total wood extract gives colour and phenols to the maturing whiskey (Paterson and Piggott, 1989). Compounds that are released into the spirit include hydrolysable tannins, such as ellagitannins and gallotannins. They help to promote oxidative reactions and ester formation from acids, such as acetic acid, and alcohols. Lignin-derived flavour compounds, such as vanillin, syringaldehyde, synapaldehyde and coniferaldehyde, give vanilla-like aroma and smoothness to the spirit. Low level sweetness is due to the release of monosaccharide sugars and glycerol (Palmer, 1997). Among these compounds, the lignin-related compounds are one of the most significant groups and are closely associated with the development of matured whiskey flavour (Nishimura and Matsuyama, 1989).

Nishimura *et al.* (1983) studied the compounds produced by the reaction of ethanol and oak wood lignin during the maturation process and found that the aromatic aldehydes, such as vanillin, syringaldehyde and synapaldehyde, occur at much higher concentrations when the spirit is matured in charred oak barrels than when it is aged in uncharred barrels. This indicates that these aromatics from lignin result from the toasting or charring of oak wood.

Another compound that shows a significant increase in concentration is ethyl acetate. This can be explained by chemical mechanisms, proposed by Reazin (1983), whereby ethanol is oxidised to acetaldehyde in the presence of oxygen. Also in the presence of oxygen, acetaldehyde is oxidised to acetic acid. Barrel wood components are also broken down to form acetic acid. The reaction of ethanol and acetic acid leads to ethyl acetate formation. Samples of a bourbon distillate were removed from a new charred whiskey barrel over a period of 56 months. It was found that the specific activity of ethyl acetate increased as the maturation time increased.

Certain factors influence the extraction of compounds from stored whiskey. They are the type of wood used, new or re-used barrels, charred or uncharred barrels, proof of stored whiskey, temperature and, of course, length of storage (Reed and Nagodawithana, 1991).

2.8 BLENDING

The pioneer of whiskey blending, Andrew Usher, first practised the art of blending in Edinburgh in the early 1860s. Pot still malt whiskey was said to be flavoured too strongly for everyday drinking, especially by people in warmer climates. By mixing malt whiskey with grain whiskey, a milder flavour with less pronounced characteristics is achieved.

Blending whiskey is considered an art that is only acquired after years of experience. The art of blending is by no means a dilution, but rather the combination of compatible whiskies to bring out the best qualities of each. The aim of blending is twofold. Firstly, blending is carried out to produce a whiskey of definite and recognisable character and, secondly to achieve consistency. It is the responsibility of the blender to decide when the different single whiskies are ready to be used. A blended whiskey consists of a mixture of different grain and malt whiskies. The percentage of each depends on the uniquely flavoured end product to be achieved and each company has its own secret recipe for a specific blend (Anonymous, 1972).

The blender can use any number of different bases in a blend. It is advantageous, however, to use a large number of individual malts in a blend in case any given malt becomes unavailable. Whenever a certain malt becomes unavailable, it is the duty of the blender to be able to replace a missing component without altering the quality and consistency of the final product.

The mixing process is a straightforward procedure. Approved whiskies are drained from the casks in the correct proportions and conveyed via stainless steel troughs to a blending vat where mixing is done with mechanical agitators and compressed air. The blender once again evaluates the mix and, if differences occur in relation to a reference standard, the blend is corrected. Water is then added to obtain the desired bottling strength. The quality of the water used for blending should be of the highest standard; otherwise problems could be experienced with precipitation in the bottle. A small amount of food grade caramel is added to the final product to achieve colour consistency, whereafter the whiskey is filtered and bottled (Booth *et al.*, 1989).

2.9 IMPROVING YEAST STRAINS FOR WHISKEY PRODUCTION

2.9.1 CHROMOSOMAL DNA AND PLOIDY

Haploid strains of *S. cerevisiae* contain 16 linear chromosomes varying between 250 and 2000 kb in length (Barre *et al.*, 1993). Most laboratory-bred strains of *S. cerevisiae* are either haploid or diploid, whereas industrial brewing and distilling strains are predominantly polyploid or aneuploid. These strains cannot be made to sporulate, or only with great difficulty, and, if spores are produced, they are of low vitality (Hinrichs and Stahl, 1993). It appears to be no accident that polyploid strains

are widely used for industrial purposes, as they are genetically more stable and are less susceptible to mutational forces than haploid or diploid strains. This characteristic enables such strains to be used with a high degree of confidence.

Little is known about the detailed genetic structure of distiller's yeasts. Generally, they are believed to be genetically close to standard laboratory strains of *S. cerevisiae* (Hansen and Kielland-Brandt, 1996). Keiding (1985) studied the genetic structure of one isolate of a distiller's yeast and found it to be tetraploid. It also contained several restriction polymorphisms. In a further study, by Codón *et al.* (1998), who characterized industrial *Saccharomyces* strains, including baker's, wine, brewer's and distiller's yeast, similar results were obtained when these strains were compared with the DNA content, chromosomal polymorphism and DNA homologies of laboratory strains. The DNA content of the distiller's yeasts varied from 1.3n-1.6n when diploid and haploid laboratory yeasts were used as controls. No uniformity was found within an industrial group. DNA homologies were examined by Southern blot analysis and the results showed that the DNA of all the industrial yeasts was highly homologous to that of the laboratory strains when different probes were used.

2.9.2 GENETIC TECHNIQUES FOR THE DEVELOPMENT OF NEW DISTILLER'S YEAST STRAINS

2.9.2.1 Clonal selection of variants

Spontaneous mutations, Ty-promoted chromosomal translocations and mitotic crossing-over or gene conversion are responsible for the distinct changes shown by a yeast strain after many generations (Snow, 1983). Selection therefore can be employed directly for strain development and the isolation of variants with desirable characteristics. The selection process, however, depends highly on the availability of screening methods to identify those variants displaying improved performance.

Slaughter (1993) briefly explains the selection procedure of glucose de-repressed variants of existing strains in his report on current research with regards to brewing and distilling yeast strains. It is known that *S. cerevisiae* takes up sugars from the medium in a definite order. The uptake of maltose and then of the higher oligosaccharides begins only after the level of glucose has fallen to a certain minimum. This effect is called catabolite de-repression. If, however, a glucose de-repressed strain is used, no preferential uptake of glucose occurs and all the sugars are utilised from the beginning of fermentation. This is achieved by selecting for resistance to the glucose analogue, 2-deoxyglucose. This sugar is transported into the cell through the same transport system as glucose, but it is not metabolised and is toxic to the cell. Growth on malt agar in the presence of 2-deoxyglucose suggests that the mutant strain shows characteristics of a glucose de-repressed strain.

2.9.2.2 Mutagenesis and selection

Mutations can be induced by the use of mutagens, which can be of a physical (e.g. UV radiation at 254 nm) or chemical (nitrosoguanidine) nature (Snow, 1983). Most mutations are recessive and are not detected easily in diploid or polyploid cells, therefore stable haploid cells must be used or mutagenesis should be carried out directly on the spores. Followed by an appropriate screening method, mutagenesis is a rational approach to strain development when only one parameter is to be changed (Pretorius, 2000). However, it is possible that unrelated genes can also be affected, causing undesirable changes in yeast performance.

2.9.2.3 Hybridisation

2.9.2.3.1 True hybridisation or mating

This method is used to cross two heterothallic diploid strains of opposite mating types, resulting in a heterozygous diploid that can be made to sporulate again, repeating the cycle. Unfortunately, it is difficult to use this process for industrial strains due to their weak sporulation (Hammond, 1996). Direct spore-cell mating by means of a micromanipulator can be used as an alternative (Pretorius, 2000).

Miklos and Sipiczki (1991) crossed a distiller's yeast with a less productive wine yeast to obtain hybrids with increased ethanol production and tolerance. The improvement might have been due to an additive complementary effect of favourable alleles of the partners (heterosis), or to the increase of ploidy, or to both. Christensen (1987) cross-bred distiller's yeast strains to obtain hybrids with an improved fermentation rate and higher ethanol tolerance. Only a few hybrids that were better than the reference strains were obtained after screening, so it is not always the case that the best performing parent strains will result in even better hybrid strains.

2.9.2.3.2 Rare mating

Industrial strains with weak or no sporulation ability can be forced to mate with haploid *Mata* and *Mat*α strains, but this cross is a rare event (Snow, 1983). Hammond and Eckersley (1984) transferred the killer property to a brewing strain by using the rare mating technique.

2.9.2.4 Spheroplast fusion

This technique also overcomes the need for opposite mating types, as in rare mating. Yeast cell walls of different parental strains are removed by lytic enzymes, creating spheroplasts that are mixed together in the presence of polyethylene glycol and Ca-ions. The regeneration of their cell walls then takes place in a selective medium (Snow, 1983).

Salek and Arnold (1994) used electro-fusion of protoplasts to obtain diploid distillery strains with higher alcohol resistance, resulting in higher alcohol production than the parental strains. The same principal as for spheroplast fusion applies here;

the only difference is the use of a commercial system for electro-fusion, consisting of helical chambers and fusion power supply. Video microscopy in combination with computerised image analysis was effective in providing information about the degree of protoplastisation. Electrofusion of *S. cerevisiae* with *S. diastaticus* was also employed to produce hybrids with an amylolytic nature.

2.9.2.5 Gene cloning and transformation

Unfortunately, however, with the methods described above, it is not only the desired properties that are united in a hybrid, but also the undesired properties contained in the genetic information of the two parent strains. DNA recombination, however, has the advantage that only a certain property can be changed, whether it be positive or negative, without affecting any other properties of the strain (Hinrichs and Stahl, 1993).

The precision and specificity of recombinant DNA technology lends itself to the introduction of genes from any donor organism into distiller's yeast, as long as the donor has GRAS status (**G**enerally **R**egarded **A**s **S**afe) (Hinchcliffe, 1992).

2.9.3 APPLICATION OF DNA TECHNOLOGY IN THE DISTILLING INDUSTRY

Characteristics of an efficient distiller's yeast include high alcohol production and tolerance, good flavour and aroma production, high fermentation rate, utilisation of higher oligosaccharides and/or starch, and low H_2S production (Benitez *et al.*, 1996). In the following discussion, we will look at strategies to improve these characteristics.

2.9.3.1 Improved utilisation of starch and higher oligosaccharides

Most efforts to improve industrial distilling yeasts using recombinant DNA techniques are focussed on the production of amylolytically active strains. Starch-containing raw materials are widely used as fermentable substrates for the production of industrial ethanol, neutral sprits, whiskey and beer (Tubb, 1986). A schematic representation of the major pathways for converting starch-containing raw material to ethanol or alcoholic beverages is given in Figure 6.

Two processes are involved in the enzymatic reduction of starch to glucose. Liquefaction is administered by α- and β-amylases and saccharification is carried out by glucoamylases and the so-called "debranching" enzymes, such as pullulanase. There are seven classes of amylolytic enzymes of microbial origin and the names, functions and examples of amylolytic microorganisms are given in Table 1 (Pretorius and Lambrechts, 1991).

In the production of Scotch whiskey, the enzymes responsible for the degradation of starch to fermentable sugars must be derived entirely from malted barley (Bringhurst *et al.*, 2001). The main objective would be to obtain a strain of *Saccharomyces* that possesses the ability to hydrolyse starch entirely, i.e. is able to synthesise and secrete α-amylase and glucoamylase with debranching activity. The presence of branched dextrins in worts has been studied and confirmed (Eneroldsen

and Bathgate, 1969), and this represents a loss of potential alcohol yield. Limit dextrinase is responsible for the hydrolisation of α-(1-6) linkages, but barley contains much lower levels of limit dextrinase than α- and β-amylase and therefore the action is limited (Walker *et al.*, 2001). One strategy therefore would be to construct a yeast strain that can secrete an enzyme with debranching activity to specifically degrade branched dextrins. The same strain could be useful in the brewing industry, where enzymes are sometimes added to the process to increase the amount of fermentable substrate. The composition of these additives, however, is not necessarily always known and they often can contain other minor enzyme activities that could have detrimental effects on the quality of the product (Lancashire, 2000).

