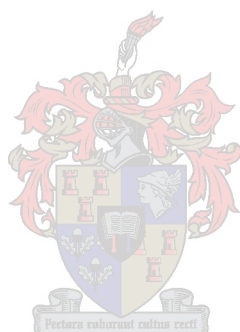# The development of yeasts for the optimal production of flavor-active esters and higher alcohols in wine and distillates

by

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Dissertation presented for the Degree of Doctor of Agricultural Sciences at Stellenbosch University.
December 2004

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation 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.

SUMMARY

Yeasts produce a broad range of aroma-active volatile esters and higher alcohols during alcoholic fermentation. Some of these esters and higher alcohols are important for the fruity flavors and therefore the final quality of wine and other fermented beverages. Esters are produced and hydrolyzed by alcohol acetyltransferases and esterases, respectively. In yeast, ester-synthesizing activities are represented by two alcohol acetyltransferases encoded by the *ATF1* and *ATF2* genes, and by an ethanol hexanoyl transferase encoded by the *EHT1* gene. Atf1p and Atf2p appear responsible for the production of ethyl acetate and isoamyl acetate, while Eht1p synthesizes ethyl hexanoate from ethanol and hexanoyl-CoA. Although a fair amount of information is available regarding the *ATF1* gene, limited information is available on the remaining alcohol acetyltransferases. Only two genes that code for esterases have been identified in yeast, namely *IAH1* and *TIP1*. It has also been shown that the balance between alcohol acetyltransferases and esterases is important for the net rate of ester accumulation. Higher alcohols are synthesized from the α-keto-acids in the branched-chain amino acid metabolic pathway by decarboxylation and reduction. The transamination of the amino acid to the respective α-keto-acid is catalyzed by mitochondrial and cytosolic branched-chain amino acid transferases, which are encoded by the *BAT1* and *BAT2* genes, respectively.

In recent years, a strong scientific and industrial interest in the metabolism of flavor-active compounds has emerged, but information regarding the roles of specific enzymes and the physiological relevance of their metabolism remains limited. The aim of this project was to investigate the physiological and metabolic consequences of changes in the expression levels of some of the key enzymes involved in aroma compound production. The consequences of these changes on the chemical composition and the fermentation bouquet of wines and distillates were also investigated.

The first part of the section on the results in this dissertation reports on the role and relative importance of the *Saccharomyces cerevisiae* enzymes involved in ester metabolism, namely Atf1p, Atf2p, Eht1p, Iah1p and Tip1p. The corresponding genes were overexpressed in a laboratory strain of *S. cerevisiae*, BY4742, and in a widely used commercial wine yeast strain, VIN13. Table wine and base wines for distillation were prepared with these VIN13 transformed strains. The ester concentrations and aroma profiles of the wines and distillates were analyzed and compared. The data indicated that the overexpression of *ATF1* and *ATF2* increased the concentrations of ethyl acetate, isoamyl acetate, 2-pheylethyl acetate and ethyl caproate, while the overexpression of *IAH1* resulted in a significant decrease in the concentrations of ethyl acetate, isoamyl acetate, hexyl acetate and 2-phenylethyl acetate. The overexpression of *EHT1* resulted in a marked increase in the concentrations of ethyl caproate, ethyl caprylate and ethyl caprate, while the overexpression of *TIP1* did not decrease the concentrations of any of the esters. In most cases, there was a correlation between the increase in esters and the decrease in higher alcohols. The data suggest that yeast balances the amount of different esters produced through alcohol acetyltransferases and esterases, and that, in some cases, these enzymes appear to overlap in function and/or influence each other's activity.

In the second part of the results section, the consequences of the deletion and the overexpression of two genes, *BAT1* and *BAT2*, which encode transaminases that contribute to the metabolism of higher alcohols, were investigated. The genes were both disrupted in a *S. cerevisiae* BY4742, and overexpressed in both this laboratory strain and in the VIN13 wine yeast strain. The effects of these modifications on the general physiology of the corresponding yeast strains and on higher alcohol metabolism were assessed in a range of growth conditions, including aerobic and anaerobic growth conditions, in the presence of glucose or raffinose as sole carbon source and growth in the presence of various concentrations of amino acids. Table wine and base wines for distillation were prepared with the modified industrial strains and the concentrations of the higher alcohols and the aroma profiles of the wine and distillates were analyzed and compared. *Bat1* deletion seemed to be lethal under the conditions that were created, and therefore only the *bat2Δ* strain, together with the *BAT1* and *BAT2* overexpression strains, were investigated. These modifications did not appear to significantly affect the general physiology of the strains. The results obtained indicated that the overexpression of *BAT1* increased the concentrations of isoamyl alcohol and isoamyl acetate, and, to a lesser extent, the concentrations of isobutanol and isobutyric acid. The overexpression of the *BAT2* gene resulted in a substantial increase in the levels of isobutanol, isobutyric acid and propionic acid production, and a modest increase in the level of propanol and isovaleric acid. Interestingly, the overexpression of *BAT2* led to a decrease in isoamyl alcohol and isoamyl acetate concentrations. Sensory analyses indicated that the wines and distillates produced with the strains in which the *BAT1* and *BAT2* genes were overexpressed had more fruity characteristics (peach and apricot aromas) than the wines produced by the wild-type strains.

This study offers new prospects for the development of wine yeast starter strains with optimized ester and higher alcohol-producing capability that could assist winemakers in their efforts to consistently produce wine to definable specifications and styles and a predetermined flavor profile.

OPSOMMING

Gedurende fermentasie produseer giste 'n wye verskeidenheid vlugtige aromatiese esters en hoër alkohole. Sommige van hierdie esters en hoër alkohole is belangrik vir die vrugtige geure en dra dus by tot die finale kwaliteit van wyn en ander gefermenteerde drankies. Esters word onderskeidelik deur alkoholasetieltranferases en esterases geproduseer en gehidroliseer. In giste word die ester-sintetiserende aktiwiteite deur twee alkoholasetieltransferases verteenwoordig wat deur die *ATF1*-en *ATF2*-gene, asook 'n etanolheksanoïeltransferase wat deur die *EHT1*-geen, gekodeer word. Dit blyk dat ATF1p en ATF2p verantwoordelik is vir die produksie van etielasetaat en isoamielasetaat, terwyl Eht1p-etielheksanoaat vanaf etanol en heksanoïel-CoA sintetiseer. Alhoewel daar 'n redelike hoeveelheid inligting t.o.v die *ATF1*-geen beskikbaar is, is daar weinig inligting oor die res van die aloholasetieltransferases. Slegs twee gene wat vir esterases kodeer, is in gis geïdentifiseer, naamlik *IAH1* en *TIP1*. Daar is ook bewys dat 'n balans tussen die alkoholasetieltransferases en esterases baie belangrik is vir die netto-tempo van ester-akkumulasie. Hoër alkohole word gesintetiseer vanaf α-ketosure in die vertakte-ketting aminosuur metaboliese pad deur dekarboksilasie en reduksie. Die transaminasie van die aminosuur na die onderkeidelike α-ketosuur word deur vertakte-ketting aminosuur transferases, geleë in die mitochondrion en sitosol, en gekodeer deur *BAT1*- en *BAT2*-gene, gekataliseer.

In die laaste paar jare het daar 'n sterk wetenskaplike, asook industrïele, belangstelling in die metabolisme van aroma-aktiewe komponente te voorskyn gekom, maar inligting in verband met die rol van spesifieke ensieme en die fisiologiese belangrikheid van hul metabolisme is egter beperk. Die doel van hierdie projek was om die fisiologiese en metaboliese gevolge van veranderinge in die ekspressievlakke van sommige sleutelensieme betrokke by aromakomponent-produksie te ondersoek. Die gevolge van hierdie veranderinge op chemiese vlakke, asook hoe die fermentasie-aroma van die wyne en distillate beïnvloed word, is ook bestudeer.

Die eerste gedeelte van die resultate rapporteer oor die rol en relatiewe belangrikheid van die *Saccharomyces cerevisiae*-ensieme betrokke by estermetabolisme, naamlik Atf1p, Atf2p, Eht1p, Iah1p en Tip1p. Die gene was ooruitgedruk in 'n laboratoriumras van *S. cerevisiae*, BY4742, asook in 'n kommersïele wyngisras, VIN13. Tafelwyne en basiswyne vir distillasie is gemaak met die getransformeerde VIN13-rasse. Die esterkonsentrasies en aromaprofiele van die wyne en distillate is ontleed en vergelyk. Die data het gewys dat die ooruitdrukking van *ATF1*- en *ATF2*-gene 'n verhoging in etielasetaat, isoamielasetaat, 2-fenieletielasetaat en etielkaproat veroorsaak het, terwyl ooruitdrukking van *IAH1* 'n betekenisvolle afname in etielasetaat-, isoamielasetaat-, heksielasetaat- en 2-fenieletielasetaat-konsentrasies veroorsaak het. Die ooruitdrukking van *EHT1* het 'n duidelike verhoging in etielkaproaat, etielkaprilaat en etielkapraat veroorsaak en die ooruitdrukking van *TIP1* het geen van die esterkonsentrasies verander nie. In die meeste gevalle was daar nie 'n korrelasie tussen die toename in esters en afname in hoër alkohole nie. Die data stel ook voor dat die gis 'n balans tussen die verskillende esters handhaaf deur middel van die alkoholasetieltrasferases en esterases, en in

sommige gevalle blyk dit dat die ensieme dieselfde funksies het en/of mekaar se aktiwiteit beïnvloed.

In die tweede gedeelte van die resultate is die oorsake van delesie en ooruitdrukking van twee gene, *BAT1* en *BAT2*, wat kodeer vir transaminases wat tot hoër alkohol metabolisme bydra, bestudeer. Die gene is uitgeslaan in *S. cerevisiae* BY4742 en ooruitgedruk in BY4742 en in die wyngisras VIN13. Die effekte van hierdie modifikasies op die algemene fisiologie van die verskillende gisrasse en op hoëralkoholmetabolisme is onder 'n verskeidenheid kondisies bestudeer, naamlik aërobies en anaërobiese groeikondisies, in die teenwoordigheid van glukose of raffinose as die enigste koolstofbron, asook in die teenwoordigheid van 'n verskeidenheid konsentrasies aminosure. Tafelwyne en basiswyne vir distillasie is gemaak met die gemodifiseerde industriële rasse en die konsentrasies van die hoër alkohole en aromaprofiele van die wyne en distillate is ontleed en vergelyk. *Bat1*-delesie was dodelik onder die kondisies, daarom is slegs die *bat2*Δ-ras tesame met die *BAT1* en *BAT2* wat in die rasse ooruitgedruk is, bestudeer. Die modifikasies het nie 'n beduidende effek op die algemene fisiologie van die rasse getoon nie. Die data het wel getoon dat die ooruitdrukking van *BAT1* 'n verhoging in isoamielalkohol- en isoamielasetaatkonsentrasies, en tot 'n mindere mate isobutielalkohol- en isobottersuur-konsentrasies, veroorsaak het. Die ooruitdrukking van *BAT2* het 'n beduidende toename in isobutanol-, isobottersuur- en propioonsuur-konsentrasies en 'n kleinere toename in propanol- en isovaleriaansuur veroorsaak. Die ooruitdrukking van *BAT2* het ook gelei tot 'n afname in isoamielalkohol- en isoamielasetaat-konsentrasies. Sensoriese analises het getoon dat die wyne en distillate wat geproduseer is met die rasse waarin die *BAT1* en *BAT2* gene ooruitgedruk is meer vrugtige eienskappe (perske- en appelkoos-aromas) getoon het as die wyne wat deur die wildetipe rasse geproduseer is.

Die studie lewer nuwe vooruitsigte vir die ontwikkeling van wyngiste met geoptimiseerde ester en hoër alkohol produserende eienskappe wat die wynmakers in staat kan stel om wyne te produseer met gedefinieerde spesifikasies en style en 'n voorafbepaalde aromaprofiel.

This dissertation is dedicated to my husband and my parents.
Hierdie proefskrif is aan my man en my ouers opgedra.

BIOGRAPHICAL SKETCH

Mariska (de Villiers) Lilly was born in Bellville, South Africa on 22 November 1974. She attended the Soutpansberg Primary School and matriculated at the Louis Trichardt High School in 1992. Mariska enrolled at the University of Stellenbosch in 1993 and obtained a BScAgric degree in Biochemistry, Genetics and Microbiology in 1996. She obtained the MScAgric degree (*cum laude*) in December 1998.

ACKNOWLEDGEMENTS

PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the *Journal of Agricultural and Food Chemistry*, to which Chapters 4 and 5 will be submitted for publication. Because the *Journal for Agricultural and Food Chemistry* is an American publication, American spelling and the style of the journal are used throughout this dissertation.

Chapter 1   **General Introduction and Project Aims**

Chapter 2   **Literature Review**

Ester and higher alcohol metabolism in yeast and its significance in alcoholic beverages

Chapter 3   **Research Results**

Approaches to the investigation of aroma compound metabolism in *Saccharomyce*

Chapter 4   **Research Results**

The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavor profiles of wine and distillates

Chapter 5   **Research Results**

The effect of increased branched-chain amino acid transaminase activity and the production of higher alcohols on the flavor profiles of wine and distillates

Chapter 6   **General Discussion and Conclusions**

CONTENTS

CHAPTER 4. THE EFFECT OF INCREASED YEAST ALCOHOL ACETYLTRANSFERASE AND ESTERASE ACTIVITY ON THE FLAVOR PROFILES OF WINE AND DISTILLATES

CHAPTER 5. THE EFFECT OF INCREASED YEAST BRANCHED-CHAIN AMINO ACID AMINO TRANSAMINASE ACTIVITY AND THE PRODUCTION OF HIGHER ALCOHOLS ON THE FLAVOR PROFILES OF WINE AND DISTILLATES

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1   INTRODUCTION

A broad range of aroma-active substances that greatly affect the complex aroma of fermented alcoholic beverages is produced during fermentation processes, which are conducted by yeast. These aroma-active compounds include esters, higher alcohols and fatty acids. Although esters are primarily responsible for the characteristic fruity odors of the fermentation bouquet, a particular aroma property can only rarely be associated with a specific ester (Van Rooyen *et al.*, 1982). Acetate esters, especially isoamyl acetate and ethyl acetate, are the most important flavor compounds in alcoholic beverages (Fujii *et al.*, 1994). Esters are secondary products produced by yeasts during fermentation and constitute one of the most important groups of compounds affecting flavor (Peddie, 1990). The concentration at which they are found in wine is generally well above their sensory threshold levels (Salo, 1970a, 1970b; De Wet, 1978). The most significant esters are acetate esters of higher alcohols: ethyl acetate (fruity, solvent-like), isoamyl acetate (isopenthyl acetate, pear-drops), isobutyl acetate (banana), ethyl caproate (ethyl hexanoate, apple) and 2-phenylethyl acetate (honey, fruity, flowery) (Thurston *et al.*, 1981); and ethyl esters of straight-chain, saturated fatty acids: ethyl butanoate, ethyl hexanoate (an odor reminiscent of apple and violets), ethyl octanoate (pineapple and pear-like odor), ethyl decanoate (floral odor) and ethyl dodecanoate (Marais, 1990). These esters are particularly important in the bouquet of young wine and still play a significant role in aged wine. A significant amount of work has been done in this field. Wagener and Wagener (1968) found highly significant correlations between the quality of a white wine and its ester content. Van Wyk *et al.* (1979) demonstrated that isoamyl acetate was strongly correlated with the characteristic aroma components of young Pinotage wines. Marais (1978) and Marais and Pool (1980) also demonstrated a significant correlation between wine quality and the ester content in Colombard, Chenin blanc and Riesling wines. Van der Merwe and Van Wyk (1981) investigated the contribution of some of the fermentation products to the odor of dry white wine. With the addition of esters to a dearomatized wine, the authors were able to reproduce the quality and intensity of the original wine. Ferreira *et al.* (1995) studied the role played by ethyl esters of fatty acids and the acetates of higher alcohols in the aroma of young wines from neutral grape varieties. It was concluded from this study that the role played by these compounds depends on the type of wine. In white wines, the main role is in the perception of tree fruit and tropical fruit notes. The authors also demonstrated that the tree fruit notes are linked to the ethyl esters and the tropical fruit notes to the acetate esters of higher alcohols. In a more recent study, Aznar *et al.* (2001) showed that the most important odorants in aged red wines from Rioja include higher alcohols, fatty acid ethyl esters and ethyl esters of isoacids.

These esters are produced from higher alcohols and acetyl-CoA by alcohol acetyltransferases. Alcohol acetyltransferases are encoded by *ATF1* (Fujii *et al.*, 1994) and *ATF2* (Nagasawa *et al.*, 1998), which are responsible for the production of ethyl acetate and isoamyl acetate (Fujii *et al.*, 1994). The ethanol hexanoyl transferase, encoded by the *EHT1*

gene (Mason and Dufour, 2000), is responsible for generating ethyl hexanoate from ethanol and hexanoyl-CoA. Although a fair amount of information is available regarding the *ATF1* gene (Fujii *et al.*, 1994, 1996a, 1996b, 1997; Fujiwara *et al.*, 1998, 1999), limited information is available on the remaining alcohol acetyltransferases. Esterases also play a role in ester accumulation. Esterases represent a diverse group of hydrolases catalyzing the cleavage of esters, but in some cases also the formation of ester bonds. Only two genes that code for esterases have been identified in yeast, namely *IAH1* (Fukuda *et al.*, 1996) and *TIP1* (Horsted *et al.*, 1998). It has been shown that the balance between alcohol acetyltransferases and esterases is important for the net rate of ester accumulation (Fukuda *et al.*, 1998).

Higher alcohols are also secondary products of alcoholic fermentation by yeasts and are the most diverse group of aroma compounds in alcoholic beverages (Amerine *et al.*, 1980). Higher or fusel alcohols have a strong, pungent smell and taste and have a significant influence on the character and taste of alcoholic beverages (Rapp and Mandery, 1986). If the concentration of these alcohols is below 300 mg/l, they usually contribute to the desirable complexity of the wine, but when it exceeds 400 mg/l the higher alcohols are regarded as a negative influence on wine quality. Generally, white wine contains lower fusel alcohol concentrations than red varieties (Guymon, 1972). Rankine (1967) determined the taste threshold levels of some prominent higher alcohols, including isoamyl alcohol and isobutanol, in wine and a model solution and concluded that different concentrations of higher alcohols capable of being formed by the yeast can influence the taste of wines.

Higher alcohols are also important precursors for ester production and the esters of higher alcohols are associated with pleasant aromas (Soles *et al.*, 1982). Isoamyl alcohol, isobutanol, propanol, active amyl alcohol, 2-phenylethyl alcohol and tyrosol are some of the most important higher alcohols affecting the flavor of alcoholic beverages. These alcohols are synthesized from the α-keto-acids in the branched-chain amino acid metabolic pathway by decarboxylation and reduction (Dickinson *et al.*, 1997). The transamination of the amino acid to the respective α-keto-acid is catalyzed by mitochondrial and cytosolic branched-chain amino acid transferases encoded by the *BAT1* and *BAT2* genes, respectively (Eden *et al.*, 1996, 2001; Kispal *et al.*, 1996). Only in recent years has there been a renewed interest in the intricacies of branched-chain amino acid biosynthesis and limited information is available in this field.

1.2 PROJECT AIMS

This study forms an integral part of an extensive research program at the Institute for Wine Biotechnology aimed at the improvement of the sensorial quality of South African wines and brandies. In a previous study (Lilly *et al.*, 2000) involving the *ATF1* gene, we proved that the manipulation of a single gene can have a significant impact on the sensorial quality of both wines and distillates. Therefore, the objective of this project was to enhance the fruity aroma of the fermentation bouquet of wines and distillates by manipulating other genes involved in

aroma compound production, thereby developing new wine styles and new products with particular characteristics.

The specific aims and approaches of this study were as follows:

(i) to clone genes involved in ester (*ATF1*, *ATF2*, *EHT1*, *IAH1* and *TIP1*) and higher alcohol (*BAT1* and *BAT2*) metabolism from *S. cerevisiae* VIN13, an industrial wine yeast strain;

(ii) to overexpress all of the abovementioned genes in VIN13 and to study the effect thereof on ester and higher alcohol production during wine fermentations and distillations;

(iii) to study the changes in the yeast's metabolism caused by the overproduction of esters and higher alcohols or the decrease in ester and higher alcohol concentrations;

(iv) to examine any change in the enologically important characteristics during fermentation and in the final products;

(v) to assess and compare the flavor profiles of the Colombard white wines and the distillates from Colombard rebate wines produced with the genetically engineered wine yeast strains with those fermented with the original wine yeast strain, VIN13; and thereby

(vi) to unravel a part of ester and higher alcohol metabolism for a better understanding of the yeast metabolism during fermentation and of how to genetically alter the genome of *S. cerevisiae* for improved fermentation performance and bouquet.

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Chapter 2

LITERATURE REVIEW

Alcohol Metabolism in Yeast and its Significance in Alcoholic Beverages

2. ESTER AND HIGHER ALCOHOL METABOLISM IN YEAST AND ITS SIGNIFICANCE IN ALCOHOLIC BEVERAGES

2.1 INTRODUCTION

Flavor is arguably a wine's most important distinguishing characteristic. Most of what is commonly described as a wine's *flavor* is, in fact, its *aroma* or, as it is called in older wines, its *bouquet*. A wine's flavor could, in its widest sense, be said to be the overall sensory impression of both aroma (as sensed both by the nose and from the mouth) and the taste compounds, and may therefore incorporate the other, more measurable aspects of acidity, sweetness, alcoholic strength, fizziness, astringency and bitterness (Noble, 1994). Many wine-tasting professionals distinguish between the simple *aroma* of the grape-fermenting must or young wine and the *bouquet* of the more complex compounds that evolve as a result of fermentation, *élevage* and aging. There is little consistency in the usage of these terms and many authorities differ about which point in a wine's life cycle represents the point at which a wine's smell stops being an aroma and becomes a bouquet. A large number of wine connoisseurs refer to grape aromas as *primary* aromas, fermentation and oak aging as *secondary* aromas and bottle-aging aromas as either *tertiary* aromas or bouquet. In this review, however, the word *flavor* is used interchangeably with *aroma* and *bouquet*.

The term *flavor compounds* includes substances in wines that can be smelled or tasted, but it is used more particularly for the volatile compounds, which are sensed olfactory and which contribute to both aroma and bouquet. These volatile aroma compounds are present in significantly smaller concentrations than those of the non-volatile taste compounds such as glycerol or various acids, some at little more than one part per trillion (Noble, 1994).

Furthermore, the flavor of both wine and distillates immediately after fermentation and distillation only approximates that of the finished product. After the sudden and dramatic changes in composition effected by fermentation and distillation, chemical constituents react slowly during aging to move to their equilibriums, resulting in gradual changes in flavor. The harmonious complexity of wine and brandy can subsequently be further increased by oxidation and volatile extraction during oak barrel aging (Cole and Noble, 1995). Given the complexity of flavor and bouquet, this review is limited mainly to the production of esters and higher alcohols and their influence on aroma (**Figure 2.1**).

2.2 SIGNIFICANCE OF ESTERS IN ALCOHOLIC BEVERAGES

The characteristic fruity odors of wine, brandy and other grape-derived alcoholic beverages are primarily due to a mixture of ethyl acetate (solvent, chemical, fruity aroma), hexyl acetate (flowery aroma), ethyl caproate (apple-like aroma), isoamyl acetate (banana-like aroma), ethyl caprylate (apple-like aroma) and 2-phenylethyl acetate (fruity, flowery flavor with a honey note) (Fujii *et al.*, 1994). These esters are formed in the yeast cells during fermentation by alcohol acyltransferase enzymes using higher alcohols and acetyl-CoA as substrates.

The esters of alcoholic beverages can be divided into three fractions of different boiling ranges. The first, light fraction contains the compounds that elute before isoamyl alcohol and the main components are ethyl, isobutyl and isoamyl esters of short-chain fatty acids. These "fruit esters" have pleasing aromas and are major factors in the perceived aroma. The middle fraction consists of compounds eluting between ethyl caproate and 2-phenylethyl alcohol, and contains the ethyl esters of caprylic and capric acids as main components. The heavy fraction comprises compounds that elute after 2-phenylethyl alcohol and contains the ethyl esters of myristic, palmitic and palmitoleic acids (Suomalainen and Lehtonen, 1979; Léauté, 1990).
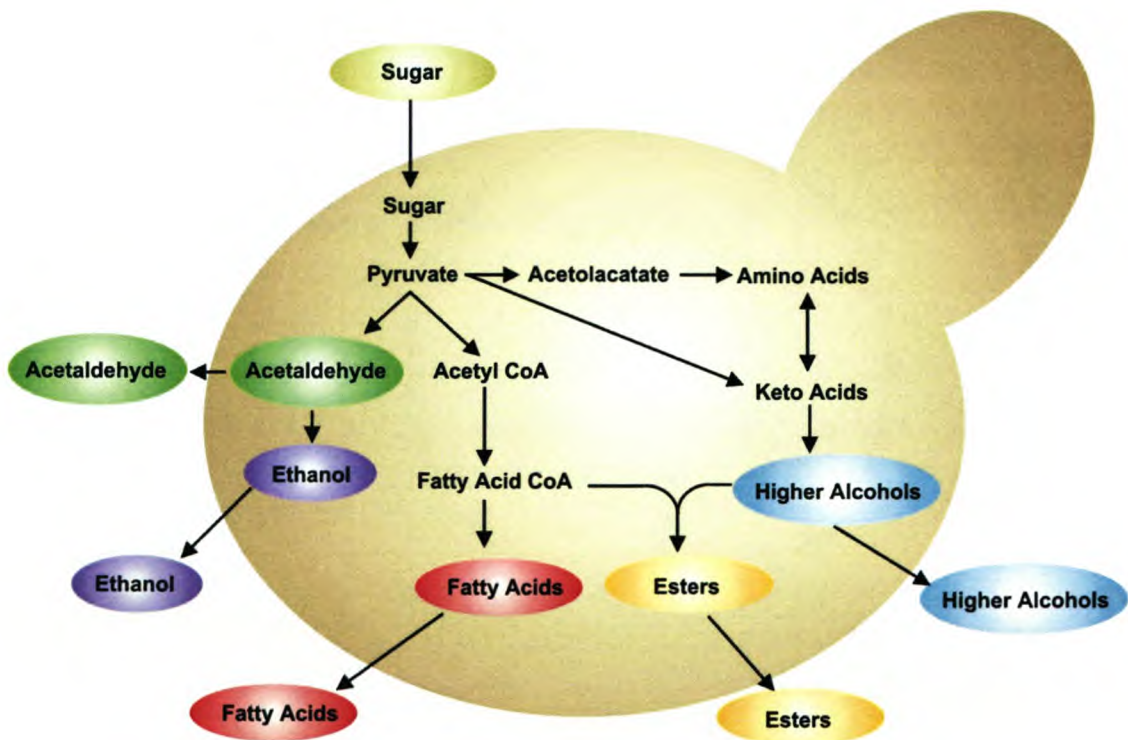
Figure 2.1 General representation of aroma compound metabolism in *Saccharomyces cerevisiae*.

2.2.1 WINE

It is well known that grapes of different varieties/cultivars display characteristic aromas that are distinctive of the wines (Schreier *et al.*, 1976; Rapp and Mandery, 1986). It can be shown that, while many volatile aroma substances arise from components of the grapes, a considerable number of these compounds are changed and a further substantial portion of wine flavor substances are formed during yeast fermentation. However, different biosynthetic pathways are interactive during the formation of the aroma of alcoholic beverages and different factors play their part in the formation of the total aroma (**Table 2.1**).

Table 2.1 Esters Produced by Yeast and their Concentrations, Threshold Values and Odors in Wine (Salo, 1970; Boulton *et al.*, 1995).

compound	conc. in wine (mg/L)	threshold value (mg/L)	odor
ethyl acetate		17.62*	varnish, nail polish, fruity, solvent, pineapple
isoamyl acetate	0.03-8.1	12.3	banana, pear
2-phenylethyl acetate	0.01-4.5	0.26*	rose, honey, fruity, flowery
ethyl isovalerate	ND-0.7		apple, fruity
isobutyl acetate	0.01-0.8		banana
ethyl butanoate	0.01-1.8	0.4	floral, fruity
	0.01-3		
ethyl 2-methyl-butanoate	ND-0.9		strawberry, pineapple
hexyl acetate			
ethyl hexanoate	trace-3.4	0.08	apple, banana, violets
ethyl octanoate	0.05-3.8	0.58	pineapple, pear
		0.258*	
ethyl decanoate	trace-2.1	0.5	floral

*Percentage-above-chance scores of 50% in grain spirit solutions of 9.4% (w/w)

The quantitative development of flavor compounds in grapes and also during yeast fermentation fluctuates considerably due to the influence of various factors, e.g. cultivar (Baumes *et al.*, 1986; Van Wyk *et al.*, 1979; Tominaga *et al.*, 1996), soil, condition and ripeness of the grapes (Houtman *et al.*, 1980a, 1980b), yeast strain (Soles *et al.*, 1982; Delteil and Jarry, 1992), pH of the must (Marais, 1978), and the nature and prevailing temperature of yeast fermentation (Daudt and Ough, 1973). Furthermore, numerous technological aspects and vinification methods (Voilley *et al.*, 1990; Gómez *et al.*, 1994), e.g. the nature of grape crushing, as well as of the mash and must treatment, and skin contact time (Falqué and Fernández, 1996) will also significantly influence the final aroma. Bacteria present on the grapes and in the must before and throughout fermentation exert their largest effect on wine flavor after completion of alcoholic fermentation, specifically during and after malolactic fermentation (Davis *et al.*, 1985). Laurent *et al.* (1994) observed significant increases in isoamyl acetate concentration after malolactic fermentation with *Oenococcus oeni*.

Storage time and temperature (Marais and Pool, 1980) must also be considered. Numerous chemical changes occur in wines during storage and these changes may drastically affect the aroma and quality of the wine. The changes could be the result of bottle or wood aging. Numerous compounds are chemically transformed and extracted from the wood, giving a new dimension to wine aroma. New aroma compounds might be formed, while the concentrations of others may increase or decrease.

In the case of esters, the final concentration of an ester in a wine is dependent on its formation during the fermentation process and its distribution between the wine and yeast (Houtman *et al.*, 1980a, 1980b).

Several studies have analyzed the ester contents of various wines. Rodríguez-Bencomo *et al.* (2002) determined the range of ester contents in commercial Spanish white wines (**Table 2.2**). The authors proposed that headspace solid-phase micro-extraction is an

appropriate technique for the quantitative analysis of several esters in dry and sweet white wines.

Table 2.2 Range of Ester Content in Commercial Spanish White Wines (Rodríguez-Bencomo *et al.*, 2002).

compound	mean +/- SD (mg/l; n=4)	
	sweet wines	dry wines
ethyl acetate	96.56 +/- 39.75	85.00 +/- 12.54
isobutyl acetate	0.07 +/- 0.02	0.07 +/- 0.04
ethyl butyrate	0.31 +/- 0.09	0.41 +/- 0.05
isoamyl acetate	1.81 +/- 0.91	2.37 +/- 0.62
ethyl hexanoate	0.87 +/- 0.41	1.06 +/- 0.19
hexyl acetate	0.06 +/- 0.04	0.14 +/- 0.14
ethyl lactate	13.5 +/- 6.6	23.00 +/- 18.88
ethyl octanoate	1.57 +/- 0.73	2.11 +/- 0.49
ethyl decanoate	0.65 +/- 0.26	0.56 +/- 0.06
benzyl acetate	0.004 +/- 0.004	0.003 +/- 0.001
2-phenylethyl acetate	0.23 +/- 0.17	0.21 +/- 0.05
ethyl dodecanoate	0.079 +/- 0.053	0.021 +/- 0.007

To gain an insight into the flavor differences of two wine varieties found mainly in Germany, namely Scheurebe and Gewürztraminer, Guth (1997) quantified 44 odor-active compounds in these wines (**Table 2.3**) by means of gas chromatography/olfactometry (GC/O). On the basis of their high odor activity values, ethyl octanoate, ethyl hexanoate, 3-methylbutyl acetate and ethyl isobutyrate are potent odorants in both varieties.

Table 2.3 Odor Threshold Values, Concentrations and Odor Activity Values of Potent Odorants in Scheurebe and Gewürztraminer Wines (Guth, 1997).

odorant	odor threshold value (mg/L)[a]	concentration (mg/L)[b]		odor activity value[c]	
		Scheurebe	Gewürztraminer	Scheurebe	Gewürztraminer
ethyl isobutyrate	15	480	150	32	10
ethyl butyrate	20	184	210	9	11
ethyl 2-methylbutyrate	1	4.5	4.4	5	4
ethyl 3-methylbutyrate	3	2.7	3.6	1	1
3-methylbutyl acetate	30	1450	2900	48	97
ethyl hexanoate	5	280	490	56	98
ethyl octanoate	2	270	630	135	315
2-phenylethyl acetate	250	262	112	1	<1
ethyl acetate	7500	22500	63500	3	8

[a]The odor threshold values were determined in water/ethanol (90 + 10, w/w). [b]The data are mean values of duplica (maximum SD: ±10%). [c]The odor activity values were calculated by dividing the concentration by the odor threshold value the compound in water/ethanol.

Aznar *et al.* (2001) identified 52 odorants and made a first evaluation of the sensory role of the most important odorants of aged wine from Rioja (Spain), which is, together with Jerez, the most emblematic area for the production of high quality Spanish red wines (**Table 2.4**). According to the estimation of the odor activity values, the most important odorants of the Rioja wine are fermentation compounds, the ethyl esters of fatty acids and the ethyl esters of

isoacids. The authors, however, concluded that the real impact of a compound must be measured in the presence of the rest of the compounds and in the same matrix.

Table 2.4 Odorants Found in the Extract of Rioja Wine (Aznar *et al.*, 2001).

odorant	odor description	flavor dilution value	SD, mg/L (s)	odor activity values
ethyl isobutyrate	fruity, strawberry	500	140 (4.88)	9.3
isobutyl acetate	strawberry	5	71 (2.74)	<0.1
ethyl butyrate	fruity, strawberry	50	124 (4.25)	6.2
ethyl 2-methylbutyrate	strawberry, berry	500	19.9 (1.7)	1.1
ethyl isovalerate	sweet fruity	50	37.7 (2.22)	12.4
ethyl pentanoate	green, mint	5	19.9 (0.89)	<0.1
ethyl hexanoate	fruity, strawberry	50	487 (21.9)	34.8
ethyl octanoate	sweet fruity	5	172 (7.41)	34.4
3-mercaptohexyl acetate	box tree, aniseed	5	nd	nd

The fresh, fruity aroma of wines derives to a great extent from the presence of the mixture of esters produced during fermentation, but esters significant to a specific grape cultivar have been identified in some cultivars. The *Vitis vinifera* cultivar Pinot noir is renowned for the production of the famous high quality Burgundy wines of the Côte d'Or region. Moio and Etievant (1995) reported that these wines are known to exhibit distinct red fruity aromas that particularly evoke the odors of small-stone fruit (plum and cherry), strawberry, raspberry, black currant, blackberry and, often, cherry stone and cherry brandy. These authors identified four odorant esters that can influence the characteristic flavor quality exhibited by the Pinot noir wines of Burgundy. Ethyl anthranilate (ethyl 2-aminobenzoate) was the most intense flavor compound among the four odorants identified in the isolated fraction. The second most intense flavor compound was ethyl cinnamate (ethyl 3-phenyl-2-propenoate), followed by ethyl 2,3-dihydrocinnamate (ethyl 3-phenylpropanoate) and, finally, methyl anthranilate (methyl 2-aminobenzoate). Ethyl anthranilate and ethyl 2,3-dihydrocinnamate were identified for the first time in wine, and methyl anthranilate was identified for the first time in a wine produced from a *V. vinifera* cultivar.