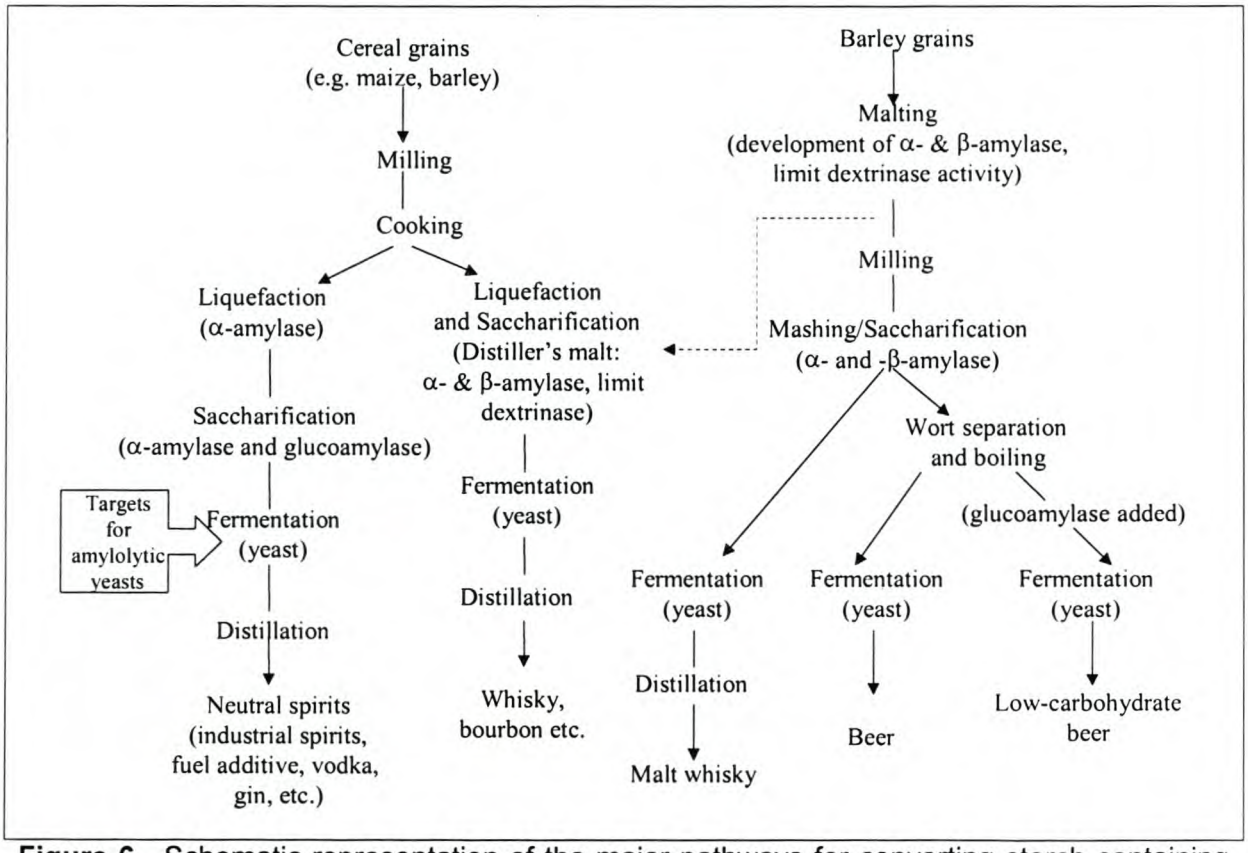

Figure 6. Schematic representation of the major pathways for converting starch-containing raw materials to ethanol or alcoholic beverages (Tubb, 1986).

For many years, several laboratories around the world have been involved in the construction and/or evaluation of amylolytic yeast strains. In the production of whiskey from maize alone, as in the production of neutral spirits, the use of a yeast strain that is able to secrete sufficient amounts of a suitable glucoamylase would reduce the need for added enzymes. The cost of α-amylase for liquefaction depends a lot on the process used, but in general it will be less than the cost of glucoamylase for saccharification (Tubb, 1986). The need therefore arose to construct a yeast strain capable of utilising dextrins that are formed as a result of the α-1,4 action of α-amylase on the starch molecule, and that possibly is also able to utilise raw starch. Extensive studies on the production of a yeast strain with glucoamylase and/or α-amylase activity have been undertaken and will be discussed in this paper.

Table 1. Starch-degrading enzymes produced by amylolytic microorganisms (Pretorius and Lambrechts, 1991).

Amylolytic enzymes[a]		Specific substrate and end products of amylolysis[b]
α-Amylase (1,4-α-D-glucan glucanohydrolase)	EC 3.2.1.1	An extracellular endoenzyme that catalyses the hydrolysis of the α-1,4-glucosidic linkages and is capable of bypassing the α-1,6-linkages, producing poly- and oligosaccharide chains of varying length (*Bacillus amyloliquefaciens, Aspergillus oryzae*)
β-Amylase (1,4-α-D-glucan maltohydrolase)	EC 3.2.1.2	An extracellular exoenzyme that hydrolyses alternate α-1,4-glucosidic linkages from the nonreducing end and is incapable of bypassing α-1,6-glucosidic linkages to produce dextrin and the β-anomeric form of maltose (*Bacillus polymyxa, Clostridium thermosulfurogenes*)
γ-Amylase/glucoamylase/ amyloglucosidase (1,4-α-D-glucan glucanohydrolase)	EC 3.2.1.3	An extracellular exoacting enzyme that splits α-1,4 and, in some cases, α-1,6-glucosidic linkages and also some 1,3-glucosidic linkages from the nonreducing ends of α-glucans to yield β-D-glucose (*Aspergillus awamori, Saccharomyces cerevisiae* var. *diastaticus*)
Pullulanase (α-dextrin 6-glucanohydrolase)	EC 3.2.1.41	An extracellular debranching enzyme that hydrolyses α-1,6-linkages of pullulan and other branched oligosaccharides to form maltotriose and dextrins respectively (*Klebsiella pneumoniae, Bacilllus stearothermophilus*)
Isoamylase (glycogen 6-glucanohydrolase)	EC 3.2.1.68	An extracellular debranching enzyme that hydrolyses α-1,6-glucosidic linkages of amylopectin, glycogen, various branched dextrins and oligosaccharides, with no activity on pullulan (*Pseudomonas amyloderamose, Lipomyces kononenkoae*)
Cyclodextrin glycosyltransferase [1, 4-α-D-glucan 4-α-D (1, 4-α-D-glucano)-transferase]	EC 2.4.1.19	An extracellular enzyme that produces a series of nonreducing cyclodextrins (rings of 6, 7 and 8 glucose units) from starch and other polysaccharides (*Bacillus macerans, Klebsiella pneumoniae*)
α-Glucosidase (α-D-glucoside glucohydrolase)	EC 3.2.1.20	An extracellular or intracellular enzyme that appears to hydrolyse short chain α-1,4 or α-1,6-linked saccharides arising from the action of other enzymes on starch (*Bacillus licheniformis, Schizosaccharomyces pombe*)

[a] Systematic names of amylolytic enzymes are listed in parentheses.

[b] Examples of amylolytic microorganisms are listed in parentheses.

S. cerevisiae is the most suitable strain for the production of alcohol due to its fast growth rate, high ethanol tolerance and its efficient ethanol-producing ability (De

Mot *et al.*, 1985). *S. cerevisiae* var. *diastaticus* is very closely related to *S. cerevisiae* and has the ability to produce an extracellular glucoamylase. Kim *et al.* (1988) reported on the transformation of a hybrid strain of *S. cerevisiae* var. *diastaticus* that has a glucoamylase activity with a plasmid containing mouse α-amylase gene. For the production of industrial ethanol, this engineered yeast strain will result in a highly efficient one-step starch conversion process. Another strategy was used by the same team to construct a stable starch-fermenting strain by introducing α-amylase copy-DNA (originally from mouse salivary glands) into the haploid *S. diastaticus* cells using an integrating vector (Kim and Kim, 1996). The haploid strain was then rare mated with a polyploid industrial strain with no amylase activity. The resulting hybrid could secrete α-amylase and glucoamylase with 100% mitotic stability. *S. diastaticus*, however, cannot be applied for the production of alcoholic beverages because it produces off-tastes. This was tested by Stewart *et al.* (1983), who found that beer produced with *S. cerevisiae* var. *diastaticus* was unpalatable, with a heavy phenolic aroma. Several attempts therefore have been made to isolate the glucoamylase gene from *S. cerevisiae* var. *diastaticus* for expression in *S. cerevisiae*. Tamaki (1978) demonstrated the presence of three unlinked genes, *STA1, STA2* and *STA3*, in *S. cerevisiae* var. *diastaticus* that produced the extracellular glucoamylase isoenzymes I, II and III respectively, thereby conferring the ability to grow on media supplemented with starch as a sole carbon source.

Yamashita and Fukui (1983) introduced the *STA1* gene of *S. cerevisiae* var. *diastaticus* into *S. cerevisiae* by using an autonomously replicating plasmid vector, conferring glucoamylase activity to the transformant. Meaden and Tubb (1985) reported on the cloning of a *DEX1* gene coding for amyloglucosidase. The gene was inserted into a multi-copy plasmid. Both ale and lager strains have been transformed with this plasmid, using *CUP1* as the selectable marker. Complete superattenuation was not achieved, as the glucoamylase lacks α-1,6 debranching activity. The *S. cerevisiae* transformants accumulated extracellular amyloglucosidase up to five times more than that accumulated by the donor strain. In 1986, it was confirmed that *STA1* is allelic to *DEX2, STA2* is allelic to *DEX1* and *STA3* is allelic to *DEX3* (Pretorius *et al.*, 1986a; Erratt and Nasim, 1986). Yamashita *et al.* (1985) also successfully cloned the *STA3* gene in *S. cerevisiae*. The *STA2* gene was cloned by Pretorius *et al.* (1986b). Pardo *et al.* (1986) studied the transcription products of *STA2* and *SGA1* genes. The *SGA1* gene codes the intracellular glucoamylase of *S. cerevisiae* and is only expressed during sporulation. It was found that the *STA2* gene differs from the *SGA1* gene in that it contains an extra piece of DNA encoding the domain for exportation of the extracellular glucoamylase.

The *STA1* gene of *S. cerevisiae* var. *diastaticus* was introduced into brewing yeasts by Sakai *et al.* (1989) and its expression was examined. The transformant with the highest glucoamylase activity and the best mitotic stability was selected and microfermentations were carried out. A comparison of the beer fermented by the host and by the transformant showed less extract in the beer fermented by the

transformant, as well as a higher alcohol concentration compared to the host strain. Other components were not significantly different. During the fermentation, however, it was noted that the fermentation rate of the transformant was slower in the early phase than that of the host, and poor plasmid stability was observed. This phenomenon was also found by Perry and Meaden (1986) in similar experiments using the *DEX1* gene. Only when the gene was integrated into the yeast chromosome was a stable transformant obtained (Park *et al.*, 1990).

Steyn and Pretorius (1991) transformed *S. cerevisiae* with a plasmid containing an α-amylase gene (*AMY*) of *Bacillus amyloliquefaciens* and the *STA2* gene of *S. cerevisiae* var. *diastaticus*. The ability to convert soluble starch to fermentable sugars was determined. A conversion efficiency of more than 93% was recorded, with 80% of the starch being assimilated within 48 hours. Vakeria and Box (1996) cloned the *STA2* gene under two different promotors, *PGK1* (phosphoglycerate kinase) and *GPD1* (sn-glycerol-3-phosphate dehydrogenase), for the construction of an amylolytic brewing strain. Growth rate and plasmid stability were both influenced by the use of different promotors. The strain with the GA expressed from the *GPD1* promotor showed lower GA production, as expected, but sufficient levels were still produced to achieve efficient substrate utilisation within a certain time period. Furthermore, this strain showed good performance and a high degree of plasmid stability as opposed to the strain expressing GA from the efficient *PGK1* promotor, which showed plasmid instability and a poor growth rate. This shows that high enzyme expression levels do not always lead to stable modified characteristics in genetically improved strains.

Various other organisms have also been employed for the cloning of heterologous amylase genes. For the improvement of an *S. cerevisiae* brewer's yeast strain, Hollenberg and Strasser (1990) cloned two genes from *Schwanniomyces occidentalis*. The *AMY1* gene, which is an α-amylase gene, and the *GAM1*, which is a glucoamylase gene, were fused to the *GAL10* and *GAL1* promoters of *S. cerevisiae* respectively. Both enzyme activities were measured and the genetically engineered strain showed slightly higher activity in both enzymes than the donor strain. The fermentation properties and end product characteristics were not examined, however. High levels of glucoamylase of *Aspergillus awamori* were successfully secreted by a strain of distiller's yeast through the introduction of an expression cassette containing glucoamylase cDNA. This glucoamylase has α(1,4) activity as well as α(1,6) activity, and the recombinant strain utilised 93% of the carbohydrate when fermentation performance was determined on 25% (w/v) soluble cornstarch (Cole *et al.*, 1988).

The glucoamylase gene from *Aspergillus niger* has been cloned by Yocum (1986) and inserted into an integrating vector, which was used to transform a brewer's yeast. This glucoamylase has both α-1,4 and α-1,6 activity. Gopal and Hammond (1992) used this transformed yeast in 100 L fermentation trials to make light beer. The fermentations were carried out successfully and the modified strain

showed dextrin removal, whereas the control strain did not show any activity. The peak activity of released glucoamylase was approximately 50% of the dosage level recommended for the use of commercial *Aspergillus*-derived glucoamylase preparations. The dextrin utilisation resulted in a definite higher alcohol concentration and taste trials indicated a slight preference for this beer against one to which commercial glucoamylase had been added. Shibuya *et al.* (1992) constructed a fusion gene using the α-amylase and glucoamylase genes of *Aspergillus shirousamii* and recorded its expression in *S. cerevisiae*. The transformant with the best enzyme activities was selected. Its fusion protein was then purified and tested for raw starch-digesting activity, which was found to be much higher than when a mixture of the two enzymes was used. A gene from the yeast *Cryptococcus* sp. that codes for a raw starch-digesting and thermostable α-amylase has been cloned and sequenced by Iefuji *et al.* (1996). However, its expression in *S. cerevisiae* has not been studied.

2.9.3.2 Genetic transformation of barley

Malted barley enzymes are of great importance for the Scotch whiskey industry to ensure the efficient utilisation of raw materials (Walker *et al.*, 2001). Mashing in the whiskey distillery process differs from that in beer brewing in that the wort is not boiled prior to fermentation. Any enzyme activity that survives the mashing process therefore will be carried over into the fermentation. α- and β-amylase are the enzymes in barley that are responsible for starch degradation. The debranching enzyme limit dextrinase also plays a role, although a limited one, because the majority of the limit dextrinase is protected in the bound form. It was believed previously that limit dextrinase is very heat labile, but it has been proven that this enzyme is indeed carried forward to the fermentation (Walker *et al.*, 2001) and it has been suggested that there is a feedback mechanism controlling the release and activation of the bound limit dextrinase. It therefore would be desirable to develop barley varieties that lack the limit dextrinase inhibitor so that the maximum amount of total limit dextrinase that survives mashing could be carried forward into the fermentation, thus maximising the conversion of fermentable carbohydrate to alcohol. Increasing the heat stability of barley enzymes would also be beneficial to decrease the loss during kilning and mashing.