Ethyl anthranilate imparts a sweet-fruity and grape-like odor. Ethyl cinnamate is responsible for a cinnamon-like, sweet-balsamic, sweet-fruity, plum and cherry-like aroma. Ethyl 2,3-dihydrocinnamate is characterized by an aroma that is very similar to ethyl cinnamate, but its contribution to the aroma is lower than that of ethyl cinnamate. Methyl anthranilate imparts a sweet-fruity, grape-like taste with a distinct floral perfumery character. This compound was first isolated from Concord grapes (Power and Chesnut, 1923). Because of the high concentration found in Concord grapes, methyl anthranilate is considered as the main substance responsible for the characteristic Concord aroma (Nelson *et al.*, 1977).

Pinot noir character varies significantly with vintage and with maturity within a given vintage (Miranda-Lopez *et al.*, 1992). These authors compared odor profiles of Pinot noir wines from grapes harvested at different maturities during 1987 and 1988. The odor profiles from those years were very different from each other, with only four active odor peaks common to both vintages. Wines made from grapes harvested at the end of the ripening period had more active odor peaks than wines from fruit harvested earlier. The 1988 wines had more active odor peaks than the 1987 wines. For the 1987 vintage, two unique peaks

found exclusively in the wine were ethyl octanoate and ethyl vanillate. The 1987 and 1988 vintage harvest dates were very different due to warm conditions during the harvest in 1987 and cool conditions in 1988 (Miranda-Lopez *et al.*, 1992). Seasonal climatic conditions influenced the maturity of the grapes, as well as the formation of potential active odor compounds and their precursors.

Tominaga *et al.* (1996) identified 3-mercaptohexyl acetate in Sauvignon blanc wine. This mercapto ester, recently found in passion fruit, exhibits an aroma reminiscent of box tree, with grapefruit and passion fruit notes. Its perception threshold in water and in model solution is around 2-4 ng/l. 3-Mercaptohexyl acetate might contribute to the typical varietal aroma of Sauvignon Blanc wines (Tominaga *et al.*, 1996).

Young red table wines of the cultivar Pinotage have a distinct fermentation ('duco') character (Van Wyk *et al.*, 1979). This bouquet is not present in either the Pinotage grapes or must, but is known to be formed during fermentation. Isoamyl acetate, when present in relatively large concentrations, was shown to be the "impact" compound for this typical bouquet. The presence of this bouquet is considered to be a positive quality-enhancing factor. During aging, this bouquet gradually decreases in intensity and finally disappears. This change is accompanied by a concurrent decrease in isoamyl acetate concentration. A similar reduction in bouquet intensity and isoamyl acetate concentration was observed when the wine was exposed to an excess of oxygen, as well as to elevated temperatures (Van Wyk *et al.*, 1979). As a rule, Pinotage vines planted on a specific soil series always yield wines with the typical bouquet, whereas vines from the same stock planted on a different soil series do not usually exhibit this bouquet. Another observation is that the maximum isoamyl acetate concentration of a wine from a specific vineyard, made by the same methods, might vary significantly from one vintage to another.

The activity of alcohol acetyltransferase and esterase in two *Saccharomyces cerevisiae* strains during biological aging has been evaluated (Plata *et al.*, 1998). Biological aging occurs after alcoholic fermentation and under the "flor" film of yeasts that grows on the surface of the wine with an ethanol content of 15.5% or higher (Domecq, 1989). The products found at the end of the aging process are correlated with the "flor" thickness and the enzyme activities tested (Plata *et al.*, 1998). Flor yeasts accelerate the disappearance of ethanol and ethyl acetate, but delay that of isoamyl alcohol and isoamyl acetate, particularly in wines aged using *S. cerevisiae* var. *capensis,* and thus enhance the aroma of the final product.

2.2.2 DISTILLATES

Carnacini *et al.* (1989) stated that, in the preparation of spirits from wine, the distillation technique is of fundamental importance in influencing the organoleptic properties of the end product. Many processes are used throughout the world for distilling wine. The continuous distillation process in plate columns tends to give a much less aromatic end product due to the alcoholic vapors undergoing rectification, which is the separation of the constituents of a liquid mixture by successive distillations. The discontinuous distillation process enhances the aromatic qualities of the original wine and it is therefore very important to know the

properties of the base wine and to use only good, high quality material (Léauté, 1990; Postel and Adam, 1990).

Aging is one of the most important factors influencing the quality and aroma of distilled beverages. A wide range of distilled beverages, such as cognac, whiskey and also many rums and liqueurs, are matured in oak barrels for long periods of time (Mosedale and Puech, 1998). During this time, physical and chemical interactions occur between the barrel, the surrounding atmosphere and the maturing distillate that transform both the flavor and composition of the alcoholic beverage.

The ester concentrations in different cognacs have been investigated (**Table 2.5**). Clear quantitative differences exist between the amounts of some constituents of German and French grape brandies, as well as French cognacs. The main esters present are the ethyl esters, among which the average concentrations of C_3-, C_5-, C_7-, and C_9-ethyl esters show a definite minimum in cognacs (Schreier et al., 1979). This clear difference in the quantitative distribution of the ethyl esters of odd-numbered acids between the various groups of grape brandies has not been described as yet. The ethyl esters of even-numbered fatty acids show a reverse behavior. The average concentrations of C_6-C_{14} even-numbered ethyl esters are two times higher in cognacs than in the two other groups of German and French grape brandies analyzed in that study. These elevated amounts, especially of ethyl caprylate, caproate and laurate, are probably caused by differences in distillation methods. According to Schreier et al. (1979), the amounts of ethyl ester can be used to analytically differentiate cognacs from other groups of grape brandies.

A special effect could be observed concerning the concentrations of isopenthyl esters in cognacs (Schreier et al., 1979). Compared to the German and French grape brandies, the cognacs showed minimal amounts of esters of short-chain fatty acids (C_3-C_5) compared to the maximal values of esters of long-chain fatty acids (C_{10}-C_{14}). Presumably, the long first distillation of cognacs liberates more hi-boiler esters from the yeast fat reserves than does continuous column distillation. Furthermore, an increase in the concentrations of long-chain compounds due to the extended aging of cognacs cannot be excluded.

Table 2.5 Cognac (1988 harvest) Gas Chromatography Analysis (Léauté, 1990).

esters	mg/l
ethyl acetate	33.4
isoamyl acetate	3.00
ethyl hexanoate	0.50
ethyl caprylate	0.80
ethyl caprate	0.20
ethyl lactate	36.1
diethyl succinate	0.20

In comparison to the two other groups of brandies, cognacs show higher amounts of ethoxy and hydroxy esters (Schreier et al., 1979). The high concentrations of ethoxy compounds could be related to the strong influence of temperature during the "Méthode Charentaise" and to the long aging process. The elevated contents of hydroxy esters,

particularly lactates, in cognacs seem to be caused by the natural fermentation conditions, including the malolactic fermentation.

Carnacini *et al.* (1989) showed that, if a certain amount of lees is left in the wine, the distillate will be smoother and more characteristic. They concluded that long- and medium-chain fatty acid ethyl esters are of particular importance for the organoleptic properties. The highest concentration, of eight- to 16-carbon fatty acid ethyl esters, can be found in the distillate of the wine containing the most lees. The ester content of distilled beverages is greatly dependent on whether or not the yeast is present at the time of distillation. Suomalainen and Nykänen (1966) found that distillation in the presence of yeast increases the amount of caprylic, capric and palmitoleic acid ethyl esters.

Wood maturation greatly influences the ester content of the final beverage. The ester increase during aging depends largely on the activity of the barrel used, e.g. the amount of acetic acid available for extraction from the barrel, and subsequent esterification with alcohol (Onishi *et al.*, 1977). Therefore, the ester increase is always greater for brandies aged in new American oak cooperage than in barrels of the same type that have been reused once or twice. Onishi *et al.* (1977) found that ester increase during brandy aging is also greatly affected by the temperature of storage. After five years, brandies stored at 20°C contained approximately twice the concentration of total esters than those stored at 15°C. However, it is difficult to separate the influence of variations in ester content due to analytical imprecision from variations in the activity of supposedly identical individual barrels. All of the acetate esters of isoamyl, *n*-hexyl and β-phenylethyl alcohols decrease quite significantly in concentration during aging. The most logical explanation for the loss of acetate esters is that ethyl alcohol is present in a far greater concentration than the other alcohols and, acting as a nucleophilic reagent, displaces the other alcohol moiety in the equilibrium reaction (Onishi *et al.*, 1977). The ethyl esters of the fatty acids, caproic, caprylic, capric and lauric, are the most abundant of the fatty acid ethyl esters present in distilled spirits. The data indicate an increase for the first three of the esters, but usually a small loss for ethyl laurate, particularly for those samples initially containing higher concentrations. The ethyl myristate and caprate esters appear to drop even more than laurate (Onishi *et al.*, 1977).

2.2.3 BEER

Esters are of much greater importance in beer flavor than higher alcohols, and a certain concentration of esters is necessary in beer for optimum aroma and flavor (Engan, 1974). But, as with all flavor components in beer, certain limits should not be exceeded. Too high a level of esters will give beer a fruity or candy-like character, and Sihto and Arkima (1963) found that a high concentration of isoamyl acetate had a negative influence. All of the flavor components are necessary, but the concentrations have to be kept below certain limits (Engan, 1974). If they are not, a single compound or a single group of compounds may dominate and destroy the flavor balance. The threshold values reported in the literature for esters in beer vary somewhat (**Table 2.6**). Engan's definition of the threshold value is "the minimum amount of a compound added to beer to change the flavor significantly" (Engan, 1974). The wort itself contains only traces of esters. The esters are secreted primarily during the main

fermentation and only small changes take place during lagering and maturation (Piendl and Geiger, 1980).

The system used for flavor terminology names and defines each of 122 separately identifiable notes that can be attributed to beer (Angelino, 1991). The system comprises eight odor classes that correspond to certain groups of aroma volatiles that occur in beer. These odor classes are: (i) aromatic, fragrant, fruity, floral, (ii) resinous, nutty, green, grassy, (iii) cereal, (iv) caramelized, roasted, (v) phenolic, (vi) soapy, fatty, diacetyl, oily, rancid, (vii) sulfury and (viii) oxidized, stale and musty. In addition, four flavor classes are defined: sour/acidic, sweet, mouth-feel and fullness, which are governed by a combined taste and odor perception (Angelino, 1991). Among the various esters, ethyl acetate is the ester with the highest concentration in beer and it has a fruity/solvent-like flavor. When present in high concentrations, it is said to give beer an increased bitter taste. Isoamyl acetate and isobutyl acetate have a banana-like flavor and ethyl caproate and ethyl caprylate an apple-like flavor. 2-Phenylethyl acetate has a fruity, flowery flavor with a honey note. These are the most important esters in beer, since the concentrations of the other esters are normally too low to give them any flavor potential (**Table 2.6**).

Table 2.6 Esters Produced by Yeast and Their Concentrations, Threshold Values and Odors in Beer (Meilgaard, 1975; Alvarez *et al.*,1994; Dufour and Malcorps, 1995).

compound	conc. in beer (mg/L)	average concentration (mg/L)	threshold value (mg/L)	odor
ethyl acetate	8-32	18.4	21-30	fruity, solvent-like
isoamyl acetate	0.3-3.8	1.72	0.6-1.2	banana, pear
ethyl caproate	0.05-0.3	0.14	0.17-0.21	apple, aniseed
ethyl caprylate	0.04-0.53	0.17	0.3-0.9	apple
2-phenylethyl acetate	0.10-0.73	0.54	3.8	roses, honey, sweet

There appears to be a correlation between the concentration of some fatty acids and their ethyl esters. Analysis of a number of different beers has shown a correlation between caproic acid (hexanoic acid) and ethyl caproate, and between caprylic acid (octanoic acid) and ethyl caprylate. All of the esters synthesized during fermentation are not necessarily present in the finished beer. Nordström (1964a) found that ethyl esters of unbranched, saturated fatty acids showed a tendency to be bound to the yeast cell and this binding increased with increased molecular weight. Ethyl caprate was found almost completely in the yeast phase. The highest of these esters expected to be found in beer in significant concentration is ethyl caprylate. However, even this ester has been found to be strongly bound to the yeast.

Apart from factors such as the yeast strain (Engan, 1974; Piendl and Geiger, 1980), the ester concentration in beer produced during fermentation is dependent on the fermentation temperature (Engan, 1974; Peddie, 1990) and method (Engan, 1974), the pitching rate (Maule, 1967; Engan, 1974), the wort composition (Engan, 1974; Anderson and Kirsop, 1974), wort aeration (Aries and Kirsop, 1977), pressure (Peddie, 1990), suspended solids present in the wort (Peddie, 1990) and lagering and storage (Engan, 1974).

Factors influencing aroma compound production can also be divided into three groups (Lyness *et al.* 1997): (i) yeast characteristics (pitching rate, strain, physiological state); (ii)

wort composition (lipids, amino acids, vitamins, inorganic nutrients, sugars, dissolved oxygen, trub, original gravity); and (iii) fermentation conditions (temperature, agitation, pH, fermentor design, pitching rate).

2.2.4 SAKÉ

Saké (Japanese rice wine) is one of the traditional alcoholic beverages in Japan. In saké brewing, rice starch is saccharified by the *Aspergillus oryzae* glucoamylase, and the glucose is then fermented to ethanol by *S. cerevisiae*. Both steps occur simultaneously (Kuriyama *et al.*, 1986a, 1986b). This fermentation system distinguishes saké brewing from alcoholic fermentations elsewhere in the world. The *ginjo*-saké is regarded as one of the high quality Japanese sakés (Inoue *et al.*, 1997). The desirable feature of this saké is its fruitlike flavor. The fruitlike flavor is due to the combinations of esters, higher alcohols, organic acids, carbonyl compounds and sulfhydryl compounds. Among them, isoamyl acetate and ethyl caproate are known as the major flavor components in saké (Kuriyama *et al.*, 1986a, 1986b). Therefore, much attention has been given to the increase of isoamyl acetate and ethyl caproate production in saké (Fukuda *et al.*, 1998; Asano *et al.*, 1999; Arikawa *et al.*, 2000).

2.2.5 OTHER

Fatty acid ethyl esters are the main components of rum aroma and play an important sensorial role in this distilled alcoholic beverage. Pino *et al.* (2002) developed a method to quantify these esters that involves separation and concentration of the esters using headspace solid-phase micro-extraction, and determination by capillary gas chromatography using flame ionization detection (**Table 2.7**).

Table 2.7 Fatty Acid Ethyl Esters (mg per liter of ethanol) in Commercial White Rums (40% v/v ethanol) (Pino *et al.*, 2002).

samples	ethyl hexanoate	ethyl octanoate	ethyl decanoate	ethyl dodecanoate
Cuban rum, 3 years old	0.15	6.80	39.17	0.75
Cuban rum, 7 years old	0.04	5.05	23.40	0.35
Cuban rum, aged	0.03	2.41	19.87	0.27
Bahamian rum	0.06	4.40	19.48	0.09
Spanish rum 1, golden	n.d.	0.65	3.79	0.03
Spanish rum 2, golden	0.04	14.14	103.33	1.59

In past years, extensive research has been devoted to whiskey flavor analysis (Nykänen, 1986; Withers *et al.*, 1995). More than 1000 components have been identified in the aroma of whiskey, including alcohols and fusel alcohols, fatty acids and esters, lactones and other carbonyl compounds, such as aldehydes and phenolics, as well as sulfur and nitrogen compounds. Fatty acid esters in particular form an essential group of aroma compounds in whiskey. Of the higher esters, ethyl E-11-hexadecenoate is interesting, because significant amounts of this compound have been found mainly in Scotch whiskey (Demyttenaere *et al.*, 2003). Fitzgerald *et al.* (2000) also used solid-phase microextraction with gas-

chromatography-mass spectrometry to determine the ester and higher alcohol concentrations in whiskeys (**Table 2.8**).

Flavor volatile compounds of sorghum malt beverage were identified on a GC-MS and 28 volatile compounds were detected (Lasekan *et al*., 1997). The sorghum malt beverage produced a characteristic nutty, sweet chocolate aroma that could not be tied to a single volatile compound.

Table 2.8 Quantitative Data for the Composition of Five Irish and Two Scotch Whiskey Samples (Fitzgerald *et al*. 2000).

components	concentration (mg/l)						
	Irish					Scotch	
	no. 1	no. 2	no. 3	no. 4	no. 5	no. 1	no. 2
methyl acetate	<12	<12	<12	<12	<12	<12	<12
ethyl acetate	<10	67	73	51	130	81	110
butan-2-ol	<21	<21	<21	<21	<21	<21	<21
propanol	<4.4	<4.4	<4.4	<4.4	<4.4	<4.4	<4.4
isobutanol	<6	<6	<6	<6	<6	<6	<6
isoamyl acetate	9	9	13	13	31	21	28
butan-1-ol	<6.9	<6.9	<6.9	<6.9	<6.9	<6.9	<6.9
2-methylbutan-1-ol	59	81	126	79	76	47	82
3-methylbutan-1-ol	168	247	314	256	217	135	225
ethyl caproate	1.1	<0.26	1.2	0.9	1.4	0.7	0.9
ethyl lactate	<10	<10	<10	<10	<10	20	<10
ethyl caprylate	0.2	0.2	0.4	0.2	0.4	0.2	0.8
ethyl caprate	0.6	0.5	0.8	0.4	0.2	<0.1	1
phenylethyl acetate	<0.5	<0.5	<0.5	<0.5	1.4	<0.5	<0.5
ethyl laurate	0.3	0.3	0.2	<0.1	<0.1	<0.1	0.4
2-phenylethanol	<9.1	<9.1	<9.1	<9.1	<9.1	<9.1	<9.1

2.3 BIOSYNTHESIS OF ESTERS

Acetate esters, especially isoamyl acetate and ethyl acetate, are the most important flavor compounds in beer and other alcoholic beverages (Fujii *et al*., 1994). Most esters found in alcoholic beverages are formed during fermentation (Engan, 1974). Esters are secondary products produced by yeasts during the metabolic metabolism of sugars and constitute one of the largest and most important groups of compounds affecting flavor (Peddie, 1990).

Many esters are formed, but the most significant esters are ethyl acetate (fruity, solvent-like), isoamyl acetate (isopenthyl acetate, pear-drops), isobutyl acetate (banana), ethyl caproate (ethyl hexanoate, apple) and 2-phenylethyl acetate (honey, fruity, flowery) (Thurston *et al*., 1981). Ester formation is linked to the lipid metabolism of yeast.
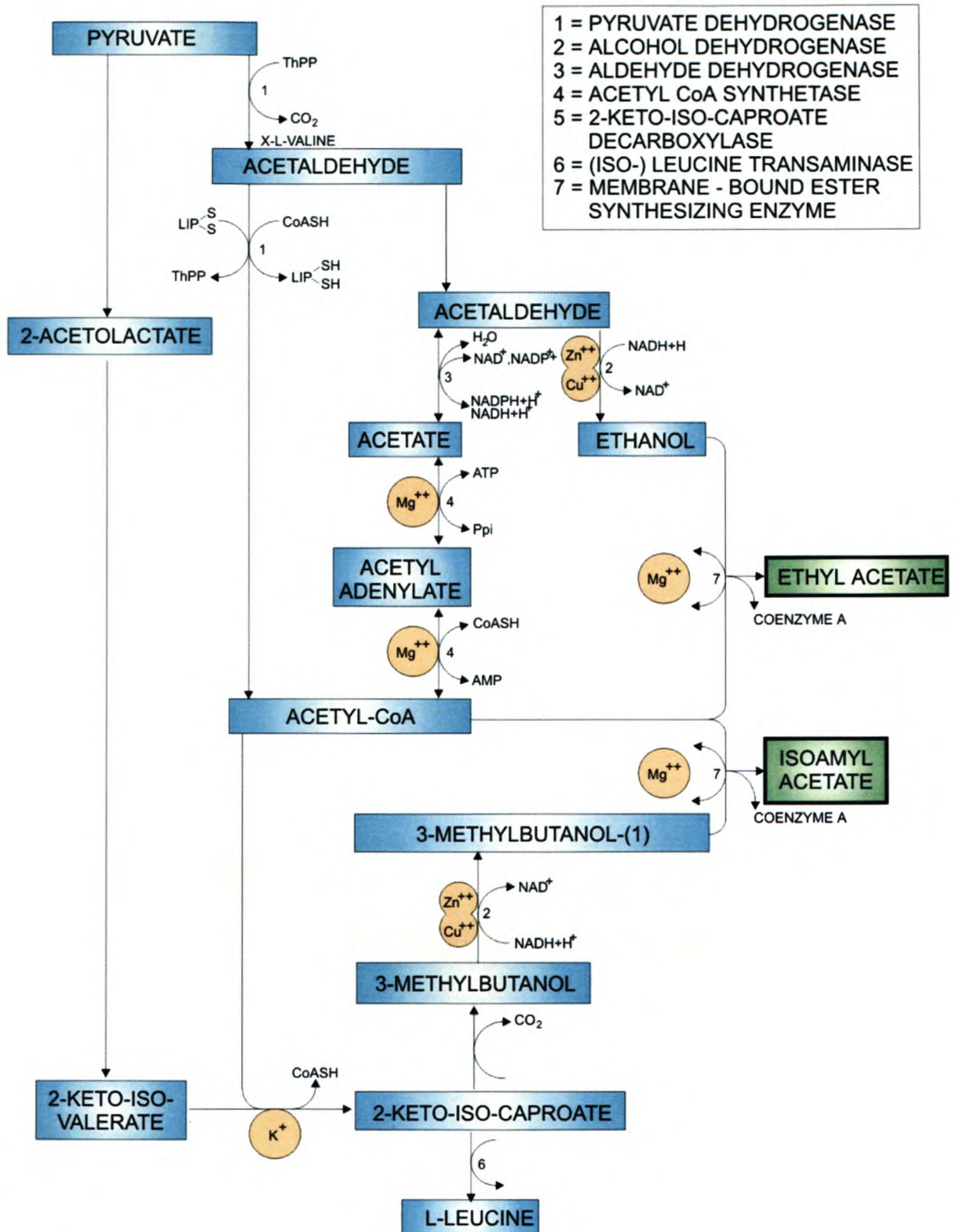
Figure 2.2 Schematic representation of the formation of ethyl acetate and isoamyl acetate (Piendl and Geiger, 1980).

Nordström (1961) found that acetate ester synthesis is an energy-requiring process that takes place inside the yeast cell and involves the high energy compound, acetyl-CoA. The only possible way to account for the high levels of esters in alcoholic beverages is via an energy-dependent biochemical reaction. This reaction is represented as follows:

$$RCOOH + ATP + CoASH \rightarrow R'CO\text{-}SCoA + AMP + PPi$$
$$RCO\text{-} SCoA + R'OH \rightarrow RCOOR' + CoASH$$
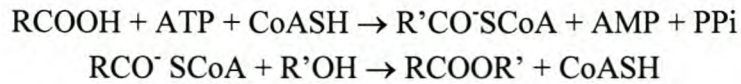
The above-mentioned reaction requires an alcohol, a fatty acid, Co-enzyme A (CoA) and an ester-synthesizing enzyme (Nordström, 1961; Peddie, 1990). Formic acid, propionic acid, isobutyric acid and isovaleric acid do not act as substrate for the formation of esters and have an inhibitory effect on ester formation (Nordström, 1964c). Ester formation is known to cease at the end of fermentation, suggesting the need for energy that must be supplied by the yeast (Nordström, 1962). The direct, enzyme-free formation of esters is an equilibrium reaction between an alcohol and an acid, as, for example, in the formation of ethyl acetate from acetic acid and ethanol ($CH_3COOH + C_2H_5OH \leftrightarrow CH_3COOC_2H_5 + H_2O$) (Engan, 1974). A great number of alcohols and acids are formed during fermentation and some are present in the raw material, e.g. grapes and wort. All of the alcohols and acids may react to form esters and therefore the theoretical number of esters in wine and beer is very large. However, direct ester formation is too slow to account for the ester concentrations found in alcoholic beverages. According to Nordström (1964a), the formation of ethyl acetate during fermentation proceeds according to the following reaction: $CH_3CO\text{-}SCoA + C_2H_5OH \leftrightarrow CH_3COOC_2H_5 + CoASH$. Nordström (1964a) proposed that alcohols become esterified by reacting with fatty acids that have undergone a previous activation by combining with coenzyme A (CoASH). He concluded that although acetyl-CoA can be formed by the oxidative decarboxylation of pyruvate (Nordström, 1964a), most of the other acyl coenzyme A (acyl-CoA) compounds come from the acylation of CoASH by the action of acyl-CoA synthetase.

It is in these activated forms that acyl-CoA compounds can act as the acyl donor. It is as a result of this need for activation that ester synthesis is an energy-requiring process. It is important to stress the central role played by acetyl-CoA in ester synthesis, since it is involved in many other reactions within the yeast cell (e.g. lipid biosynthesis, amino acid biosynthesis, fatty acid biosynthesis and the TCA cycle) (Nordström, 1964c). Factors that govern the anabolism and catabolism of this compound could be crucial for ester production (Peddie, 1990). It has been shown that acetate esters are synthesized by an enzyme called alcohol acetyltransferase (Atf1p refers to the gene product and AAT to the enzyme activity), which uses an alcohol and acetyl-CoA as substrates (Peddie, 1990). However, esterases also play a role in ester accumulation. Esterases represent a diverse group of hydrolases catalyzing the cleavage of esters, but in some cases also the formation of ester bonds. It has been shown that the balance between ester-synthesizing enzymes and esterases is important for the net rate of ester accumulation (Fukuda et al., 1998). The enzymes involved in ester breakdown are termed esterases and catalyze the reaction $RCOOR^1 + H_2O \rightarrow R^1OH + RCOOH$ (Peddie, 1990). In *Hansenula mrakii*, an isoamyl acetate-synthesizing esterase was described and detected in the soluble fractions of the cell extracts (Inoue et al., 1997). It was found that *H. mrakii* could produce a large amount of isoamyl acetate when cultured at both 15 and 30°C under aerobic conditions. Kashima et al. (1998) also isolated and characterized the *EST1*

gene from the bacterium *Acetobacter pasteurianus*. The results indicated that ethyl acetate production is mostly catalyzed by the intracellular esterase, esterase-1, which uses ethanol and acetic acid as substrates (Kashima *et al.*, 2000). Nardi *et al.* (2002) also stated that the EstA esterase is responsible for the main capacity of *Lactococcus lactis* to synthesize short-chain fatty acid esters *in vitro*.

Thurston *et al.* (1982) has suggested that there are two inductions of ester synthesis during wine fermentation. At the beginning of fermentation, ester synthesis is very slow due to the high metabolic demand for acetyl-CoA for yeast growth (Yoshioka and Hashimoto, 1983). At this time, oxygen and acetyl-CoA are rapidly consumed for the production of unsaturated fatty acids and sterols. Immediately following this stage, an equilibrium is established between acetyl-CoA consumption for fatty acid and sterol synthesis and for ester production. This represents the first induction for ester synthesis and occurs after eight hours of wine fermentation. When fatty acid and sterol synthesis finally stop, a peak occurs in the cellular acetyl-CoA levels and also in the acetyl charge [the ratio between (acetyl-CoA) and (acetyl-CoA + CoASH)] and, at this point, the second induction of ester synthesis takes place. This happens at about the midpoint of fermentation (between 20 and 30 hours) and is relatively short-lived. However, this synthesis contributes significantly to the overall ester level. At the point of maximum specific rate of ethyl acetate synthesis, about 80% of the CoASH is in the acetyl form.

The expression of the *ATF1* gene, encoding the major *S. cerevisiae* alcohol acetyltransferase, is low, almost not detectable, during the exponential growth phase, and it is only after cell growth slows that a rapid increase in *ATF1* expression occurs (Lyness *et al.* 1997). One explanation for this observation is that there is a rapid increase in fatty acid synthesis during exponential growth in *S. cerevisiae* and, as a result, the demand for fatty-acyl-CoA residues is high. When fatty acid synthesis slows and the cells enter the stationary growth phase, the fatty-acyl-CoA residues can be utilized as substrates by alcohol acetyltransferase to produce the acetate esters.

2.4 REGULATION OF ESTER SYNTHESIS

All conditions promoting lipid and protein synthesis will reduce the pool of acetyl-CoA and consequently the amount available for the synthesis of the esters (Dufour and Malcorps, 1994). In agreement with this hypothesis, Thurston *et al.* (1981, 1982) reported a large increase in the specific rate of ester production in the latter half of fermentation that is concurrent with the cessation of lipid synthesis. This is due to the transient increase of available acetyl-CoA. The alcohol acetyltransferase enzyme activities increase during the active growth phase to reach a maximum at the end of the exponential phase, followed by a decrease during the stationary phase (Dufour and Malcorps, 1994). Dufour and Malcorps (1994) also suggested that ester synthesis is not inhibited by unsaturated fatty acids or oxygen, but is rather modulated by the repression or induction of enzyme synthesis or processing. As both unsaturated fatty acids and oxygen have a similar effect, the regulation of ester synthesis

could be linked to lipid metabolism. The similar behavior of all examined ester-synthesizing enzymes indicates the involvement of the same enzymatic system and the existence of a common regulatory mechanism.

Malcorps *et al.* (1991) showed that the maximum AAT-specific activity decreased with increasing headspace volume, which had a negative effect on the total amount of esters produced during fermentation. On the other hand, adding oxygen-free medium to the fermentor before yeast growth ceased had a positive effect on the induction of AAT activity. Acetyl-CoA was not the limiting factor in the *in vitro* assay. The inhibitory effect of oxygen on *in vivo* ester synthesis was not caused by lower amounts of acetyl-CoA, due to its use to sustain yeast growth and membrane synthesis. The low specific activity of AAT is mainly responsible for the low ester production in the presence of oxygen. This mechanism explains the low ester production in continuous culture compared with that in batch fermentation, due to the long-lasting "repression" of Atf1p by the incoming oxygen. Malcorps *et al.* (1991) also concluded that the addition of lipids could be performed late in the process to restore membrane properties without significantly affecting the final ester content of the product. The added lipids only prevent the late induction of AAT activity, without exerting any effect on the enzyme already present. Unsaturated fatty acids synthesized too early would be rapidly diluted by growth, resulting in a short repression of Atf1p, whereas late-synthesized unsaturated fatty acids would only repress the last part of the activity to be induced (Malcorps *et al.*, 1991).

2.5 PHYSIOLOGICAL ROLES FOR ESTER SYNTHESIS

Esters could simply be overspill products from the metabolism of sugar during fermentation and might be of no advantage to the yeast cell, simply leaving the cell by diffusion. However, there might be a specific reason for ester production. Rainbow (1970) suggested that fatty acids with a chain length of between C_8 and C_{14} are toxic to the yeast. These acids are known to exhibit strong antimicrobial activity. Esters might therefore be formed to remove these toxic fatty acids from the yeast cell (Nordström, 1962, 1964b). Esters of shorter chain fatty acids (C_2 - C_6) would be produced by the same detoxification process. Lyness *et al.* (1997) found quite significant differences in the biomass of the parental strain and a modified yeast strain with multiple copies of *ATF1*. There is little difference in the first 24 hours of growth; however, if the culture is incubated for 48 hours, the difference in biomass is approximately three-fold. This would suggest that increased copies of *ATF1* could lead to increased removal of medium-chain fatty acyl-CoA residues, which means that the cell is healthier and grows to a higher biomass.

Thurston (1982) suggested that another reason for ester formation could be to maintain a balance between acetyl-CoA and CoASH. According to this hypothesis, yeast synthesizes esters to redress any imbalance in the CoA:acetyl-CoA ratio caused by the cessation of the lipid synthesis pathway through fermentation (Thurston *et al.*, 1981, 1982). However, preliminary evidence presented by Calderbank and Hammond (1994) on *in vitro* AAT activity

suggests that acetyl-CoA may be a poor substrate for Atf1p, compared with long-chain fatty-acyl-CoA compounds. The latter were better inhibitors of isoamyl acetate synthesis than were their short-chain counterparts. If long-chain fatty-acyl-CoA compounds are indeed better substrates for Atf1p than acetyl-CoA, this raises the possibility that the enzyme might be primarily responsible for triglyceride or phospholipid synthesis, and that ester synthesis might be a minor function (Calderbank and Hammond, 1994).

As several enzymes are involved in the synthesis of esters, this could suggest different metabolic roles for long-chain and medium-chain alcohol acetylation, as well as for the esterification of long-chain and medium-chain fatty acids with ethanol and for ethyl acetate synthesis (Dufour and Malcorps, 1994). A possible role of ethyl acetate synthesis could be to regenerate free coenzyme A from acetyl-CoA without releasing acetic acid. The physiological role of medium-chain aliphatic ester synthesis (isoamyl acetate, ethyl caproate) in yeast remains undetermined. According to Dufour and Malcorps (1994), ethyl caproate could be a byproduct of the synthesis of long-chain saturated fatty acid ethyl esters that could mimic the role of triglycerides under anaerobic growth. It has also been suggested that short-chain and medium-chain saturated fatty acid ethyl esters might be formed to remove the corresponding toxic acids from the yeast cells (Peddie, 1990). As far as the isoamyl acetate synthase is concerned, an interesting property is its ability, just like unsaturated fatty acids, to acetylate 12-DL-hydroxystearic acid, a compound that is able to support yeast growth under anaerobic conditions (Light *et al.*, 1962).

Throughout an anaerobic fermentation of a lipid-free synthetic medium carried out with a *S. cerevisiae* strain selected for winemaking, Bardi *et al.* (1998) monitored medium-chain fatty acid and ethyl ester production and, at the same time, measured the growth and esterasic activity of the intact cells. No correlation was found between the concentration of each fatty acid and its ethyl ester, and no evidence exists that ester synthesis reduces the toxicity of medium-chain fatty acids. According to Bardi *et al.* (1998), esterasic activity does not show any correlation with ester synthesis, but it is related to the release of medium-chain fatty acids. The authors also proposed that ester synthesis is a consequence of the arrest of lipid biosynthesis resulting from a lack of oxygen. Under these conditions, an excess of acyl coenzyme A is produced and acyl esters are formed as secondary products of reactions aimed at recovering free coenzyme A.

Fujiwara *et al.* (1998) showed that *ATF1* transcriptional regulation by unsaturated fatty acids occurs by the same mechanism as that regulating the *OLE1* gene encoding Δ-9 fatty acid desaturase. Fatty acids with a lower melting point resulted in higher repression of *ATF1* and *OLE1*. The authors suggested that there might be a mechanism that monitors the fluidity of the membrane and transmits the signal to the *ATF1* and *OLE1* regulatory circuit. Also, AATase is localized in the cell membrane (Yoshioka and Hashimoto, 1981) and AATase is reported to acetylate 12-DL-hydroxystearic acid (Malcorps and Dufour, 1992). From these observations, Fujiwara *et al.* (1999) came to the conclusion that the *ATF1* gene might have another, more important, function in the regulation of membrane fluidity.

2.6 ENZYMES INVOLVED IN ESTER METABOLISM

2.6.1 ALCOHOL ACETYLTRANSFERASE

The synthesis of acetate esters in yeast is catalyzed by alcohol acetyltransferases (AATases) and utilizes available alcohols and acetyl-CoA (Nordström, 1961). Thus far, four different AATase genes have been cloned, namely *ATF1*, *ATF2*, *LgATF1* and *EHT1* (Fujii *et al.*, 1994; Nagasawa *et al.*, 1998; Yoshimoto *et al.*, 1998; Mason and Dufour, 2000).

The *ATF1* gene was cloned from *S. cerevisiae* and brewery lager yeast, *Saccharomyces pastorianus* (Fujii *et al.*, 1994). The structural gene consists of a 1,575-bp open reading frame that encodes a peptide of 525 amino acids with a calculated molecular weight of 61059 Da. The gene is located on chromosome XV. Although the yeast AATase is considered to be a membrane-bound enzyme, the results of a hydrophobicity analysis suggested that the gene product does not have a membrane-spanning region. Transformants carrying multiple copies of the *ATF1* gene exhibited a six- to 15-fold increase in AATase activity compared to the control. The concentrations of ethyl acetate and isoamyl acetate present in cultured supernatants obtained from transformant cultures were also greater than the concentrations present in the control. Transformants carrying multiple copies of the *S. pastorianus ATF1* gene showed a 27-fold increase in isoamyl acetate concentration and a nine-fold increase in ethyl acetate concentration. Transformants with multiple copies of the Lg-*ATF1* gene showed a 17-fold increase in isoamyl acetate concentration and a two-fold increase in ethyl acetate concentration. The production of ethanol and other higher alcohols did not change.

In order to determine the precise role of Atf1p, Fujii *et al.* (1996b) disrupted the *ATF1* gene in *S. cerevisiae*. The results of AATase assays using isoamyl alcohol as substrate revealed that, although the AATase activity of the null mutant was dramatically reduced, 20% of the activity was retained. However, when ethanol was used as substrate, more than 80% of the activity was retained. These results suggest that Atf1p plays a major role in isoamyl acetate production, but that it has a relatively minor role in acetate ester production.

One of the most interesting features of acetate ester synthesis by *S. cerevisiae* is that the production of acetate esters is greatly reduced by aeration or the addition of unsaturated fatty acids to the medium, although the production of higher alcohols, which are the precursors of acetate esters, is not (Ishikawa and Yoshizawa, 1979; Thurston *et al.*, 1982; Yoshioka and Hashimoto, 1983; Malcorps *et al.*, 1991). Atf1p has been suggested to be responsible for this phenomenon (Malcorps *et al.*, 1991). Fujii *et al.* (1997) have indeed shown that expression of the *ATF1* gene is greatly reduced by aeration and by the addition of unsaturated fatty acids. The degree of reduction in AAT activity and in the level of *ATF1* mRNA were closely related in both conditions. When the *ATF1* promoter was replaced with a strong constitutive promoter, no reduction of AAT activity was observed after aeration or the addition of unsaturated fatty acids (Fujii *et al.*, 1997). Further experiments using the *lacZ* reporter gene also suggested that repression by aeration and unsaturated fatty acids was controlled at the transcriptional level. The results of this experiment suggested that a 150-bp 5' flanking sequence (-50 to -200) plays an important role in both types of regulation.

More recently, Fujiwara *et al.* (1998) studied the co-regulation of *S. cerevisiae ATF1* and *OLE1* by unsaturated fatty acids. It was noted that the degree of *ATF1* transcriptional repression was related to the melting point of the unsaturated fatty acids added to the medium. The *OLE1* gene has been reported to be repressed under similar conditions than the *ATF1* gene. Therefore, it is considered that *ATF1* and *OLE1* transcription is regulated in response to cell membrane fluidity (Fujiwara *et al.*, 1998). As has been reported for *OLE1*, the repression of *ATF1* by unsaturated fatty acids was relieved in a disruptant carrying a double mutation of *faa1* and *faa4*, two fatty acid activation genes.

However, the *ATF1* transcript in this double gene disruptant remained repressed by oxygen. Thus, the transcriptional repression of *ATF1* by oxygen is not only through the production of unsaturated fatty acids, but also through an oxygen-dependent signal. AATase activity has been reported to be inhibited by a variety of lipids, especially by cell membrane components, such as ergosterol, phosphatidylinositol and phosphatidylcholine, in addition to unsaturated fatty acids. However, no effect was observed on *ATF1* expression. Thus, unsaturated fatty acids, but not all lipids, cause the repression of *ATF1* expression. These results suggest that *ATF1* transcription is co-regulated by the same mechanism as the *OLE1* gene and that unsaturated fatty acids and oxygen repress the *ATF1* transcript by different regulation pathways (Fujiwara *et al.*, 1998).

The regulatory pathway through which oxygen regulates *ATF1* is similar to the one that regulates a set of hypoxic genes that are repressed under aerobic conditions and induced in limited oxygen conditions (Fujiwara *et al.*, 1999). Oxygen control of gene expression is essentially mediated by two factors, the activator Hap1p and the repressor Rox1p, which are activated or synthesized under aerobic conditions. Rox1p requires the general repressor complex Tup1p-Ssn6p to repress transcription of its target genes (Balasubramanian *et al.*, 1993; Zhang *et al.*, 1991). Under aerobic conditions, heme accumulates in the cell and serves as an effector for the transcriptional activator Hap1p. The heme-Hap1p complex activates transcription of the *ROX1* gene and the expression of the hypoxic genes is repressed (Zhang *et al.*, 1991; Lowry and Zitomer, 1988). Under anaerobic conditions, the heme level falls, the heme-dependent-Hap1p complex represses *ROX1* expression and, as a result, the hypoxic genes are derepressed. A series of analyses of the *ATF1* promoter identified an 18-bp element that is essential for transcriptional activation (Fujiwara et al., 1999). Fujiwara *et al.* (1999) identified two pathways for transcriptional regulation of the *ATF1* gene: (i) when oxygen levels increase, heme accumulates to levels sufficient to induce *HAP1* expression and Hap1p induces *ROX1* expression (Zitomer and Lowry, 1992). Rox1p binds to a 18-bp binding sequence in the *ATF1* promoter and the general repressor Tup1p-Ssn6p complex acts in cooperation with Rox1p to produce transcriptional repression (Fujiwara *et al.*, 1999); (ii) the transcriptional activator Rap1p binds to the 18-bp binding sequence to activate transcription of the *ATF1* gene. The 18-bp element is also involved in unsaturated fatty acid repression, although the factor that transmits the fatty acid signal remains unknown (Fujiwara *et al.* 1999) (**Figure 2.3**).
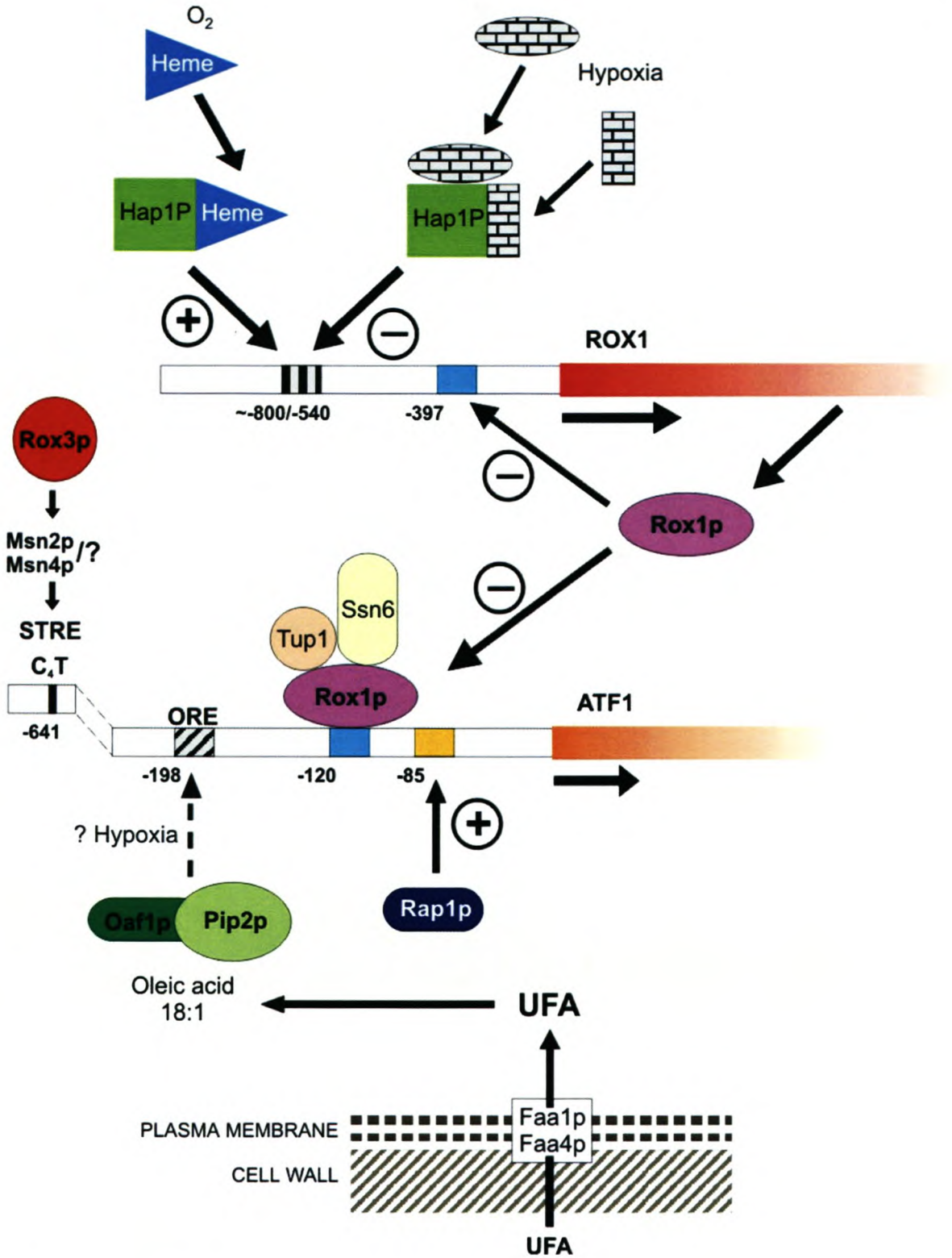
Figure 2.3 Model for *ATF1* transcriptional regulation. Key: + indicates positive regulation of transcription; - indicates negative regulation of transcription;? indicates speculation (Mason and Dufour, 2000).

Another DNA motif found upstream of the *ATF1* ORF is the sequence $^{-650}$CCCCT (Mason and Dufour, 2000). The C$_4$T sequence (STRE: stress response element) is associated with global stress response genes induced by stress conditions, such as heat shock, osmotic stress,

oxidative stress, acid pH, glucose or nitrogen starvation and DNA-damaging agents (Mager and De Kruijff, 1995). Signal transduction pathways detect various stress conditions and activate the Rox3p effector, which then activates transcription of the stress response genes. The effects of stress conditions on *ATF1* transcription have not been measured directly, but a two- to three-fold increase in AATase activity on the second day of a barley *shochu* yeast fermentation was observed as a consequence of heat shock treatment (Kajiwara *et al.*1997). Omori *et al.* (1997) also found that heat shock-resistant yeast mutants of *S. cerevisiae shochu* yeast BAW-6, obtained after two rounds of heat shock selection (45°C), showed a two-fold increase in the production of isoamyl acetate, ethyl caproate and ethyl caprate. The selection, by heat shock, of yeast mutants with elevated AATase activity suggests either that this characteristic specifically contributes to enhanced thermotolerance, or that increased AATase production is a serendipitous consequence of a generalized stress response (Mason and Dufour, 2000).

Another sequence within the region between nt-50 to nt-200 of the *ATF1* promoter was identified as an ORE (oleate response element) sequence targeted by the transcription factors Oaf1p and Pip2p (oaf2p) (Karpichev and Small, 1998). These oleate-activated transcription factors increase the transcription of peroxisomal protein genes that are induced when the yeast is grown on fatty acids as the sole carbon source. However, *ATF1* does not encode a peroxisomal component and no changes were observed in *ATF1* mRNA transcription in the *oaf1Δ*, *pip2Δ* or *oaf1Δ /pip2Δ* mutants. Given the repressive effect of UFAs on *ATF1* expression, it seems more likely that the ORE would be utilized in a negative regulatory context in *ATF1* transcription (Mason and Dufour, 2000).

Fujii *et al.* (1996a) determined the nucleotide sequences of alcohol acetyltransferase genes isolated from lager brewing yeast, *Saccharomyces pastorianus*. *S. pastorianus* has one *ATF1* gene and another homologous gene, the Lg-*ATF1* gene. There was a high degree of homology between the amino acid sequences deduced for the *ATF1* protein and the Lg-*ATF1* protein (75.7%), but the N-terminal region has a relatively low degree of homology. Further analyses suggested that the Lg-*ATF1* gene might originate from the non-*S. cerevisiae* genome of *S. pastorianus*, which is similar to that of *S. bayanus* and is located on a 850 kb chromosome. Yoshimoto *et al.* (1998) found that, as in the case of the *ATF1* gene, the transcription of the Lg-*ATF1* gene is also repressed by the addition of oxygen and unsaturated fatty acids.

Nagasawa *et al.* (1998) cloned the *ATF2* gene, which encodes alcohol acetyltransferase II (AATase II), from saké yeast, *S. cerevisiae* Kyokai No.7. The *ATF2* gene, situated on chromosome 7, encodes a 535 amino acid protein with a molecular weight of 61,909 daltons. The deduced amino acid sequences of *ATF2* show 36.9% homology with that of *ATF1*. The hydrophobicity profiles for the Atf1p and Atf2p are similar. A transformant carrying multiple copies of the *ATF2* gene showed 2.5-fold higher AATase activity than the control. A Southern analysis showed that *S. cerevisiae* and *S. pastorianus* (Yoshimoto *et al.* 1999) have one *ATF2* gene, while *S. bayanus* has none. AATase II activity is also regulated by oxygen in the same manner as AATase activity, but AATase II activity is activated by unsaturated fatty

acids. The *ATF2* promoter region contains no STRE or ORE elements (Mason and Dufour, 2000).

Cauet *et al.* (1999) found that yeast contains an enzymatic activity capable of esterifying pregnenolone and related steroids to their corresponding acetate esters. The protein was identified and encoded by *ATF2*. The enzyme shows a significant specificity towards steroids and accepts short-chain acyl-CoA esters, but not long-chain acyl-CoA esters such as oleoyl-CoA. Cauet *et al.* (1999) also obtained an *atf2Δ* mutant that had lost all Atf2p activity, resulting in its capacity to esterify pregnenolone and its growth rate being significantly reduced. These results indicate that pregnenolone is toxic for yeast and that pregnenolone esterification mediated by Atf2p largely reduces this toxicity. Atf2p acts together with the ABC transporters Pdr5p and/or Snq2p, which are involved in the transport of pregnenolone. Consequently, Cauet *et al.* (1999) suggest that the Atf2p represents a detoxification mechanism that acts in concert with the plasma membrane pumps to reduce the inhibitory effect of 3β-hydroxysteroids on yeast growth. It seems unlikely that yeasts are naturally in contact with steroids and have developed a detoxification system especially for these compounds, but APAT might detoxify structurally-related compounds that are present in plants, for instance phytochemicals such as flavonoids. Some of these have structural similarities to steroids and display hormone-like activity (Miksicek, 1993; Baker, 1998).

Mason and Dufour (2000) have suggested the existence of a fourth ester-synthesizing gene. The *EHT1* gene encodes the ethanol hexanoyltransferase enzyme, which produces ethyl hexanoate using ethanol and hexanoyl-CoA as substrates. Eht1p shows only 17% homology with the alcohol acetyltransferases Atf1p and Atf2p and does not include the conserved motif, WRLICLP, of the AATase enzymes Atf1p, LgAtf1p and Atf2p (Mason and Dufour, 2000).

2.6.2 ISOAMYL ACETATE-HYDROLYZING ESTERASE

The *S. cerevisiae IAH1* (*EST2)* gene, encoding a carboxylesterase, has a 714-bp open reading frame (238 amino acids) and the protein has a molecular weight of 27304.66 daltons. The gene is located on chromosome XV (Fukuda *et al.,* 1996). The Iah1p lacks the consensus sequence (Gly-Xaa-Ser-Xaa-Gly) that is observed in serine-type esterases or lipases, but a similar sequence (Ala-Cys-Ser-Ala-Gly) is present. The activity of the serine-type esterase (as well as the serine-type lipase) is inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl flourophosphate (DFP). The *EST2* esterase activity is slightly inhibited by PMSF and completely inhibited by DFP. An *iah1* mutant produced a 19-fold higher concentration of isoamyl acetate compared to the parental strain under saké brewing conditions. The Iah1p is therefore likely to play a crucial role in the hydrolysis of isoamyl acetate in saké mash and other conditions.

2.6.3 EXTRACELLULAR ESTERASE TIP1P

Horsted *et al.* (1998) isolated an extracellular esterase from the brewer's yeast *Saccharomyces pastorianus.* Inhibition by diisopropyl fluorophosphates showed that the enzyme has a serine active site and it weighs 16.9 kDa. The optimal pH activity is in the range of four to five, but the enzyme is stable over a pH range from three to seven. Esterase activity was present in the

beer before pasteurization, and a low level of activity was still present after pasteurization. This might influence the ester level of the finished beer during storage, causing a change in the beer flavor. Caprylic acid, which is present in beer, completely inhibited the esterase. The substrate preference towards esters of ρ-nitrophenol indicated that the enzyme prefers esters of fatty acids from four to sixteen carbon atoms, but ρ-nitrophenyl laurate (C_{12}) was hydrolyzed at the highest rate. The esterase also has lipolytical activity. The N-terminal sequence analysis of the esterase yielded a sequence which is identical to the deduced amino acid sequence of the *S. cerevisiae TIP1* gene. The sequence of the Tip1p lacks the conserved pentapeptide Gly-X-Ser-X-Gly with the active site serine, found in most other esterases, but a homologous sequence [49]Gly-X-Ser-Phe[53] is present. The esterase preparation did not appear to contain significant amounts of other proteins besides Tip1p, and Horsted *et al.* (1998) therefore concluded that the *TIP1* gene is the structural gene for the esterase. The gene is located on chromosome II, and was previously identified as a cold shock-inducible protein and a cell wall protein, but the function of the protein was not determined since the *tip1* mutant had no observable phenotype (Kondo and Inouye, 1991; Van der Vaart *et al.*, 1995). The *TIP1* gene is induced by cold shock and anaerobic growth conditions (Kondo and Inouye, 1991; Donzeau *et al.*, 1996). Horsted *et al.* (1998) concluded that this expression pattern of Tip1p is in agreement with lager beer fermentation, where the primary fermentation is carried out at low temperatures under anaerobic conditions, which indicates that a *TIP1* homologue of *S. pastorianus* might be expressed under these conditions.

2.7 SIGNIFICANCE OF HIGHER ALCOHOLS IN ALCOHOLIC BEVERAGES

Higher alcohols are secondary products of alcoholic fermentation by yeasts and are the largest group of aroma compounds in alcoholic beverages (Amerine *et al.*, 1980). Higher or fusel alcohols have a strong, pungent smell and taste and have a significant influence on the character and taste of alcoholic beverages (Rapp and Mandery, 1986). Below 300 mg/l they contribute to the desirable complexity of the wine, but above 400 mg/l the higher alcohols have a negative influence on wine quality. Higher alcohols are also important precursors for ester production and the esters of higher alcohols are associated with pleasant aromas (Soles *et al.*, 1982). Higher alcohols are also extremely important in wine distillates and are much more concentrated (Boulton *et al.*, 1995).

Higher alcohols are divided into two categories, aliphatic and aromatic (Nykänen *et al.*, 1977). The aliphatic alcohols include propanol, isoamyl alcohol, isobutanol and active amyl alcohol; the aromatic alcohols consist of 2-phenylethyl alcohol and tyrosol (**Table 2.9**).

Many factors influence the final concentration of higher alcohols in alcoholic beverages. In wine, viticultural conditions and the use of different yeast strains during fermentation contribute considerably to the variations (Guidici *et al.*, 1990). The amino acid concentration of the medium is also an important factor influencing higher alcohol production (Schulthess and Ettlinger, 1978). The total production of higher alcohols increases with increasing concentrations of the corresponding amino acids. Ethanol, fermentation temperature, must

pH, aeration, level of solids, grape variety, maturity and skin contact time also affect the higher alcohol concentration present in the final product (Fleet and Heard, 1993).

Table 2.9 Some Higher Alcohols Produced by Yeast and Their Concentrations, Threshold Values and Odors in Wine (Adapted from Lambrechts and Pretorius, 2000).

compound	amino acids	concentration in wine (mg/l)	threshold value (mg/l)	odor
propanol	threonine / 2-amino-butyric acid	9-68 125	500** 800***	stupefying
butanol	?	0.5-8.5		fusel odor
isobutanol	valine	9-28(100) 140	500** 75* 200***	alcoholic
active amyl alcohol	isoleucine	15-150	65***	marzipan
isoamyl alcohol	leucine	45-490	300** 7* 70***	marzipan
hexanol	?	0.3-12	5.2* 4***	
tyrosol	tyrosine			bees wax, honey-like
tryptophol	tryptophan			
phenylethyl alcohol	phenylalanine	10-180	7.5* 125***	floral, rose

*Percentage-above-chance-scores of 50% in grain spirit solutions of 9.4%(w/w)
**In a wine solution
***In beer

2.8 BIOSYNTHESIS OF HIGHER ALCOHOLS

The branched-chain higher alcohols, isoamyl alcohol, active amyl alcohol and isobutanol, are synthesized during fermentation through the Ehrlich pathway, which involves the degradation of the branched-chain amino acids, leucine, isoleucine and valine. The branched-chain amino acid biosynthetic pathways are illustrated in **Figure 2.4**. The branched-chain amino acid uptake in *S. cerevisiae* is mediated by at least three transport systems: the general amino acid permease Gap1p, the branched-chain amino acid permease Bap2p, and one or more unknown permeases (Didion *et al.*, 1996). Transcriptional regulation of *BAP2* is mainly subject to the presence of certain amino acids in the medium. Didion *et al.* (1996) suggested that Gap1p and Bap2p are not active at the same time. The *BAP2* promoter contains one or two Gcn4 binding sites and one putative Leu3p binding site, but none of the three sites are needed for induction by leucine. Induction by leucine is accompanied by an increase in branched-chain amino acid uptake. There is still a leucine-inducible increase in branched-chain amino acid uptake in a *gap1*Δ *bap2*Δ strain, indicating that *BAP2* shares leucine induction with at least one remaining branched-chain amino acid-transporting permease (Didion *et al.*, 1996).

The branched-chain alcohols are synthesized from the α-keto-acids during fermentation in the branched-chain amino acid metabolic pathway, by decarboxylation and by reduction (Dickinson *et al.*, 1997). These α-keto-acids are formed via two major pathways, the catabolic or Ehrlich pathway (**Figure 2.5**), involving the degradation of the corresponding

amino acid, leucine to isoamyl alcohol, isoleucine to active amyl alcohol and valine to isobutanol (Ehrlich, 1904), and anabolically, involving the *de novo* synthesis of branched-chain amino acids through their biosynthetic pathway from glucose.
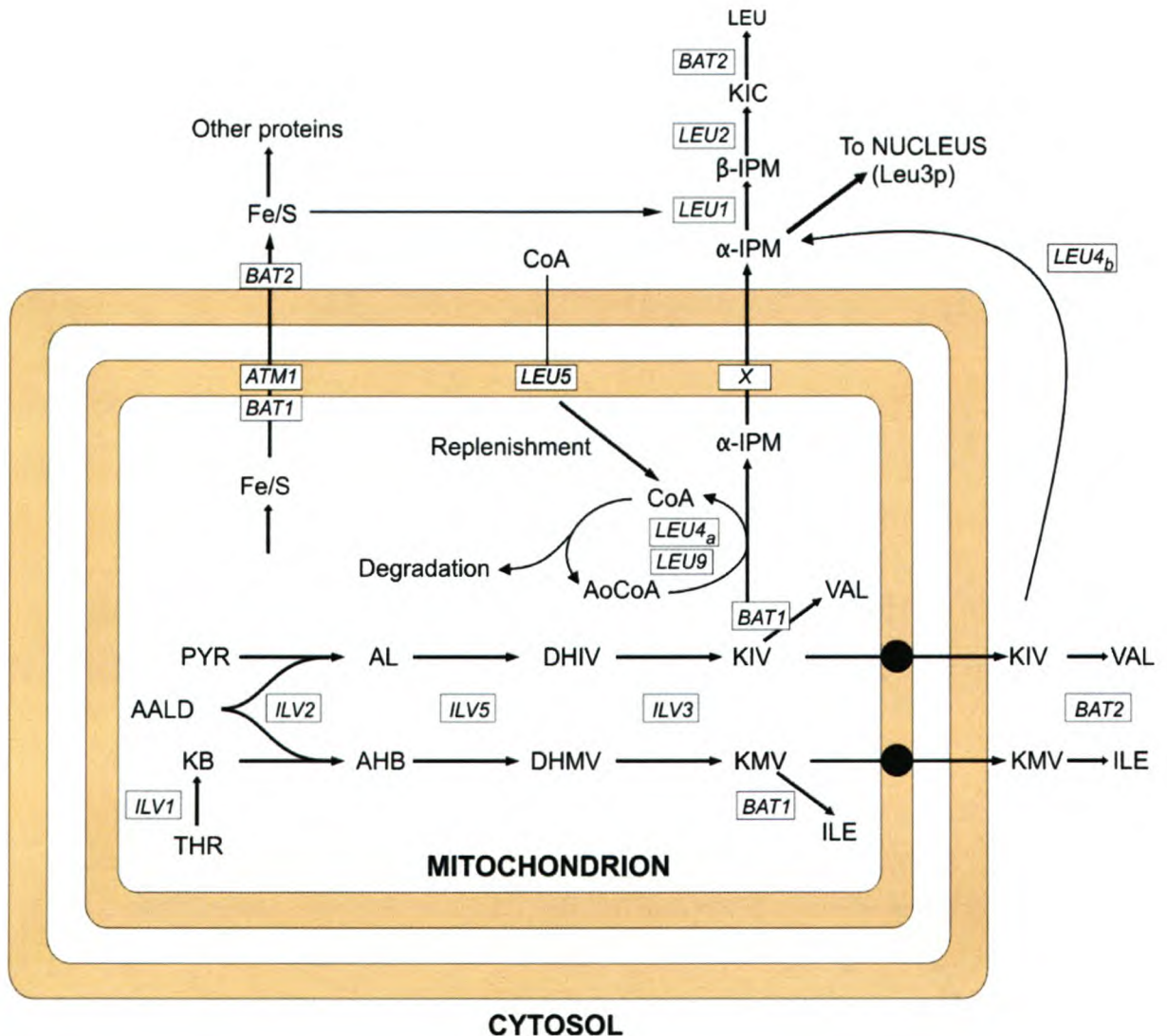
Figure 2.4 Compartmentalization of the branched-chain amino acid biosynthetic pathways of *S. cerevisiae* (Kohlhaw, 2003). The boxed characters refer to the genes involved directly or indirectly in the pathways. Their protein products are as follows: *ILV1*, threonine deaminase; *ILV2*, acetohydroxy acid synthasecatalytic subunit; *ILV3*, dihydroxy acid dehydratase; *BAT1*, mitochondrial branched-chain amino acid transaminase; *BAT2*, cytosolic branched-chain amino acid transaminase; *LEU4*a, α-isopropylmalate synthase I, long form; *LEU4*b, α-isopropylmalate synthase, short form; *LEU9*, α-isopropylmalate synthase II; X, hypothetical α-isopropylmalate transporter; *LEU1*, isopropylmalate isomerase; *LEU2*, β-isopropylmalate dehydrogenase; *LEU5*, protein necessary for the accumulation of CoA within the mitochondria; presumably an importer of CoA or precursor thereof; *ATM1*, ABC transporter involved in exporting Fe-S clusters to the cytosol; the implied interaction between Bat1p and Atm1p is hypothetical. Abbreviations: KB, α-ketobutyrate; AALD, active acetaldehyde; PYR, pyruvate; AL, acetolactate; AHB, α-aceto-α-hydroxybutyrate; DHIV, α,β-

dihydroxyisovalerate; DHMV, α,β-dihydroxy-β-methylvalerate; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; α-IMP, α-isopropylmalate; β-IMP, β-isopropylmalate; kIC, α-ketoisocaproate; Fe/S, iron-sulfur cluster. Not shown for reasons of clarity: transport of leucine and/or KIC back into the mitochondria.
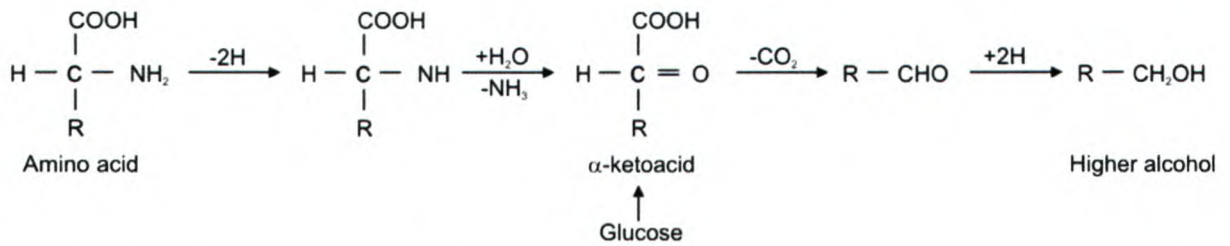
Figure 2.5 Ehrlich pathway for higher alcohol formation (Ehrlich, 1904).

The first step in the catabolism of branched-chain amino acids is transamination to form the respective α-keto-acids (α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine) (Dickinson and Norte, 1993). This reaction is catalyzed by mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by the *BAT1* and *BAT2* genes respectively (Eden *et al.*, 1996, 2001; Kispal *et al.*, 1996). A pyruvate decarboxylase then converts the resulting keto-acid to the corresponding branched-chain aldehyde with one carbon less atom, and the alcohol dehydrogenase catalyzes the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol (Derrick and Large, 1993). Alternatively, the aldehyde might be oxidized to an acid (**Figure 2.6**).

Hammond (1993) and Oshita *et al.* (1995) reported the influence of nitrogen concentration on the production of branched-chain higher alcohols. At low levels of assimilable nitrogen, the biosynthetic pathway predominates, whereas the Ehrlich pathway becomes predominant at high levels as a result of feedback and/or repression of key enzymes in the biosynthetic pathway.
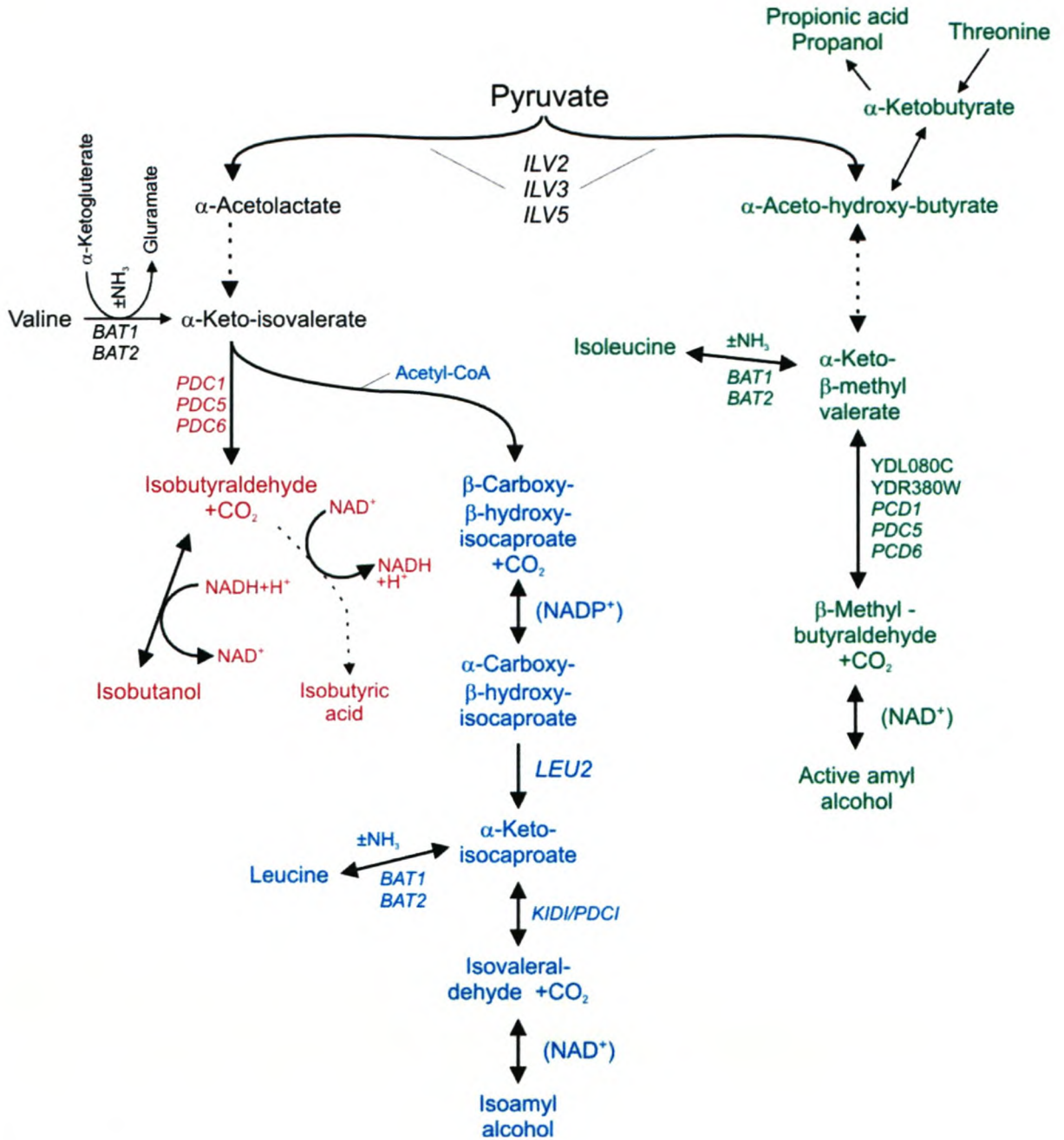
Figure 2.6 Branched-chain amino acid catabolism and the production of the respective higher alcohols.

2.9. ENZYMES INVOLVED IN HIGHER ALCOHOL METABOLISM

2.9.1 BRANCHED-CHAIN AMINO ACID AMINOTRANSFERASE

The mitochondrial and cytosolic branched-chain amino acid aminotransferases are encoded by the *BAT1* and *BAT2* genes, respectively (Eden *et al*., 1996, 2001; Kispal *et al*., 1996). The *BAT1* gene is highly expressed during the logarithmic growth phase and is down-regulated

during the stationary phase, while the *BAT2* gene shows an inverse pattern of expression (Eden *et al.*, 1996). The amino acid sequence of Bat1p (43.6 kDa, including its mitochondrial presequence) and Bat2p (41.6 kDa) show 77% homology and both proteins share about 55% homology with the branched-chain amino transferases from other species (Kispal *et al.*, 1996). Eden *et al.* (2001) produced *S. cerevisiae* mutants in which these two genes had been deleted. Analysis of the production of higher alcohols by the different yeast strains suggested different roles of the aminotransferase activities in the synthetic pathways of these compounds. From a series of fermentations, Eden *et al.* (2001) concluded that the *bat2* mutation results in a drastic decrease in the production of isobutanol and that Bat2p is responsible for the transamination of valine to α-ketoisovalerate, the precursor of isobutanol.

The formation of α-keto acids is regulated by three enzymes: β-isopropylmalate (encoded by *LEU2*), a decarboxylase (encoded by *PDC1* and *KID1*) and the branched-chain aminotransferase (encoded by the *BAT* genes). Yoshimoto *et al.* (2002) investigated the regulation of these three genes. They concluded that isoamyl alcohol production correlated with the expression of the *LEU2* and *BAT* genes, but not with that of the *PDC1* and *KID1* genes, and that the expression of the *LEU2* and *BAT* genes is co-regulated by the nitrogen source. The data suggested that isoamyl alcohol production influenced by the nitrogen source depends on the transcription of L-leucine biosynthetic and branched-chain amino acid aminotransferase-encoding genes. Amino acid biosynthesis is regulated at both the transcriptional and translational levels (Hinnebusch, 1990). It has been reported that the *LEU1*, *LEU2* and *LEU4* genes require a positive regulator encoded by *LEU3* and a Leu3p-dependent protein-DNA complex has been shown to specifically form with a G+C-rich palindromic decanucleotide sequence, 5'-CCGGNNCCGG-3', which is present in the 5'-noncoding regions of all three of these genes (Friden and Schimmel, 1988). These *LEU* genes also contain the recognition sequence 5'-TGACTC-3' for the transcriptional activator Gcn4p, which is responsible for general amino acid control (Arndt and Fink, 1986). Computer analysis of the promoters of the *BAT* genes showed that the *BAT2* promoter does not contain the exact sequences, but Yoshimoto *et al.* (2002) detected a similar sequence, 5'-<u>TGACAC</u>-3', at position -323 to -318 and -293 to -288, and 5'-<u>CCGGAACC</u>AT-3' at position -366 to -357. The *BAT1* promoter contained both recognition sequences at position -171 to -166 and -147 to -138. Natarajan *et al.* (2001) also reported that both the *BAT* genes are Gcn4p targets. Yoshimoto *et al.* (2002) concluded that, although it is unclear if *BAT2* gene expression is regulated through these sequences, genes in the leucine metabolic pathway, including the *BAT* genes, could be co-regulated by Leu3p and/or Gcn4p. *BAT1* and *ILV5* are also regulated by the cAMP-dependent protein kinase Tpk1p (Robertson *et al.*, 2000). The enzymes encoded by these two genes play other important roles besides their catalytic function in the branched-chain amino acid pathways. The stimulatory effect of Tpk1p is aimed at these other functions, which are associated with mitochondrial integrity.