2.9.3.3 Increased fermentation rate/higher alcohol production

The main sugars produced in malt mashing are maltose and maltotriose. Glucose derepression is a common feature in brewing and distiller's yeast, giving preference to the uptake of wort glucose before the uptake of maltose and maltotriose starts (Lancashire, 2000). One strategy would be to construct a strain with no preference for sugar uptake. A yeast strain has been successfully engineered in which the activity of the maltose transporter gene *MAL6T* has been increased by overexpression of the gene (Kodama *et al.*, 1995).

Another strategy would be to make use of the technique of multiple gene deletions. Industrial yeasts have at least two copies of each gene per genome and it usually is essential to disrupt all the alleles of a given gene to create a specific phenotype. This process is readily performed in industrial strains and one such strain has been successfully engineered by creating *petite* mutants that lack functional mitochondrial DNA (incapable of growth through respiration) (Panoutsopoulou *et al.*, 2001). This strain has the ability to produce 40% more alcohol per volume than the reference strain. The two *PET191* alleles of a wine yeast (essential for respiration) were deleted by using of a specially designed replacement cassette to create a respiratory-deficient *petite* mutant, after which the cassettes were excised from the genome following the expression of a *cre* recombinase gene on the multi-copy plasmid YEP351-*cre*-cyh. This plasmid was maintained in the mutant by making use of selective pressure and then removed when both genes had been deleted successfully.

2.9.3.4 Increased flavour production / decrease in off-flavour production

During recent years, several attempts have been made to increase flavour components to improve or change the characteristics of alcoholic beverages. Fukuda *et al.* (1990) reported on the breeding of a brewing yeast producing a large amount of β-phenylethylalcohol that appears to have rose-like flavours. This compound is said to be an important aroma compound and whiskey contains about 10-15 ppm of β-phenylethylalcohol.

The majority of volatile sulphur compounds found in alcoholic beverages are not synthesised by yeast (Berry and Watson, 1987), although the yeast can produce significant quantities of hydrogen sulphide. Raw materials are a major source, particularly the malt and hops used in beer production. Small quantities of volatile sulphur compounds may have a desirable effect on the characteristics of particular beverages, but, if present in significant quantities, they are perceived as off-flavours. These compounds have extremely low odour thresholds and impart a range of aromas, such as rubbery, burnt, rotten egg, rotten fish and catty. The sulphur compounds produced by yeast are formed as by-products during the biosynthesis of the amino acids cysteine and methionine from inorganic sulphate. Yeast also produces dimethyl sulphide with a threshold value of 50 μg/l (Ingledew, 1995) if the precursors S-methyl-methionine and dimethyl sulphoxide are present.

Yeast strains can also vary in their production of sulphur dioxide and hydrogen sulphide (Berry and Watson, 1987). Walker and Simpson (1993) investigated the production of volatile sulphur compounds by ale and lager brewing strains of *S. cerevisiae* and found that the lager strains produced significant concentrations of hydrogen sulphide, methanethiol and methyl thioacetate. The ale strains, however, produced little, if any, of these compounds. It therefore may be possible to select yeast strains that produce low levels of these sulphur compounds during fermentation.

The enzyme cysteine synthetase, which is used in the biosynthesis of cysteine, requires the vitamin pantothenate (Berry and Watson, 1987). When it is absent or present at low levels, excessive quantities of hydrogen sulphide are formed. The same effect was investigated by Slaughter and Jordan (1986), when they tried to closely define the conditions needed for the production of hydrogen sulphide by *S. cerevisiae* NC1108. They found that hydrogen sulphide was produced mainly at the end of the cell growth phase. Above 0.2 mg/l of pantothenate, little or no hydrogen sulphide was produced, but, at concentrations in the range of 0.0125 to 0.125 mg/l pantothenate, significant production of hydrogen sulphide occurred.
The hydrogen sulphide that is formed during fermentation can form ethanethiol during distillation. Ethanethiol, on the other hand, can be readily oxidised to evil smelling diethyl disulphide (Suomalainen and Lehtonen, 1979). The threshold value for diethyl sulphide is approximately 0.4 μg/l (Ingledew, 1995).

Tezuka *et al.* (1992) reported on the suppression of hydrogen sulphide production in a brewing yeast by cloning the *NHS5* gene from an *S. cerevisiae* strain and expressing it in the brewer's yeast. Considerably less H_2S was formed by the transformants.

2.9.3.5 Yeast strains with other novel traits

2.9.3.5.1 Effluent treatment

Liquid effluents from maltings, breweries and distilleries contain high organic loads originating from dissolved carbohydrates, alcohol from the mashing waste and a large content of suspended solids (Armitt, 1981). Traditionally, many companies have discharged effluent with little further thought, but environmental awareness and legislation have forced them to meet discharge conditions and, if these conditions are not met, companies face high discharge costs and even prosecution (Paterson and Cooke, 1995). Effluent strength is measured by the Chemical Oxygen Demand (COD), which gives a measure of the oxygen needed for the oxidation of a broad range of substances and therefore the load that the effluent will place upon a treatment plant or river. Different effluent treatment methods are available, for example, the active sludge process. It could be possible to construct a yeast strain that could consume the raw material more efficiently to produce saleable products, i.e. yeast cell mass or ethanol (Korhola, 1987). This would result in lower effluent CODs and lower discharge costs.

2.9.3.5.2 Anti-contaminant yeast

As previously mentioned, the presence of large numbers of lactic acid bacteria in a mash can result in lower alcohol yield. By introducing genes for lysozyme or nisin production into the genome of distiller's yeast, undesired contaminants will be excluded from the fermentation, which would result in reliable high alcohol yield fermentations (Korhola, 1987). The presence of lactic acid bacteria, however, can

contribute to the flavour of the whiskey, as has already been shown. Thus, by broadening the product range of distiller's yeast through the addition of anti-contaminant yeast, distilleries will have a choice of whether to allow these bacteria in a mash or to exclude them from the process.

2.10 CONCLUSION

The development of DNA technology over the past 25 years has provided tools for the application of these techniques to the food and drink manufacturing industries (Lancashire, 2000). For a genetic engineered strain to be used in the industry, a number of criteria need to be met (Gopal and Hammond, 1992). These are sufficient expression levels of the foreign gene, mitotic stability of the strains (no loss of the gene function should occur after several generations) and, very importantly, that the fermentation process and end products should not be worse off than the original process and end products. From a legal point of view, approval from a number of statutory bodies is also required and this, together with a positive public perception, can only be achieved by successful application of this technology.

Genetic engineering is a powerful tool and potentially has wide application in the malting, brewing and distilling industries. Advanced genetic techniques are already being utilised in commercial food production, and these uses will multiply as public acceptance grows.

2.11 LITERATURE CITED

Anonymous. 1972. Scotch Whisky. *Questions and Answers*. Scotch Whisky Association, Edinburg, Scotland.

Armitt JDG. 1981. Brewery and Malthouse Effluents and their Management. In*: Brewing Science. Volume 2*. Edited by: Pollock, JRA. pp 551-627. Academic Press, London.

Barbour EA and Priest FG. 1988. Some effects of *Lactobacilus* contamination in Scotch whisky fermentations. *J. Inst. Brew.* **94**:89-92.

Barre P, Vezinhet, F, Dequin, S and Blondin, B. 1993. Genetic improvement of wine yeast. In: *Wine Microbiology and Biotechnology.* Edited by: Fleet, GH. pp 265-287. Harwood, Switzerland.

Bathgate GN and Cook R. 1989. Malting of barley for Scotch whiskies. In: *The Science and Technology of Whiskies*. Edited by: Piggott JR, Sharp R and Duncan REB. pp 19-63. Horwood, UK.

Benítez T, Gasent-Ramírezm JM, Castrejón F and Codón, AC. 1996. Development of New Strains for the Food Industry. *Biotechnol. Prog.* **12**:149-163.

Berry DR. 1979. The physiology of whisky fermentation. *Brewers' Guardian* **6**:25-29.

Berry DR. 1984. The physiology and microbiology of Scotch whisky fermentations. *Prog. Ind. Microbiol.* **19**:199-243.

Berry DR, Lavery M and Sim GB. 1987. Amylase activities during malt whisky production. *Crit. Rev. Biotech.* **5**:143-157.

Berry DR and Watson DC. 1987. Production of organoleptic compounds. In: *Yeast Biotechnology*. Edited by: Berry DR, Russell I and Stewart GG. pp 345-368. Allen & Unwin, London.

Booth M, Shaw W and Morhalo L. 1989. Blending and bottling. In: *The Science and Technology of Whiskies*. Edited by: Piggott JR, Sharp R and Duncan REB. pp 295-326. Horwood, UK.

Bringhurst TA, Broadhead AL, Brosnan JM, Pearson SY and Walker JW. 2001. The identification and behaviour of branched dextrins in the production of Scotch whisky. *J. Inst. Brew.* **107**:137-149.

Christensen BE. 1987. Cross-breeding of distillers' yeast by hybridization of spore derived clones. *Carlsberg Research Commun*. **52**:253-262.

Codón AC, Benitez T and Korhola, M. 1998. Chromosomal polymorphism and adaptation to specific industrial environments of *Saccharomyces* strains. *Appl. Microbiol. Biotechnol*. **49**:154-163.

Cole GE, McCabe PC, Inlow D, Gelfand DH, Ben-Bassat A and Innis MA. 1988. Stable expression of *Aspergillus awamori* glucoamylase in distiller's yeast. *Biotechnology* **6**:417-421.

De Mot R, Van Dijck K, Denkers A and Verachtert H. 1985. Potentialities and limitations of direct alcoholic fermentations of starch material with amylolytic yeasts. *Appl. Microbiol. Biotechnol.* **22**:222-226.

Eneroldsen BS and Bathgate GN. 1969. Structural analysis of wort dextrins by means of β-amylase and the debranching enzyme, pullulanase. *J. Inst. Brew*. **75**:433-443.

Erratt JA and Nasim A. 1986. Allelism within the DEX and STA gene families in *Saccharomyces diastaticus*. *Mol. Gen. Genet*. **202**:255-256.

Fukuda K, Watanabe M, Asano K, Ueda H and Ohta S. 1990. Breeding of brewing yeast producing a large amount of β-phenylethyl alcohol and β-phenylethyl acetate. *Agric. Biol. Chem*. **54**:269-271.

Geddes PA. 1986. The production of hydrogen sulphide by *Lactobacillus* spp. in fermenting wort. In: *Proceedings of the Second Aviemore Conference on Malting, Brewing and Distilling.* Edited by: Campbell I and Priest FG. pp 364-370. Institute of Brewing, London.

Geddes PA and Riffkin HL. 1989. Influence of lactic acid bacteria on aldehyde, ester and higher alcohol formation during Scotch whisky fermentations. In: *Distilled Beverage Flavour*. Edited by: Piggott JR and Paterson A. pp 193-199. Horwood, UK.

Gopal CV and Hammond JRM. 1992. Use of genetically modified yeasts for beer production. In: *Proceedings of the Fifth International Brewing Technology Conference, Harrogate*. pp 297-306. Brewing Technology Services Ltd, London.

Hansen J and Kielland-Brandt MC. 1996. Modification of biochemical pathways in industrial yeasts. *J. Biotechnol*. **4**:1-12.

Hammond JRM. 1994. Yeast and fermentation in the 12st century. In*: Proceedings of the Fourth Aviemore conference on malting, brewing and distilling*. Edited by: Campbell I and Priest FG. pp 85-99. Institute of Brewing, London.

Hammond JRM. 1996. Yeast genetics. In: *Brewing Microbiology*. Edited by: Priest FG and Campbell I. pp 45-82. Chapman and Hall, London.

Hammond JRM and Eckersley KW. 1984. Fermentation properties of brewing yeast with killer character. *J. Inst. Brew*. **90**:167-177.

Hinchcliffe E. 1992. Cambridge Prize Lecture. Developing new strains of yeast. *J. Inst. Brew*. **98**: 27-31.

Hinrichs J and Stahl U. 1993. Genetic engineering in the brewing industry. *Brauwelt International* **4**:293-298.

Hollenberg CP and Strasser AWM. 1990. Improvement of baker's and brewer's yeast by gene technology. *Food Biotechnology* **4**:527-534.

Hough JS. 1985. *The Biotechnology of Malting and Brewing*. Cambridge University Press, UK.

Iefuji H, Chino M, Kato M and Iimura Y. 1996. Raw starch-digesting and thermostable α-amylase from the yeast *Cryptococcus* sp. S-2: purification, characterization, cloning and sequencing. *Biochem J*. **318**:989-996.

Ingledew WM. 1995. The Biochemistry of Alcohol Production. In: *The Alcohol Textbook*. Edited by: Lyons TP, Kelsall DR and Murtagh JE. pp 55-79. Alltech Inc., UK.

Keiding AK. 1985. Genetic and molecular characterization of a distiller's yeast. *Carlsberg Research Commun*. **50**:95-125.

Keim CR. 1995. Wet Milling of Grain for Alcohol Production. In: *The Alcohol Textbook*. Edited by: Lyons TP, Kelsall DR and Murtagh JE. pp 1-25. Alltech Inc., UK.

Kim K, Park CS and Mattoon JR. 1988. High-Efficiency, One-Step Starch Utilization by Transformed *Saccharomyces* Cells Which Secrete Both Yeast Glucoamylase and Mouse α-Amylase. *Appl. Environ. Microb*. **54**:966-971.

Kim T and Kim K. 1996. The Construction of a Stable Starch-Fermenting Yeast Strain Using Genetic Engineering and Rare-Mating. *Appl. Biochem. and Biotechn*. **59**:39-51.

Kodama Y, Fukui N, Ashikara T, Shibano Y, Marioka-Fujimoto K, Hiraki Y and Nakatani K. 1995. Improvement of maltose fermentation efficiency: constitutive expression of MAL genes in brewing yeasts. *J. Am. Soc. Brew. Chem.* **53**:24-29.

Korhola M. 1987. Yeast in the Alcohol Industry. *Proceedings of the 21st Congress of the European Brewery Convention, Madrid.* pp 105-121.

Korhola M, Harju K and Lehtonen M. 1989. Fermentation. In: *The Science and Technology of Whiskies.* Edited by: Piggott JR, Sharp R and Duncan REB. pp 89-117. Horwood, UK.

Lancashire B. 2000. Application of DNA Technology for yeast strain and fermentation process development. *The Brewer.* February. pp 69-75.

Lyons TP. 1995. The Production of Scotch and Irish Whiskies. In: *The Alcohol Textbook.* Edited by: Lyons TP, Kelsall DR and Murtagh JE. pp 127-156. Alltech Inc., UK.

Makanjuola DB and Springham DG. 1984. Identification of lactic acid bacteria isolated from different stages of malt whisky distillery fermentations. *J. Inst. Brew.* **90**:13-19.

Makanjuola DB and Springham DG. 1990. Comparison between the effects of added lactic acid and bacteria on laboratory scale malt whisky fermentations. *Microbiologie-Aliments-Nutrition* **8**:189-196.

Meaden PG and Tubb RS. 1985. A plasmid vector system for the genetic manipulation of brewing strains. *Proceedings of the 20th Congress of the European Brewery Convention, Helsinki.* pp 219-226.

Miklos I and Sipiczki M. 1991. Breeding of a distiller's yeast by hybridization with a wine yeast. *Appl. Microbiol. Biotechnol.* **35**:638-642.

Nagodawithana TW. 1986. Yeasts: Their role in modified cereal fermentations. In*: Advances in Cereal Science and Technology* Vol. 8. American Association of Cereal Chemists. pp 15-104. St Paul, Minn.

Nicol D. 1989. Batch distillation. In: *The Science and Technology of Whiskies.* Edited by: Piggott JR, Sharp R and Duncan REB. pp 118-149. Horwood, UK.

Nishimura K and Matsuyama R. 1989. Maturation and maturation chemistry. In: *The Science and Technology of Whiskies.* Edited by: Piggott JR, Sharp R and Duncan REB. pp 235-263. Horwood, UK.

Nishimura K, Ohnishi M, Masuda M, Koga K and Matsuyama R. 1983. Reactions of wood components during maturation. In: *Flavour of distilled beverages.* Edited by: Piggott JR. pp 241-255. Unwin, UK.

Palmer GH. 1997. Scientific Review of Scotch Malt Whisky. *Ferment* **10**:367-379.

Panek RJ and Boucher AR. 1989. Continuous distillation. In: *The Science and Technology of Whiskies.* Edited by: Piggott JR, Sharp R and Duncan REB. pp 150-181. Horwood, UK.

Panoutsopoulou K, Hutter A, Jones P and Gardner DCJ. 2001. Improvement of Ethanol Production by an Industrial Yeast Strain via Multiple Gene Deletions. *J. Inst. Brew.* **107**:49-53.

Pardo JM, Polaina J and Jiménez A. 1986. Cloning of the STA2 and SGA genes encoding glucoamylases in yeasts and regulation of their expression by the STA10 gene of *Saccharomyces cerevisiae*. *Nucleic Acids Research* **14**:4701-4718.

Park CS, Park YJ, Lee YH, Park KJ, Kang HS and Pek UH. 1990. The novel genetic manipulation to improve the plasmid stability and enzyme activity in the recombinant brewing yeast. *Technical Quarterly of the Master Brewers Association of the Americas* **27**:112-116.

Paterson A and Piggott JR. 1989. The contributions of the process to flavour in Scotch malt whisky. In: *Distilled Beverage Flavour.* Edited by: Piggott JR and Paterson A. pp 151-170. Horwood, UK.

Paterson I and Cooke, PJ. 1995. Water and effluent treatment in juice processing. In: *Production and packaging of non-carbonated fruit juices and fruit beverages.* Edited by: Ashurst PR. pp 386-421. Chapman & Hall, Glasgow.

Perry C and Meaden P. 1986. Properties of a genetically engineered dextrin-fermenting strain of brewers' yeast. *J. Inst. Brew.* **94**:64-67.

Priest FG and Pleasants JG. 1988. Numerical taxonomy of some leuconostocs and related bacteria isolated from Scotch whisky distilleries. *J. Appl. Bacteriol.* **64**:379-387.

Pyke M. 1965. The manufacture of Scotch grain whisky. *J. Inst. Brew.* **71**:209-218.

Pretorius IS. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16**:675-729.

Pretorius IS and Lambrechts MG. 1991. The Glucoamylase Multigene Family in *Saccharomyces cerevisiae* var. *diastaticus*: An Overview. *Crit. Rev. Biochem. Mol. Biol.* **26**:53-76.

Pretorius IS, Chow T and Marmur J. 1986a. Identification and physical characterization of yeast glucoamylase structural genes. *Mol. Gen. Genet.* **203**:36-41.

Pretorius IS, Chow T, Modena D and Marmur J. 1986b. Molecular cloning and characterization of the STA2 glucoamylase gene of *Saccharomyces diastaticus*. *Mol. Gen. Genet.* **203**:29-35.

Ramsay CM and Berry DR. 1984. The effect of inoculum level on the formation of higher alcohol, fatty acids and esters in the malt whisky fermentation. *Food Microbiology* **1**:111-115.

Reazin GH. 1983. Chemical analysis of whisky fermentation. In: *Flavour of distilled beverages*. Edited by: Piggott JR. pp 225-240. Horwood, UK.

Reed G and Nagodawithana TW. 1991. Distillers' yeasts. In: *Yeast Technology* Volume 5. pp 225-260. Van Nostrand Reinhold, New York.

Sakai K, Fukui S, Yabuuchi S, Aoyagi S and Tsumura Y. 1989. Expression of the *Saccharomyces diastaticus* STA1 Gene in Brewing Yeasts. *Am. Soc. Brew. Chem.* **47**:87-91.

Salek AT and Arnold WM. 1994. Construction of ethanol-resistant, osmophilic industrial strains of *Saccharomyces* sp. *Chem. Mikrobiol. Technol. Lebensm.* **16**:165-183.

Sears AE. 1995. The Role of Enzymes in Alcohol Production. In: *The Alcohol Textbook*. Edited by: Lyons TP, Kelsall DR and Murtagh JE. pp 81-88. Alltech Inc., UK.

Sharp R and Watson DC. 1979 Improving yeast strains for whisky production. *Brewing and Distilling International* **9**:50-51, 53.

Shibuya I, Tamura G, Shima H, Ishikawa T and Hara S. 1992. Construction of an α-Amylase / Glucoamylase Fusion Gene and Its Expression in *Saccharomyces cerevisiae*. *Biosci. Biotech. Bioch.* **56**:884-889.

Slaughter JC. 1993. Current research into brewing and distilling yeast strains. *Brewing and Distilling International* **4**:26-27.

Slaughter JC and Jordan B. 1986. The production of hydrogen sulphide by yeast. In: *Proceedings of the Second Aviemore Conference on Malting, Brewing and Distilling*. Edited by: Campbell I and Priest FG. pp 308-310. Institute of Brewing, London.

Snow R. 1983. Genetic improvement of wine yeast. In: *Yeast Genetics – Fundamental and Applied Aspects.* Edited by: Spencer JFT, Spencer DM and Smith ARW. pp 439-459. Springer-Verlag, New York.

Stewart GG and Russell I. 1986. Centenary Review. One hundred years of yeast research and development in the brewing industry. *J. Inst. Brew.* **92**:537-558.

Stewart GG, Panchal CJ and Russell I. 1983. Current developments in the genetic manipulation of brewing yeast strains. A Review. *J. Inst. Brew.* **89**:170-188.

Steyn AJC and Pretorius IS. 1991. Co-expression of a *Saccharomyces diastaticus* glucoamylase-encoding gene and a *Bacillus amiloliquefaciens* α-amylase-encoding gene in *Saccharomyces cerevisiae*. *Gene* **100**:85-93.

Suomalainen H and Lehtonen M. 1979. The production of aroma compounds by yeast. *J. Inst. Brew.* **85**:149-156.

Tamaki H. 1978. Genetic Studies of Ability to Ferment Starch in *Saccharomyces*: Gene Polymorphism. *Mol. Gen. Genet.* **164**:205-209.

Ter Heide R. 1986. The flavour of distilled beverages. In: *Food Flavours. Part B. The flavour of beverages*. Edited by: Morton ID and Macleod AJ. pp 239-336. Elsevier Science, Amsterdam.

Tezuka H, Mori T, Okumura Y, Kitabatake K and Tsumura Y. 1992. Cloning of a Gene Suppressing Hydrogen Sulphide Production by *Saccharomyces cerevisiae* and Its Expression in a Brewing Yeast. *Am. Soc. Brew. Chem.* **50**:130-133.

Tubb RS. 1986. Amylolytic yeasts for commercial applications. *Tibtech* **4**:98-104.

Vakeria D and Box W. 1996. Characterization of amyloytic brewing yeast. *J. Inst. Brew.* **102**:27-32.

Varnam AH and Sutherland JP (Editors). 1999. Alcoholic Beverages: III. Distilled Spirits. In: *Beverages. Technology, Chemistry and Microbiology.* Chapter 9. pp 400-448. Aspen Publishers, Great Britain.

Walker JW, Bringhurst AL, Broadhead JM, Brosnan JM and Pearson SY. 2001. The Survival of Limit Dextrinase during Fermentation in the Production of Scotch Whisky. *J. Inst. Brew.* **107**:99-106.

Walker MD and Simpson WJ. 1993. Production of volatile sulphur compounds by ale and lager brewing strains of *Saccharomyces cerevisiae*. *Lett. Appl. Microbiol.* **16**:40-43.

Watson DC. 1981. The development of specialized yeast strains for use in Scotch malt whisky fermentations. In: *Current Developments in Yeast Research.* Edited by: Stewart GG and Russell I. pp 57-62. Pergamon Press Inc., Toronto.

Watson DC. 1983. Factors influencing the congener composition of malt whisky new spirit. In: *Flavour of distilled beverages. Origin and Development*. Edited by: Piggott JR. pp 79-92. Horwood, UK.

Wilkin GD. 1989. Milling, Cooking and Mashing. In: *The Science and Technology of Whiskies*. Edited by: Piggott JR, Sharp R and Duncan REB. pp 64-88. Horwood, UK.

Yamashita I and Fukui S. 1983. Molecular Cloning of a Glucoamylase-producing Gene in the Yeast *Saccharomyces*. *Agric. Biol. Chem*. **47**:2689-2692.

Yamashita I, Maemura T, Hatano Y and Fukui S. 1985. Polymorphic Extracellular Glucoamylase Genes and Their Evolutionary Origin in the Yeast *Saccharomyces diastaticus*. *J. Bacteriol.* **161**:574-582.

Yocum RR. 1986. Genetic engineering of industrial yeasts. *Proceedings of BioExpo. '86.* pp 171-180.

Chapter 3

RESEARCH RESULTS

Characterisation of whiskey yeast and the expression of different amylolytic genes of *Lipomyces kononenkoae* in distiller's yeast

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CHAPTER 3

Characterisation of Whiskey Yeast and the Expression of different Amylolytic Genes of *Lipomyces kononenkoae* in Distiller's Yeast.

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Nineteen whiskey yeast strains collected from different sources were characterised according to the following performance criteria: fermentation time, experimental alcohol yield, H_2S formation and genotypes. The sifting process resulted in four strains that outperformed the rest, which were further tested for off-flavour production. After fermentation, the mash was double distilled for quality and presented t+6o a tasting panel. All samples showed positive notes and no off-flavours were detected. All 4 strains were transformed with the *LKA1*$_{P\text{-}T}$ cassette and with the *LKA1-LKA2*$_{P\text{-}T}$ cassette, both under the *PGK1* promoter and terminator. The *LKA1* and *LKA2* genes were previously isolated from *Lipomyces kononenkoae*. The wine yeast VIN13 was included for its fast fermentation rate. Thirty-five transformants were obtained and further selected according to their α-amylase activity, growth in 2% starch medium and their ability to ferment whiskey mash. Whiskey yeast transformants with both the *LKA1* and *LKA2* genes showed a much faster exponential phase than those with only the *LKA1* gene when their ability to grow in 2% starch medium was assessed. Transformants obtained from the wine yeast, VIN13, showed the opposite effect with *LKA1* transformants having a faster growth rate than the VIN13[LKA1/2] transformant. The selected transformants, 310[LKA1/2](3), 310[LKA1/2](6), VIN13[LKA1](1), VIN13[LKA1](8) and VIN13[LKA1/2](1), were evaluated for their ability to ferment whiskey mash. The VIN13 transformant VIN13[LKA1](8), produced a significantly higher alcohol yield than the non-transformed strain. An alcohol yield of up to 65.74% was obtained, compared to 1.5% by the non-transformed strain.