Cells deficient in Bat1p and Bat2p do not grow on minimal media unless supplied with all three branched-chain amino acids and, even then, growth remains sluggish. It is hypothesized that Bat1p in particular might be essential in iron homeostasis by being involved in the efficient transfer of Fe-S clusters from the mitochondria, where the clusters are

synthesized, to the cytosol. This process also involves the mitochondrial ABC transporter Atm1p (Lill *et al.*, 1999; Kispal *et al.*, 1999). The *BAT1* gene was isolated as a suppressor of a temperature-sensitive *ATM1* mutant and elevated levels of Bat1p were able to stabilize the Atm1p mutant at the non-permissive temperature. A disturbance in the maintenance of the iron homeostasis process can lead to severe accumulation of iron in the mitochondria, with subsequent damage and loss of mitochondrial DNA.
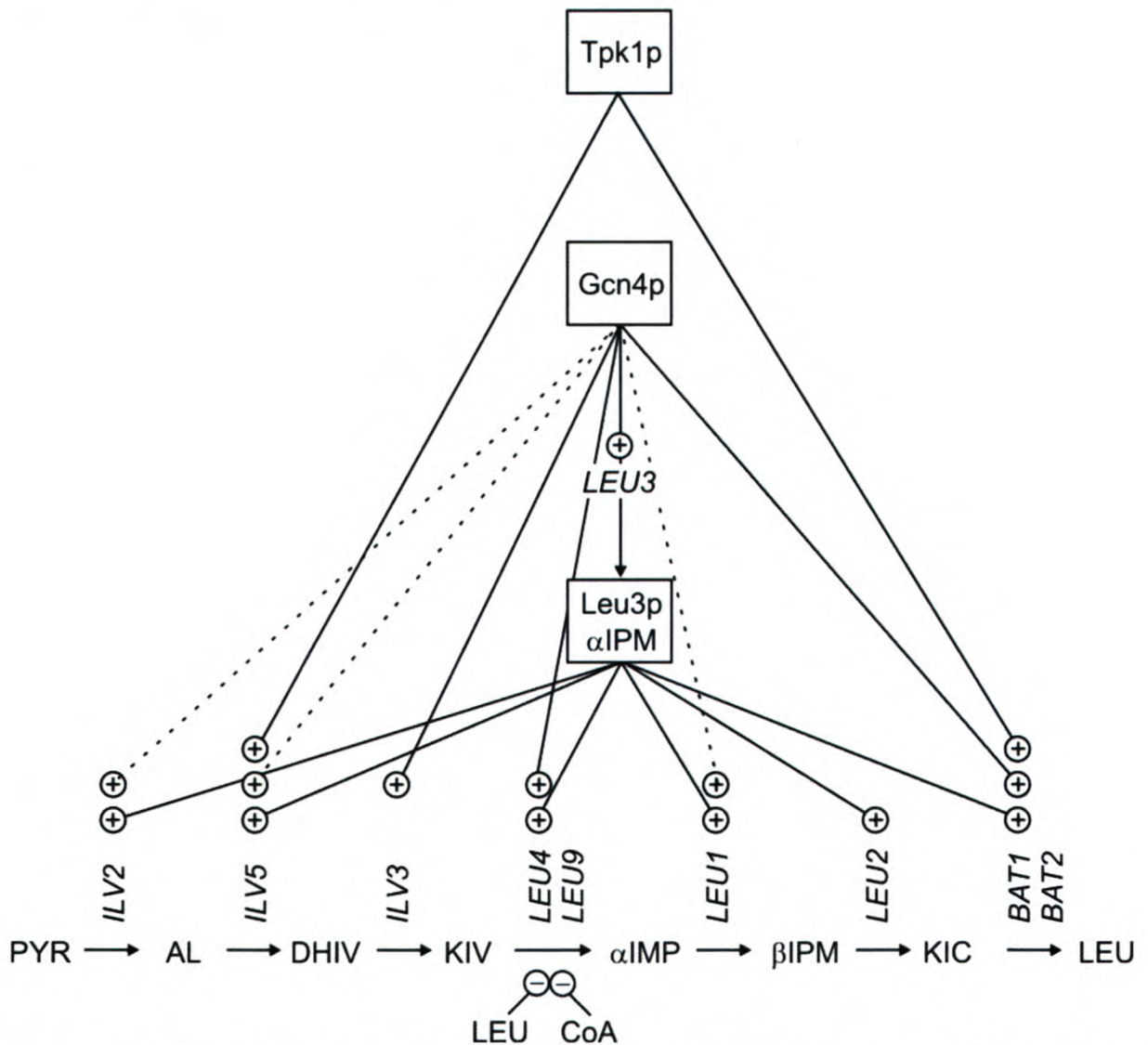
Figure 2.7 Major regulatory mechanisms impacting on the extended leucine pathway of *S. cerevisiae* (Kohlhaw, 2003).

The catabolism of the three branched-chain amino acids (leucine, isoleucine and valine) in *S. cerevisiae* occurs via the Ehrlich pathway, as already hypothesized 100 years ago (Ehrlich, 1904). However, the process turned out to be much more complex than imagined, because yeast uses at least three aminotransferases, five decarboxylases and six dehydrogenases and the precise combination of enzymes used at a particular stage depends upon the amino acid, the carbon source, as well as the growth stage of the culture.

2.10 YEAST STRAIN DEVELOPMENT FOR IMPROVED AROMA IN ALCOHOLIC BEVERAGES

2.10.1 SAKÉ

A special technique called *ginjozukuri*, which uses highly polished rice and low temperature fermentation, is usually used to brew saké rich in esters. Even with this technique, it is not easy to brew richly-flavored saké. Such procedures also increase saké-brewing costs. It is therefore considered important to develop a yeast strain that will produce saké with high levels of aroma compounds (Ichikawa *et al.*, 1991).

Isoamyl alcohol and isoamyl acetate have been recognized as important constituents of saké flavor. To enhance the flavor of saké, Ashida *et al.* (1987) developed yeast mutants resistant to 5,5,5-trifluoro-DL-leucine, a leucine analogue, that produce higher levels of isoamyl acetate and isoamyl alcohol. This is obtained through the elimination of feedback inhibition and repression of α-isopropylmalate synthase by leucine accumulation. Furthermore, Hirata *et al.* (1992) transformed saké yeast with extra copies of the *LEU4* gene (encoding α-isopropyl malate synthase), which catalyzes an early reaction leading to the production of isoamyl alcohol. The excess isoamyl alcohol was then esterified to isoamyl acetate by the action of Atf1p (Minetoki *et al.*, 1993; Yoshioka and Hashimoto, 1981). Asano *et al.* (1999) also produced saké yeast strains that produce higher concentrations of isoamyl acetate than the parental strain by isolating econazole-resistant mutants. Econazole is an imidazole antimycotic agent that interacts directly with unsaturated fatty acids in the cell membrane by inhibiting ergosterol synthesis and decreasing the ratio of unsaturated to saturated fatty acids. Alcohol acetyltransferase, which catalyzes isoamyl acetate production, is inhibited by unsaturated fatty acids (Fujii *et al.*, 1997). The *S. cerevisiae* Kyokai No. 701 mutants produced 1.4- to 2.4-fold higher isoamyl acetate concentrations and 1.4- to 2.4-fold lower AATase activities than the parental strain (Asano *et al.*, 1999).

Another component that is beneficial to saké flavor is ethyl caproate (apple flavor). The substrates needed by the esterase and alcohol acyltransferase for the production of ethyl caproate are ethanol and caproic acid or caproyl-CoA. Caproic acid or caproyl-CoA is the rate-limiting factor for the synthesis, since there is sufficient ethanol in the saké mash. The increased production of this compound has been achieved by Ichikawa *et al.* (1991) using a saké yeast mutant resistant to cerulenin, an inhibitor of fatty acid synthetase. The concentration of caproic acid increased five-fold in the saké produced with the mutant strain. The mutant strain also produced five times more ethyl caproate and the concentrations of the caprylic acid, capric acid and ethyl caprylate also increased. In a sensory evaluation, this wine was characterized as having an excellent fruity flavor.

By using genetic engineering, Asano *et al.* (2000) accomplished the same goal. Cerulenin is a specific inhibitor of fatty acid synthetase (Omura, 1976). *S. cerevisiae* has two metabolic pathways for the synthesis of long-chain acyl-CoAs that are incorporated into membranes. Firstly, a *de novo* pathway involving fatty acid synthetase and, secondly, a pathway that involves the utilization of exogenous fatty acids by acyl-CoA synthetases

encoded by at least five fatty acid activation genes (*FAA*) (Johnson *et al.*, 1994). The product of the *FAA1* gene is required for the activation of exogenous fatty acids and interchangeable with the FAA4-encoded gene product. The products of these two genes account for 99% of the cellular myristoyl-CoA and palmitoyl-CoA synthetase activities in the yeast cell (Knoll *et al.*, 1995). Acyl-CoA formed by the activation of exogenous fatty acids represses the *de novo* pathway by inhibiting acetyl-CoA carboxylase, which plays a crucial role in the regulation of fatty acid synthesis (Kamiryo et al., 1976). Asano *et al.* (2000) disrupted the *FAA1* gene in saké yeast, Kyokai No. 701. In a saké brewing test, the fermentation ability of the disruptant was inferior to the wild type, but the production of ethyl caproate was 1.6-fold higher.

β-Phenylethyl alcohol and β-phenylethyl acetate (rose-like flavor) are also known to be important flavor components in beer (Peddie, 1990; Piendl and Geiger, 1980), as well as in saké (Fukuda *et al.*, 1990a, 1990b). β-Phenylethyl alcohol is synthesized by the decarboxylation of phenylpyruvate, which is a precursor to phenylalanine. The first step of aromatic amino acid synthesis in *S. cerevisiae* is catalyzed by two functional isozymes of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthases (DAHP synthase). One of these enzymes is sensitive to feedback inhibition by phenylalanine (phenylalanine-dependent DAHP synthase) and the other to feedback inhibition by tyrosine (tyrosine-dependent DAHP synthase). To increase the levels of β-phenylethyl alcohol and β-phenylethyl acetate in saké, Fukuda *et al.* (1990a) isolated saké yeast mutants that are resistant to the phenylalanine analogues *o*-fluoro-DL-phenylalanine (OFP) or *p*-fluoro-DL-phenylalanine (PFP) from saké yeast, *S. cerevisiae* Kyokai No.9. These mutants produced over 5.5 times more β-phenylethyl acetate and over 6.3 times more β-phenylethyl alcohol than did their parental strains (Fukuda *et al.*, 1990a, 1990b). Fukuda *et al.* (1992) also transformed *S. cerevisiae* used to make saké with a YCp vector containing a mutated *ARO4* gene (*ARO4-OFP*) that encodes 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase without feedback inhibition by tyrosine. The transformed strain showed feedback-insensitive DAHP synthase activity, was *o*-fluoro-DL-phenylalanine (OFP) resistant and increased the production of β-phenylethyl alcohol and tyrosine. This strain brewed saké with fine aromatic characteristics, taste and body (Fukuda *et al.*, 1992).

Most of the mutant saké yeast strains producing a higher concentration of flavor compounds and organic acids have been isolated by positive selection for mutants resistant to the analogues of objective compounds. This procedure is effective and labor saving, but it is not always of value because suitable analogues are not available for all metabolites related to flavor compounds. Therefore, Arikawa *et al.* (2000) isolated mutants with increased flavor compounds by global selection using a diploid saké yeast, *S. cerevisiae* Kyokai No. 901, which was mutated with ethylmethane sulfonate (EMS). These mutants were grown in test tubes and the amounts of flavor compounds produced in the culture broth were determined by gas chromatography. Arikawa *et al.* (2000) selected 205 mutants that produced at least 1.5-fold higher concentrations of ethyl caproate and isoamyl acetate than the parental strain. These mutant strains are valuable for practical saké fermentation.

Genetic engineering is another method used in producing yeast strains to improve alcoholic beverage aroma. The *EST2* gene, encoding an isoamyl acetate-hydrolyzing esterase,

was disrupted in a diploid strain of *S. cerevisiae* UT-1, which is derived from the industrial saké yeast Kyokai no. 701, by using two disruption plasmids (Fukuda *et al.*, 1998). Small-scale saké brewing was carried out using these strains and their brewing properties were compared. The fermentation profiles of the strains were largely similar, as were the components, except that the modified strains produced approximately two-fold more isoamyl acetate than the wild-type K-701. These results suggest that the *EST2* gene product plays a crucial role in isoamyl acetate hydrolysis in saké mash (Fukuda *et al.*, 1998) and that the modified strains, which are deficient in Est2p esterase, are suitable for saké brewing.

Fukuda *et al.* (1998) constructed yeast strains with different numbers of copies of the *ATF1* and the *IAH1* genes and used these strains in small-scale saké fermentations. The isoamyl acetate concentration increased with an increasing ratio of alcohol acetyltransferase/esterase activity. It was concluded that the balance of these two enzymes is important for isoamyl acetate accumulation in saké mash.

2.10.2 BEER

Because the desirable flavor components of saké are very similar to those of beer and the flavor enhancements have successfully been made by researchers with the *S. cerevisiae* saké yeast, Lee *et al.* (1995) experimented to determine whether the lager yeast, *Saccharomyces pastorianus*, would yield similar results. The bottom-fermenting brewing yeast was induced to produce higher levels of desirable alcohols and esters (isoamyl acetate and phenylethyl acetate) in beer by selection on media containing the amino acid analogues 5,5,5,-trifluoro-DL-leucine (TFL) and fluoro-DL-phenylalanine (TPA), respectively. The isolated mutants had apparently lost their ability to regulate amino acid biosynthesis by means of feedback repression of the key enzymes in these pathways, resulting in the overproduction of the corresponding keto-acids. After decarboxylation and reduction, the resulting alcohols were esterified by the Atf1p. Three- and nine-liter laboratory fermentations showed that the selected mutants produced substantially greater amounts of higher alcohols, as well as their corresponding esters, in beer when compared with the parental strain. The chemical analysis of the beer at the end of primary fermentation indicated that the TFL mutant strains produced 2.2 to 4.2 times more isoamyl alcohol and 2.2 to 4.6 times more isoamyl acetate than did the control yeast, while the other alcohols and esters remained at about the same levels.

Flavor and aroma analyses were performed for nine-liter fermentations with various TFL isolates. Panelists detected a much higher estery character (banana aroma in particular) in the test beers than in the control, coinciding with a preference for these beers. Sensory results clearly indicated that overproduction of isoamyl acetate enhances the fruity character of beer. On the other hand, the higher level of production of fusel alcohol (isoamyl alcohol) by the TFL isolates is considered detrimental to beer quality. Thus, the amplification of AAT activity during fermentation should reduce the isoamyl alcohol level by converting it to isoamyl acetate. It can be seen that important beer esters and alcohols are derived from the corresponding amino acids and feedback inhibition of key enzymes in the biosynthesis of the latter plays a crucial role in the regulation of alcohol and ester production (Lee *et al.*, 1995). One common negative character shown by the isolates thus far is the slower attenuation rate

by the TFL or TPA mutants compared to the parental strain. However, it would be possible to screen for faster-growing mutants among the isolates, because it was observed that the growth rates varied considerably among the isolates.

Verstrepen *et al.* (2003b) deleted or overexpressed the *ATF1*, *Lg-ATF1* and *ATF2* genes in a laboratory strain and a commercial brewing strain. The analysis of the fermentation products confirmed that the expression levels of *ATF1* and *ATF2* greatly affect the production of ethyl acetate and isoamyl acetate, and are also responsible for the formation of a broad range of less volatile esters, such as propyl acetate, isobutyl acetate, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate and phenylethyl acetate. The data obtained with the *Lg-ATF1* overexpression strain indicate that the Lg-Atf1p plays only a very limited role in the synthesis of volatile esters. With respect to the esters studied, Atf2p seemed to play only a secondary role compared to the Atf1p. Verstrepen *et al.* (2003b) also showed that the Atf1p and Atf2p together are responsible for the total cellular isoamyl alcohol acetyltransferase activity. The overexpression of different alleles of *ATF1* and *ATF2* led to different ester production rates, indicating that differences in the aroma profiles of yeast strains might be partially due to mutations in their *ATF1* genes.

Verstrepen *et al.* (2003a) also overexpressed the *ATF1* and *ATF2* genes in the commercial lager yeast strain, CMBS 33. The beers were evaluated by a flavor profile panel and the organoleptic differences between the different fermentation products were clearly demonstrated. The *ATF1* and *ATF2* overexpression strains produced beers with greatly increased fruitiness. The banana, pineapple, and solvent-like flavors were particularly strong in these beers. It was also noted that the results of the flavor profile almost perfectly matched the quantitative headspace analysis.

2.10.3 WINE

Lilly *et a*l. (2000) investigated the effect of increased alcohol acetyltransferase activity on the sensory quality of Chenin blanc wines and distillates from Colombard base wines by overexpressing the *ATF1* gene in a commercial wine yeast strain of *S. cerevisiae*, VIN13. The levels of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate in the wines increased three- to 10-fold, 3.8- to 12-fold and two- to 10-fold respectively. The concentrations of ethyl caprate, ethyl caprylate and hexyl acetate only showed minor changes, whereas the acetic acid concentration decreased by more then half of that of the control fermentation. These changes had a pronounced effect on the solvent or chemical aroma (associated with ethyl acetate and isoamyl acetate) and the herbaceous and heads-associated aromas of the final distillate, and the solvent or chemical and fruity or flowery characters of the Chenin blanc wines.

2.11 CONCLUSIONS

Differences in wine yeast strains with respect to their fermentation behaviors have been noted since the earliest selection of pure cultures over a century ago (Kunkee, 1984). However, the question as to the dependency of the sensory characteristics of the resulting wine upon the

specific yeast strain used for the fermentation does not seem to have been answered satisfactorily. This is due largely to the fact that many of the flavor and aroma compound interactions comprise complex, synergistic and antagonistic perception effects that are not linearly related to the compound concentration levels found in the respective alcoholic beverages. Our limited understanding of the physiological role of yeast ester and higher alcohol synthesis also detracts from our ability to fully control ester and higher alcohol production during fermentation. To gain a clear understanding of ester and higher alcohol metabolism, one needs to take a closer look at the individual genes involved, and their functions and regulatory mechanisms. From this review it is clear that esters and higher alcohols play a key role in the sensorial quality of the fermented alcoholic beverages and that these aroma compounds are synthesized via the yeasts' complex metabolic pathways. Some of the genes involved in these pathways have been cloned and characterized and this is the start to unraveling the complex interactions of these genes and their enzymes. These insights could lead to better management of ester and higher alcohol levels in alcoholic beverages.

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Chapter 3

RESEARCH RESULTS

Approaches to the Investigation of Aroma Compound Metabolism in *Saccharomyces cerevisiae*

3. APPROACHES TO THE INVESTIGATION OF AROMA COMPOUND METABOLISM IN SACCHAROMYCES CEREVISIAE

3.1 INVESTIGATION OF AROMA COMPOUND METABOLISM IN *S. CEREVISIAE*

In the course of this work, various approaches were implemented to improve our understanding of flavor chemistry in wine yeast strains. However, several of these approaches failed. To ensure completeness of the present dissertation, these failed approaches are briefly summarized in the following paragraphs.

The initial focus of this study was to contribute to a better understanding of the complexity of flavor chemistry in wines and brandy through the unraveling of the metabolism of the aroma-active compounds, esters and higher alcohols, by identifying, cloning and characterizing new genes involved in these complex pathways in *S. cerevisiae*. The first approach that we investigated was based on the work done by Lilly *et al.* (2000). In that study, it was found that, when a wine yeast that overexpressed *ATF1* was grown on minimal selective media, the plates had an intense banana-pineapple flavor. The idea was then to search for genes involved in ester synthesis by constructing a cDNA expression library that would be screened through the sniffing of the plates. mRNA was isolated from VIN13 at different stages throughout wine fermentation, pooled together and used to synthesize cDNA. These cDNA fragments were cloned into a plasmid containing the constitutive regulatory elements of the *PGK1* gene. The library was then transformed into a laboratory yeast strain and each colony was streaked out on a separate plate of minimal selective media and screened by sniffing the plates. Out of eight thousand colonies screened, forty-two different-smelling yeasts were identified, but after sequencing it was determined that the cDNA library did not contain functional genes. It was then decided to screen a 2μ-based genomic library of *S. cerevisiae* (laboratory strain), but no different-smelling yeast plates could be identified. This approach might have failed because ethyl and isoamyl acetate esters are the most abundant and pronounced aroma compounds, while the other compounds are present at much lower concentrations and insufficient amounts of these compounds might have been produced by the transformed yeast to be able to smell the aroma. It is also possible that the aroma trait is multigenic.

It was then decided to shift the focus of the investigation to the identification of genes involved in higher alcohol metabolism, since esters are produced by enzymatically combining acetyl-CoA and the corresponding higher alcohol. It was first necessary to establish a method for the identification of such genes. It was decided to use microarray technology for this purpose. A synthetic must with different amino acid concentrations was optimized and it was observed that, when fermented with VIN13, the isoamyl alcohol concentration increased if leucine was in excess. The same effects were observed with valine and isobutanol, and with phenylalanine and 2-phenylethyl alcohol. It was also observed that when glutamate was used as nitrogen source instead of ammonium chloride and diammonium phosphate, the effect increased for isoamyl alcohol and isobutanol, but decreased for 2-phenylethyl alcohol. To test

whether this method would work, known genes in the higher alcohol pathways were identified and used as probes in northern blots with RNA isolated from fermentations to see if these genes are also transcribed at a higher level than normal. The signals were detected at higher intensities and it was decided to search for genes involved in 2-phenylethyl alcohol production, since none had been characterized at that stage. The synthetic must containing ammonium chloride and diammonium phosphate as nitrogen source was fermented with VIN13 for 24 hours and RNA was isolated at this stage as a control. A 500 mg/l phenylalanine solution was then added and the fermentation was left for another six hours, after which the RNA was isolated from the cells. Microarray analysis was performed using these two sets of RNA. The microarray analysis was repeated four times for each set of RNA, but the data were not completely reproducible because the incorporation percentage of the radioactive isotopes differed with each experiment. Nevertheless, 49 open reading frames (ORFs) were identified to be either induced or repressed after the addition of phenylalanine to the fermentation. The corresponding mutants were selected from an *S. cerevisiae* yeast deletion library and used in fermentation studies. The concentration of 2-phenylethyl alcohol in the synthetic must during fermentation was determined for all the strains by GC analysis. Only two ORFs that might have an effect on higher alcohol metabolism were identified. These genes were cloned and constitutively expressed, but there were no significant differences in higher alcohol concentration when the yeast strains were used in fermentation studies.

The third option that was used was to try to identify genes involved in restoring the redox balance in the cell, since the last reaction in higher alcohol production from the aldehyde involves the recycling of the NAD^+ molecule and thereby may be used to maintain the redox balance in the cell. Mutant yeast strains in the FY23 and W303 genetic background were constructed in which both the *GPD1* and *GPD2* genes, which are mainly responsible for redox balance, were deleted. When grown anaerobically, the control laboratory strains grew normally, but the mutant strains did not grow. The mutant strains were transformed with several genomic and cDNA libraries from *S. cerevisiae*, as well as with a genomic library from *Saccharomyces pastorianus* and *Neurospora crassa*. For each library transformed, ten thousand colonies were screened. Several colonies were identified that did grow anaerobically when replica plated from the aerobically-grown colonies. These yeast colonies were selected and the plasmids were isolated and transformed into *Escherichia coli*. The inserts in the plasmids were sequenced and the plasmids were transformed back into FY23 and W303 in which the *GPD1* and *GPD2* genes had been deleted. At this stage, the mutant strains with the transformed plasmids lost the initial phenotype, indicating that factors other than the gene or the plasmids had been responsible for the observed phenotypes.

It was then decided to select the known genes involved in ester metabolism, i.e. *ATF1*, *ATF2*, *EHT1*, *IAH1* and *TIP1*, and in higher alcohol metabolism, i.e. *BAT1* and *BAT2*, to manipulate and further characterize their involvement in aroma compound production and to evaluate their effect during wine fermentation and distillation on a chemical as well as sensorial level.

3.2 LITERATURE CITED

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Chapter 4

RESEARCH RESULTS

The Effect of Increased Yeast Alcohol Acetyltransferase and Esterase Activity on the Flavor Profiles of Wine and Distillates

4. THE EFFECT OF INCREASED YEAST ALCOHOL ACETYLTRANSFERASE AND ESTERASE ACTIVITY ON THE FLAVOR PROFILES OF WINE AND DISTILLATES

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4.1 ABSTRACT

The characteristic fruity odors of a wine's fermentation bouquet are primarily derived from the fermentative activity of the wine yeast, *Saccharomyces cerevisiae*, and the synthesis of esters and higher alcohols. The *ATF1*- and *ATF2*-encoded alcohol acetyltransferases of *S. cerevisiae* are responsible for the synthesis of ethyl acetate and isoamyl acetate esters, while the *EHT1*-encoded ethanol hexanoyl transferase is responsible for synthesizing ethyl caproate. However, esters such as these might be degraded by *S. cerevisiae*'s *IAH1*- and *TIP1*-encoded esterases. The objective of this study was (i) to overexpress genes encoding ester-synthesizing and ester-degrading enzymes in a widely used industrial wine yeast strain (VIN13); (ii) to prepare table wine and base wines for distillation with these transformed strains; and (iii) to analyze and compare the ester concentrations and aroma profiles of wines and distillates prepared with these transformants. The data indicated that the overexpression of *ATF1* and *ATF2* increased the concentrations of ethyl acetate, isoamyl acetate, 2-pheylethyl acetate and ethyl caproate, while the overexpression of *IAH1* resulted in a significant decrease in the concentrations of ethyl acetate, isoamyl acetate, hexyl acetate and 2-phenylethyl acetate. The overexpression of *EHT1* resulted in a marked increase in the concentrations of ethyl caproate, ethyl caprylate and ethyl caprate, while the overexpression of *TIP1* did not decrease the concentrations of any of the esters. In most cases, there was a correlation between the increase in esters and decrease in higher alcohols. The data suggest that yeast balances the amounts of different esters produced through alcohol acetyltransferases and esterases, and that, in some cases, these enzymes appear to overlap in function and/or influence each other's activity. This study offers prospects for the development of wine yeast starter strains with optimized ester-producing capability that could assist winemakers in their effort to consistently produce wine to definable specifications and styles.

4.2 INTRODUCTION

During the primary (alcoholic) fermentation of grape sugars, the wine yeast, *Saccharomyces cerevisiae*, produces ethanol, carbon dioxide and a number of byproducts, including esters, of

which alcohol acetate and C_4 to C_{10} fatty acid ethyl esters are found in the highest concentrations in wine and brandy (Nykänen & Suomalainen, 1983; Schreier, 1979). Volatile esters are only present in trace amounts in fermented beverages, but are extremely important for the flavor and aroma profiles of these products. The characteristic fruity odors of wine, brandy and other grape-derived alcoholic beverages are primarily due to a mixture of ethyl acetate, hexyl acetate, ethyl caproate (apple-like aroma), isoamyl acetate (banana-like aroma), ethyl caprylate (apple-like aroma) and 2-phenylethyl acetate (fruity, flowery flavor with a honey note) (Fujii *et al.*, 1994). These esters are formed in the yeast cells by enzymes called alcohol acetyltransferases using higher alcohols and acetyl-CoA as substrates. Higher alcohols, such as isobutanol, isoamyl alcohol and 2-phenylethyl alcohol, can also have an impact on the aroma of wine and are formed as part of the branched-chain amino acid metabolism. The role of ester production in yeast metabolism is unclear, but several hypotheses have been proposed. One school of thought suggests that esters might be formed to remove toxic fatty acids from the yeast cell (Nordström, 1962; 1964), whereas another proposes that esters could simply be overspill products from the yeast's sugar metabolism during fermentation and might be of no advantage to the yeast cell (Peddie, 1990). However, it has been shown that fatty acids with a chain length of C_8 to C_{14} are toxic to the yeast and exhibit strong antimicrobial activity and that the effect is intensified if these fatty acids are unsaturated (Bardi *et al.*, 1998). Esters of shorter chain fatty acids (C_2 to C_6) would be produced through the same detoxification process. Another reason for ester formation could be to reduce the acetyl charge, as it is essential for the yeast cell to maintain a balance between acetyl-CoA and CoA-SH (Thurston *et al.*, 1982). Yeast might synthesize esters to redress any imbalance in the CoA-SH:acetyl-CoA ratio caused by the cessation of the lipid synthesis pathway through fermentation (Thurston *et al.*, 1981; 1982). This is supported by the fact that the genetic regulation of the *ATF1* gene is repressed by oxygen and unsaturated fatty acids (Peddie, 1990). Furthermore, it has been suggested that Atf2p activity represents a detoxification mechanism to reduce the inhibitory action of steroids on the growth of yeast cells (Cauet *et al.*, 1999).

Several enzymes are involved in the formation of esters, of which the *ATF1*-encoded alcohol acetyltransferase (AATase I) is the best studied and has the most activity in *S. cerevisiae* (Fujii *et al.*, 1994; 1996; 1997; Malcorps & Dufour, 1987; Malcorps *et al.*, 1991). It was also demonstrated that the manipulation of the expression of *ATF1* alone could alter the ester production significantly during wine fermentation, thereby adjusting the aroma profiles of wine and distillates considerably (Lilly *et al.*, 2000). A second alcohol acetyltransferase (AATase II) encoded by the *ATF2* gene has also been characterized (Nagasawa *et al.*, 1998). Atf1p and Atf2p are responsible for the production of ethyl acetate and isoamyl acetate during fermentation (Fujii *et al.*, 1996; Nagasawa *et al.*, 1998). Another enzyme involved in ethyl acetate and isoamyl acetate production is Lg-Atf1p, an AATase found in lager yeast that is homologous to Atf1p (Yoshimoto *et al.*, 1998). A fourth ester-synthesizing enzyme, ethanol hexanoyl transferase (Eht1p), is responsible for generating ethyl hexanoate from ethanol and hexanoyl-CoA. This alcohol acetyltransferase, encoded by the *EHT1* gene, has not been studied in any detail (Mason & Dufour, 2000). It has also been

shown that the balance between ester-synthesizing enzymes and esterases such as Iah1p, which hydrolyzes isoamyl acetate, is important for the net rate of ester accumulation (Fukuda *et al.*, 1996; 1998). Furthermore, the *TIP1* gene product has been identified as a novel esterase from *Saccharomyces carlsbergensis* (Horsted *et al.*, 1998).

To investigate the role of different alcohol acetyltransferases and esterases in the ester metabolism of yeast, we overexpressed the *ATF1*, *ATF2*, *EHT1*, *IAH1* and *TIP1* genes in the commercial wine yeast strain, VIN13, and analyzed the concentration of esters produced during microvinification trials. The data confirmed that Atf1p is the major and Atf2p the minor producer of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate. As expected, overexpression of the ethanol hexanoyl transferase, Eht1p, resulted in higher hexyl acetate concentrations, but surprisingly, it also increased the concentrations of esters produced by Atf1p and Atf2p. In addition, Atf1p and Atf2p resulted in an increase in hexyl acetate, suggesting that the enzymes have overlapping functions and/or that they are cross-regulated. Under our conditions, Tip1p did not appear to have esterase activity. However, Iah1p had very strong esterase activity towards isoamyl acetate and hexyl acetate. All the enzymes that were overexpressed had a major effect on the concentration of higher alcohols, which, in most cases, decreased in concentration. We further investigated the aroma profiles of Colombard table wines and distillates prepared from Colombard base (rebate) wines produced by these strains. We showed that the ester aroma profile can be largely controlled in these products by manipulating the expression of alcohol acetyltransferases and esterases. Wines and distillates produced by some of the transformed strains had more fruity, herbaceous and floral characteristics than the wines produced by the wild-type strain. In the future, these strains could be developed further as aids to enable winemakers and distillers to adjust the flavor profiles of their products in order to satisfy the different sensory preferences of consumers.

4.3 MATERIALS AND METHODS

4.3.1 MICROBIAL STRAINS, MEDIA AND CULTURE CONDITIONS

All yeast and bacterial strains used in this study and their relevant genotypes are listed in **Table 4.1**. *Escherichia coli* cells were grown in Luria-Bertani broth at 37°C (Sambrook *et al.*, 1989). *S. cerevisiae* cells were grown at 30°C in either a synthetic medium, SCDSM [containing 0.67% yeast nitrogen base without amino acids (Difco), 0.13% amino acid stock solution (Ausubel *et al.*, 1994) lacking valine and isoleucine and supplemented with 0.5% glucose and 400 µg sulfometuron methyl (Dupont) per ml dissolved in *N*-,*N*-,dimethylformamide], or in a rich medium, YPD (containing 1% yeast extract, 2% peptone and 2% glucose). Solid media contained 2% agar (Difco). The bacteria were grown at 37°C and the yeast at 30°C.

4.3.2 RECOMBINANT DNA METHODS AND PLASMID CONSTRUCTION

Standard procedures for the isolation and manipulation of DNA were used throughout this study (Ausubel *et al.*, 1994). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity

DNA polymerase (Roche) were used in the enzymatic manipulation of DNA according to the specifications of the supplier.

Gene constructs and plasmids are shown in **Figure 4.1**. Primers (**Table 4.2**) were synthesized to amplify the coding regions of the different genes by means of the polymerase chain reaction (PCR) technique. To identify possible cloning artifacts, all inserts were sequenced. The gene sequences obtained for VIN13 were identical to the available laboratory sequences. Genomic DNA from the commercial wine yeast strain, VIN13, was used as template to amplify the coding sequences of the respective genes. A multicopy, episomal *S. cerevisiae-E. coli* shuttle vector pHVXII (Volschenk *et al.*, 1997), containing the promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences of the yeast phosphoglycerate kinase gene (*PGK1*), was used for subcloning the respective full-length open reading frames (ORFs). PCR-generated 1608-bp *ATF2*, 1356-bp *EHT1*, 633-bp *TIP1*, and 717-bp *IAH1* fragments were digested with *Bgl*II, *Xho*I, *Eco*RI and *Xho*I, and *Eco*RI and *Bgl*II respectively and subcloned into pHVXII, thereby generating plasmids pATF2-m, pEHT1-m, pTIP1-m and pIAH1-m. The *Hin*dIII-*Hin*dIII fragments containing the $PGK1_P$, the ORFs of interest and the $PGK1_T$ were obtained from the respective multicopy plasmids and inserted into the unique *Hin*dIII site of plasmid pDLG42, generating single-copy integrating *S. cerevisiae-E. coli* shuttle plasmids pATF2-s, pEHT1-s, pTIP1-s and pIAH1-s. Plasmid pDLG42 contained the dominant selectable *SMR1-410* marker gene (Casey *et al.*, 1988), a mutant allele of an endogenous gene of *S. cerevisiae* conferring resistance to the herbicide sulfometuron methyl, i.e. Sm^R. Plasmids pATF2-s, pEHT1-s, pTIP1-s and pIAH1-s were linearized with *Apa*I in the *SMR1-410* terminator region for integration into the genome of the VIN13 wine yeast strain.