3.1 INTRODUCTION

In the production of whiskey, the criteria for an acceptable yeast strain are the following: 1) The yeast strain should be genetically stable, 2) it must ferment the wort or mash in a reasonable period of time until all the substrates present are converted to ethanol, and 3) the fermented medium should be palatable, with no off-flavours such as sulphur compounds (Stewart, 1985). *Saccharomyces cerevisiae* is commonly used for the production of alcohol because of its fast growth rate, high ethanol tolerance and its efficient ethanol-producing ability (De Mot *et al*, 1985). For the production of ethanol from starch, enzymes are essential to hydrolyse starch molecules to glucose units, which are utilised by the yeast. Unfortunately, *S. cerevisiae* lacks the ability to produce such enzymes (Hinrichs and Stahl, 1993).

Starch-containing raw materials are widely available for the production of potable ethanol and maize and barley are used most commonly in the production of whiskey (Tubb, 1986). The hydrolysis of starch to glucose and higher oligosaccharides is accomplished by amylolytic enzymes and, in the case of South African whiskey, which is made entirely from maize, commercial enzymes are employed for this conversion, representing a significant expense in the production process. In the production of Scotch whiskey, however, the enzymes for the conversion process are derived entirely from the malted barley (Bringhurst *et al.*, 2001). The process for the production of grain whiskey is similar to that for the production of industrial and fuel ethanol from starchy biomass. It involves three steps: (1) liquefaction of starch by an α-amylase, (2) enzymatic saccharification of low molecular weight liquefaction products (dextrins) to produce glucose and (3) fermentation of glucose (Laluce and Mattoon, 1984). During grain whiskey production, starch is first gelatinised by cooking, which liquefies the starch molecules to make them more acceptable for enzyme attack. A thermostable α-amylase, which catalyses the hydrolysis of α(1-4) linkages in the starch molecule, is added at the same time to speed up the process. Saccharification is achieved by using a glucoamylase for the hydrolysis of α(1-6) bonds, yielding glucose units, which are utilised during fermentation. The ideal yeast strain therefore would secrete an effectively synergistic combination of enzymes to convert raw starch at an efficient rate. Several attempts have been made to introduce amylolytic enzymes from different organisms into yeast to increase the fermentability of the starch-derived carbohydrates, for example the amyloglucosidases from *S. diastaticus* (Hammond, 1994) and *Schwanniomyces* sp. (Lancashire *et al.*, 1989; Dohmen *et al.*, 1990) and the α-amylase from wheat (Lancashire, 1986).

Only a few of the more than 150 starch-degrading species of yeast secrete a combination of enzymes consisting of α-amylase and glucoamylase (De Mot, 1990). The strain *L. kononenkoae* IGC4052B is one such strain that secretes a group of highly effective amylases, including an α-amylase, glucoamylase (Spencer-Martins and Van Uden, 1979) and isoamylase (Spencer-Martins, 1982). These amylases are known for their raw starch degradation and therefore would be of great industrial

importance to reduce the energy intensive and costly pre-cooking, as well as to replace the use of commercial enzyme preparations (Steyn *et al.*, 1996).

Steyn *et al.* (1995) have cloned and characterised the α-amylase-encoding gene of *L. kononenkoae* (*LKA1*) and the gene was expressed in *S. cerevisiae* under control of the phosphoglycerate kinase (*PGK1*) promoter. The optimum pH and temperature of the *LKA1* gene are 4.0 and 40°C respectively (Steyn and Pretorius, 1995). Recently, a second α-amylase gene from *L. kononenkoae* IGC4052B (*LKA2*) was cloned and characterised and then expressed in *S. cerevisiae* (Eksteen *et al.*, 2003). The optimum pH of *LKA2* is 3.5 and the optimum temperature is 60°C. The reaction specificity of *LKA2* is still unclear, however, and therefore it was decided not to transform whiskey strains with *LKA2* alone. However, by transforming the whiskey strains with the *LKA1-LKA2*$_{P\text{-}T}$ cassette, it would be interesting to see whether the inclusion of the *LKA2* gene makes any difference to the enzyme activity of the transformed strains.

The aim with this paper was to characterise 19 whiskey yeast strains according to their ability to ferment the mash to dryness within a certain period of time, produce palatable spirit with no off-flavours and low or no production of H_2S, and to give a high alcohol yield (expressed as a percentage of the theoretical yield). The best performers were then transformed with two α-amylase genes from *L. kononenkoae*, namely *LKA1* on its own and in combination with *LKA2*, and their ability to ferment a starch-containing mash was compared with the commercial process.

3.2 MATERIALS AND METHODS

3.2.1 WHISKEY YEAST STRAINS

The yeast strains used for the selection process and their origin are listed in Table 1.

All the strains are of the species *S. cerevisiae* and were characterised according to industrial processes. Table 2 depicts the bacterial strains and plasmids used, together with their genotypes and sources.

3.2.2 GROWTH MEDIA AND CULTURE CONDITIONS

E. coli were grown in Luria Bertani broth (LB) (Sambrook *et al.* 1989). Bacterial transformants were cultured in LB medium containing ampicillin at a concentration of 100 μg/ml. Yeast strains were cultured in YPD medium containing 1% yeast extract, 2% bacto-peptone and 2% glucose. *S. cerevisiae* transformants were cultured and selected on synthetic complete (SC) medium containing 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco), including 50 μg/ml SMM as the selectable marker. Plates were incubated at 30°C in the dark for 4-5 days. Growth on SMM plates indicated resistance towards SMM and possible transformants. Colonies were further screened on starch plates for positive transformants.

Table 1. Whiskey yeast strains.

Whiskey strain reference	Name/Origin
WH299	HE1000; Lalvin (Canada) / Brewer's strain
WH300	DCL Scotland
WH301	DCL Scotland
WH302	DGI 338; ex Anchor
WH303	Anchor; ex Maurivin
WH304	No. 269; ex NCP
WH305	No. 270; ex NCP
WH306	No. 271; ex NCP
WH308	DGI 227; ex Anchor
WH309	DGI 335; ex Anchor
WH310	DY10; ex Anchor
WH311	NCYC 87; NRRL Y-567
WH312	NCYC 431; NRRL Y-132
WH313	NRRL Y-978
WH314	NRRL Y-567
WH315	NRRL Y-684
WH316	NRRL Y-635
WH317	CBS 1311
WH319	M15; ex Anchor

NCYC – National Collection of Yeast Cultures, England.
NRRL – ARS Culture Collection, USA.
CBS – Centraalbureau voor Schimmelcultures, Netherlands.

Table 2. Bacterial strains and plasmids used for cloning.

STRAIN OR PLASMID	GENOTYPE	SOURCE/REFERENCE
PLASMIDS:		
pIPLKA1	*Ap*^{R} *Tc*^{R} *PGK1*$_P$ *LKA1 PGK1*$_T$ *URA3*	Eksteen *et al.*(2003)
pIPLKA1/2	*Ap*^{R} *Tc*^{R} *PGK1*$_P$ *LKA1 PGK1*$_T$ *PGK1*$_P$ *LKA2 PGK1*$_T$ *URA3*	Eksteen *et al.*(2003)
pDLG31	*Ap*^{R} *PGK1*$_p$*LKA1-PGK1*$_T$ *SMR1*-410	Gundllappali Moses *et al.*, 2002
pIPLKA1SMR1	*Ap*^{R} *Tc*^{R} *PGK1*$_P$ *LKA1 PGK1*$_T$ *URA3 SMR1*-410	This study
pIPLKA1/2SMR1	*Ap*^{R} *Tc*^{R} *PGK1*$_P$ *LKA1 PGK1*$_T$ *PGK1*$_P$ *LKA2 PGK1*$_T$ *URA3* SMR1-410	This study
BACTERIAL STRAINS:		
E. coli DH5α	*sup*E44 Δ*lac*U169(ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	aGIBCO/Bethesda

3.2.3 STARCH PLATE ASSAYS

Colonies were picked from SMM plates and blotted onto starch plates containing 20 g/L soluble starch, 6.7 g/L yeast nitrogen base without amino acids and 20 g/L agar. Plates were incubated for 2-3 days at 30°C and then incubated for 3-4 days at 4°C and checked for clear zones around the colonies.

3.2.4 CHEF ANALYSIS

Cells were grown in 100 ml of YPD medium to the late logarithmic phase and prepared for CHEF analysis according to the embedded-agarose procedure of Carle and Oslen (1985). Separation of the chromosomes was performed according to the technique described by Van der Westhuizen *et al.* (1999) with the following modifications: samples were run on a 2% agarose gel in the CHEF pulse field gel electrophoresis system CHEF-DR11 (Bio-Rad laboratories, Richmond, USA) for 42 h; first for 14 h with a switching interval of 60 seconds, then for 28 h with a switching interval of 90 sec. The temperature of the 0.5 x TBE electrophoresis buffer was maintained at 14°C, the pump speed was set at 55 and the volts at 200.

3.2.5 SCREENING FOR H_2S FORMATION

Cultures were grown in YPD broth for 24 h at 30°C on an orbital shaker. Bismuth citrate plates were prepared according to the method by Jiranek *et al.* (1995). A diluted yeast suspension was spread plated onto the agar plates and the plates were incubated at 30°C for 72 h. A low H_2S-producing colony was identified by its white colour, whereas a high H_2S-producing colony had a black colour.

3.2.6 FERMENTATION TIME

Fermentation time was monitored for all the yeast strains by preparing a whiskey mash on small scale and inoculating the strains at a concentration of 1 x 10^7 cells/ml in a total of 400 ml of mash. A 32% (v/v) maize/water slurry was made up by adding 90.5 g of maize (Meadow Feeds, SA) (starch content = 65%) to 220 ml of double distilled H_2O. The starch content of the maize was measured according to the method in the Polarimeter Manual (Polarimeter Polax-D, Cat. No. 5221, Japan). Spezyme Delta AA (Genencor International), a commercial α-amylase enzyme, was added at a concentration of 0.15% of dry starch content. The slurry was kept at 90°C for 60 min, with stirring, then cooled down to room temperature, and the pH was set at 4.0-4.4 with 10% H_2SO_4. AMG 300L, a commercial amyloglucosidase enzyme (Enzymes SA), was added at a concentration of 0.11% of dry starch content and made up to a final volume of 400 ml with double distilled water. Fermentation took place at 30°C and was monitored by HPLC (Hewlett Packard 1100 series) until all the fermentable sugars present were converted to alcohol. A Bio-Rad HPX-87C column for carbohydrate analysis was used, making use of degassed double distilled water

as mobile phase, with a flow speed of 0.6 ml/min and the oven temperature set at 80°C. Detection was made possible by the use of a Refractive Index detector.

3.2.7 DETERMINING ALCOHOL YIELD

After fermentation, the mash was distilled for quantification of the alcohol and the experimental yield expressed as a percentage of the theoretical yield (Thomas *et al.*, 1996). All tests for alcohol yield were done in duplicate. Alcohol was measured with a density meter (DMA58, AP Paar, Austria).

Theoretical yield:

(i) Determine weight of starch: (weight of maize) g x % starch content = (weight of starch) g

(ii) Amount of glucose derived enzymatically g starch x 1.111 = (weight of sugar) g (The factor of 1.111 is derived by taking into account that one molecule of water is used for each glycosidic bond hydrolysed)

(iii) For a 0.511 g theoretical alcohol yield per g of glucose: g sugar x 51.1% = (weight of alcohol)

(iv) Determining volume of alcohol

$$\text{Density} = \frac{\mathbf{m}(g)}{\mathbf{V}(ml)} \qquad (\mathbf{D} = 28.97)$$

Experimental yield: **V** = **Alc** (%) of distillate **x Vol.** of distillate (ml) (Thomas *et al.*, 1996).

3.2.8 SENSORY ANALYSIS AND GAS CHROMATOGRAPHIC ANALYSIS

Four litres of mash were prepared per sample and, after fermentation, the samples were distilled and 1200 ml of spirit was collected. This portion was distilled for a second time, the first 10 ml were discarded and only the "heart" fraction of ± 200 ml was collected. These samples were broken down with distilled water to 20% absolute alcohol and presented to a tasting panel for their remarks on off-flavours and positive notes. The panel consisted of 10 judges with brandy and whiskey distillate tasting expertise and they were asked to determine whether there were any differences between the distillates and WH310, which was taken as the control sample. The samples were evaluated at room temperature. GC analysis was done on the samples for higher alcohol, ester and fatty acid profiles. The HP 6890 Series GC was used and components were separated on a HP-Innowax column (30 m x 0.25 mm x 0.5 μm film). The samples were introduced by making use of an automatic liquid sampler and data was collected by HP Chemstation. The conditions are given in Table 3.

3.2.9 GROWTH IN 2% STARCH

Strains were inoculated in 50 ml of a 2% starch medium, consisting of 2% soluble starch (Sigma) and 6.7 g/L yeast nitrogen base without amino acids (Difco), to an OD_{600} of 0.2, using 500 ml baffled Erlenmeyer flasks. The samples were put on a shaker at 150 rpm and OD readings were taken every 12 h.

Table 3. GC conditions for the separation of esters and higher alcohols.