4.3.3 TRANSFORMATION

All bacterial transformations and the isolation of DNA were carried out according to standard protocols (Sambrook *et al.*, 1989). VIN13 was transformed by means of electroporation (Ausubel *et al.*, 1994). YPD (10 ml) was inoculated with yeast cells, and the cells were incubated at 30°C until the stationary phase. A 500 ml volume of YPD was then inoculated with 10 ml of the preculture and incubated until the mid-logarithmic growth phase was reached [absorbance at 600 nm (A_{600}) of 1.3 to 1.5]. The cells were harvested, washed with 80 ml of sterile water, resuspended in 10 ml of a 10x TE-buffer, pH 7.5, and 10 ml of a 10x lithium acetate stock solution, and incubated at 30°C while shaking gently (Ausubel *et al.*, 1994). After 45 min, 2.5 ml of 1 M 1,4-dithio-threithol (DTT) solution was added and the mixture incubated for another 15 min. The solution was then diluted to 500 ml with water and centrifuged. The cells were first washed with 250 ml ice-cold water and 30 ml of ice-cold 1 M sorbitol, and then suspended in 0.5 ml 1 M sorbitol. In a sterile, ice-cold 1.5 ml tube, 40 μl of the concentrated yeast cells were added to 5 – 15 μg DNA and transferred to an ice-cold 0.4 cm gap electroporation cuvette. The EasyjecT + 450 V Twin pulse (EquiBio) apparatus was used for electroporation. The pulse program was as follows: voltage, 1500 V; capacity, 25 μF; shunt, 201 Ω. The yeast cells were then immediately plated onto SCDSM agar plates and incubated at 30°C for at least three days.

4.3.4 SOUTHERN BLOT ANALYSIS

Genomic DNA was isolated from the control yeast strain, VIN13, as well as from the corresponding transformed *S. cerevisiae* strains [(VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pTIP1-s) and VIN13(pIAH1-s)], using the standard mechanical method (Ausubel *et al.*, 1994). VIN13(pATF2-s) genomic DNA was digested with *Eco*RV and *Stu*I, VIN13(pEHT1-s) genomic DNA was digested with *Stu*I and *Nru*I, and the genomic DNA of the remaining strains was digested with only *Eco*RV. The DNA fragments were separated by agarose gel electrophoresis and transferred to a Hybond-N nylon membrane (Amersham), and Southern blot hybridization was performed using the DIG Luminescent Detection kit (Roche Molecular Biochemicals). The *ATF2*, *EHT1*, *IAH1* and *TIP1* ORFs were labeled with the digoxigenin molecule using PCR and used as probes.

4.3.5 PRODUCTION AND ANALYSIS OF TABLE WINE

The wine yeast strains, VIN13, VIN13(pATF1-s), VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pTIP1-s) and VIN13(pIAH1-s) were each inoculated (2 x 10^6 cells/ml) into 4.5 liters of Colombard grape juice and fermented at 15°C until dry (<1 g/l residual sugar). The wine was then cold stabilized, filtered and bottled according to standard practices for white wine production. All fermentations were done in triplicate and wine samples were scanned using a WineScan FT 120 instrument (Foss, Denmark) that employs a Michelson interferometer that was used to generate the FT-IR (Fourier infra-red) spectra (**Table 4.3**). The samples (7 ml) were pumped through the CaF_2-lined cuvette (optical path length 37 μm) that is housed in the heater unit of the instrument. The temperature of the samples was brought to exactly 40°C before analysis. Samples were scanned from 5011-929 cm^{-1} at 4 cm^{-1} intervals, which includes a small section of the near infra-red (NIR). The frequencies of the NIR beam transmitted by a sample were recorded at the detector and used to generate an interferogram. The latter was calculated from a total of 10 scans before being processed by Fourier transformation and corrected for the background absorbance of water to generate a single beam transmittance spectrum. Two transmittance spectra for each sample were generated in order to calculate the absolute repeatability of the spectral measurements. The calculation of the absolute repeatability has been described (WineScan FT120 Type 77110 and 77310 reference Manual, Foss, Denmark, 2001). The transmittance spectra were finally converted into linearized absorbance spectra through a series of mathematical procedures.

4.3.6 PRODUCTION AND ANALYSIS OF BASE WINE AND SMALL-SCALE DISTILLATION

The wine yeast strains VIN13, VIN13(pATF1-s), VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pTIP1-s) and VIN13(pIAH1-s) were each inoculated (2 x 10^6 cells/ml) into 15 liters of Colombard grape juice, to which no sulfur dioxide had been added, and fermented at 15°C until dry. These fermentations were done in triplicate. Routine WineScan analysis was performed on the base wines just after alcoholic fermentation (**Table 4.3**). Three 5-liter round-bottom flasks were each filled with 4.5 liters of base wine and yeast lees derived from

the original 15-liter base wine fermentation volume. Two copper plates and 3 g of copper sulfate were added to the base wine and heated in heating mantles. The distillation flow rate was maintained at 5 ml/min, and the distillate was collected until 30% v/v alcohol was reached. The same procedure was followed with the second distillation, except that the first 40 ml of distillate, collected at a flow rate of 2 ml/min, was discarded. The flow rate was then adjusted to 5 ml/min and the heart was collected until 70% v/v alcohol was reached.

4.3.7 GAS-CHROMATOGRAPHIC ANALYSIS

To each wine sample (10 ml of Colombard table or base wine), 0.8 ml of internal standard [4-methyl-2-pentanol, 230.2 mg/l, 12% (v/v) ethanol] and 6.5 ml of solvent (diethyl ether) were added. The tube was then mechanically rotated at 60 rpm for 30 min. The top layer of ether was separated and the extracts were analyzed. For the 70% distillates, a 5 ml sample was taken and 0.25 ml of 4-methyl-2-pentanol (2 g/l, 70% (v/v) ethanol) was added. After mixing, 2 μl of the sample were injected into the gas chromatograph (GC). The extractions were done in triplicate.

The analysis of volatile compounds was carried out on a Hewlett Packard 5890 Series II gas chromatograph coupled to an HP7673 autosampler and injector and an HP 3396A integrator. The column used was a Lab Alliance organic coated fused silica capillary with dimensions of 60 m x 0.32 mm i.d. with a 0.5 μm coating thickness; hydrogen was used as the carrier gas for an FID detector held at 250°C. The injector temperature was 200°C, the split ratio 20:1, the flow rate 15 ml/min, and the injection volume was 3 μl. The oven temperature program was as follows: 35°C (10 min) to 230°C (0 min) at 3°C/min. For the distillate analysis, the conditions were as described above, except for a different oven program and 2 μl injection volume: 30°C (5 min) to 80°C at a speed of 2°C/min, and 80°C (0 min) to 230°C at 3°C/min. For each of the compounds measured, a specific amount was measured for the standard used to calibrate the machine. The internal standard and the chemicals were sourced from Merck (Cape Town, South Africa).

Table wine extractions were done after bottling. Extractions from the base wine were made after alcoholic fermentation, but before distillation. Samples from the distillates were taken after the second distillation.

4.3.8 SENSORY EVALUATION

The table wines and distillates were sensorially evaluated for different fruity aromas, as well as for flowery and solvent or chemical intensity, by a panel of six experienced judges. The wines and distillates were evaluated on a percentage scale from 0 to 100, where 0 represented the absence of a specific flavor and 100 represented a very high intensity of the flavor.

4.3.9 STATISTICAL ANALYSIS

The statistical differences between the GC results for the wines and 70% distillates produced by the modified and the control yeast strains were determined using ANOVA and the Bonferroni post-hoc test (**Tables 4.6 and 4.7**). A 5% significance level ($p \leq 0.05$) was used to

judge significant differences. The sensory evaluation data were also statistically evaluated in the same way. Cluster analysis was used to select subsets of tasters that appeared to conform the most.

4.4 RESULTS

4.4.1 CONSTITUTIVE EXPRESSION OF *ATF1*, *ATF2*, *EHT1*, *TIP1* AND *IAH1* IN VIN13

With the aim of assessing the effects of acetyltransferases and esterases throughout wine fermentation, the *ATF2*, *EHT1*, *IAH1* and *TIP1* genes were cloned from a widely used commercial wine yeast strain, VIN13, and placed under the constitutive control of the *PGK1* regulatory sequences to generate plasmids pATF2-s, pEHT1-s, pIAH1-s and pTIP1-s (**Figure 4.1**). Previously, we constructed the $PGK1_P$-*ATF2*-$PGK1_T$ gene cassette in a similar way (Lilly *et al.*, 2000). To allow for stable maintenance of the gene constructs in non-selective grape juice medium, VIN13 was transformed with linearized plasmids to facilitate direct integration into the *ILV2* gene. Integration of the respective plasmids into the genomes of the Sm^R transformants VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pIAH1-s) and VIN13(pTIP1-s) were confirmed by Southern blot analysis (**Figure 4.2**). Two hybridization bands of 1854 and 2656 bp were obtained for the *Stu*I-digested *ATF2* gene of the control host yeast strain, VIN13, whereas four hybridization bands of 1854, 2656, 7762 and 8397 bp, corresponding to the *Stu*I-digested wild-type *ATF2* gene and the integrated $PGK1_P$-*ATF2*-$PGK1_T$ gene cassette, were obtained with the recombinant wine yeast strain VIN13(pATF2-s) (**Figure 4.2A**). Two hybridization bands of 2744 and 2910 bp were obtained for the *Nru*I-digested wild-type *EHT1* gene in VIN13, and three hybridization bands of 2744, 2910 and 6267 bp were detected in VIN13(pEHT1-s), corresponding to the 2744 and 2910 bp fragments of the wild-type *EHT1*, and the 6267 bp fragment of the overexpression cassette (**Figure 4.2B**). Two single hybridization bands of 4419 and 5901 bp, corresponding to the wild-type genes *IAH1* and *TIP1*, respectively, were obtained in the host strain of VIN13, and additional bands of 15267 and 15183 bp were obtained in VIN13(pIAH1-s) and VIN13(pTIP1-s), respectively, corresponding to the integration cassettes (**Figures 4.2C** and **4.2D**). Therefore, all transformed VIN13 strains carried two copies of each of the *ATF1*, *ATF2*, *EHT1*, *TIP1* and *IAH1*; the original copy of which the transcription was directed under its authentic promoter and terminator sequences, and a second copy that was expressed under the direction of the constitutive *PGK1* promoter and terminator sequences. The host (control) strain, VIN13, and the transformed strains [VIN13(pATF1-s), VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pIAH1-s) and VIN13(pTIP1-s)] were used to produce Colombard table wine and rebate wine for distilling purposes.

4.4.2 CHEMICAL COMPOSITION OF EXPERIMENTAL WINES AND DISTILLATES

The concentrations of certain esters, higher alcohols and acids were determined for the wines and distillates (**Tables 4.4** and **4.5**). The concentration of most of the esters increased in both the table and base wines prepared with VIN13(pATF1-s) when compared with the control wines (**Figure 4.3**). The concentrations of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and hexyl acetate, as well as of ethyl caproate, ethyl caprylate and ethyl caprate, increased as described in Lilly *et al.* (2000). The concentration of isoamyl acetate and 2-phenylethyl acetate in the table and base wines produced with VIN13(pATF2-s) increased 1.5- to 1.8-fold and 1.3- to 1.5-fold respectively, in comparison with the wines fermented with the untransformed VIN13 host strain. The ethyl acetate concentration in the table wine produced with VIN13(pATF2-s) increased 1.3-fold compared to the control, but remained the same for the base wines. The ethyl caproate concentration showed a slight increase in the table and base wines produced with VIN13(pATF2-s) in comparison with that in the wines prepared with VIN13. In comparison with the control wines, the table wine fermented with VIN13(pEHT1-s) showed a 1.3- to 1.5-fold increase in the concentrations of ethyl acetate, ethyl caproate, ethyl caprylate, ethyl caprate and hexyl acetate and the base wine produced with VIN13(pEHT1-s) presented a 1.5- and 1.7-fold decrease in 2-phenylethyl acetate and isoamyl acetate concentrations respectively. The concentration of ethyl caprylate also increased slightly in the base wine fermented with VIN13(pEHT1-s). The table and base wines produced with VIN13(pIAH1-s) showed a significant decrease in ester concentrations when compared to the control wines. The concentration of isoamyl acetate decreased 11.4- to 15.6-fold and hexyl acetate decreased 13-fold in the wines fermented with VIN13(pIAH1-s). Ethyl acetate and 2-phenylethyl acetate concentrations decreased by 1.6- to 1.8-fold and 3.4- to 3.9-fold, respectively, in the wines fermented with VIN13(pIAH1-s). The table and base wines fermented with VIN13(pTIP1-s) showed no significant difference in ester concentrations when compared with the wines fermented with VIN13.

The wines produced with VIN13(pATF1-s) showed a decrease in all of the corresponding higher alcohols of which the ester concentrations increased, but the decrease in higher alcohol concentrations does not directly correlate with the quantity of esters produced (**Figure 4.4**). In all of the experimental wines produced with the transformed VIN13 strains, the *n*-butanol concentration decreased two-fold or more. The concentration of 2-phenylethyl alcohol decreased 1.2-fold in the table and base wines fermented with VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pTIP1-s) and VIN13(pIAH1-s). The propanol concentration only decreased slightly in the wines produced with VIN13(pATF2-s) when compared to the wines fermented with VIN13, but decreased 1.4-fold in the wines fermented with VIN13(pTIP1-s) and VIN13(pIAH1-s). The isobutanol concentration increased in all the wines fermented with the transformed strains; the biggest increase was in the wines fermented with VIN13(pTIP1-s). The isoamyl alcohol concentration remained the same in the wines produced with VIN13(pATF2-s), VIN13(pTIP1-s) and VIN13(pIAH1-s), but the concentration decreased 1.4-fold in the wines fermented with VIN13(pEHT1-s). The hexanol concentration increased 1.5-fold in the wines fermented with VIN13(pIAH1-s). The decrease or increase in alcohol

concentrations corresponded to the increase or decrease in the ester concentrations. The acetic acid concentrations in the wines produced by the transformed strains were drastically decreased in comparison to those fermented with the control strains.

4.4.3 EFFECT OF DISTILLATION ON THE ESTER CONTENT OF THE REBATE WINES

After the base wine had undergone double distillation, the content changed because only the aroma compounds present in the 'heart' fraction were collected, while other components, such as some acids, were discarded in the 'heads' and 'tails' fractions. The distillate of the VIN13(pATF1-s)-fermented base wine still contained a higher content of all the acetate esters, as well as of ethyl caproate, ethyl caprylate and ethyl caprate. The distillate produced from VIN13(pATF2-s)-fermented base wine showed a 1.6-fold increase in 2-phenylethyl acetate concentration, as well as a 1.2-fold increase in ethyl caproate, ethyl caprylate and ethyl caprate concentrations and only a slight increase in ethyl acetate and isoamyl acetate concentrations. The concentration of 2-phenylethyl acetate decreased 1.5- and 4.5-fold, respectively, in the distillates produced from the VIN13(pEHT1-s)- and VIN13(pIAH1-s)-derived base wines. The distillate produced from the base wine fermented with VIN13(pIAH1-s) showed a 1.5-fold decrease in ethyl acetate concentration, a 15-fold decrease in isoamyl acetate concentration and still contained no hexyl acetate. A 1.7-fold decrease in isoamyl acetate concentration in the distillate produced from the VIN13(pEHT1-s)-fermented base wine was observed and this finding is consistent with the decrease in the base wines.

With the exception of isobutanol, the concentration of all the higher alcohols in the distillate produced from the VIN13(pATF1-s)-fermented base wine decreased correspondingly to the increased ester concentrations. The concentration of *n*-butanol decreased by 1.7-fold and more in the distillate produced from base wines prepared with the transformed strains. The concentration of 2-phenylethyl alcohol, propanol and isoamyl alcohol in the distillate produced from the VIN13(pEHT1-s)-fermented base wine decreased 1.7-, 1.5- and 1.4-fold, respectively, but the hexanol concentration of the distillate produced with the VIN13(pIAH1-s)-fermented base wine increased 1.6-fold in correlation with the drastic decrease in hexyl acetate concentration.

The distillation process played an important role in the differences in the aroma compound concentrations between the rebate wine and the 70% distillate. Most of the aroma compounds were concentrated during the first distillation, but some were also partly lost during the second distillation (**Figure 4.5**). All of the ester concentrations increased when the rebate wine was double distilled. The concentration of ethyl caprate increased the most of all the esters, with a 25-fold increase in concentration, and the ethyl caprylate concentration increased 7.5-fold in the distillate compared to in the rebate wine. Ethyl acetate was less concentrated and only a 2.8-fold increase was obtained in the distillate. All of the other ester concentrations also increased between 3.8- and 4.8-fold when the rebate wine was double distilled. A seven-fold increase in the concentration of isobutanol and propanol was observed in the distillates, while *n*-butanol, isoamyl alcohol and hexanol were concentrated six-fold. 2-

Phenylethyl alcohol was the only higher alcohol of which the concentration decreased during the distillation process. The concentrations of the fatty acid esters were increased and that of acetic acid decreased 14-fold. Decanoic acid was concentrated seven-fold, hexanoic acid 4.9-fold, octanoic acid four-fold and *i*-butyric acid 1.7-fold.

4.4.4 SENSORIAL ATTRIBUTES OF THE EXPERIMENTAL WINES

All of the sensory results presented here are statistically significant. The fruity aroma, which is usually associated with the combination of many esters, was detected in a higher intensity in the table wines fermented with VIN13(pATF1-s) and VIN13(pEHT1-s) (**Figure 4.6**). The intensity of the fruity aroma for the rest of the wines fermented with the transformed strains remained the same as that of the control wine. The peachy aroma was stronger in all of the wines produced with the transformants than in the control, except for the wine fermented with VIN13(pATF2-s), in which no peachy flavor was detected. The peachy aroma was significantly more intense in the wines fermented with VIN13(pEHT1-s) and VIN13(pTIP1-s). The apricot aroma was only detected in the wines fermented with VIN13(pEHT1-s) and VIN13(pIAH1-s). An apple aroma was detected in all of the wines except the wine fermented with VIN13(pATF1-s). The apple aroma was much more intense in the wines fermented with VIN13(pEHT1-s) and VIN13(pTIP1-s) than in the control wine. A banana aroma was absent in the wines fermented with VIN13 and VIN13(pIAH1-s), but was quite prominent in the VIN13(pATF2-s)-fermented wine. The estery/synthetic fruit flavor was overpowering in the wines fermented with VIN13(pATF1-s), but much more subtle in the VIN13(pATF2-s)- and VIN13(pTIP1-s)-fermented wines and completely absent in the VIN13(pIAH1-s)-fermented wine. The intensity of the guava aroma in the wines fermented with VIN13(pATF2-s) and VIN13(pEHT1-s) was more prominent than in the control wine and the citrus aroma was only detected in the wine fermented with VIN13(pEHT1-s). The floral aroma was also only detected in the wines fermented with VIN13 and VIN13(pTIP1-s).

In most cases, similar changes were observed in the distillates. However, some interesting differences were also observed. The fruity aroma, associated with the combination of all the esters, was detected in a higher intensity in all of the distillates prepared from base wines that were produced with the transformed strains, but was the most intense in the distillates derived from base wines that were produced with VIN13(pATF1-s), VIN13(pIAH1-s) and VIN13(pATF2-s) (**Figure 4.7**). The peach and apricot aromas were stronger in the distillates of VIN13(pATF2-s)- and VIN13(pIAH1-s)-fermented wines and the apple aroma was more prominent in the distillates produced with VIN13(pATF2-s)- and VIN13(pATF1-s)-fermented base wines. As previously described by Lilly *et al.* (2000), the estery/synthetic fruit aroma was overpowering in the distillate produced from VIN13(pATF1-s), but much more subtle in the distillates of VIN13(pATF2-s) wines. The herbaceous aromas remained the same as in the control distillate, except for a decrease in intensity in the distillates produced from VIN13(pATF1-s) and VIN13(pIAH1-s), and the floral aroma was less prominent in all of the distillates derived from based wine that had been fermented with the transformants.

4.5 DISCUSSION

In this work, five known ester-synthesizing (the *ATF1*- and *ATF2*-encoded alcohol acetyltransferases and the *EHT1*-encoded ethanol hexanoyl transferase) and ester-degrading enzymes (the *IAH1*- and *TIP1*-encoded esterases) were investigated by overexpressing the *ATF1, ATF2, EHT1, IAH1* and *TIP1* genes in an industrial wine strain, VIN13. Most of our findings regarding the experimental wines and distillates prepared with the transformed VIN13 strains were in agreement with observations made with regard to beer and saké (Verstrepen *et al.*, 2003; Fukuda *et al.*, 1998). We confirmed that Atf1p is responsible for the production of the majority of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate in wine (**Figure 4.3**). However, here we show that Atf1p also has an effect on ethyl caproate, ethyl caprylate and hexyl acetate. This could be due to the broad specificity of the enzyme or because increased levels of esters might influence the expression of Eht1p, which has been implicated in the synthesis of hexyl acetate. These effects would have to be investigated further by using strains deleted for the different alcohol acetyltransferases genes in combination with their overexpression equivalents. We confirmed that Atf2p plays a role in ethyl acetate, isoamyl acetate and 2-phenylethyl acetate ester production, but it appears to be less efficient than Atf1p. However, Atf2p also slightly increased the concentrations of ethyl caproate, ethyl caprate and hexyl acetate, but it had no effect on ethyl caprylate production as in the case of Atf1p. Interestingly, overexpression of the *EHT1* gene slightly increased the concentration of all of the esters, with ethyl caprate and ethyl caprylate concentrations increasing the most. Again, it is unclear whether Eht1p has a broad specificity or whether the esters it produces have a stimulatory effect on the expression of Atf1p and Atf2p. The individual enzymes of Atf1p, Atf2p and Eht1p would have to be purified and enzyme assays would have to be conducted on various substrates to establish whether or not this is the case. In the case of ester hydrolysis, overexpression of the *IAH1* gene, as expected, led to a decrease in ester concentration. However, a decrease in the concentrations of hexyl acetate, isoamyl acetate and 2-phenylethyl acetate was most prominent. It therefore appears that Iah1p has a higher affinity for these substrates, although its effect on the regulation of alcohol acetyltransferases cannot be ruled out. The other esterase, Tip1p, did not have a significant effect on ester concentration. Previously, it was suggested that the *S. cerevisiae* Tip1p did not have the same activity as the *S. carlsbergensis* Tip1 enzyme (Horsted *et al.*, 1998). Under the conditions used in the present study, *S. cerevisiae*'s Tip1p did not seem to have any significant esterase activity. Therefore, in the VIN13 wine yeast, Atf1p, Atf2p, Eht1p and Iah1p appear to be the major enzymes involved in determining the ester profile of the wine produced.

Overexpression of the alcohol acetyltransferase and esterase genes also had an interesting effect on the concentrations of higher alcohols, which act as substrates for ester synthesis. Overexpression of *ATF1, ATF2, EHT1, IAH1* and *TIP1* resulted in an overall increase in the concentration of isobutanol and a decrease in the concentration of *n*-butanol and 2-phenylethyl alcohol. The increase in isobutanol concentrations is interesting, as both isobutanol and isoamyl alcohol are derived from α-keto-isovalerate. When *ATF1* was overexpressed, isoamyl alcohol levels dropped in correlation with an increase in the formation

of isoamyl acetate. However, this led to an increase in isobutanol concentration, indicating that the regulation of ester formation probably affects the branched-chain amino acid metabolism as well. In this work, we could not find a clear correlation between an increase and decrease in esters and a corresponding decrease and increase in higher alcohols for all the overexpressed genes tested. The reason why there is a major increase in hexanol concentration when Eht1p and Iah1p are overexpressed and a decrease in hexanol concentration when Atf1p, Atf2p and Tip1p are overexpressed is unclear. Overexpression of *TIP1* also had a marked effect (either a major increase or decrease) on the concentration of higher alcohols, but not on the ester concentrations. It therefore seems that there is a complex regulation between esters, higher alcohols and branched-chain amino biosynthesis that needs further investigation.

One of the aims of this work was to develop better ester aroma profiles for wines and distillates. The small increase in ethyl acetate concentration when fermenting with VIN13(pATF2-s) instead of VIN13(pATF1-s) might be advantageous to the wine and brandy industry, since Lilly *et al*. (2000) showed that excessively high concentrations of ethyl acetate did not improve the fermentation bouquet and aroma of the wines and distillates. The sensory evaluation of the wines and distillates produced from VIN13(pATF2-s) proved that the lower concentration of ethyl acetate, the more the other fruity aromas could be detected. Such strains might therefore provide wines with higher complexity. The Colombard wines fermented with VIN13(pATF2-s) had a much less estery/synthetic fruit character than the table wine fermented with VIN13(pATF1-s), but more intense apple, banana and guava aromas were observed. On the other hand, distillates produced from VIN13(pATF2-s) base wine had a more intense peach, apricot and apple aroma than the distillates produced from base wines fermented with VIN13 or VIN13(pATF1-s). The perceived intensity of certain fruity aromas is therefore not linked to the total ester concentration, but rather to specific ratios thereof.

In this study, we also confirmed the involvement of Eht1p in medium-chain fatty acid ethyl ester synthesis, as hypothesized by Mason and Dufour (2000) and Verstrepen *et al*. (2003). Statistical analysis of the data showed a significant increase in ethyl caproate, ethyl caprylate and ethyl caprate concentrations in the table wines fermented with VIN13(pEHT1-s), and the wines presented a definite apple aroma that is usually associated with these esters. The base wines and corresponding distillates only showed a slight increase in ethyl caprylate concentration. The overall concentration of esters is higher in the table wine than in the rebate wine for all of the strains. The reason for this might be the difference in the winemaking practices. In order to let the lees settle to the bottom of the fermentation vessel, the table wine was left on the lees for a week after the wine had fermented dry; it was then racked and underwent a three-week cold stabilization at 4°C, after which it was filtered and bottled. On the other hand, the base wine was double distilled immediately after alcoholic fermentation. This difference suggests that a significant amount of esters is produced and released from the wine yeasts after the end of alcoholic fermentation.

It was shown that the *IAH1* gene encodes an isoamyl acetate-hydrolyzing esterase that hydrolyzes isoamyl acetate (Fukuda *et al*., 1996; 1998; 2000). In this study, we found that the

isoamyl acetate concentration decreased when the *IAH1* gene was overexpressed in VIN13. We also found that the 2-phenylethyl acetate concentration decreased drastically, and that the ethyl acetate concentration also decreased in the wines. The data suggest that Iah1p hydrolyzed hexyl acetate completely in all of the different wines. The Iah1p enzyme therefore does not only hydrolyze isoamyl acetate and ethyl acetate, but also 2-phenylethyl acetate and, to a large extent, hexyl acetate. However, the sensory evaluations of the wines and distillates produced with VIN13(pIAH1-s) were contradictory to the GC-data. The table wine fermented with VIN13(pIAH1-s) was rated as more fruity, with a stronger banana, guava and estery/synthetic fruit aroma than the control wine fermented with VIN13, even though the VIN13-fermented wine contained much higher concentrations of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and hexyl acetate. The same fruity, peach, apricot, estery/synthetic flavors were more intense in the distillate produced with VIN13(pIAH1-s) than in the control distillate. The concentration of higher alcohols could play a role in this regard, as *IAH1* overexpression resulted in a significant increase in hexanol and isobutanol that could impact on the flavor. This increase in the concentration of higher alcohols could be due to ester degradation. The ratio of esters and higher alcohols could also have an effect on the perception of fruity aromas.

Previously, an extracellular esterase was isolated from the brewer's yeast, *S. carlsbergensis*, which prefers esters of fatty acids from 4 to 16 carbon atoms (Horsted *et al.*, 1998). The overexpression of the *TIP1* gene in VIN13 did not result in a drastic decrease in any of the esters that were analyzed in this study. Only a slight decrease was detected in ethyl acetate, 2-phenylethyl acetate, ethyl caproate, ethyl caprylate and ethyl caprate concentrations. However, the overexpression of *TIP1* had a major effect on the concentration of higher alcohols. It resulted in the biggest decrease in *n*-butanol concentration and the highest increase in isobutanol concentration. It therefore seems that Tip1p could perform other functions in *S. cerevisiae*.

In conclusion, this report does not claim to advance the field appreciably closer to the goal of producing wine to definable specifications and styles in the sense of technological enablement; rather, its significance hinges on the fact that this study has resulted in progress towards laying the foundation for the possible development of wine yeast starter strains with optimized ester-producing capability.