Injection	Split (ratio 50:1), 1 µl, temp. 250°C
Carrier	He, 30 cm/sec (average velocity), ramped flow mode: initial flow: 1.3 ml/min (20 min), post flow: 4.2 ml/min
Oven	35°C (10 min) to 40°C at 1°C/min to 190°C at 20°C/min to 255°C (5 min) at 30°C/min
Detector	FID at 300°C, 45 ml/min He make-up, 40 ml/min H_2 and 450 ml/min air
Sample preparation	5 ml sample + 0.5 ml Internal Standard (4-Methyl-2-Pentanol)

3.2.10 ENZYME ASSAYS: α-AMYLASE ACTIVITY

The intra- and extracellular amylolytic activity produced were determined by measuring the reducing sugar released from the starch by a colorimetric method in which the reduction of 3,5-dintroaminosalicylic acid to nitroaminosalicylic acid is determined. Amylolytic activity was determined at an OD_{600} of 2 ± 0.25 and at an OD_{600} of 6 ± 0.25. Cells were grown in 50 ml of YPD medium to the OD of interest and one ml of the culture was filtered through a Whatman 0.45 µm filter of which the weight was pre-determined by heating the filter in a microwave for 10 min at 30% strength, cooling the filter in a dessicator and weighing it. After the culture was filtered, the filter was washed 3x with distilled water. The filter was once again heated for 10 min at 30% strength and cooled in a dessicator for 10 min before being weighed again. The difference in weight represents the yield in terms of dry biomass weight. For the intracellular preparation, the cells were spun down at 5 K for 5 min at room temperature. The pellet was dissolved in 5 ml of a 50 mM Tris (pH7.5), 10 mM NaCl buffer. Glass beads were added and the mixture was vortexed for 2.5 min, spun down for 2 min at 6 K and the supernatant was collected in a clean tube. For the assay, each reaction mixture contained 200 µl of the supernatant, 100 µl of a citrate/phosphate buffer (pH4.0) and 700 µl of a 0.5% soluble starch substrate. The mixture was kept at 42°C for 30 min after which the reaction was terminated by adding 3,5-dinitrosalicylic acid reagent, and it was then boiled at 100°C for 15 min. After the mixture had cooled down, 100 µl of the reaction mixture was added to 900 µl of distilled H_2O and the reducing sugar was determined colorimetrically at 540 nm. One unit of enzyme was defined as the amount that liberated 1 µmol of glucose per minute per ml of enzyme sample. A standard glucose curve for colorimetric assay was constructed, with glucose as the reducing sugar. The colorimetric values were fitted onto a linear curve with a slope of $y = 0.2233x + 0.0021$.

3.2.11 CLONING AND EXPRESSION IN INDUSTRIAL STRAINS

Standard methods were used for DNA restriction digests, DNA purification, DNA ligation, DNA isolation, plasmid transformation into *E. coli* and agarose-gel eletrophoresis (Sambrook *et al.*, 1989). The *SMR1* gene was isolated from pDLG31 with *Bam*HI and ligated into pIPLKA1, creating pIPLKA1SMR1. The *SMR1* gene was also cloned into pIPLKA1/2, creating pIPLKA1/2SMR1. The resultant plasmids are shown in Fig. 1. Industrial yeast transformations were performed according to the method of Gietz and Schiestl (1995). T4 DNA ligase and all the restriction endonucleases were purchased from Roche Biochemical Products, SA.

3.2.12 AMPLIFICATION

DNA was amplified by the polymerase chain reaction (PCR) technique. The PCR was conducted in a 50 µl volume containing 25 mM $MgCl_2$, 1 mM of each of the DIG-labelled nucleotides, 250 µM of each primer, 2.6 U of Expand High Fidelity mix (Roche Biochemical Products) and 2-10 ng of template DNA. The programme was conducted as follows: Step 1: initial denaturation at 94°C for 2 min. Step 2 (30 cycles): denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 68°C for 90 sec and denaturation at 94°C for 30 sec. Step 3: 55°C for 5 min, 68°C for 5 min, then cooled down to 4°C. PCR products were purified by ethanol precipitation. Restriction enzyme digestion of PCR products was performed overnight at 37°C. Primers were obtained from Roche Biochemical Products South Africa. The following oligodeoxyribonucleotides were used as primers:

LKA1 5′ (5′ -ATG TTG CTG ATC AAC TTT TTC ATC GCT- 3′)

LKA1 3′ (5′ -TCT CTA CAT GGA GCA GAT TCC AGA GCC - 3′)

3.2.13 SOUTHERN BLOT ANALYSIS

Southern hybridisation was performed with the DIG Labelling Kit from Roche Biochemical Products (South Africa), using the method described in the DIG Application Manual. Genomic DNA was digested with *Nsi*I restriction enzyme and the *LKA1* gene was used as probe to confirm integration.

3.2.14 WHISKEY FERMENTATION

The starch content of the maize was determined at 60% and 13.4 g maize was mixed with 400 ml of water to reach a final starch content of 20 g/L. The pH of the slurry was measured at 6.0-6.4. The slurry was heated to 90°C while stirring and kept at this temperature for 20 min to ensure full liquefaction of the starch, after which the mash was cooled down to room temperature and the yeast was inoculated at a rate of 1×10^7 cells/ml. The yeast was prepared by inoculating a culture from a slant into 50 ml of YPD medium and growing it overnight at 30°C on a shaker. The weight loss was determined every day for 10 consecutive days until an insignificant loss was

reported. The fermented slurry was distilled and the first 200 ml were collected. An alcohol test was carried out on this by making use of a DMA. The final alcohol yield was then determined as described previously.

3.3 RESULTS

3.3.1 CHARACTERISATION OF WHISKEY STRAINS

The whiskey yeast strains in Table 1 have been collected over a period of time and have never been characterised by comparing their chromosomal patterns. CHEF analysis showed a difference in the chromosomal pattern between all strains except for strains WH311 and WH314, which possibly could be the same strain (data not shown).

On an industrial scale, the whiskey mash fermentation is completed within 65 h. The strains therefore were selected on their ability to ferment the mash to dryness within 65 h. All strains were successful in utilising the sugars within this time.

The formation of H_2S during the fermentation of whiskey mash is highly unacceptable, since it has a negative effect on the quality of the final distillate. All strains therefore were tested for their ability to produce H_2S. Strains WH316, WH311 and WH314 produced more H_2S than any other strain according to the plate test for H_2S formation. These colonies turned dark brown to black on the bismuth citrate agar plates and were excluded from further evaluation. The degree of H_2S formation by the whiskey strains is shown in Table 4.

High alcohol yield is one of the most important criteria when selecting for a whiskey yeast strain, because of the potential financial impact. If more alcohol can be produced from the same amount of raw material, the process would be very economical. The industrial whiskey strain that is currently being used in South Africa, WH310, produced a final alcohol yield of 96.44%. This amount can never be 100%, as some of the glucose taken up by the yeast is used for growth (biomass production), cell maintenance and the production of small amounts of byproducts, such as glycerol (Thomas *et al.*, 1996). Yeast strains, however, differ in their metabolic activities and their ability to produce a high alcohol yield. All strains producing similar or higher alcohol per volume than WH310 were selected for further work. These strains that were selected are highlighted in Table 5.

Table 4. Degree of H_2S formation by distiller's yeast.

Cream colonies (-)	Light brown colonies (+)	Dark brown colonies (++)	Very dark brown to black colonies (+++)
WH300, WH302, WH303, WH304, WH305, WH306, WH309, WH313, WH319	WH299, WH301, WH308, WH310, WH312, WH315, WH317,	WH316	WH311, WH314

Table 5. Final alcohol yield produced by whiskey strains (first distillate).

Whiskey strain	Final alcohol yield* (%)
WH299	93.75
WH300	92.81
WH301	90.55
WH302	95.72
WH303	98.62
WH304	93.04
WH305	93.65
WH306	91.67
WH308	93.00
WH309	94.54
WH310	96.44
WH311	95.12
WH312	95.59
WH313	96.06
WH314	95.18
WH315	95.72
WH316	94.67
WH317	94.43
WH319	98.12

* The experimental alcohol yield as a percentage of the theoretical alcohol yield

Table 6. GC results of second distillates of four selected yeast species.

YEAST NO.	WH303	WH310	WH313	WH319
%AA/V	77.69	76.30	77.00	78.40
RESULTS OF GASCHROMATOGRAPHIC ANALYSIS (IN g/hl AA)				
Acetaldehyde	10.30	13.11	11.69	11.48
Ethyl acetate	37.33	39.32	36.36	45.92
Ethyl butyrate	n.d.	n.d.	n.d.	n.d.
Isoamyl acetate	6.44	3.93	2.60	2.55
Methanol	6.44	5.24	6.49	6.38
n-Propanol	73.37	45.87	59.74	84.18
Isobutanol	289.61	235.91	207.80	332.91
n-Butanol	1.29	1.31	1.30	1.28
Isoamyl alcohol	592.10	639.58	612.99	540.82
TOTAL ESTERS	43.76	43.25	38.96	48.47
TOTAL HIGHER ALC.	956.36	922.67	881.82	959.18
TOTAL VOLATILES*	1000.13	965.92	920.78	1007.65

*excluding acetaldehyde and methanol

Five members of the tasting panel preferred WH303 to any other sample, using descriptive notes such as nuttiness and smokiness. However, WH313 and WH319 were found to be closest to the control sample by most members, who used terms such as clean and fruity. No off-flavours were detected. The conclusion was that all the samples had positive notes, with few differences in the organoleptic characteristics. The fatty acid, ester and higher alcohol content of these four samples were also similar (see Table 6 for the GC analysis), and it therefore was decided that all four strains should be transformed with *LKA1* and *LKA1/2*.

3.3.2 CONSTRUCTION OF AN *LKA1* AND AN *LKA2* RECOMBINANT WHISKEY YEAST STRAIN

Figure 1 shows the resultant plasmids pIPLKA1SMR1 and pIPLKA1/2SMR1 that were obtained by cloning the *SMR*1 gene into pIPLKA1 and pIPLKA1/2 respectively. Both plasmids were linearised with *Stu*1 (restriction site in *URA*3) and used for yeast transformations. It was decided to transform the wine yeast, VIN13, as well, because of its fast fermentation characteristics. Transformation of the whiskey strains resulted in 35 transformants, which were put through a selection process to obtain the best performers. No transformants of the combination 319LKA1/2 and 313LKA1/2 were obtained.

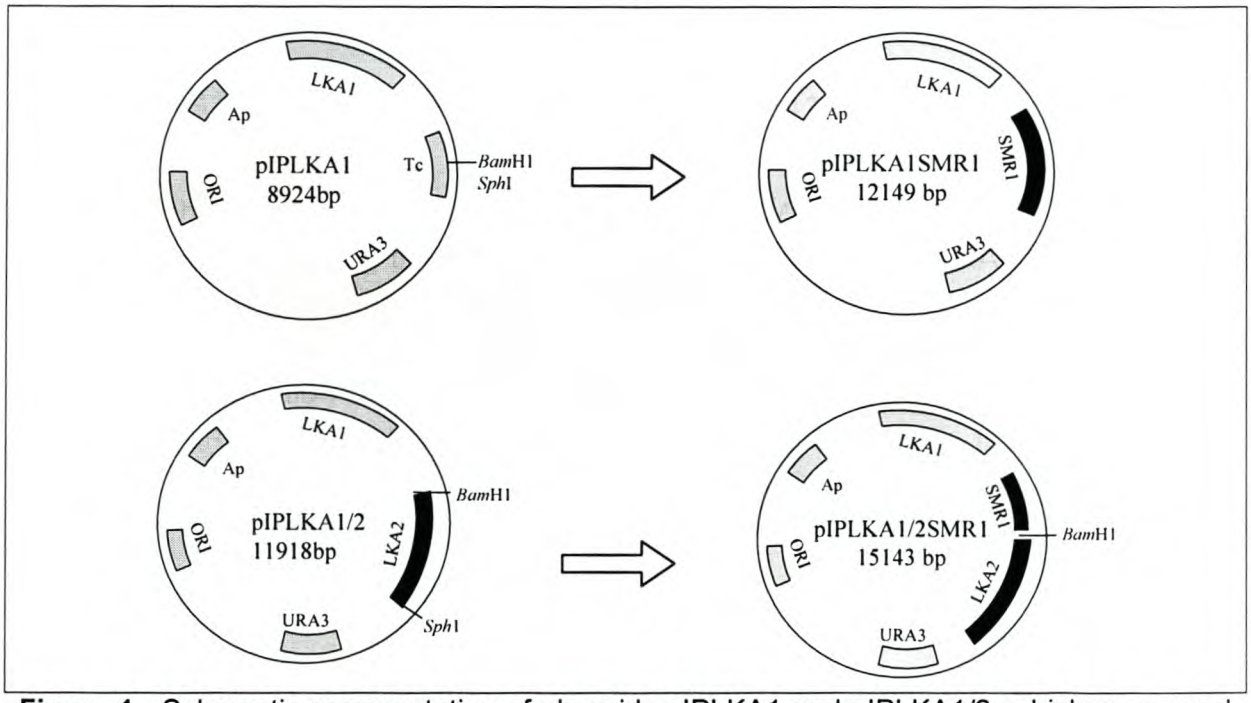

Figure 1. Schematic representation of plasmids pIPLKA1 and pIPLKA1/2, which were used to construct recombinant plasmids pIPLKA1SMR1 and pIPLKA1/2SMR1.

3.3.3 AMYLOLYTIC ACTIVITY OF THE TRANSFORMANTS

The amylolytic activity of all the transformants was measured and Tables 7 and 8 provide a summary of the intra- and extracellular activities obtained.