4.6 ACKNOWLEDGEMENTS

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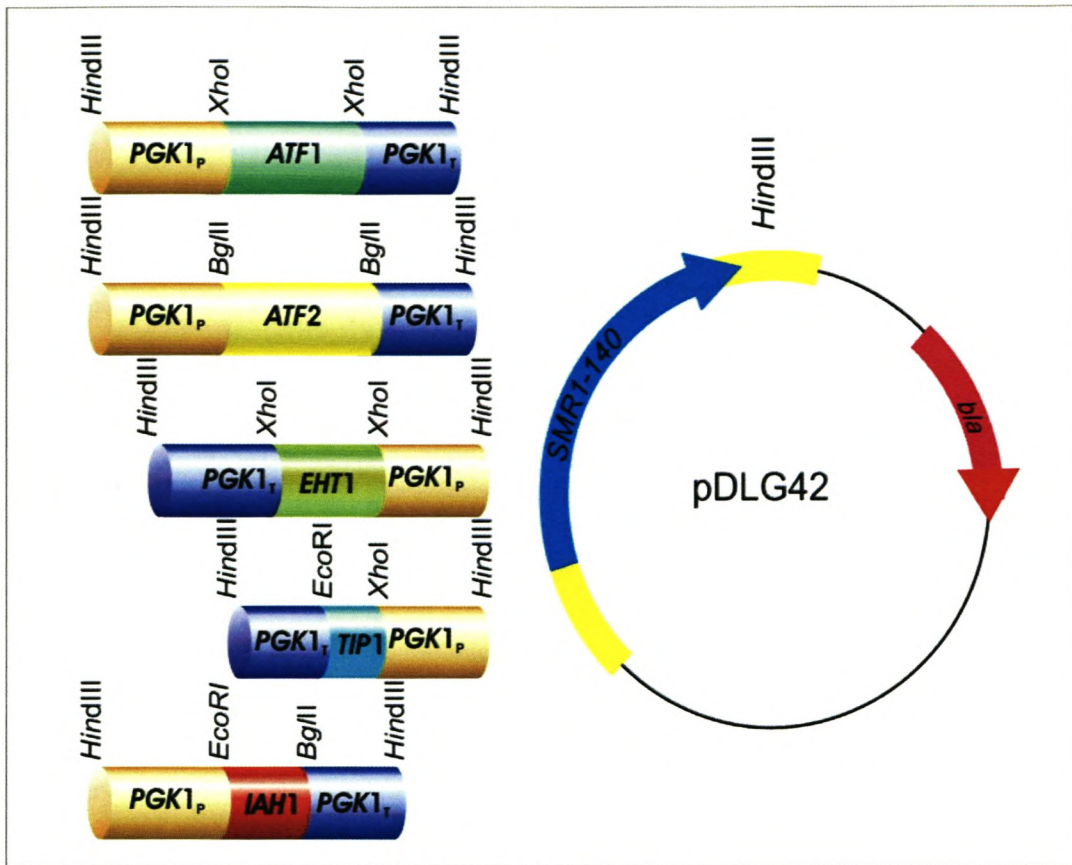
Figure 4.1. Maps of the different gene constructs and the plasmid pDLG42.
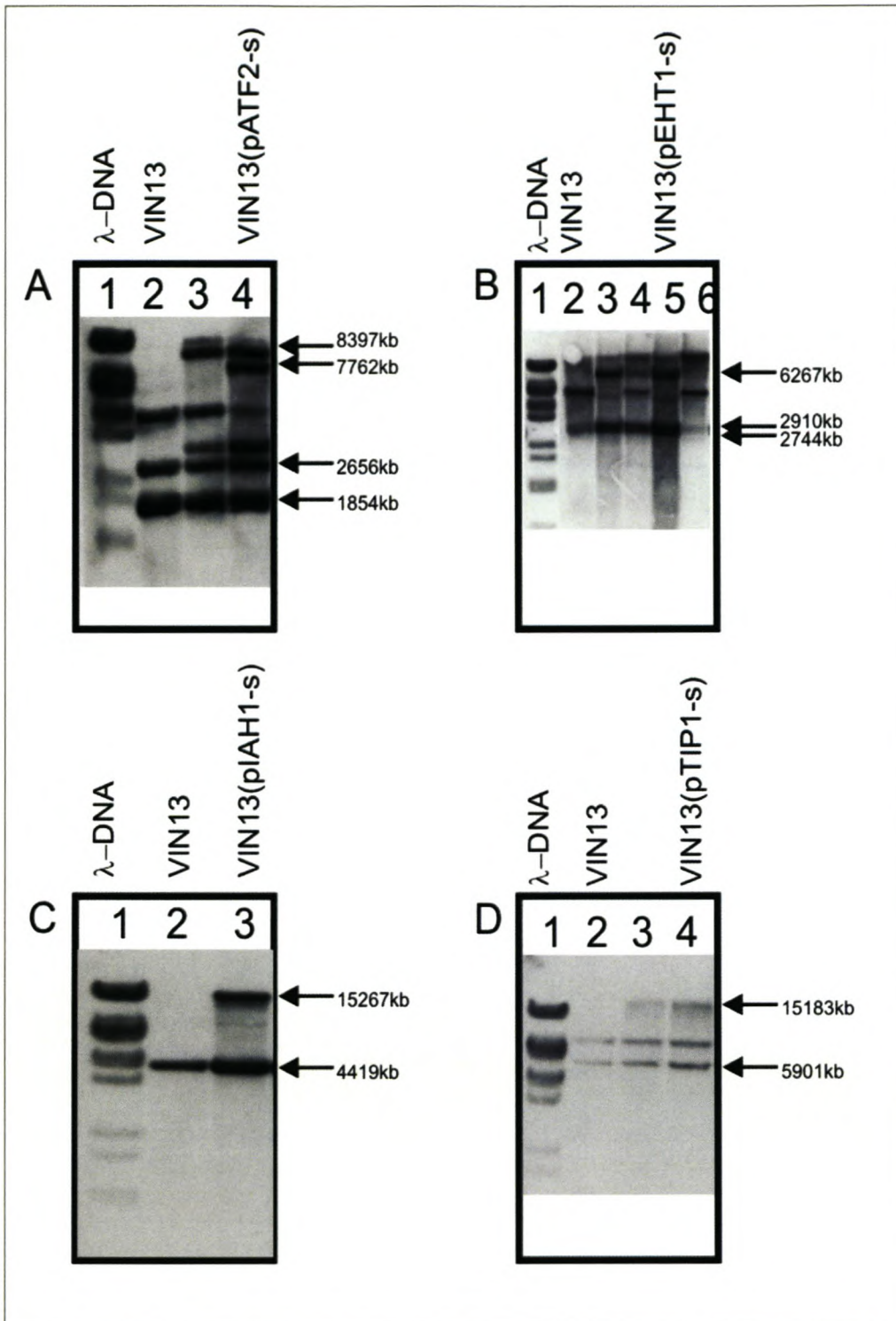
Figure 4.2. Genomic DNA analysis of *ATF2*, *EHT1*, *IAH1* and *TIP1*. Lanes were loaded with *Bst*EII-digested lambda DNA (lanes 1) or digested genomic DNA of the yeast strains VIN13 (control strain), VIN13(pATF2-s) (**A**), VIN13(pEHT1-s) (**B**), VIN13(pIAH1-s) (**C**) and VIN13(pTIP1-s) (**D**).
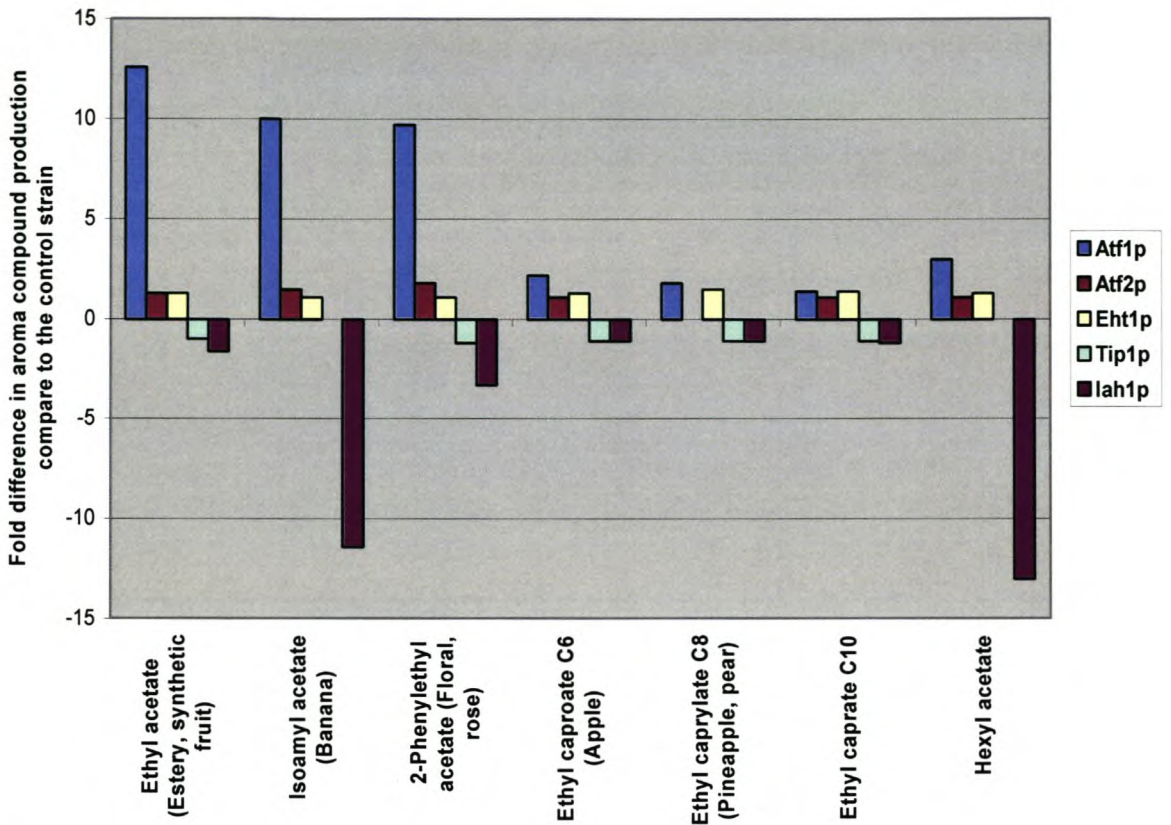
Figure 4.3. Comparison of the fold difference in ester production between the modified wine yeast strains and the control host strain, VIN13, where the aroma compound concentration of VIN13 is calculated at 0.
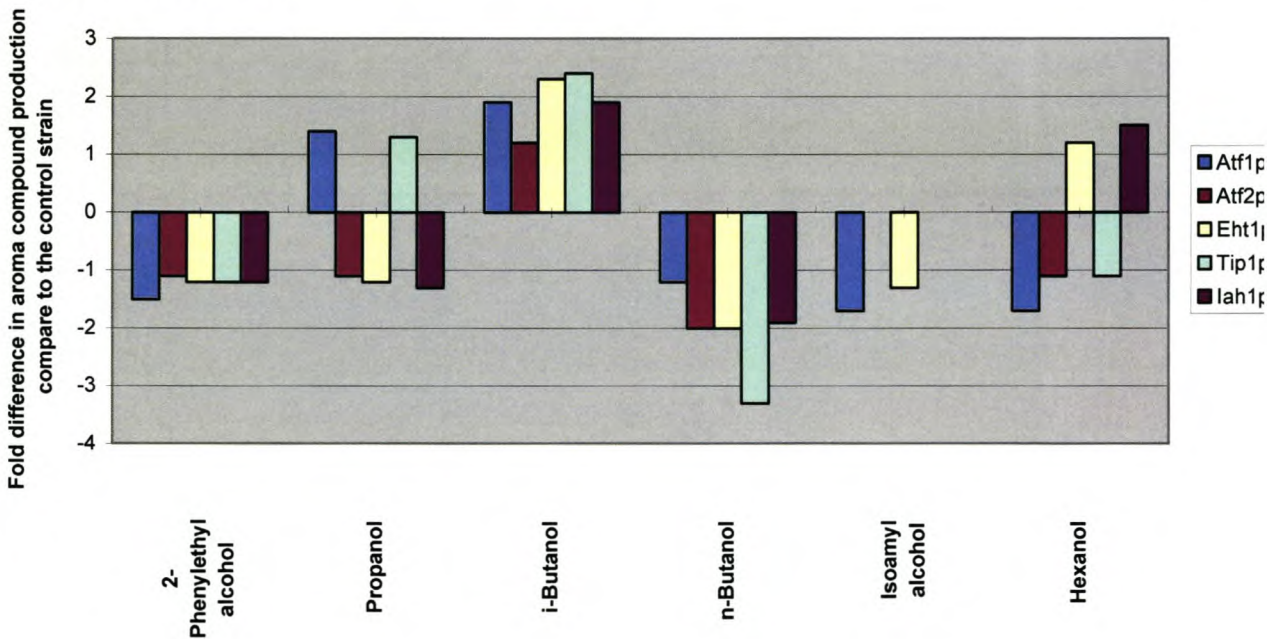
Figure 4.4. Comparison of the fold difference in higher alcohol production between the modified wine yeast strains and the control wine yeast strain, VIN13, where the aroma compound concentration of VIN13 is calculated at 0.
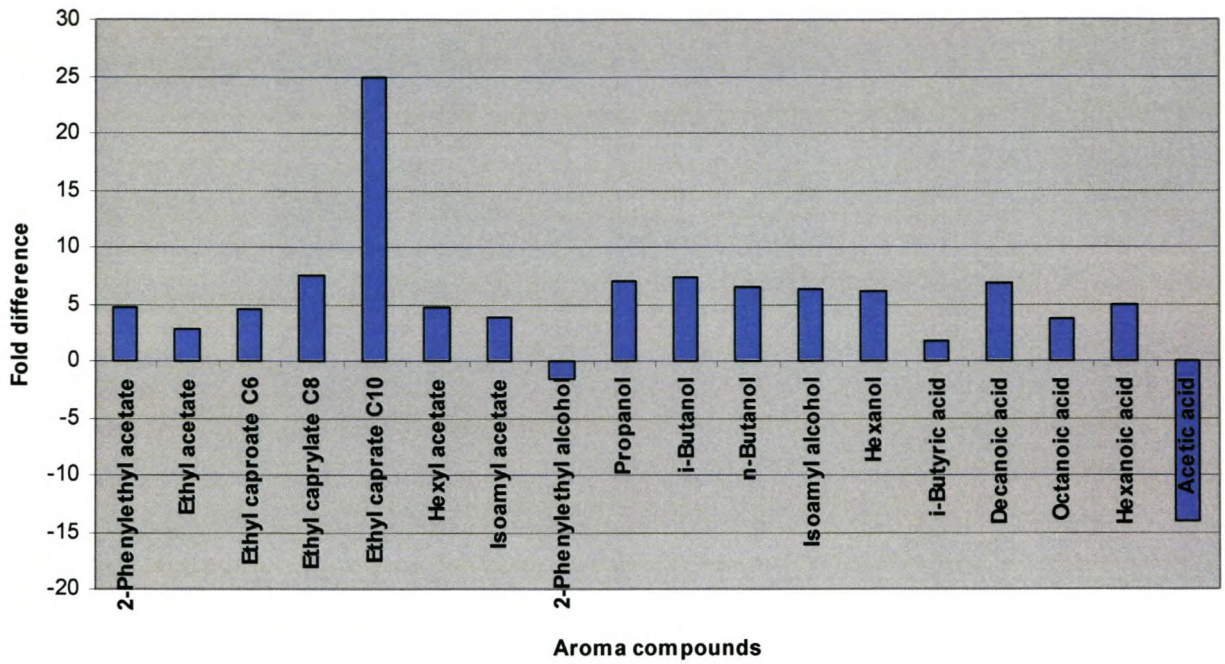
Figure 4.5. Fold difference in the aroma compound concentrations of the 70% distillates (average from all distillates) after the second distillation compared to the concentrations in the Colombard rebate wines from which the distillates were produced.
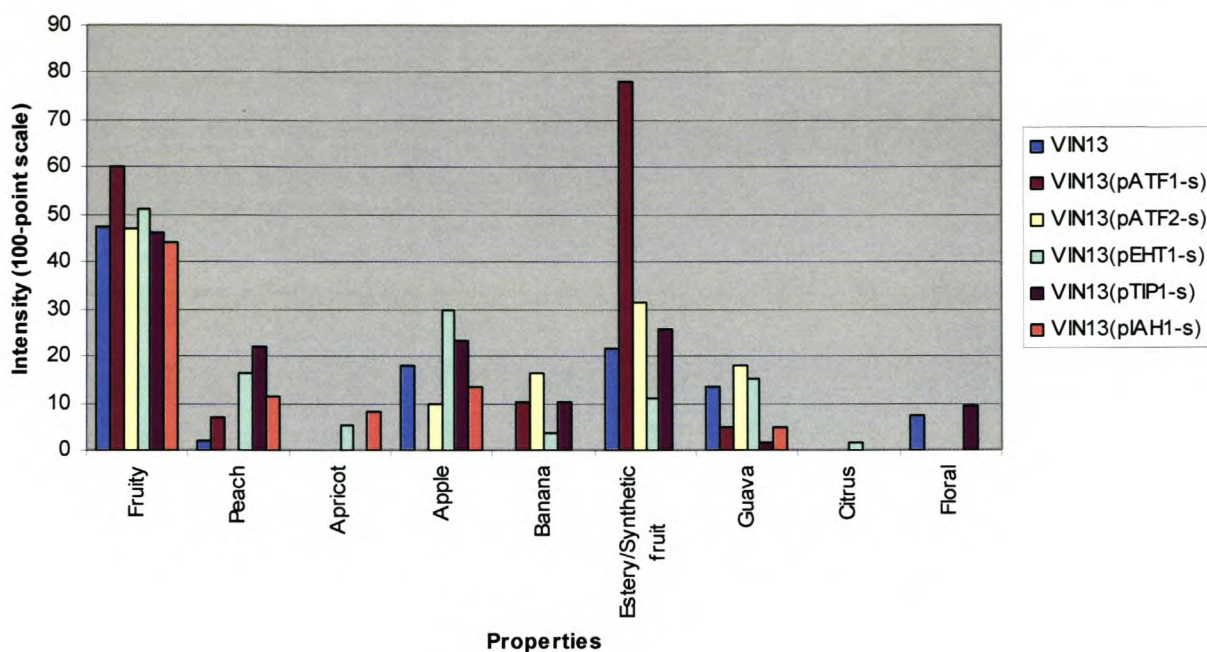
Figure 4.6. The aroma property intensities of the Colombard wine fermented with yeast strains VIN13 (control) and VIN13(pATF1-s), VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pTIP1-s) and VIN13(pIAH1-s).
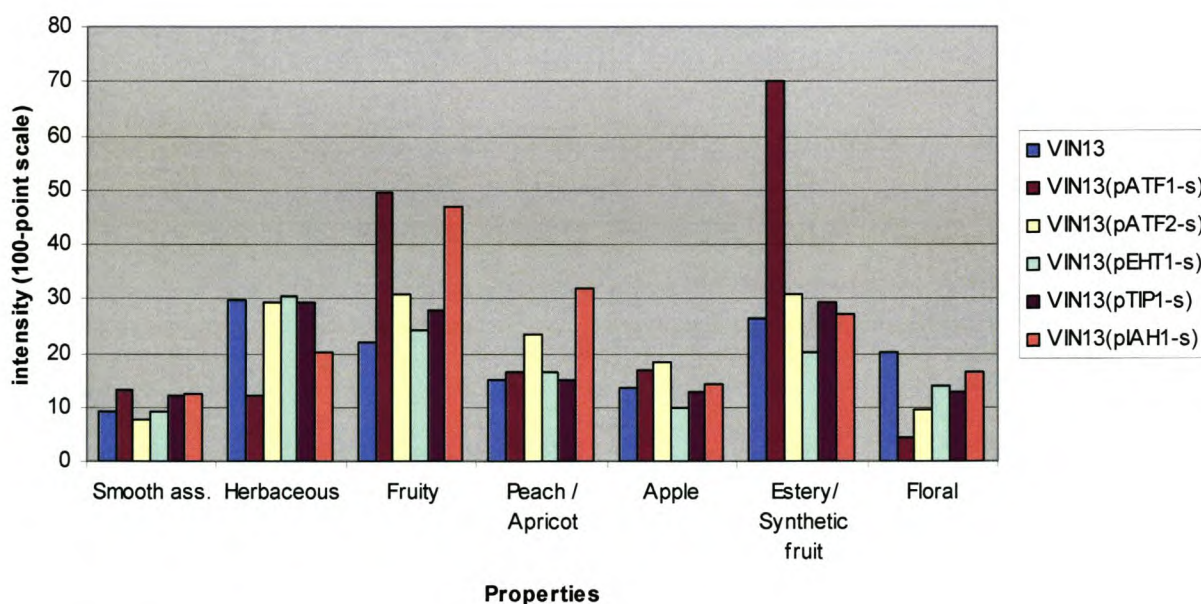
Figure 4.7. The aroma property intensities of the distillates, distilled from Colombard base wines fermented with the untransformed VIN13 strain (control), and the VIN13 transformants VIN13(pATF1-s), VIN13(pATF2-s), VIN13(pEHT1-s), VIN13(pTIP1-s) and VIN13(pIAH1-s).

Table 4.1. Microbial Strains and Plasmids Used in This Study

strain or plasmid	genotype or construct	reference or source
Escherichia coli DH5a	F' endA1 hsdR17 (r_k^- m_k^+) supE44 thi-1 recA1 gyrA (Nal^r) relA1 D(laclZYA-argF)U169 deoR [F80dlac DE(lacZ)M15]	GIBCO-BRL/Life Technologies
Saccharomyces cerevisiae industrial strain		
VIN13	commercial wine yeast strain	Anchor Yeast, Cape Town, South Africa
transformants		
VIN13(pATF1-s)	SMR1-140 PGK1 $_P$-ATF1-PGK1 $_T$	Lilly et al. 2000
VIN13(pATF2-s)	SMR1-140 PGK1 $_P$-ATF2-PGK1 $_T$	this study
VIN13(pEHT1-s)	SMR1-140 PGK1 $_P$-EHT1-PGK1 $_T$	this study
VIN13(pTIP1-s)	SMR1-140 PGK1 $_P$-TIP1-PGK1 $_T$	this study
VIN13(pIAH1-s)	SMR1-140 PGK1 $_P$-IAH1-PGK1 $_T$	this study
plasmids		
pHVXII	bla LEU2 PGK1 $_P$-PGK1 $_T$	Volschenk et al. 1997
pDLG42	bla SMR1-140	Le Grange, 1999
pATF1-s	bla SMR1-140 PGK1 $_P$-ATF1-PGK1 $_T$	Lilly et al. 2000
pATF2-s	bla SMR1-140 PGK1 $_P$-ATF2-PGK1 $_T$	this study
pEHT1-s	bla SMR1-140 PGK1 $_P$-EHT1-PGK1 $_T$	this study
pTIP1-s	bla SMR1-140 PGK1 $_P$-TIP1-PGK1 $_T$	this study
pIAH1-s	bla SMR1-140 PGK1 $_P$-IAH1-PGK1 $_T$	this study

Table 4.2. Primers Synthesized to Amplify the Genes

primer name	sequence	enzyme
ATF1'F	5'-GATC**CTCGAG**ATGAATGAAATCGATGAGAA-3'	*Xho* I
ATF1'R	5'-GATC**CTCGAG**GTAAGGGCCTAAAAGGAGAG-3'	*Xho* I
ATF2'F	5'-GATC**AGATCT**ATGGAAGATATAGAAGGATA-3'	*Bgl* II
ATF2'R	5-GATC**AGATCT**TTAAAGCGACGCAAATTCGC-3'	*Bgl* II
EHT1'F	5'-TCGA**CTCGAG**ATGTCAGAAGTTTCCAAATGGCC-3'	*Xho* I
EHT1'R	5'-TCGA**CTCGAG**TCATACGACTAATTCATCA-3'	*Xho* I
TIP1'F	5'-AATT**GAATTC**ATGTCCGTTTCCAAGATTGCT-3'	*Eco* RI
TIP1'R	5'-TCGA**CTCGAG**TTATAACAATAAAGCAGCTGC-3'	*Xho* I
IAH1'F	5'-AATT**GAATTC**ATGGATTACGAGAAGTTTCT-3'	*Eco* RI
IAH1'R	5'-GATC**AGATCT**ATTCAAGACATTATGTTATA-3'	*Bgl* II

* The enzyme sites are indicated in boldface and the region homologous to the corresponding genes are underlined

Table 4.3. FOSS-Analysis of the Colombard White Wine after Bottling and the Colombard Base Wine After Alcoholic Fermentation but Before Distillation.

parameters	Colombard white wine						Colombard base wine					
	VIN13	VIN13 (pATF1-s)	VIN13 (pATF2-s)	VIN13 (pEHT1-s)	VIN13 (pTIP1-s)	VIN13 (pIAH1-s)	VIN13	VIN13 (pATF1-s)	VIN13 (pATF2-s)	VIN13 (pEHT1-s)	VIN13 (pTIP1-s)	VIN13 (pIAH1-s)
pH	3.42	3.46	3.46	3.42	3.42	3.40	3.74	3.81	3.73	3.74	3.74	3.71
volatile acid g/l	0.26	0.39	0.38	0.22	0.24	0.23	0.32	0.52	0.25	0.29	0.26	0.20
total acids g/l	5.38	5.51	5.51	5.34	5.31	5.27	5.20	5.28	5.04	5.26	5.06	4.97
malic acid g/l	2.82	2.94	2.96	2.79	2.76	2.62	2.99	3.07	3.06	3.36	3.09	2.97
lactic acid g/l	-0.18	-0.11	-0.12	-0.17	-0.19	-0.33	-0.07	0.10	0.01	-0.13	-0.08	-0.05
glucose g/l	-0.16	0.02	0.07	0.14	-0.04	-0.23	-0.06	-0.29	-0.56	1.01	-0.09	-0.31
fructose g/l	2.08	3.25	3.60	3.45	1.88	1.96	1.27	1.57	0.88	10.31	0.98	0.78
glycerol g/l	5.48	5.84	5.87	5.23	5.38	5.08	5.28	5.30	4.66	5.67	5.10	4.90
ethanol %v/v	11.49	12.00	11.99	11.03	11.33	11.44	11.96	12.73	11.50	12.13	12.03	11.67

Table 4.4. Yeast Strain Effect on the Concentration of Major Volatiles in Colombard White Wines. Values in Bold Differ Significantly from VIN13 on a 5% Significance Level.

component	concentration (mg/liter)					
	Colombard white wine after bottling					
	VIN13	VIN13 (pATF1-s)	VIN13 (pATF2-s)	VIN13 (pEHT1-s)	VIN13 (pTIP1-s)	VIN13 (pIAH1-s)
acetic acid	109.62	49.55	107.22	61.27	84.92	105.47
decanoic acid	3.18	**6.22**	2.91	**5.58**	3.03	2.77
hexanoic acid	6.40	**11.68**	6.24	**12.39**	6.63	6.07
i-butyric acid	1.06	2.08	1.74	2.46	2.27	1.68
n-butyric acid	0.69	**1.38**	0.61	**1.00**	0.61	0.53
i-valeric acid	0.69	0.99	0.66	0.80	0.67	0.61
n-valeric acid	0.53	0.54	0.39	0.38	0.37	1.32
octanoic aicd	9.63	**15.90**	9.26	**17.00**	9.89	9.07
propionic acid	0.79	5.95	5.52	0.20	0.23	0.22
acetate	0.00	0.00	0.00	0.00	68.73	0.00
2-phenylethyl acetate	0.39	**3.80**	**0.72**	0.43	0.33	**0.12**
diethyl succinate	0.16	0.32	0.24	0.23	0.18	0.18
ethyl acetate	71.77	**906.81**	**93.77**	**92.76**	68.73	**45.35**
ethyl butyrate	0.00	1.93	0.19	0.00	0.00	0.00
ethyl caproate C6	1.86	**4.03**	2.12	**2.50**	1.77	1.67
ethyl caprylate C8	1.63	**3.01**	1.70	**2.50**	1.52	1.45
ethyl caprate C10	1.60	**2.26**	1.44	**2.18**	1.41	1.30
ethyl lactate	1.34	2.24	1.27	1.53	1.44	1.24
hexyl acetate	1.17	**3.49**	1.28	**1.52**	1.14	**0.00**
iso-amyl acetate	8.52	**85.56**	**12.45**	9.79	8.61	**0.75**
acetoin	0.63	2.89	1.34	2.03	0.17	0.12
methanol	24.57	**50.65**	27.10	**32.68**	24.41	23.95
2-phenylethyl alcohol	9.62	**10.95**	9.33	**8.45**	7.98	8.32
propanol	32.88	**45.57**	30.69	30.43	25.57	25.55
iso-butanol	5.62	**10.47**	**7.00**	**13.00**	13.54	10.68
n-butanol	0.98	**0.79**	**0.49**	**0.48**	**0.30**	**0.51**
iso-amyl alcohol	129.57	**76.84**	131.00	**101.40**	132.70	138.57
hexanol	1.62	**0.96**	1.58	**1.93**	1.50	**2.42**

Table 4.5. Yeast Strain Effect on the Concentration of Major Volatiles in Colombard Base Wines and the Respective 70% Distillates. Values in Bold Differ Significantly from VIN13 on a 5% Significance Level.

component	concentration (mg/liter)											
	Colombard base wine after alcoholic fermentation						70% distillate after second distillation					
	VIN13	VIN13 (pATF1-s)	VIN13 (pATF2-s)	VIN13 (pEHT1-s)	VIN13 (pTIP1-s)	VIN13 (pIAH1-s)	VIN13	VIN13 (pATF1-s)	VIN13 (pATF2-s)	VIN13 (pEHT1-s)	VIN13 (pTIP1-s)	VIN13 (pIAH1-s)
acetic acid	212.29	**58.80**	**65.54**	168.31	**101.94**	**90.29**	14.82	0.48	0.00	11.83	1.09	0.00
decanoic acid	5.37	7.15	8.09	6.48	5.73	5.60	41.98	42.15	49.66	38.52	44.56	40.67
hexanoic acid	7.55	**9.47**	**9.44**	**10.39**	8.46	**8.80**	34.35	50.82	49.75	37.29	43.69	41.74
i-butyric acid	0.62	0.74	0.84	0.69	0.66	0.58	1.00	1.04	1.17	1.36	1.39	0.97
n-butyric acid	1.77	1.56	1.81	1.83	1.79	1.84	1.48	1.06	1.44	1.71	1.43	1.20
i-valeric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
n-valeric acid	0.70	0.51	0.79	0.35	0.47	0.00	0.00	0.00	0.00	0.00	0.00	0.00
octanoic acid	11.40	**13.80**	**15.06**	**14.50**	13.39	14.62	49.54	51.04	62.96	46.70	57.43	57.31
propionic acid	31.01	28.74	35.55	24.41	**15.73**	**19.97**	0.00	0.00	0.00	0.00	0.00	0.00
acetate	0.00	0.00	0.00	0.00	0.00	0.00	89.54	66.75	80.98	127.96	77.37	81.36
2-phenylethyl acetate	0.55	**2.33**	**0.81**	**0.36**	0.52	**0.14**	2.66	**11.44**	**4.31**	**1.80**	2.41	**0.59**
diethyl succinate	1.14	1.06	1.11	0.89	1.09	1.01	2.55	2.16	2.59	1.69	2.78	2.39
ethyl acetate	95.54	**532.99**	92.50	77.32	90.10	**52.81**	250.10	**1393.53**	258.60	212.61	245.75	**162.00**
ethyl butyrate	0.31	0.08	0.47	0.00	0.61	0.61	0.70	0.00	0.45	0.00	1.33	1.70
ethyl caproate C6	2.14	**2.52**	2.32	2.11	2.22	2.30	8.96	10.09	10.88	9.34	8.73	10.44
ethyl caprylate C8	2.11	**2.44**	2.12	**2.41**	2.04	1.87	13.71	15.82	17.79	15.52	14.88	15.93
ethyl caprate C10	1.42	1.75	1.37	1.26	1.15	0.97	29.63	34.04	38.80	28.56	31.71	28.78
ethyl lactate	0.00	0.00	0.00	0.00	0.00	0.00	2.25	2.45	2.61	1.88	2.55	2.42
hexyl acetate	1.35	**2.06**	1.29	1.13	1.33	**0.00**	6.51	**9.18**	6.72	5.58	5.91	**0.00**
iso-amyl acetate	10.01	**44.60**	**12.71**	**5.92**	12.06	**0.64**	39.17	**163.83**	52.45	**23.23**	43.36	**2.68**
acetoin	0.00	0.18	0.00	4.58	0.00	0.00	0.00	0.00	0.00	2.42	0.00	0.00
methanol	43.41	53.28	**30.50**	40.77	**32.65**	**30.50**	216.87	223.65	213.66	215.27	213.78	225.10
2-phenylethyl alcohol	13.67	**9.36**	**10.90**	**9.83**	**11.60**	**11.61**	8.50	**5.85**	7.71	**5.03**	8.76	7.38
propanol	41.66	**30.07**	34.28	**28.57**	**29.26**	**30.54**	256.69	**199.85**	266.97	**177.67**	210.51	243.77
iso-butanol	8.87	9.86	10.27	9.22	**13.77**	10.53	61.60	69.01	81.88	64.17	100.08	84.50
n-butanol	1.15	**0.54**	**0.56**	**0.35**	**0.31**	**0.58**	6.56	**3.07**	**3.56**	**2.18**	**2.08**	**3.89**
iso-amyl alcohol	121.54	**72.61**	114.72	**86.95**	129.56	131.85	716.02	**429.38**	756.56	**509.55**	758.27	861.47
hexanol	2.07	**1.00**	1.78	2.32	1.93	**3.05**	12.34	**6.04**	11.40	13.87	11.17	**19.34**

Table 4.6. Table with p-values Showing the Statistical Differences Between Wines and Distillates Produced by Control and Modified Strains with Respect to Certain Fermentation Buoquet Volatiles. If p<0.05 the Difference is Significant.

Component	Statistical difference				
	Colombar white wine after bottling				
	VIN13 vs VIN13(pATF1-s)	VIN13 vs VIN13(pATF2-s)	VIN13 vs VIN13(pEHT1-s)	VIN13 vs VIN13(pTIP1-s)	VIN13 vs VIN13(pIAH1-s)
Ethyl acetate	0.01	0.01	0.01	1.00	0.01
Isoamyl acetate	0.01	0.01	0.25	1.00	0.01
Isoamyl alcohol	0.01	1.00	0.01	1.00	1.00
2-Phenylethyl acetate	0.01	0.01	1.00	0.29	0.01
2-Phenylethyl alcohol	0.01	1.00	0.01	0.01	0.01
Hexyl acetate	0.01	1.00	0.01	1.00	0.01
Hexanol	0.01	1.00	0.02	1.00	0.01
Propanol	0.01	1.00	1.00	0.01	0.01
Isobutanol	0.01	0.03	0.01	0.01	0.01
n-Butanol	0.01	0.01	0.01	0.01	0.01
Acetic acid	1.00	1.00	1.00	1.00	1.00
Ethyl caproate C6	0.01	1.00	0.01	1.00	1.00
Ethyl caprylate C8	0.01	1.00	0.01	1.00	1.00
Ethyl caprate C10	0.01	1.00	0.01	1.00	0.13

Table 4.7. Table with p-values Showing the Statistical Differences Between Wines and Distillates Produced by Control and Modified Strains with Respect to Certain Fermentation Buoquet Volatiles. If p<0.05 the Difference is Significant.

Component	Colombar base wine after alcoholic fermentation					70% Distillate after second distillation				
	VIN13 vs VIN13(pATF1-s)	VIN13 vs VIN13(pATF2-s)	VIN13 vs VIN13(pEHT1-s)	VIN13 vs VIN13(pTIP1-s)	VIN13 vs VIN13(pIAH1-s)	VIN13 vs VIN13(pATF1-s)	VIN13 vs VIN13(pATF2-s)	VIN13 vs VIN13(pEHT1-s)	VIN13 vs VIN13(pTIP1-s)	VIN13 vs VIN13(pIAH1-s)
Ethyl acetate	0.01	1.00	0.18	1.00	0.01	0.01	1.00	1.00	1.00	0.01
Isoamyl acetate	0.01	0.14	0.01	0.77	0.01	0.01	1.00	1.00	1.00	0.01
Isoamyl alcohol	0.01	1.00	0.01	1.00	1.00	0.01	1.00	0.01	1.00	0.08
2-Phenylethyl acetate	0.01	0.01	0.01	1.00	0.01	0.01	0.01	0.01	1.00	0.01
2-Phenylethyl alcohol	0.01	0.01	0.01	0.02	0.01	0.04	1.00	0.01	1.00	1.00
Hexyl acetate	0.01	1.00	0.32	1.00	0.01	0.01	1.00	1.00	1.00	0.01
Hexanol	0.01	0.19	0.40	1.00	0.01	0.01	1.00	1.00	1.00	0.01
Propanol	0.01	0.19	0.01	0.01	0.01	0.03	1.00	0.01	0.18	1.00
Isobutanol	1.00	1.00	1.00	0.01	1.00	1.00	0.23	1.00	0.01	0.10
n-Butanol	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Acetic acid	0.01	0.01	1.00	0.20	0.09	0.01	0.01	1.00	0.01	0.01

Chapter 5

RESEARCH RESULTS

The Effect of Increased Yeast Branched-Chain Amino Acid Transaminase Activity and the Production of Higher Alcohols on the Flavor Profiles of Wine and Distillates

5. THE EFFECT OF INCREASED YEAST BRANCHED-CHAIN AMINO ACID TRANSAMINASE ACTIVITY ON THE PRODUCTION OF HIGHER ALCOHOLS ON THE FLAVOR PROFILES OF WINE AND DISTILLATES

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5.1 ABSTRACT

The *BAT1*- and *BAT2*-encoded branched-chain amino acid transaminases (BCAATases) of *Saccharomyces cerevisiae* catalyze the last step of the biosynthesis and the initial step of the degradation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine. This degradation leads to the formation of α-keto-acids, which are precursors for the biosynthesis of higher alcohols. Some of these yeast-derived higher alcohols can exert a pronounced influence on the flavor of wine and distillates. The objectives of this study were (i) to delete the *BAT1* and *BAT2* genes in a laboratory strain of *S. cerevisiae*, BY4742; (ii) to overexpress the *BAT1* and *BAT2* genes in strain BY4742, as well as in a widely used industrial wine yeast strain, VIN13; (iii) to assess the physiological and metabolic consequences of these modifications; (iv) to prepare table wine and base wines for distillation with the modified strains; and (v) to analyze and compare the concentrations of the higher alcohols and the aroma profiles of wines and distillates prepared with these transformants. As has previously been reported, the deletion of *BAT1* results in a slow growth phenotype and therefore only the *bat2*Δ strain, together with the *BAT1* and *BAT2* overexpression strains, were investigated. The results obtained indicated that the overexpression of *BAT1* increases the concentration of isoamyl alcohol and isoamyl acetate and, to a lesser extent, the concentration of isobutanol and isobutyric acid. The overexpression of the *BAT2* gene resulted in a substantial increase in the level of isobutanol, isobutyric acid and propionic acid production, while the deletion of this gene led to a decrease in the production of these compounds. Most of these changes remained unaffected by the various growth conditions investigated. Sensory analyses indicated that the wines and distillates produced with the strains in which the *BAT1* and *BAT2* genes were overexpressed had more fruity characteristics (peach and apricot aromas) than the wines produced by the wild-type strains. This study presents prospects for the development of wine yeast starter strains with optimized higher alcohol-producing capability that could assist winemakers in their endeavor to produce wine with specific flavor profiles

5.2 INTRODUCTION

The term "higher alcohol" refers to those alcohols that possess more than two carbon atoms and have a higher molecular weight and boiling point than ethanol. Higher alcohols, also known as fusel alcohols, are quantitatively the largest group of aroma compounds in many alcoholic beverages (Amerine *et al.*, 1980). They are recognized by a strong, pungent smell and taste and have a significant effect on the sensorial quality and character of wine and brandy (Rankine, 1967; Rapp and Mandery, 1986). Higher alcohols are composed of aliphatic and aromatic alcohols (Nykänen and Nykänen, 1977). The aliphatic alcohols include propanol, isobutanol, active amyl alcohol and isoamyl alcohol, while phenylethyl alcohol is considered to be one of the most important aromatic alcohol contributing to wine flavor. Isoamyl alcohol, active amyl alcohol and isobutanol are also known as branched-chain alcohols because they are the degradation products of the branched-chain amino acids, leucine, isoleucine and valine.

During fermentation, the wine yeast *Saccharomyces cerevisiae* produces flavor-active higher alcohols that originate from the degradation of imported branched-chain amino acids (BCAA) or from endogenous biosynthesis. The BCAA uptake in *S. cerevisiae* is mediated by at least three transport systems, i.e. the general amino acid permease, Gap1p, the branched-chain amino acid permease, Bap2p, and one or more unknown permeases (Didion *et al.*, 1996). The amino acids are converted to the corresponding α-keto-acids by transamination (α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine) (Dickinson and Norte, 1993). This transamination reaction is catalyzed by mitochondrial and cytosolic branched-chain amino acid aminotransferases (BCAAT), which are encoded by the *BAT1* and *BAT2* genes, respectively (Eden *et al.*, 1996, 2001; Kispal *et al.*, 1996). Alternatively, these α-keto-acids can be generated through the *de novo* synthesis pathway from glucose (Dickinson *et al.*, 1997). Branched-chain alcohols are then synthesized from the corresponding α-keto-acids by decarboxylation and reduction (Dickinson *et al.*, 1997). The first reaction is catalyzed by a pyruvate decarboxylase, which converts the α-keto-acid to the corresponding branched-chain aldehyde by the removal of one carbon atom. This is followed by a reaction catalyzed by an alcohol dehydrogenase that leads to the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol (Derrick and Large, 1993). Alternatively, the aldehyde might be oxidized in an NAD^+-dependent reaction to an acid (**Figure 5.1**). Therefore, it is likely that the redox state of a yeast cell is important for determining the fate of the α-keto-acid.

The physiological function of higher alcohol production by yeast is unclear, although many hypotheses have been postulated. One school of thought postulates that oxidative deamination provides the yeast cell with a mechanism for obtaining nitrogen when the pool has become depleted (Vollbrecht and Radler, 1973). A second hypothesis proposes that higher alcohol production contributes to the maintenance of the redox balance in the cell, since the final reduction step in higher alcohol production involves the reoxidation of $NADH + H^+$ to NAD^+ (Van Dijken and Scheffers, 1986; Quain, 1988; Zoecklein *et al.*, 1995). Others believe that the yeast cells contain enough acetaldehyde to maintain the redox balance and that the formation of higher alcohols is not considered to be an important means for the reoxidation of

NADH (Boulton *et al.*, 1995). Finally, it has been suggested that higher alcohol production might act as a detoxification process of the intracellular medium of α-keto-acids and aldehydes, or as a means of regulating the metabolism of amino acids (Ribéreau-Gayon *et al.*, 2000).

The objective of this study was to gain greater understanding of the role that the *BAT1*- and *BAT2*-encoded branched-chain amino acid transaminases (BCAATases) of *S. cerevisiae* play during alcoholic fermentation and their influence on the concentration of higher alcohols and the flavor profile of wine and distillates.

5.3 MATERIALS AND METHODS

5.3.1 MICROBIAL STRAINS, MEDIA AND CULTURE CONDITIONS

All yeast and bacterial strains used in this study and their relevant genotypes are listed in **Table 5.1**. *Escherichia coli* cells were grown in Luria-Bertani broth at 37°C (Sambrook *et al.*, 1989). *S. cerevisiae* cells were grown at 30°C in synthetic medium SCDSM [containing 0.67% yeast nitrogen base without amino acids (Difco), 0.13% of an amino acid stock solution (Ausubel *et al.*, 1994) lacking valine and isoleucine and supplemented with 0.5% glucose and 400 µg/l sulfometuron methyl (Dupont) per ml dissolved in *N*-,*N*-,dimethylformamide], as well as in a rich medium, YPD (containing 1% yeast extract, 2% peptone and 2% glucose). The SCD and SCR media contained 0.67% yeast nitrogen base without amino acids plus 60 µg/ml leucine, 20 µg/ml uracil, 20 µg/ml histidine and 30 µg/ml lysine, as well as either 4% glucose or 4% raffinose. SCD^{complete} and SCR^{complete} contained 0.13% amino acid stock (Ausubel *et al.*, 1994) and the SCD^{leu,ile,val} and SCR^{leu,ile,val} media contained the same as SCD^{complete} and SCR^{complete}, with an additional 100 mg/l leucine, isoleucine and valine. The SCD^{leu}, SCD^{ile}, SCD^{val} media were SCD to which 150 mg/l leucine, 135 mg/l isoleucine and 225 mg/l valine were added, respectively. Solid media contained 2% agar (Difco).

S. cerevisiae strains were precultured overnight in 50 ml of SCD or SCR media at 30°C. The cells were then inoculated into 120 ml of SCD, SCD^{complete} and SCD^{leu,ile,val} or SCR, SCR^{complete} and SCR^{leu,ile,val} at an optical density (measured at a wavelength of 600 nm; OD$_{600}$) of 0.1, and were grown aerobically or anaerobically at 30°C. The OD$_{600}$ value was determined and gas-chromatographic (GC) analysis was performed after 6 h (time point 1), 24 h (time point 2), 30 h (time point 3) and 48 h (time point 4) of growth. All experiments were performed in triplicate.

To assess the effect of amino acid concentration on the formation of higher alcohols, strains BY4742 and VIN13 were precultured overnight in 50 ml of SCD medium at 30°C. The cells were then inoculated into 120 ml of SCD^{leu}, SCD^{ile} and SCD^{val} at an OD$_{600}$ of 0.1 and grown aerobically at 30°C. The OD$_{600}$ value was determined and GC analysis was performed.

5.3.2 RECOMBINANT DNA METHODS AND PLASMID CONSTRUCTION

Standard procedures for the isolation of DNA were used throughout this study (Ausubel *et al.*,

1994). Restriction enzymes, T4 DNA ligase and Expand High-Fidelity DNA polymerase (Roche) were used in the enzymatic manipulation of DNA according to the specifications of the supplier.

The primers listed in **Table 5.2** were used to amplify the coding regions of the different genes by means of the polymerase chain reaction (PCR) technique. To identify possible cloning artifacts, all inserts were sequenced. Genomic DNA from the VIN13 strain was used as template for the amplification of the *BAT1* and *BAT2* coding sequences. A multicopy, episomal *S. cerevisiae-E. coli* shuttle vector pHVXII (Volschenk *et al.*, 1997), containing the promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences of the yeast phosphoglycerate kinase gene (*PGK1*), was used for subcloning the full-length *BAT1* and *BAT2* open reading frames (ORFs). PCR-generated 1182-bp *BAT1* and 1131-bp *BAT2* fragments were digested with *Eco*RI and *Xho*I and subcloned into pHVXII, thereby generating pBAT1-m and pBAT2-m. The *Hin*dIII-*Hin*dIII fragments containing either the $PGK1_P$-*BAT1*-$PGK1_T$ or $PGK1_P$-*BAT2*-$PGK1_T$ gene cassettes were obtained from the respective multicopy plasmids and inserted into the unique *Hin*dIII site of plasmid pDLG42, generating single-copy integrating *S. cerevisiae-E. coli* shuttle plasmids pBAT1-s and pBAT2-s (**Figure 5.2**). All plasmid inserts were sequenced and compared to the corresponding genomic sequence. Vector pDLG42 contained the dominant selectable *SMR1-410* marker gene (Casey *et al.*, 1988), a mutant allele of an endogenous gene of *S. cerevisiae* conferring resistance to the herbicide sulfometuron methyl, i.e. Sm^R. Plasmids pBAT1-s and pBAT2-s were linearized with *Apa*I in the *SMR1-410* terminator region for integration into the genome of the BY4742 laboratory strain and the VIN13 wine yeast strain.

5.3.3 TRANSFORMATION

All bacterial transformations and the isolation of DNA were carried out according to standard protocols (Ausubel *et al.*, 1994). Industrial wine yeast strains were transformed by means of electroporation. YPD (10 ml) was inoculated with yeast cells and the cells were incubated at 30°C until the stationary phase. A 500 ml volume of YPD was then inoculated with 10 ml of the preculture and incubated until the mid-logarithmic growth phase was reached (an OD_{600} of 1.3 to 1.5). The cells were then harvested, washed with 80 ml of sterile water, resuspended in 10 ml of a 10x TE-buffer, pH 7.5, and 10 ml of a 10x lithium acetate stock solution, and incubated at 30°C while shaking gently. After 45 min, 2.5 ml of 1 M dithiothreitol (DTT) solution was added and the cells were incubated for another 15 min. The solution was then diluted to 500 ml with water and centrifuged. The cells were first washed with 250 ml of ice-cold water and 30 ml of ice-cold 1 M sorbitol and then suspended in 0.5 ml of 1 M sorbitol. In a sterile, ice-cold 1.5-ml tube, 40 µl of the concentrated yeast cells were added to 5 – 15 µg of DNA and transferred to an ice-cold 0.4 cm gap electroporation cuvette. The EasyjecT + 450 V Twin pulse (EquiBio) apparatus was used for electroporation. The pulse program was as follows: voltage, 1500 V; capacity, 25 µF; shunt, 201 Ω. The yeast cells were then immediately plated out on SCDSM and incubated at 30°C for at least three days.

5.3.4 SOUTHERN BLOT ANALYSIS

Genomic DNA was isolated from the control (host) yeast strains, VIN13 and BY4742, as well as from the corresponding transformed *S. cerevisiae* strains VIN13(pBAT1-s), VIN13(BAT2-s), BY4742(pBAT1-s) and BY4742(pBAT2-s), using the standard method (Ausubel *et al.*, 1994). The genomic DNA of VIN13(pBAT1-s) and BY4742(pBAT1-s) was digested with *Eco*RV, while the genomic DNA of VIN13(pBAT2-s) and BY4742(pBAT2-s) was digested with *Eco*RV and *Stu*I. The DNA fragments were separated by agarose gel electrophoresis and transferred to a Hybond-N nylon membrane (Amersham), and Southern blot hybridization was performed using the DIG Luminescent Detection kit (Roche Molecular Biochemicals). The *BAT1* and *BAT2* ORFs were labeled with the digoxigenin molecule using PCR and then used as probes.

5.3.5 PRODUCTION AND ANALYSIS OF TABLE WINE

The wine yeast strains, VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s), were each inoculated (2×10^6 cells/ml) into 4.5 liters of Colombard grape juice and fermented at 15°C until dry (<1 g/l residual sugar). The wine was then 'cold stabilized', filtered and bottled according to standard practices for white wine production. All fermentations were done in triplicate and wine samples were scanned using a WineScan FT 120 instrument (Foss, Denmark) that employs a Michelson interferometer that was used to generate the FT-IR (*F*ourier *t*ransformation *i*nfra-*r*ed) spectra. The samples (7 ml) were pumped through the CaF_2-lined cuvette (optical path length 37 μm) that is housed in the heater unit of the instrument. The temperature of the samples was brought to exactly 40°C before analysis. Samples were scanned from 5011-929 cm^{-1} at 4 cm^{-1} intervals, which includes a small section of the near infra-red (NIR). The frequencies of the NIR beam transmitted by a sample were recorded at the detector and used to generate an interferogram. The latter is calculated from a total of 10 scans before being processed by Fourier transformation and corrected for the background absorbance of water to generate a single beam transmittance spectrum. Two transmittance spectra were generated for each sample in order to calculate the absolute repeatability of the spectral measurements. The calculation of the absolute repeatability has been described (WineScan FT120 Type 77110 and 77310 Reference Manual, Foss, Denmark, 2001). The transmittance spectra were finally converted to linearized absorbance spectra through a series of mathematical procedures.

5.3.6 BASE WINE PRODUCTION AND SMALL-SCALE DISTILLATION

Wine yeast strains VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) were inoculated (2×10^6 cells/ml) into 15 liters of Colombard grape juice to which no sulfur dioxide had been added and fermented at 15°C until dry (<1 g/l residual sugar). Fermentations were done in triplicate. Routine WineScan analysis was performed on the base wines just after alcoholic fermentation. Three 5-liter round-bottom flasks were each filled with 4.5 liters of base wine and yeast lees derived from the original 15-liter base wine fermentation volume. Two copper plates and 3 g of copper sulfate were added to the base wine and heated in heating mantles.

The distillation flow rate was maintained at 5 ml/min and the distillate was collected until 30% v/v alcohol was reached. The same procedure was followed with the second distillation, except that the first 40 ml (the so-called 'heads' fraction) of the distillate, collected at a flow rate of 2 ml/min, was discarded. The flow rate was then adjusted to 5 ml/min and the 'heart' fraction was collected until 70% v/v alcohol was reached.

5.3.7 GAS-CHROMATOGRAPHIC ANALYSIS

To each sample (10 ml of Colombard table or base wine), 0.8 ml of internal standard (4-methyl-2-pentanol, 230.2 mg/l, 12% (v/v) ethanol) and 6.5 ml of solvent (diethyl ether) were added. The tube was then mechanically rotated at 60 rpm for 30 min. The top ether layer was separated and the extracts were analyzed. For the 70% distillates, a 5 ml sample was taken and 0.25 ml of 4-methyl-2-pentanol (2 g/l, 70% (v/v) ethanol) was added. After mixing, 2 µl of the sample were injected into the gas chromatograph. The extractions were done in triplicate.

The analysis of volatile compounds was carried out on a Hewlett Packard 5890 Series II gas-chromatograph coupled to an HP7673 auto-sampler and injector and an HP 3396A integrator. The column used was a Lab Alliance organic coated fused silica capillary with dimensions of 60 m x 0.32 mm i.d., with a 0.5 µm coating thickness; hydrogen was used as the carrier gas for an FID detector held at 250°C. The injector temperature was 200°C, the split ratio was 20:1, the flow rate was 15 ml/min and the injection volume was 3 µl. The oven temperature program was as follows: 35°C (10 min) to 230°C (0 min) at 3°C/min. For the distillate analysis, the conditions were as described above, except for a different oven program and a 2 µl injection volume: 30°C (5 min) to 80°C at a speed of 2°C/min, and 80°C (0 min) to 230°C at 3°C/min. For each of the compounds measured, a specific amount was measured for the standard used to calibrate the machine. The internal standard and the chemicals were sourced from Merck (Cape Town, South Africa).

Table wine extractions were done after bottling. Extractions from the base wine were made after alcoholic fermentation, but before distillation. Samples from the distillates were taken after the second distillation. The isoamyl alcohol and active amyl alcohol compounds could not be separated with the extraction and GC analyses, which means that the isoamyl alcohol concentration throughout this paper also includes the active amyl alcohol concentration.

The results from the chemical analyses of the wines and distillates are presented in **Tables 5.3** to **5.8**.

5.3.8 SENSORY EVALUATION

The table wines and distillates were sensorially evaluated for different fruity aromas, as well as for flowery and solvent or chemical intensity, by a panel of six experienced judges. The wines and distillates were evaluated on a percentage scale from 0 to 100, where 0 represented the absence of a specific flavor and 100 represented a very high intensity of the flavor. The results from the sensorial analyses of the wines and distillates are presented in **Table 5.9**.

5.3.9 STATISTICAL ANALYSIS

The statistical differences between the GC results for the wines and the 70% distillates produced by the control and modified yeast strains were determined using the Bonferroni test, by which the ρ value is determined; if $\rho \leq 0.05$, the difference is significant. The sensory evaluation data were statistically evaluated by cluster analysis by determining the taster reliability and the ρ values for the different aroma properties.

5.4 RESULTS

5.4.1 CONSTITUTIVE EXPRESSION OF *BAT1* AND *BAT2* IN A LABORATORY (BY4742) AND INDUSTRIAL (VIN13) YEAST STRAIN

In order to determine the effect of the yeast branched-chain amino acid transaminases on the concentration of higher alcohols and the flavor profile of the wine and distillates, the *BAT1* and *BAT2* were cloned from a widely used commercial wine yeast strain, VIN13, and placed under the regulatory sequences of the constitutively expressed *PGK1* gene. The $PGK1_P$-$BAT1$-$PGK1_T$ and $PGK1_P$-$BAT2$-$PGK1_T$ gene cassettes were inserted separately into a yeast integrating plasmid to generate plasmids pBAT1-s and pBAT2-s (**Figure 5.2**). Linear copies of these *SMR1*-carrying plasmids were integrated into the genomic *ILV2* gene of the BY4742 laboratory strain and the VIN13 industrial strain, thereby generating transformants BY4742(pBAT1-s), BY4742(pBAT2-s), VIN13(pBAT1-s) and VIN13(pBAT2-s). The integration of a single copy of either the $PGK1_P$-$BAT1$-$PGK1_T$ or $PGK1_P$-$BAT2$-$PGK1_T$ gene cassette into each of these transformants was confirmed by Southern blot analysis (**Figure 5.3**).

In addition to the strains in which *BAT1* or *BAT2* were overexpressed, we also investigated BY4742 strains in which these two genes had been disrupted. However, only the BY4742*bat2*Δ strain was available from EUROSCARF, and we failed to generate a BY4742*bat1*Δ strain, presumably because the *bat1* deletion resulted in a non-viable strain. Therefore, we only assessed the effect of the disruption of *BAT2* and the overexpression of *BAT1* and *BAT2* on higher alcohol production.

5.4.2 EFFECT OF THE DISRUPTION OF *BAT2* AND THE OVEREXPRESSION OF *BAT1* AND *BAT2* ON FERMENTATION PERFORMANCE

The growth rate and final cell density (OD_{600}) of strains BY4742, BY4742(pBAT1-s), BY4742(pBAT2-s), BY4742*bat2*Δ, VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) were determined under various growth conditions, including aerobic and anaerobic conditions with either glucose or raffinose as a carbon source, and in the presence of different amino acid concentrations. In addition, the industrial strains VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s) were also assessed under winemaking conditions. No significant difference in either the fermentation rate or final cell numbers (OD_{600}) was observed between the wild-type strains (BY4742 and VIN13) and their modified equivalents (BY4742(pBAT1-s), BY4742(pBAT2-s), BY4742*bat2*Δ, VIN13(pBAT1-s) and VIN13(pBAT2-s)) under any of the conditions tested. Neither was there any significant difference in the concentration of

ethanol, glycerol, lactic acid, malic acid, volatile acids and total acids in the table and rebate wines produced with the modified VIN13 strains (**Table 5.7**). It therefore appears that neither the disruption of *BAT2* nor the overexpression of *BAT1* and *BAT2* had any obvious negative metabolic effect. The modification of *BAT1* and *BAT2* expression also did not seem to influence the fermentation performance of the wine yeast strain.

5.4.3 EFFECT OF THE DISRUPTION OF *BAT2* AND THE OVEREXPRESSION OF *BAT1* AND *BAT2* ON THE ACCUMULATION OF HIGHER ALCOHOLS.

The laboratory and industrial strains in which the *BAT1* and *BAT2* genes had been modified were tested under different culture conditions, i.e. (i) in media containing glucose and raffinose; (ii) with and without various additional amino acids; and (iii) under aerobic and anaerobic conditions. The data for each of the conditions are presented in **Tables 5.3** to **5.6**.

The data presented in **Table 5.3** suggest that isobutanol production by the laboratory strain BY4742 is not affected significantly by the growth conditions, since similar concentrations were found whether this strain had been grown aerobically or anaerobically, or with glucose or raffinose as sole carbon source. Compared to strain BY4742, a two-fold reduction in isobutanol production was observed when the strain BY4742*bat2Δ* was used in all of the tested conditions, suggesting that the Bat2p-dependent activity contributes significantly to isobutanol production. The contribution of *BAT2* appeared to be unaffected by glucose concentration or oxygen availability.

The overexpression of *BAT2* in BY4742 resulted in a significant increase in isobutanol concentration, further supporting the role of *BAT2* in the accumulation of this metabolite in *S. cerevisiae*. The overexpression of *BAT1* also resulted in increased levels of isobutanol concentrations, but the total amounts were significantly lower than in the case of *BAT2* overexpression. Contrary to the BY4742 reference laboratory strain, the levels of isobutanol produced were considerably higher when the *BAT1* and *BAT2* overexpression strains were grown anaerobically. The isobutanol concentrations increased 3.6- and 1.4-fold in BY4742(pBAT2-s) and BY4742(pBAT1-s), respectively, when grown aerobically, and 10.8- and three-fold when grown under anaerobic conditions. While the VIN13 transformants [VIN13(pBAT1-s) and VIN13(pBAT2-s)] followed a similar trend as the laboratory transformants [BY4742(pBAT2-s) and BY4742(pBAT1-s)] regarding the effect of the constitutive expression of *BAT1* and *BAT2* on isobutanol production, the strains produced two times less isobutanol when grown anaerobically compared to when they were grown aerobically.

When increased levels of valine, the direct precursor of isobutanol, were added to the media at two different concentrations, a significant increase in isobutanol concentration was observed in all of the strains and under most of the conditions used. The only exception was when the strains were grown anaerobically in a raffinose medium; the isobutanol concentrations remained approximately the same for all the strains, whether they were grown in the presence or absence of valine. The relative contribution of the overexpressed *BAT1* and *BAT2* genes to the increases in isobutanol production were unaffected by the addition of valine. Under the same growth conditions, VIN13, VIN13(pBAT1-s) and VIN13(pBAT2-s)

continued to produce two-fold less isobutanol when grown anaerobically in glucose or raffinose compared to when grown aerobically. Interestingly, BY4742 and BY4742*bat2Δ* showed a similar tendency under these conditions.

The isobutyric acid concentrations (**Table 5.4**) were affected similarly to the isobutanol concentrations by the different strains under all the conditions tested. However, in this case, the concentrations were significantly lower when grown anaerobically. This is most likely due to the fact that biosynthesis is an oxidative process that requires NAD^+.

There was no significant difference in isoamyl alcohol concentration produced by the BY4742 wild-type, overexpression [BY4742(pBAT2-s) and BY4742(pBAT1-s)] or deletion (BY4742*bat2Δ*) strains when they were grown in glucose or raffinose media, with or without oxygen (**Table 5.5**). However, a 1.5-fold decrease in isoamyl alcohol concentration was observed for the VIN13 wild-type and overexpression strains [VIN13(pBAT1-s) and VIN13(pBAT2-s)] when grown in raffinose compared to glucose medium. In comparison to VIN13, VIN13(pBAT1-s) showed a 1.4-fold increase in isoamyl alcohol concentration under all the conditions, and VIN13(pBAT2-s) and BY4742(pBAT2-s) showed a 1.3-fold increase in isoamyl alcohol concentration. VIN13(pBAT1-s) also produced higher isoamyl alcohol concentrations than VIN13(pBAT2-s) when grown anaerobically. The same tendencies were also observed when different leucine and isoleucine concentrations were assessed.

The isovaleric acid concentration remained approximately the same for the wild-type and *BAT1* overexpression strains under all of the culture conditions. A two- to three-fold increase in isovaleric acid was detected in the strains in which the *BAT2* gene was overexpressed and under all of the different growth conditions (**Table 5.6**), while a 1.3-fold decrease in isovaleric acid concentration was measured with the BY4742*bat2Δ* strain under all of the conditions.

To assess the effect of amino acid concentrations on the formation of higher alcohols, the BY4742 laboratory strain and the VIN13 industrial strain (data not shown) were grown aerobically in minimal media containing high concentrations of leucine, isoleucine and valine, respectively. The growth and the concentration of the aroma compounds were evaluated over a 54 h period (data not shown). Both yeast strains reached the stationary phase after 24 h. Interestingly, the two most important higher alcohols, isoamyl alcohol and propanol, as well as isobutyric acid and isovaleric acid, also reached a maximum concentration after 24 h and the concentration remained constant for the remainder of the time. However, the concentration of isobutanol and 2-phenylethyl alcohol increased throughout the monitored period, indicating continued production during the stationary phase.

After 54 h of growth, the media were analyzed using gas chromatography (**Figures 5.4-5.7**). The addition of high concentrations of valine to the medium (SCD^{val}) caused a 2.6- and 1.7-fold increase in isobutanol concentration, a 3.6- and five-fold increase in isobutyric acid concentration, a 1.8- and 1.4-fold increase in propanol concentration, and a 2.8- and 6.2-fold increase in propionic acid concentration in the VIN13 and BY4742 culture media, respectively, in comparison to the other two media, SCD^{leu} and SCD^{ile}. The isoamyl alcohol concentration increased 3.4- and 3.1-fold during growth in VIN13 and BY4742, respectively, when high concentrations of leucine were added to the medium (SCD^{leu}). The addition of

isoleucine to the medium (SCD^{ile}) also resulted in a 2.2- and 1.8-fold increase in isoamyl alcohol concentration compared to the medium (SCD^{val}) with added valine. The addition of high concentrations of leucine to the medium (SCD^{leu}) also caused a 1.3- and two-fold increase in 2-phenylethyl alcohol concentration in the VIN13 and BY4742 culture media, respectively. The addition of leucine (SCD^{leu}) and isoleucine (SCD^{ile}) to the media caused a similarly significant 3.8- and three-fold increase in isovaleric acid concentration compared to the medium (SCD^{val}) with added valine. The concentrations of the other compounds that were evaluated did not show any significant differences.

5.4.4 EFFECT OF THE OVEREXPRESSION OF *BAT1* AND *BAT2* ON AROMA COMPOUND CONCENTRATIONS IN WINE

The concentrations of certain esters, higher alcohols and acids that are important to wine aroma were determined for the Colombard table and base wines and the corresponding distillates (**Table 5.8**). The table wine fermented with VIN13(pBAT1-s) showed an overall decrease in ester concentration compared to the control wine. The concentration of 2-phenylethyl acetate, ethyl caprylate and hexyl acetate decreased 1.3-fold, the concentration of ethyl acetate and ethyl caproate decreased 1.4-fold and the ethyl caprate concentration decreased 1.6-fold compared to the concentration in the table wine fermented with VIN13. However, a 1.2-fold increase in isoamyl acetate concentration was obtained in the table wine fermented with VIN13(pBAT1-s) compared to the control wine. The table wine produced with VIN13(pBAT2-s) showed a slight increase in total ester concentration. The diethyl succinate concentration increased three-fold, ethyl acetate and hexyl acetate concentrations increased 1.2-fold and the concentration of ethyl lactate increased 1.6-fold in the wines fermented with VIN13(pBAT2-s). However, the isoamyl acetate concentration of the VIN13(pBAT2-s)-fermented table wine decreased 1.2-fold. The ester concentrations of the base wines did not differ significantly, except for a 1.3-fold increase in diethyl succinate concentration in both the wines fermented with VIN13(pBAT1-s) and VIN13(pBAT2-s) in comparison to the VIN13-fermented base wine. A considerable increase in isoamyl acetate concentration in the VIN13(pBAT1-s)-fermented base wine and a 1.4-fold decrease in isoamyl acetate concentration in the base wine fermented with VIN13(pBAT2-s) were also observed. These results correlated with those of the corresponding distillates.

The table wines, base wines and distillates produced with VIN13(pBAT2-s) contained the highest fusel alcohol concentration when compared to the products produced with VIN13(pBAT1-s), which, in turn, had a higher fusel alcohol concentration than the control wines and distillates. The table wine fermented with VIN13(pBAT1-s) showed a 1.5-fold decrease in 2-phenylethyl alcohol concentration, and a 1.4-fold, 1.8-fold and 4.5-fold decrease in hexanol, propanol and *n*-butanol concentration, respectively. These wines also presented a two-fold increase in isobutanol concentration and a 1.4-fold increase in isoamyl alcohol concentration. A 1.2- and 2-fold decrease in isoamyl alcohol and *n*-butanol concentrations were obtained in the table wines fermented with VIN13(pBAT2-s) when compared to the control wines. An increase in propanol, 2-phenylethyl alcohol and hexanol concentration, as well as a 14.5-fold increase in isobutanol concentration, was observed in the VIN13(pBAT2-

s)-fermented table wine in comparison to the control wines. A three- and 2.4-fold decrease in *n*-butanol concentration and a 1.7- and 9.7-fold increase in isobutanol concentration were detected in the base wines fermented with VIN13(pBAT1-s) and VIN13(pBAT2-s), respectively. A 1.4-fold increase in isoamyl alcohol concentration was measured in the base wines fermented with VIN13(pBAT1-s), and there was a decrease in isoamyl alcohol concentration in the VIN13(pBAT2-s)-fermented base wines. A decrease in 2-phenylethyl alcohol concentration was observed in both the wines fermented with the modified strains when compared to the control, there was a 1.3-fold decrease in propanol concentration in the VIN13(pBAT1-s)-fermented base wines and an increase in the propanol concentration of the VIN13(pBAT2-s)-fermented base wine. These results correlated with the data of the distillates of the corresponding base wines.

There was an overall decrease in acetic acid concentration in the wines and distillates produced with the transformed wine yeast strains. The isobutyric acid concentration increased between 1.2- and 1.7-fold in the products produced with VIN13(pBAT1-s) and between 5.1- and 5.8-fold in the VIN13(pBAT2-s)-derived products compared to those products produced with VIN13. The table wines fermented with VIN13(pBAT2-s) showed a 1.7-fold increase in isovaleric acid and an eight-fold increase in propionic acid concentration. The VIN13(pBAT1-s)-fermented table wines showed a 2.3-fold decrease in *n*-valeric acid concentration and a 2.8-fold increase in propionic acid concentration. Isovaleric acid, *n*-valeric acid and propionic acid were not detected in the distillates.

5.4.5 EFFECT OF THE OVEREXPRESSION OF *BAT1* AND *BAT2* ON THE FLAVOR PROFILE OF WINE

The overall fruity aroma was more prominent in the VIN13(pBAT1-s)-fermented table wine than in the control wine, but was detected least in the VIN13(pBAT2-s)-fermented table wine (**Figure 5.8**). The peach, apricot and banana flavors were more prominent in the wines produced with the VIN13(pBAT1-s) and VIN13(pBAT2-s) transformants than in the VIN13-fermented control wine. The estery, synthetic fruit and guava characters were also more intense in the table wine fermented with VIN13(pBAT1-s). A floral aroma was only detected in the VIN13-fermented table wine.

The 'smooth' and 'herbaceous' attributes were less intense in the distillates produced with VIN13(pBAT1-s) and VIN13(pBAT2-s) than in the VIN13-fermented control wine (**Figure 5.9**). Furthermore, the distillates produced from the base wines fermented with VIN13(pBAT1-s) and VIN13(pBAT2-s) had stronger fruity, peach, apricot, apple and synthetic fruit aromas than the control distillate.

5.5 DISCUSSION

In this study, we overexpressed the *S. cerevisiae BAT1* and *BAT2* genes in a laboratory yeast strain (BY4742) and in a widely used commercial wine yeast strain (VIN13), and assessed the effect of different carbon sources, amino acid concentrations and oxygen on the production of different aroma compounds under controlled laboratory conditions and during wine

fermentations. The data show that Bat2p plays an important role in isobutanol and isobutyric acid production and, indirectly, also affects isovaleric acid production (**Figures 5.4** to **5.7**). Bat1p also contributes to isobutanol and isobutyric acid production, but to a lesser extent than Bat2p. Under anaerobic conditions, Bat1p contributes to isoamyl alcohol production. Our data show that *S. cerevisiae* uses valine as a precursor in the production of isobutanol and isobutyric acid, and that valine indirectly also plays a role in propanol and propionic acid production. The amino acids leucine and isoleucine contribute directly to isoamyl alcohol production and indirectly to isovaleric acid production. Leucine also indirectly contributes to 2-phenylethyl alcohol production.

To assess the effect of oxygen on higher alcohol metabolism, the yeast strains were grown in aerobic and anaerobic growth conditions. Although acetate ester synthesis is greatly reduced by aeration (Fujii *et al.*, 1997), we found that the production of higher alcohols was in some cases affected by the presence or absence of oxygen. These differences sometimes were strain dependent. A possible explanation for the difference between the laboratory and industrial strains might be linked to the NAD^+/NADH ratios of the two strains. Bakker *et al.* (2001) found that quantitative differences in the redox metabolism among different *S. cerevisiae* strains could be significant. In addition, biomass formation is accompanied by a net production of NADH. The formation of 1 g of yeast dry biomass from glucose and ammonia is accompanied by the net reduction of 10 mmol NAD^+ to NADH (Verduyn *et al.*, 1990). The cell density (OD_{600}) was measured for the two strains during growth in the different conditions (data not shown) and the VIN13 strains produced three times more biomass when grown aerobically than when grown under anaerobic conditions. Since the reaction catalyzed by Bat1p and Bat2p requires NADH, this difference could explain why the VIN13 strains produced more isobutanol when grown aerobically. Indeed, the BY4742 strains produced similar amounts of biomass when grown under both conditions.

To assess the effect of different carbon sources on higher alcohol production, the cells were grown in media containing glucose or raffinose. The data show that glucose does not drastically affect higher alcohol production, but, interestingly, the fold difference in isobutanol and isobutyric acid concentration in the VIN13(pBAT1-s) and BY4742(pBAT1-s) strains compared to VIN13 and BY4742 was greater when they were grown in the raffinose-containing media than when grown in glucose-containing media, whereas, for the VIN13(pBAT2-s) and BY4742(pBAT2-s) strains, the fold difference was greater when they were grown in the glucose media (**Figures 5.4** to **5.7**). The data also indicate that the VIN13(pBAT2-s) and BY4742(pBAT2-s) strains produced a significant increase in isobutanol, isobutyric acid and isovaleric acid concentrations. When the *BAT2* gene was deleted, the BY4742*bat2Δ* strain still produced the same isoamyl alcohol concentration in the SCD and SCR media, but a slight decrease in concentration was observed in the other media, as well as decreased amounts of isobutanol, isobutyric acid and isovaleric acid compared to the control strain BY4742. When the *BAT1* gene is overexpressed in these strains, the VIN13(pBAT1-s) and BY4742(pBAT1-s) strains produced increased amounts of isobutanol and isobutyric acid, but less than when *BAT2* is overexpressed in these strains. VIN13(pBAT1-s) also produced an increased isoamyl alcohol concentration under all the

conditions, whereas BY4742(pBAT1-s) did not show an increase in isoamyl alcohol concentration under any of the growth conditions. With the VIN13(pBAT1-s) strain, the fold difference in isoamyl alcohol concentration under anaerobic conditions was greater than with the VIN13(pBAT2-s) strain under these conditions, but under aerobic conditions the fold difference in isoamyl alcohol concentration of VIN13(pBAT2-s) was greater than that of VIN13(pBAT1-s). It is therefore clear that the modification of the expression levels leads to results that are dependent on the genetic background of the strains.

The assessment of the effect of amino acid concentration on the formation of higher alcohols suggested that the addition of a high concentration of valine to the media increased the isobutanol, isobutyric acid, propanol and propionic acid concentrations significantly. The addition of leucine to the media increased isoamyl alcohol and 2-phenylethyl alcohol concentrations, and the addition of either leucine or isoleucine caused a significant increase in isovaleric acid concentration. The data therefore suggest that the addition of high concentrations of individual BCAAs to the growth medium results in increased levels of the corresponding higher alcohols and acids. However, increases in other higher alcohols were also observed, indicating some more complex metabolic interaction.

A significant increase in isoamyl acetate and isoamyl alcohol concentrations was detected in the wines fermented with VIN13(pBAT1-s). These wines and distillates also showed an increase in isobutanol and isobutyric acid concentrations when compared to the control wines fermented with VIN13. However, the wines and distillates produced with VIN13(pBAT2-s) showed a decrease in isoamyl acetate and isoamyl alcohol concentrations, but a significant increase in isobutanol and isobutyric acid concentrations when compared to the products produced by the control strains. With regard to this difference in isoamyl alcohol concentration between the two modified strains, in the case of *BAT2* overexpression the metabolic flux from α-keto-isovalerate could be in the direction of isobutanol, whereas in the *BAT1* overexpression strain the flux is shifted towards isoamyl alcohol and, subsequently, isoamyl acetate production (**Figure 5.10**). The table wine fermented with VIN13(pBAT2-s) also showed an increase in propanol, isovaleric acid and propionic acid concentrations, respectively. According to these results, it is possible to conclude that the overexpression of the *BAT1* gene in VIN13 resulted mainly in increased production of isoamyl acetate and isoamyl alcohol and, to a lesser extent, of isobutyric acid and isobutanol in the wines and distillates. It is also possible to state that the overexpression of the *BAT2* gene was mainly responsible for the production of isobutanol, isobutyric acid, propionic acid and, to a lesser extent, of propanol and isovaleric acid under winemaking conditions. The overexpression of the *BAT2* gene in VIN13 also resulted in a significant decrease in isoamyl acetate and isoamyl alcohol concentrations in the wines and distillates. The sensory evaluation showed that the wines and distillates produced with the modified strains had a stronger peach and apricot aroma; the wines also had a more prominent banana flavor and the distillates a stronger fruitier, apple aroma than the wines and distillates produced with VIN13. These fruity aromas could originate from the corresponding aldehydes.

The results of the sensory evaluation were unexpected, since the wine produced with the two transformed strains contained high levels of either isoamyl alcohol or isobutanol.

Although the concentrations of the respective aldehydes were not measured, it is tempting to speculate that the overexpression of *BAT1* and *BAT2* would also result in an increase in the corresponding aldehydes. The Oxford Chemicals website (www.oxfordchemicals.com) describes isobutyraldehyde, the precursor of isobutanol, as having an apple, banana and soft fruit aroma and isovaleraldehyde as being fruity and peachy. Acetaldehyde was also described as having an apple, peach, fruity and ester-like odor. These aromas, which were detected in the wines produced with the VIN13 transformants, could therefore be ascribed to the indirect increase in the corresponding aldehydes that are perceived as apple, banana, peach and soft fruit aromas. Considering both the different growth conditions and the fermentation data of the VIN13 wine yeast strain, it can be concluded that the *BAT1* gene is mainly responsible for the production of isoamyl alcohol from leucine and the indirect production of isoamyl acetate from the higher alcohol. Under the anaerobic growth conditions, the VIN13(pBAT1-s) strain produced higher amounts of isoamyl alcohol than VIN13(pBAT2-s) and, under the fermentation conditions, VIN13(pBAT1-s) was mainly responsible for the increase in isoamyl alcohol and isoamyl acetate concentrations. Overexpression of the *BAT1* gene also resulted in an increase in isobutanol and isobutyric acid concentrations, but to a lesser extent than that caused by *BAT2* overexpression. The *BAT2* gene might be mainly responsible for the production of isobutanol and isobutyric acid from valine and also play a role in the production of propanol and propionic acid, as well as of isovaleric acid, since the overexpression of this gene led to significant increases in these aroma compounds. The deletion of the *BAT2* gene in BY4742 led to a significant decrease in isobutanol, isobutyric acid and iso-valeric acid concentrations (**Figure 5.8**).