Table 7. Intracellular, extracellular and total cellular activity of the control strains measured in units/ml, where 1 U = μmol glucose released per minute per gram of cells at pH 4.4 and 30°C.

Control strains	OD2 +/- 0.25			OD6 +/- 0.25		
	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total
303	1	74	**75**	3	23	**26**
310	0	62	**62**	5	27	**32**
313	0	142	**142**	3	28	**31**
319	0	94	**94**	5	23	**28**
VIN13	0	142	**142**	12	25	**37**

Table 8. Intracellular, extracellular and total cellular activity of the transformants measured in units/ml, where 1 U = μmol glucose released per minute per gram of cells at pH 4.4 and 30°C.

Transformant strains	OD2 +/- 0.25			OD6 +/- 0.25		
	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total
303LKA1(6)	108	298	**406**	65	50	**115**
303LKA1(8)	60	234	**294**	53	35	**88**
303LKA1/2(8)	71	544	**615**	88	82	**170**
310LKA1(1)	75	305	**380**	66	58	**124**
310LKA1/2(2)	101	231	**332**	89	55	**144**
310LKA1/2(3)	78	413	**491**	70	56	**126**
310LKA1/2(4)	101	264	**365**	65	53	**118**
310LKA1/2(5)	94	254	**348**	67	51	**118**
310LKA1/2(6)	96	210	**306**	70	61	**131**
313LKA1(2)	163	185	**348**	120	64	**184**
313LKA1(3)	150	148	**298**	105	44	**149**
313LKA1(4)	106	261	**367**	73	53	**126**
313LKA1(5)	111	208	**319**	70	37	**107**
313LKA1(6)	95	157	**252**	66	37	**103**
313LKA1(7)	67	123	**190**	49	38	**87**
319LKA1(10)	81	117	**198**	103	55	**158**
319LKA1(18)	87	110	**197**	88	50	**138**
319LKA1(8)	107	266	**373**	74	47	**121**
319LKA1(16)	89	124	**213**	73	47	**120**
319LKA1(15)	78	110	**188**	77	47	**124**
319LKA1(6)	70	233	**303**	74	51	**125**
319LKA1(20)	103	154	**257**	80	42	**122**
319LKA1(7)	63	157	**220**	81	46	**127**
319LKA1(19)	127	195	**322**	66	46	**112**
319LKA1(12)	81	155	**236**	76	46	**122**
319LKA1(17)	100	133	**233**	93	54	**147**
VIN13LKA1(1)	220	207	**427**	188	89	**277**
VIN13LKA1(10)	143	70	**213**	135	58	**193**
VIN13LKA1(9)	195	259	**454**	203	90	**293**
VIN13LKA1(7)	316	285	**601**	235	124	**359**
VIN13LKA1(4)	225	293	**518**	212	100	**312**
VIN13LKA1(5)	134	258	**392**	134	50	**184**
VIN13LKA1(2)	210	275	**485**	204	89	**293**
VIN13LKA1(8)	414	361	**775**	262	157	**419**
VIN13LKA1/2(1)	117	185	**302**	149	58	**207**

The extracellular activity of the control strains shown here is the effect of the glucose still left in the YPD medium. These values were subtracted from the enzyme activities shown by the transformants.

The transformants in the grey blocks were selected for their enzyme activities and their ability to grow in 2% starch medium was assessed. These strains were also subjected to Southern blot analysis to confirm the integration of the *LKA1* gene, or of the *LKA1* and *LKA2* genes, into the genome (results not shown).

3.3.4 GROWTH IN 2% STARCH MEDIUM

Strains 319[LKA1](15), 319[LKA1](10), 313[LKA1](4), 313[LKA1](5), 303[LKA1](6), 303[LKA1/2](8), 310[LKA1](1), 310[LKA1/2](3), 310[LKA1/2](6), VIN13[LKA1](1), VIN13[LKA1](8) and VIN13[LKA1/2](1) were selected on the basis of their high enzyme activities, cultivated in a 2% starch medium and their growth curves were determined (Fig 2).

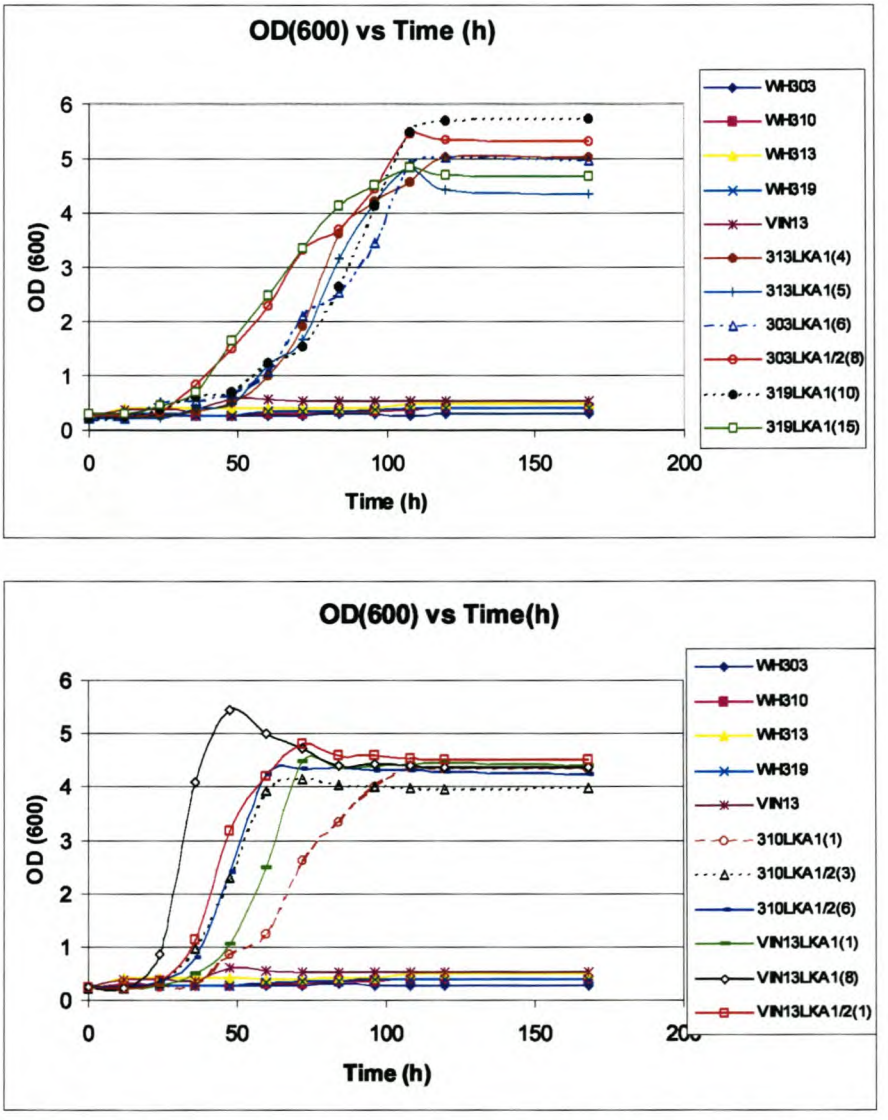

Figure 2. Growth curves of transformants and controls in 2% starch medium.

3.3.5 WHISKEY FERMENTATIONS

The strains that were selected on their ability to grow in 2% starch medium, were: 310[LKA1/2](3), 310[LKA1/2](6), VIN13[LKA1/2](1), VIN13[LKA1](1) and VIN13[LKA1](8). Fermentation curves were drawn up to see whether there is a relationship between enzyme production and fermentation rate (Fig 3).

Table 9 gives a summary of the results of the final alcohol yield produced by the transformants, as well as by the industrial whiskey strains with commercial enzymes. CHEF analysis was also performed on these transformants to show that the strains were not different from the parent strains in their chromosome pattern (results not shown).

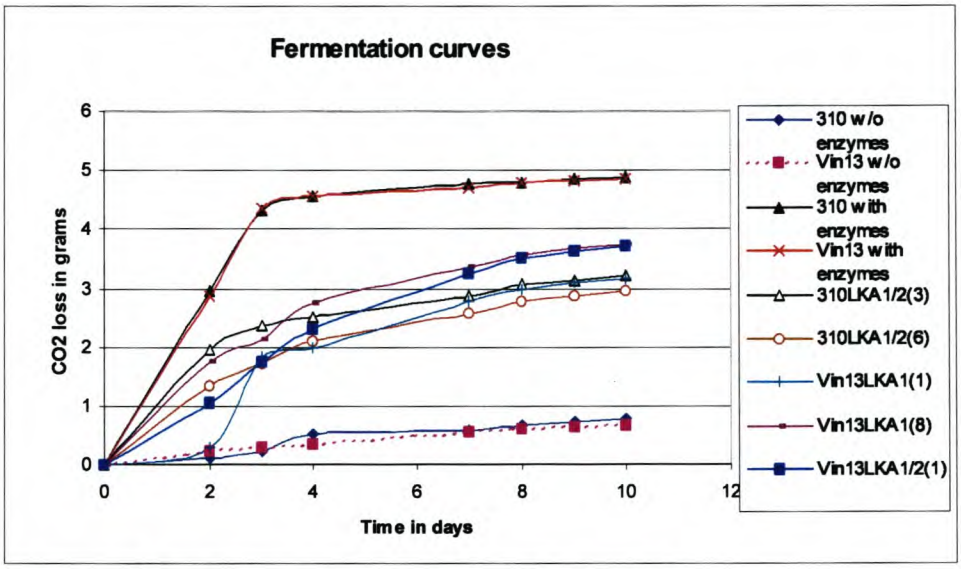

Figure 3. Fermentation curves of transformants in a maize slurry.

Table 9. Final alcohol yield as a percentage of the theoretical yield of control strains and recombinant strains.

Strain	Maize starch content	Theoretical yield (ml Alcohol)	Experimental yield (ml Alcohol)	Final yield (%)
310 + commercial enzymes	60%	5.78	5.56	96.1
VIN13 + commercial enzymes	60%	5.78	5.47	94.65
310 (No enzymes)	60%	5.78	0.08	1.38
VIN13 (No Enzymes)	60%	5.78	0.088	1.5
310LKA1/2(3)	60%	5.78	3.09	53.46
310LKA1/2(6)	60%	5.78	2.73	47.23
VIN13LKA1(1)	60%	5.78	3.25	56.31
VIN13LKA1(8)	60%	5.78	3.8	65.74
VIN13LKA1/2(1)	60%	5.78	3.35	62.46

3.4 DISCUSSION

The first part of the experiment involved the selection of a suitable whiskey yeast strain that had all the requirements needed for the effective fermentation of whiskey mash. Strain WH310 is currently being used on a commercial scale, but, during the screening process, it became clear that there are other strains in the collection that perform even better than the commercial strain. Two strains produced a higher alcohol yield than WH310, namely WH303 and WH319. This criterion is one of the most important as it can have a big financial impact on whiskey production. The double distillate also showed positive notes when presented to a tasting panel, which means that it is possible to test these two strains on a commercial scale to see whether the effect is the same. Further investigations will be conducted.

Genetic transformations with *LKA1* and *LKA1/2* resulted in 35 transformants, which had to be screened and selected for the best enzyme activity. The enzyme activities did not vary much between transformants, but, on average the VIN13 transformants showed the highest total enzyme activity at OD 2 and at OD 6 (Table 8). This definitely shows that the secretion signals of VIN13 are much stronger than those of the whiskey strains. If the total extracellular activities of all the transformants at OD 2 and OD 6 are compared, it is clear that the total activity per gram of cells decreases at OD 6. With the increase of biomass from OD 2 to OD 6, it therefore is evident that the total activity decreases or stays constant during the growth period. Growth curves were drawn up for the selected strains in 2% starch medium and Fig. 2 shows that the fastest growing strain is VIN[13LKA1](8), followed by VIN13[LKA1/2](1). In their study, Eksteen *et al.* (2003) found that both genes expressed in a laboratory strain consumed up to 80% of soluble starch in a batch fermentation, as opposed to 45% by the strain transformed with only *LKA1*. It therefore was expected that the transformants with both *LKA1* and *LKA2* genes would grow faster and give better starch breakdown than those with only the *LKA1* gene. This was not the case with the VIN13 transformants, however, as VIN13[LKA1/2](1) had a much slower exponential phase than VIN13LKA1(8). The latter transformant also had a higher alcohol yield in a maize slurry fermentation. With regard to the whiskey yeast transformants, 310[LKA1/2](3) and 310[LKA1/2](6) showed the expected improved growth rate, in comparison to that of transformant 310[LKA1](1) (Fig 2).

The recombinant strains were tested in whiskey fermentations, but the industrial process unfortunately could not be simulated with recombinant strains, because the commercial enzymes added to the process helped to prevent the thickening of the maize slurry when the temperature reached the gelatinisation temperature of the starch. Therefore, the percentage of starch in the mash had to be decreased to prevent clogging, which can lead to uneven liquefaction. Instead of using maize with a total content of 135.75 g/L starch, only 20 g/L starch could be used in the mashing and fermentation. The alcohol yield did not vary much between the transformant strains, with VIN13[LKA1](8) giving the highest yield at 65.74% (Table 9). The

fermentations took 10 days to complete in comparison to the 3 days for the controls with commercial enzymes. Nevertheless, there is a big difference in the final alcohol yield of whiskey strain WH310 without any added commercial enzymes and that of transformant 310[LKA1/2](3). WH310 cannot produce alcohol from starch, but 310[LKA1/2](3) can produce an alcohol yield of 53.46%. The reason for the incomplete conversion of starch to glucose and the subsequent formation of ethanol could be that insufficient amounts of the enzyme were secreted into the medium. To test this possibility, fermentations were conducted with 100x more inoculum with two transformed strains, namely VIN13[LKA1/2](1) and 310[LKA1/2](3). The final alcohol yield for the 100x higher inoculum of VIN13[LKA1/2](1) was 76.30% (compared to 62.46%) and for the 100x higher inoculum of 310[LKA1/2](3) it was 69.90% (compared to 53.46%). It seemed as if the higher inoculum resulted in more enzyme being secreted, leading to higher conversion of starch and higher levels of alcohol being produced. A further possible stumbling block could be caused by the secretion of enzyme into the medium. More efficient secretion signals could possibly solve this problem and should be investigated.

The possibility that the commercial enzymes have amylolytic activity that differs from that of the enzymes produced by the *LKA1* and *LKA2* genes is not excluded. If the specific activity of the *LKA1* and *LKA2* enzymes is such that they attack sites in the starch molecule to produce more oligosaccharides than glucose, it will result in incomplete saccharification, which will lead to incomplete fermentation and lower alcohol yield. To check whether the enzyme activity from the *LKA1* and *LKA2* enzymes is sufficient to liquefy starch, the enzyme activity of the commercial enzyme was tested with the DNS method. The number of units of commercial α-amylase used in the industrial process was tested at pH 6.6 and 30°C (Spezyme AA Product Information sheet, Genencor, Int.). The results showed 22 U of enzyme activity, compared to 28 U (extracellular) for VIN13[LKA1](8), the transformant with the highest enzyme activity. One U is defined as the amount of μmol glucose released per minute per ml of enzyme. According to this result, enough enzyme is present for liquefaction. This could support the statement that the specific activity of the *LKA1* and *LKA2* enzymes differs from that of the commercial enzyme.

The fermentation curves of the transformants follow each other closely and, from the results, there seems to be a link between enzyme production and fermentation rate. VIN13[LKA1](8), which had much higher extracellular enzyme activity than all the other strains (775 U), also fermented faster, but VIN13[LKA1/2](1), of which the enzyme activity was less than half that of VIN13[LKA1](8), namely 302 U, gave the second highest alcohol yield and its fermentation curve lies close to that of VIN13[LKA1](8). There is no supportive explanation for this phenomenon.

Fermentations were also conducted in duplicate with 310[LKA1/2](3) in a normal industrial small-scale fermentation, using 50% and 80% of the amount of the glucoamylase, to see whether the transformant strain might produce a higher final alcohol yield. As a control, strain WH310 was used in a normal mashing with the

correct amount of enzyme. There was no difference in the alcohol yield between the control strain and the transformant, but the fermentations with 50% and 80% less glucoamylase took 5 days to complete, in comparison to 3 days for the control experiment. This experiment shows that 50% of the glucoamylase used is enough for complete saccharification, although the process takes longer to complete. Further investigations with regard to reducing the amount of glucoamylase will be conducted. To see whether pH has any influence on the activity of the enzymes, fermentations were also conducted with a maize slurry at pH 4.0 (using H_2SO_4), but the fermentation rate and final alcohol yield were not influenced by lower pH.

For the further evaluation of amylolytic strains in mash fermentations with a higher starch concentration, high-pressure steam will be necessary to increase the temperature of the mash rapidly. Unfortunately, this device was not available, but it will be essential if amylolytic whiskey strains need to be evaluated on a small scale in the future.

3.5 LITERATURE CITED

Bringhurst TA, Broadhead AL, Brosnan JM, Pearson SY and Walker JW. 2001. The Identification and Behaviour of Branched Dextrins in the Production of Scotch Whisky. *J. Inst. Brew.* **107**:137-149.

Carle GF and Olsen MV. 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA.* **82**: 3756-3760.

De Mot R. 1990. Conversion of starch by yeasts. In: *Yeast: biotechnology and biocatalysis*. Edited by: Verachtert H and De Mot R. Marcel Dekker, New York, pp 163-221.

De Mot R, Van Dijck K, Denkers A and Verachtert H. 1985. Potentialities and limitiations of direct alcoholic fermentations of starch material with amylolytic yeasts. *Appl. Microbio. Biotechnol.* **22**:222-226.

Dohmen RJ, Strasser AWM, Dahlems UM and Hollenberg CP. 1990. Cloning of the *Schwanniomyces occidentalis* gene (*GAM1*) and its expression in *Saccharomyces cerevisiae*. *Gene* **95**:111-121.

Eksteen JM, Steyn AJC, Van Rensburg P, Cordero Otero RR and Pretorius IS. 2003. Cloning and characterization of a cDNA and genomic copy of a second α-amylase gene (*LKA2*) from *Lipomyces kononenkoae* IGC4052B and its expression in *Saccharomyces cerevisiae*. *Yeast* **20**:69-78.

Gietz RD and Schiestl RH. 1995. Transforming Yeast with DNA. *Methods in Molecular and Cellular Biology* **5**:255-269.

Gundllappali Moses SB, Cordero Otero RR, La Grange DC, Van Rensburg P and Pretorius IS. 2002. Different genetic backgrounds influence the secretory expression of the *LKA1*-encoded *Lipomyces kononenkoae* α-amylase in industrial strains of *Saccharomyces cerevisiae*. *Biotechnology Letters* **651**: 651-656.

Hammond JRM. 1994. Yeast and fermentation in the 21st century. In: *Proceedings of the Fourth Aviemore Conference on Malting, Brewing and Distilling*. Edited by: Priest FG and Campbell I. pp 85-99. Institute of Brewing, London.

Hinrichs J and Stahl U. 1993. Genetic engineering in the brewing industry. *Brauwelt International* **4**:293-298.

Jiranek V, Langridge P and Henschke PA. 1995. Validation of Bismuth-Containing Indicator Media for Predicting H_2S-producing Potential of *Saccharomyces cerevisiae* Wine Yeasts Under Enological Conditions. *Am. J. Enol. Vitic.* **46**:269-272.

Laluce C and Mattoon JR. 1984. Development of Rapidly Fermenting Strains of *Saccharomyces diastaticus* for Direct Conversion of Starch and Dextrins to Ethanol. *Applied and Environmental Microbiology*, July, pp 17-25.

Lancashire WE. 1986. Modern genetics and brewing technology. *Brewer* **72**: 345-348.

Lancashire WE, Carter AT, Howard JJ and Wilde RJ. 1989. Superattenuating brewing yeast. *Proc. of the Eur. Brew. Conv. Congress*. Zürich. pp 491-498.

Sambrook J, Fritsch EF and Maniatis T. 1989. Molecular cloning. A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Spencer-Martins I. 1982. Extracellular isoamylase produced by the yeast *Lipomyces kononenkoae*. *Appl. Environ. Microbiol*. **44**:1253-1257.

Spencer-Martins I and Van Uden N. 1979. Extracellular amylolytic system of the yeast *Lipomyces kononenkoae*. *Eur. J. Appl. Microbiol*. **6**:241-250.

Steyn AJC and Pretorius IS. 1995. Characterization of a novel α-amylase from *Lipomyces kononenkoae* and expression of its gene (*LKA1*) in *Saccharomyces cerevisiae*. *Curr. Genet*. **28**:526-533.

Steyn AJC, Marmur J and Pretorius IS. 1996. Cloning, Mapping and Characterization of a Genomic Copy of the *Lipomyces kononenkoae* α-Amylase-Encoding Gene (*LKA1*). *Yeast* **12**: 925-937.

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

Stewart GG. 1985. New Developments in Ethanol Fermentation. *Am. Soc. Brew. Chem*. **43**: 61-65.

Thomas KC, Hynes SH and Ingledew WM. 1996. Practical and Theoretical Considerations in the Production of High Concentrations of Alcohol by Fermentation. *Proc. Biochem*. **31**: 321-331.

Tubb RS. 1986. Amylolytic yeasts for commercial applications. *Tibtech* **4**:98-104.

Van der Westhuizen TJ, Augustyn OPH and Pretorius IS. 1999. Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from South African vineyards in the cooler, coastal regions of the Western Cape in South Africa. *S. Afr. J. Enol. Vitic*. **21**: 3-9.

Chapter 4

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4

4.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

Whiskey is the matured distilled product of fermented cereal grains. Malt whiskies are made entirely from malted barley, whereas grain whiskies are made from grains such as maize or wheat. Blended whiskies are a mixture of both (Berry, 1987). In current grain whiskey fermentations with maize as raw material, raw starch is gelatinised by cooking, liquefied by treatment with α-amylase and then saccharified by addition of glucoamylase (Cole *et al.*, 1988). *S. cerevisiae* is widely used for production of ethanol from starch-containing raw materials with the addition of amylolytic enzymes, as *S. cerevisiae* lacks the amylase enzymes necessary for starch utilisation. Added enzyme preparations are expensive and it therefore would be of great financial importance if the yeast could produce the enzymes needed for the conversion of starch to fermentable sugars.

Whiskey industries strive to manufacture whiskey in the most economical and efficient manner and to gain the maximum amount of organoleptically acceptable whiskey from a given quantity of cereal raw material (Dolan, 1979).

In Chapter 1, the importance and commercial interest of a yeast strain that is able to ferment starch from raw materials, such as grain and wheat, is outlined. The ideal recombinant amylolytic strain would be a genetically modified strain of *S. cerevisiae* with amylolytic activity, as *S. cerevisiae* has a high ethanol tolerance, fast fermentation rate and is widely applied in the food and beverage fermentation industries.

The differences between Scotch malt and Scotch grain whiskies are discussed in Chapter 2 and the whiskey-making process is discussed briefly. The rest of the chapter included ways to improve distiller's yeast by means of genetic techniques and the available strategies, focusing on the amylolytic activity of the yeast.

Research results are given in Chapter 3 and the most important conclusions will now be summarised briefly with further suggestions. At Distell (South Africa), whiskey is being made by fermenting the maize slurry with WH310, which is known commercially as DY10. In this study, a collection of 19 different whiskey strains were screened for certain characteristics important for whiskey yeast strains. It became clear that there are other strains in the collection that could perform better in a whiskey fermentation than the control strain, WH310. Strains WH303, WH313 and WH319 are less likely to form H_2S than WH310. With regard to the alcohol yield produced in a mash fermentation, WH303 and WH319 gave a higher alcohol yield than WH310. This was tested on a small scale, but it is recommended that these strains are tested in a large-scale fermentation to see whether the effect is the same. There were no significant differences between the second distillates of WH303, WH310, WH313 and WH319 with regard to the GC analysis, and most members of the tasting panel preferred WH303 to any other sample, although there were no negative comments on any of the samples.

The transformant strains showed significantly higher enzyme activity than the control strains. It was also shown that some of the transformants with both the *LKA1* and *LKA2* genes had lower enzyme activity than the respective transformants with the *LKA1* gene alone. The same was found by Eksteen *et al.* (2003) in a plate assay, in which the *LKA1/2* transformant showed smaller zones on starch plates than the *LKA1* transformant. This suggests that the *LKA2* gene has an inhibitory effect on enzyme production in the presence of *LKA1*. Transformants of VIN13, which is a wine yeast, showed higher enzyme activity on average than any of the whiskey transformants. This could suggest that VIN13 has a better secretion system for these enzymes, although the intracellular activity is also higher in VIN13 transformants than in the whiskey transformants, which suggests that more enzyme is in fact being produced. The transformant strains that were selected for whiskey fermentations, 310[LKA1/2](3), 310[LKA1/2](6), VIN13[LKA1](1), VIN13[LKA1](8) and VIN13 [LKA1/2](1), showed a faster exponential phase when cultivated in 2% starch, reaching the stationary phase after ± 70 h, except for VIN13[LKA1](8), which only took 50 h to reach the stationary phase. The VIN13 transformants also gave the highest alcohol yield in a whiskey fermentation. It is not clear what the organoleptic contribution of VIN13 will be to the final distillate, since VIN13 is a wine yeast and is not generally used for whiskey production. Further testing is necessary if transformants of VIN13 are to be used for whiskey production.

A significant improvement in alcohol yield was obtained with the transformants in comparison to the WH310 control and VIN13 control without added enzymes. Although optimum alcohol yield was not achieved by the transformants, this study paves the way for further research on the creation of industrial yeast strains with raw starch-degrading enzymes that could be used in a whiskey fermentation without the use of commercial enzymes.

Further work should concentrate on ways to increase the amount of amylolytic enzymes available to degrade starch by developing strains with stronger secretion signals. To test these strains in an industrial whiskey fermentation, a device is needed for the starch to be liquified by using steam to prevent thickening of the maize slurry. A whiskey recombinant strain with the ability to ferment a maize slurry, without liquefaction of the starch through heat application, would be ideal.

4.2 LITERATURE CITED

Berry DR. 1987. Amylase activities during malt whisky production. *Crit. Rev. Biotech.* **5**:143-157.

Cole GE, McCabe PC, Inlow D, Gelfand DH, Ben-Bassatt A and Innis MA. 1988. Stable expression of *Aspergillus awamori* glucoamylase in distiller's yeast. *Biotechnol.* 6:417-421.

Dolan TCS. 1979. Bacteria in whisky production. *The Brewer.* February. pp 60-64.

Eksteen JM, Steyn AJC, Van Rensburg P, Cordero Otero RR and Pretorius IS. 2003. The evaluation and comparison of recombinant *Saccharomyces cerevisiae* strains expressing α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomyces fibuligera*. *Biotechnology and Bioengineering* (submitted).