5.6 ACKNOWLEDGEMENTS

5.7 LITERATURE CITED

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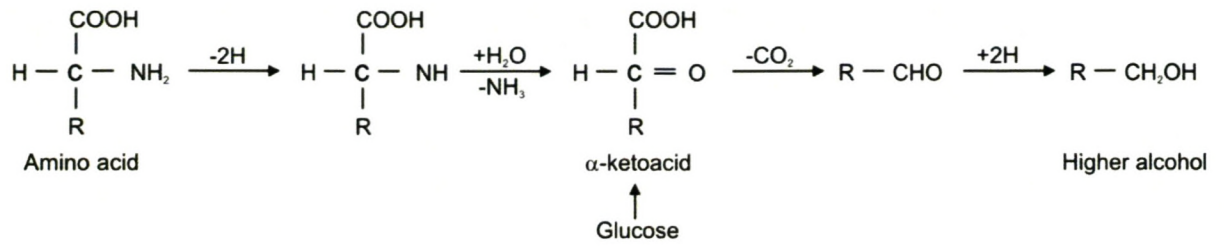
Figure 5.1. The Ehrlich pathway for higher alcohol production (Ehrlich, 1904).
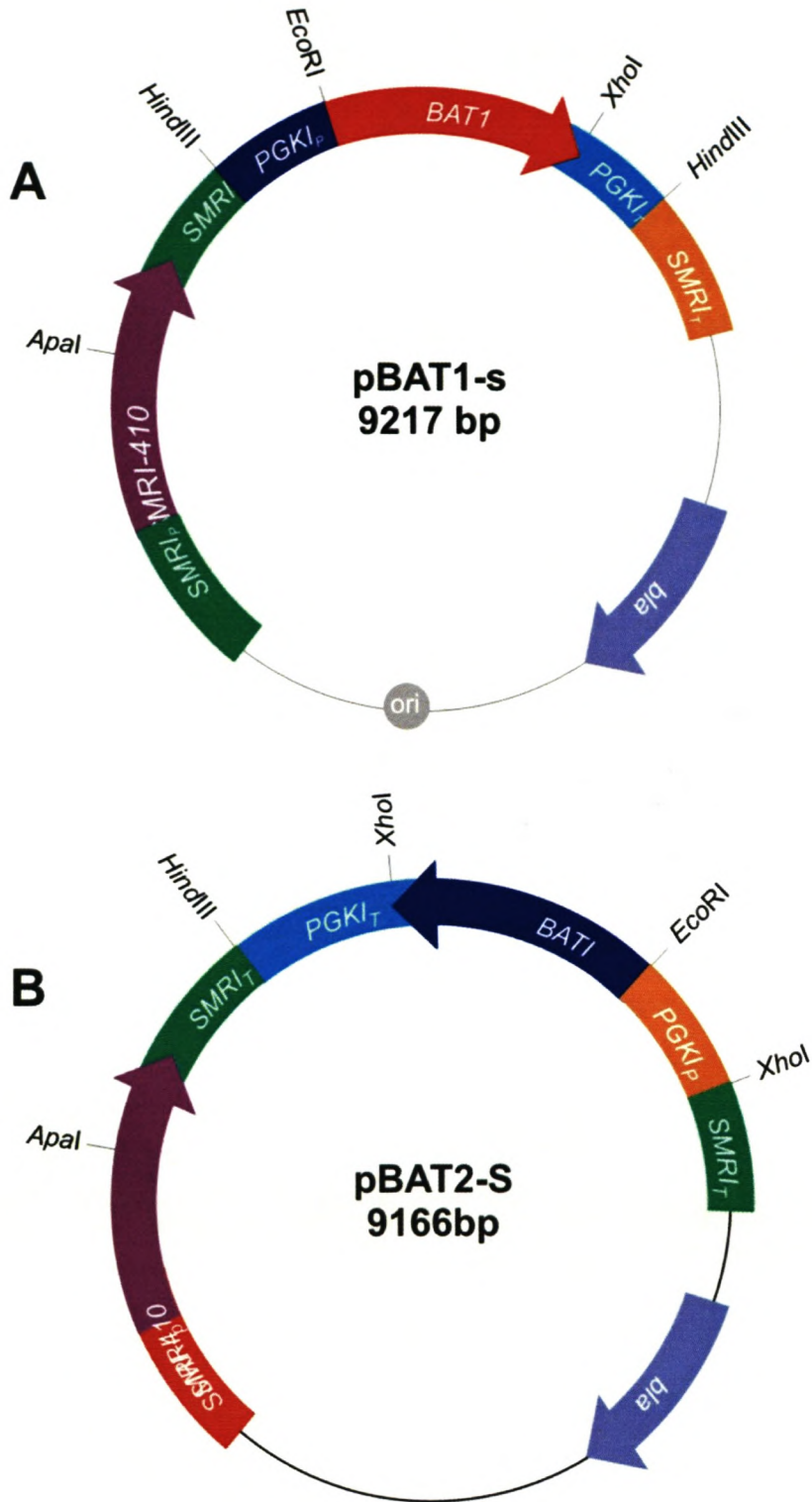
Figure 5.2. Restriction maps of the different gene constructs (*PGK1$_P$-BAT1-PGK1$_T$* and *PGK1$_P$-BAT2-PGK1$_T$*) and plasmids pBAT1-s and pBAT2-s.
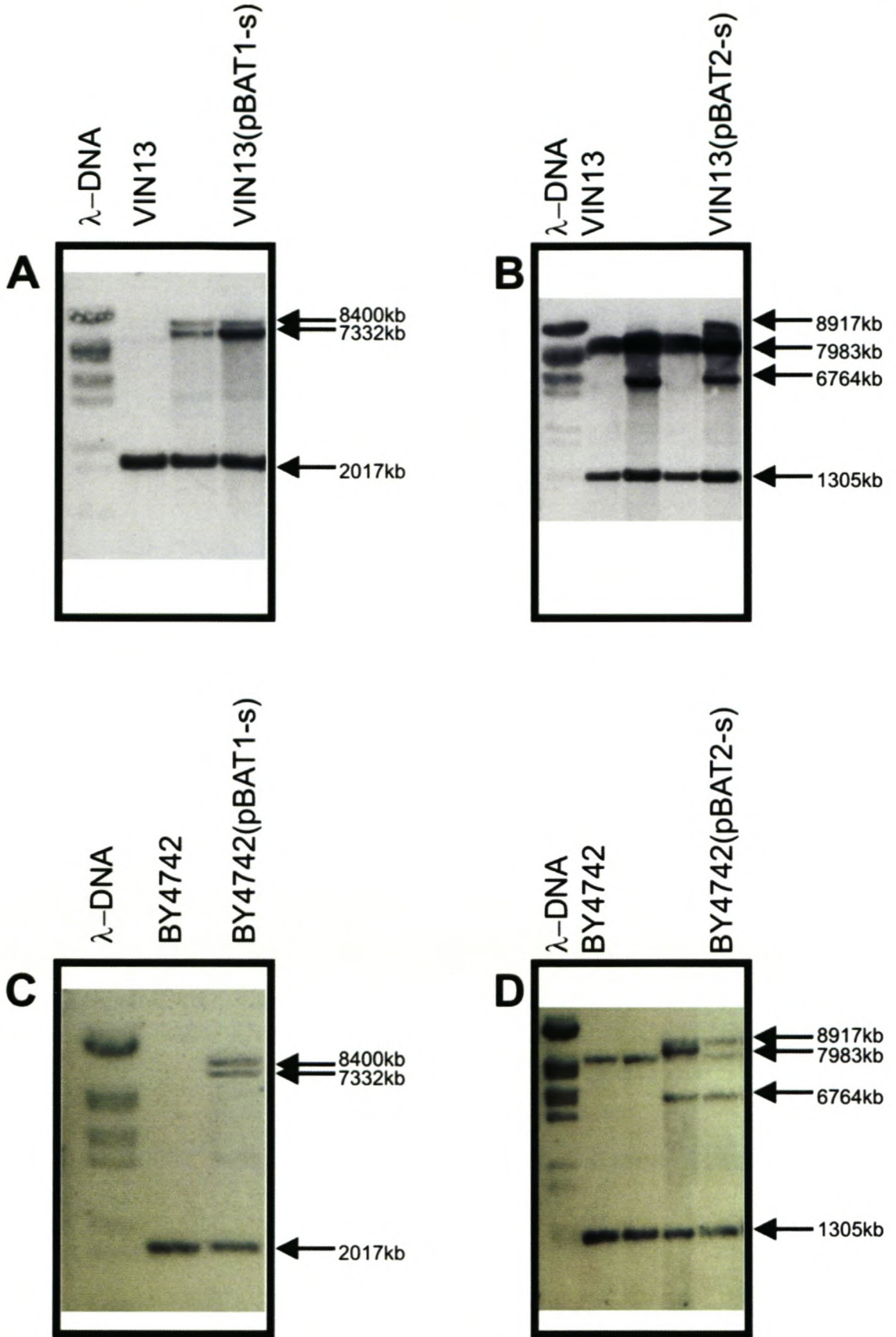
Figure 5.3. Southern blot analysis of the *BAT1* and *BAT*. Lanes were loaded with *Bst*EII-digested lambda DNA (lanes 1).
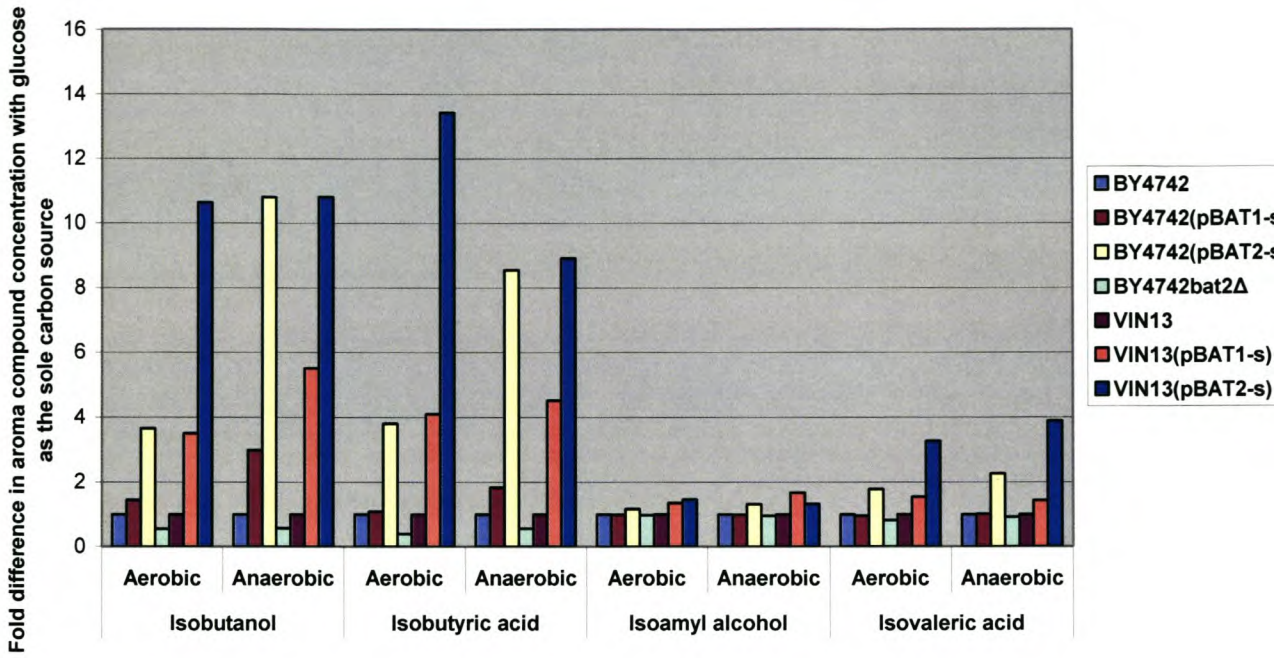
Figure 5.4. The fold difference in aroma compound concentration (mg/l) produced by the different strains with glucose as the sole carbon source.
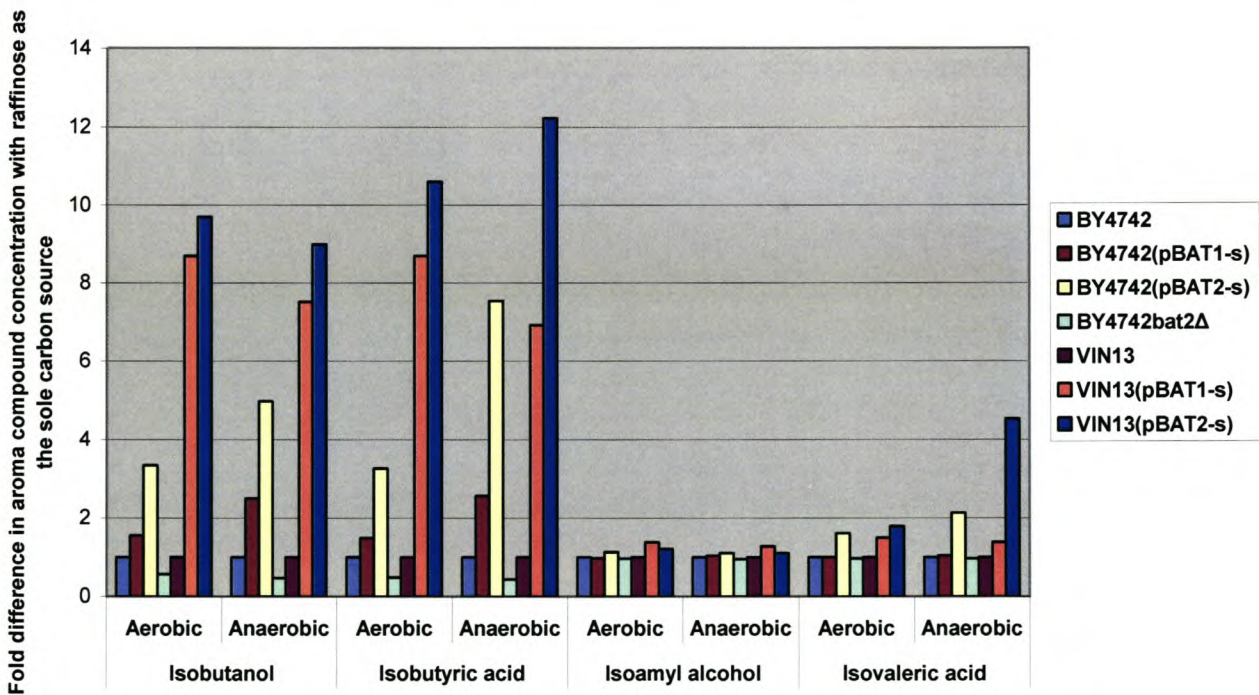
Figure 5.5. The fold difference in aroma compound concentration (mg/l) produced by the different strains with raffinose as the sole carbon source.
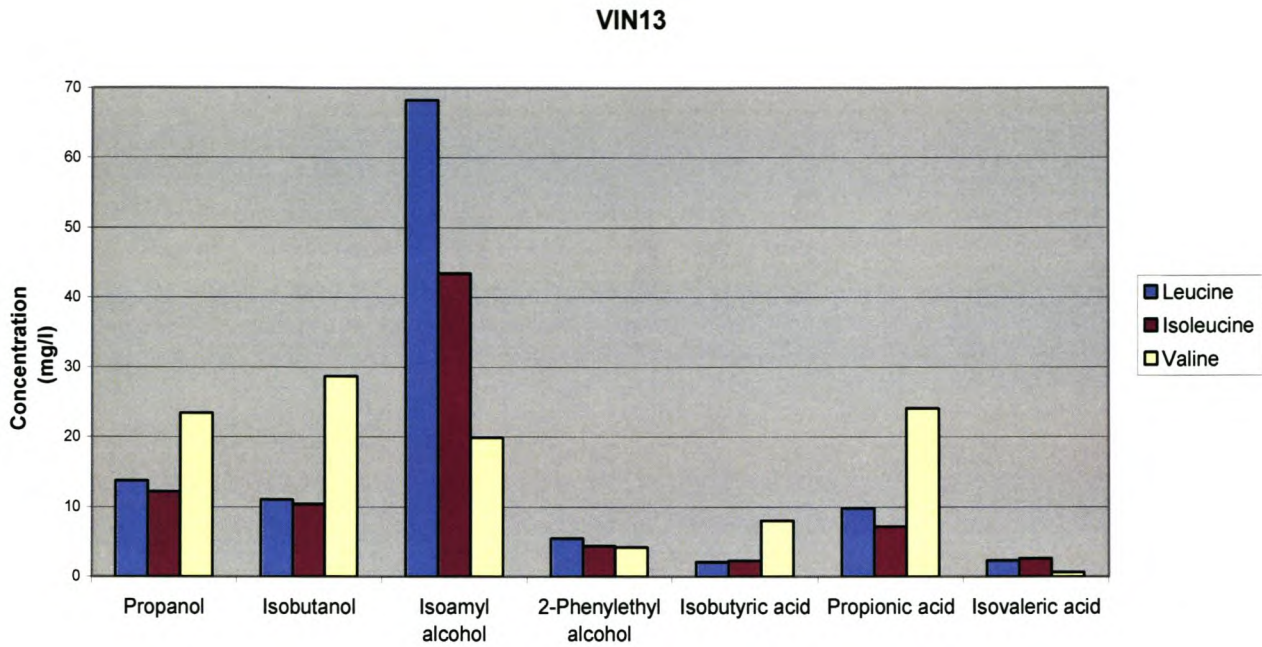
VIN13

Figure 5.6. Higher alcohols and fatty acids produced by VIN13 when specific amino acids were added to the fermentation media.

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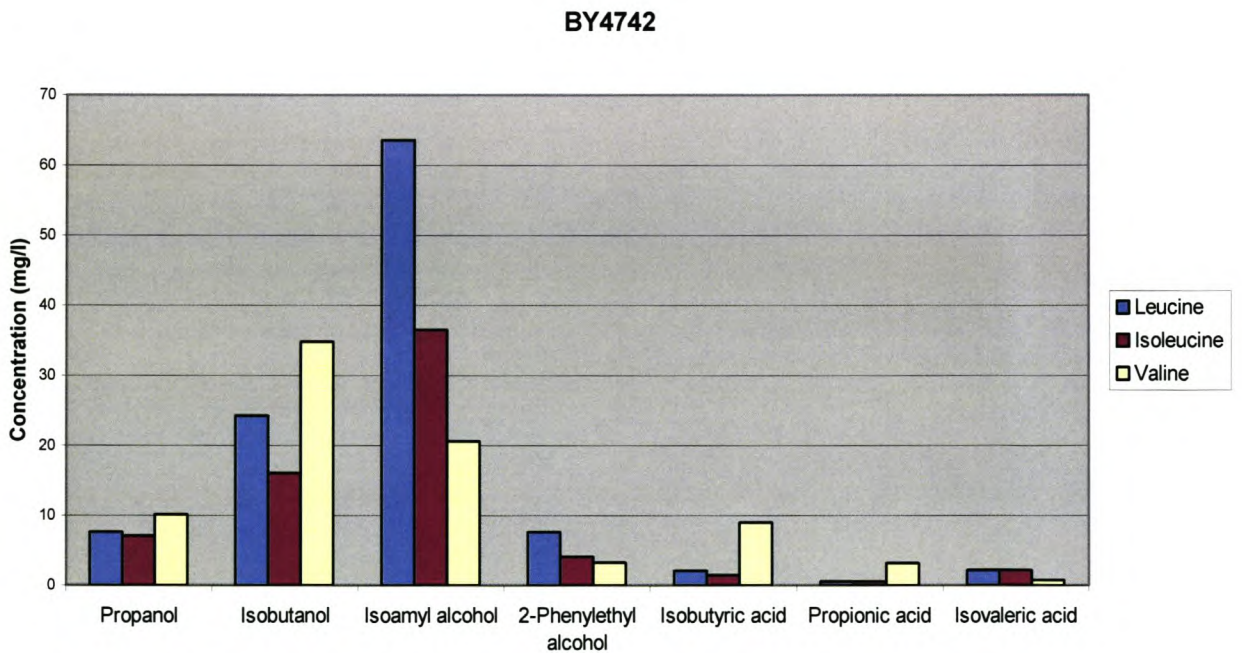
BY4742

Figure 5.7. Higher alcohols and fatty acids produced by BY4742 when specific amino acids were added to the fermentation media.
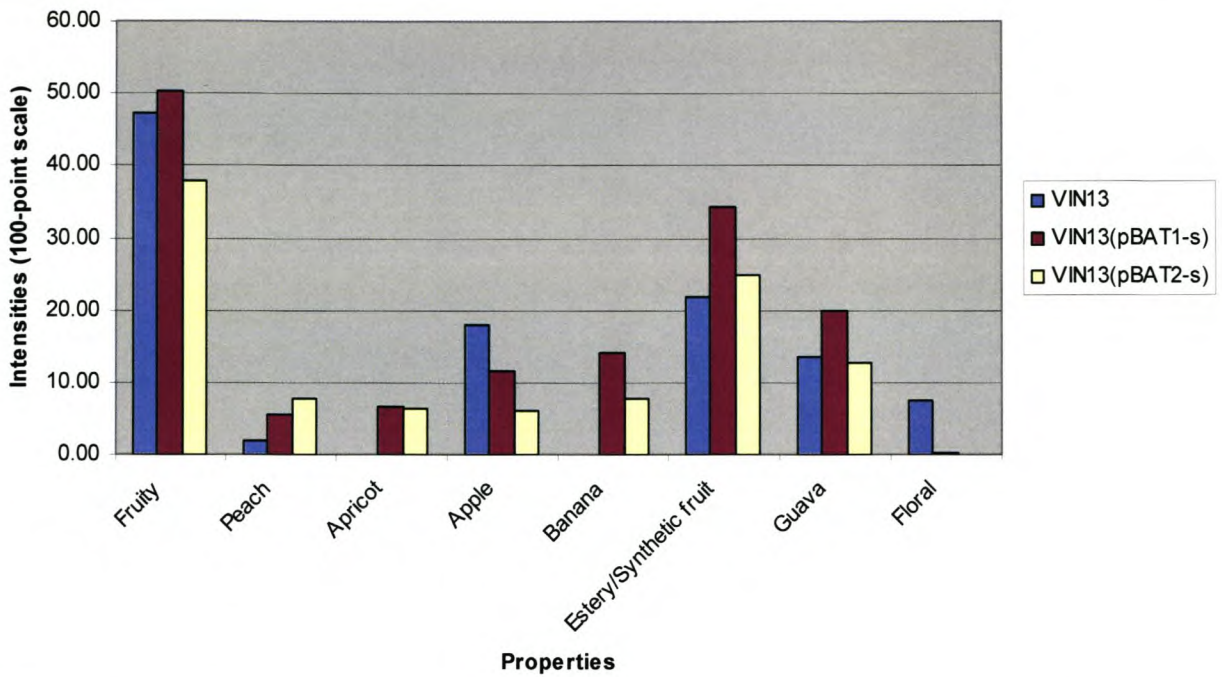
Figure 5.8. The aroma property intensities of the Colombard wine fermented with yeast strains VIN13 (control strain) and VIN13(pBAT1-s) and VIN13(pBAT2-s) (modified yeast strains).
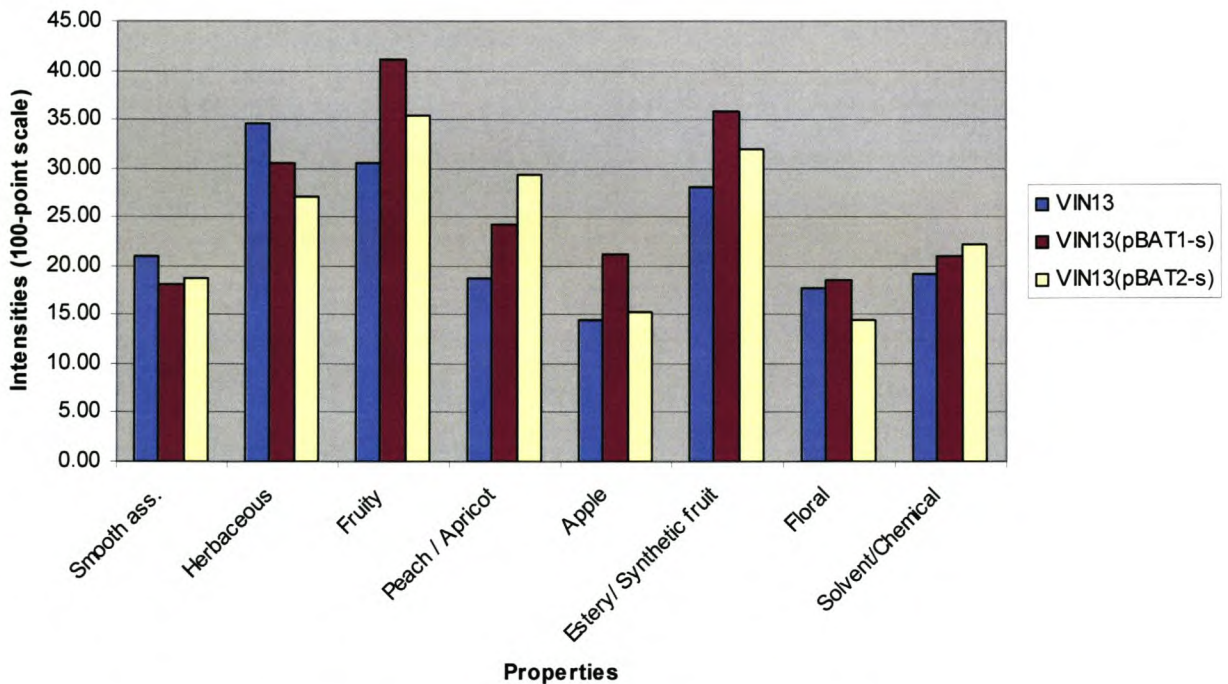
Figure 5.9. The aroma property intensities of the distillates, distilled from Colombard base wines fermented with yeast strains VIN13 (control strain) and VIN13(pBAT1-s) and VIN13(pBAT2-s) (modified yeast strains).
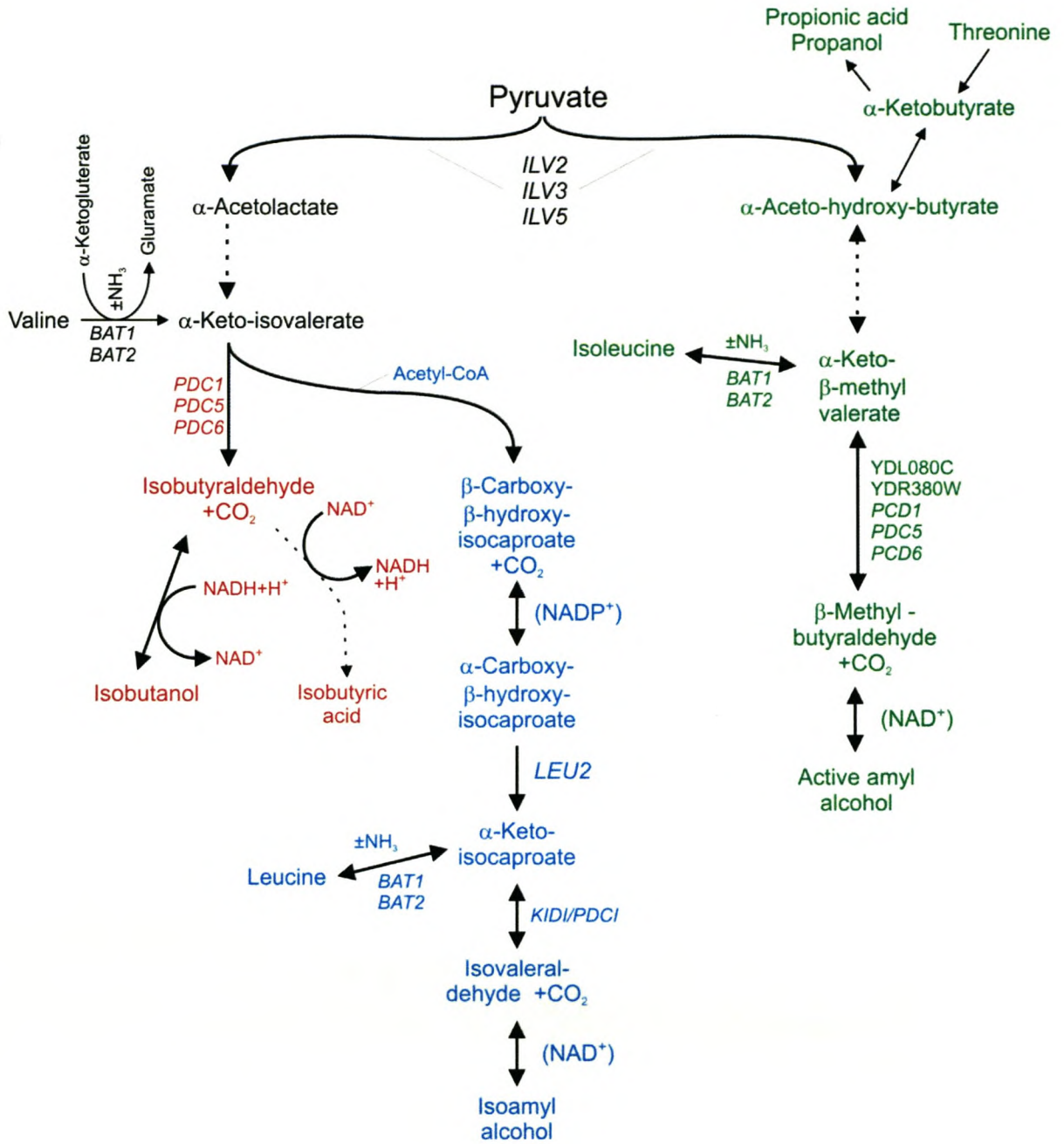
Figure 5.10. Hypothesis of higher alcohol production involving the *BAT1* and *BAT2* genes.

Table 5.1. Microbial Strains and Plasmids Used in This Study.

strain or plasmid	genotype or construct	reference or source
		GIBCO-BRL/Life Technologies
Escherichia coli DH5a	F' *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* D(*laclZYA-argF*)*U169 deoR* [F80d*lac* DE(*lacZ*)M15]	
Saccharomyces cerevisiae		
industrial strain		
VIN13	commercial wine yeast strain	Anchor Yeast, Cape Town, South Africa
transformants		
VIN13(pBAT1-s)	*SMR1-140 PGK1 $_P$-BAT1-PGK1 $_T$*	this study
VIN13(pBAT2-s)	*SMR1-140 PGK1 $_P$-BAT2-PGK1 $_T$*	this study
laboratory strain		
BY4742	*MATa his3D1 leu2D0 lys2D0 ura3D0*	Euroscarf
transformants		
BY4742(pBAT1-s)	*MATa his3D1 leu2D0 lys2D0 ura3D0 SMR1-140 PGK1 $_P$-BAT1-PGK1 $_T$*	this study
BY4742(pBAT2-s)	*MATa his3D1 leu2D0 lys2D0 ura3D0 SMR1-140 PGK1 $_P$-BAT2-PGK1 $_T$*	this study
BY4742Dbat2	*MATa his3D1 leu2D0 lys2D0 ura3D0 bat2D0*	Euroscarf
BY4742Dbat1	*MATa his3D1 leu2D0 lys2D0 ura3D0 bat1D0*	this study
plasmids		
pHVXII	*bla LEU2 PGK1 $_P$-PGK1 $_T$*	Volschenk *et al*. 1997
pDLG42	*bla SMR1-140*	Le Grange, 1999
pBAT1-s	*bla SMR1-140 PGK1 $_P$-BAT1-PGK1 $_T$*	this study
pBAT2-s	*bla SMR1-140 PGK1 $_P$-BAT2-PGK1 $_T$*	this study

Table 5.2. Primers Synthesized to Amplify the Genes.

primer name	sequence	enzyme
BAT1'F	5'-AATT**GAATTC**ATGTTGCAGAGACATTCCTTG-3'	*Eco* RI
BAT1'R	5'-TCGA**CTCGAG**TTAGTTCAAGTCGGCAACAGT-3'	*Xho* I
BAT2'F	5'-AATT**GAATTC**ATGACCTTGGCACCCCTAGAC-3'	*Eco* RI
BAT2'R	5'-TCGA**CTCGAG**TCAGTTCAAATCAGTAACAAC-3'	*Xho* I

* The enzyme sites are indicated in boldface and the region homologous to the corresponding genes are underlined

Table 5.3. Isobutanol Concentration (mg/l) Produced by the Different Strains in the Different Growth Conditions.

yeast strain	glucose (without valine or leucine addition; 59.76 mg/l leucine)		raffinose (without valine or leucine addition; 59.76 mg/l leucine)		glucose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		raffinose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		glucose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)		raffinose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
BY4742	11.13	9.87	8.69	9.74	47.86	34.83	19.02	12.65	26.39	19.25	14.31	6.96
BY4742(pBAT1-s)	16.08	29.41	13.48	24.39	57.24	72.06	29.80	30.24	38.32	56.14	28.05	24.95
BY4742(pBAT2-s)	40.60	106.64	29.03	48.47	76.00	149.01	64.32	40.61	73.70	126.87	68.65	36.52
BY4742Dbat2	6.09	5.64	4.94	4.61	36.28	20.43	12.26	7.81	14.03	10.52	8.34	3.47
VIN13	12.64	4.68	6.71	3.79	13.49	6.66	6.66	5.19	14.51	8.39	7.36	4.96
VIN13(pBAT1-s)	44.27	25.77	58.38	28.49	49.49	25.93	36.34	23.82	50.01	33.91	40.48	27.06
VIN13(pBAT2-s)	134.52	50.57	65.14	34.07	141.42	84.90	62.57	43.52	134.58	90.36	60.86	44.06

Table 5.4. Isobutyric Acid Concentration (mg/l) Produced by the Different Strains in the Different Growth Conditions.

yeast strain	glucose (without valine or leucine addition; 59.76 mg/l leucine)		raffinose (without valine or leucine addition; 59.76 mg/l leucine)		glucose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		raffinose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		glucose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)		raffinose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
BY4742	2.43	0.99	2.68	1.35	6.51	1.94	2.83	1.72	4.11	1.48	2.37	1.30
BY4742(pBAT1-s)	2.66	1.81	4.00	3.46	7.31	3.71	4.47	3.60	5.85	3.80	4.48	3.72
BY4742(pBAT2-s)	9.24	8.46	8.74	10.18	14.21	11.75	12.72	8.05	17.22	12.51	13.71	8.89
BY4742Dbat2	0.97	0.55	1.30	0.59	3.73	0.99	1.58	0.75	1.99	0.72	1.41	0.58
VIN13	2.51	0.61	2.72	0.51	3.09	0.87	2.31	0.87	3.51	0.76	2.81	0.83
VIN13(pBAT1-s)	10.27	2.75	23.66	3.53	13.07	2.44	13.70	4.19	13.23	3.52	17.22	5.56
VIN13(pBAT2-s)	33.72	5.44	28.83	6.24	44.70	8.62	30.32	9.53	44.82	9.32	32.13	9.70

Table 5.5. Isoamyl Alcohol Concentration (mg/l) Produced by the Different Strains in the Different Growth Conditions.

yeast strain	glucose (without valine or leucine addition; 59.76 mg/l leucine)		raffinose (without valine or leucine addition; 59.76 mg/l leucine)		glucose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		raffinose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		glucose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)		raffinose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
BY4742	27.78	29.32	27.88	28.60	13.28	13.37	16.25	7.45	22.39	17.68	29.98	11.90
BY4742(pBAT1-s)	27.53	29.11	27.28	29.62	12.43	11.42	14.29	6.97	21.28	16.05	29.21	11.62
BY4742(pBAT2-s)	32.65	38.68	31.38	31.65	18.10	21.35	24.62	10.42	36.83	29.75	51.67	18.46
BY4742Dbat2	27.17	28.25	27.04	27.25	9.89	9.20	12.78	6.05	15.29	16.75	21.82	9.70
VIN13	42.42	41.55	28.44	30.83	22.12	24.25	11.87	12.85	35.91	28.99	24.53	16.50
VIN13(pBAT1-s)	57.23	69.29	39.25	39.41	41.80	50.26	23.27	22.23	40.09	38.86	27.98	22.70
VIN13(pBAT2-s)	62.09	55.08	34.36	34.34	44.17	47.13	24.57	20.62	73.79	65.47	43.00	28.77

Table 5.6. Isovaleric Acid Concentration (mg/l) Produced by the Different Strains in the Different Growth Conditions.

yeast strain	glucose (without valine or leucine addition; 59.76 mg/l leucine)		raffinose (without valine or leucine addition; 59.76 mg/l leucine)		glucose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		raffinose (125.94mg/l valine; 50.38 mg/l leucine; 34.98 mg.l isoleucine)		glucose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)		raffinose (225.94mg/l valine; 150.38 mg/l leucine; 134.98 mg/l isoleucine)	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
BY4742	1.41	0.92	1.84	2.00	0.49	0.28	0.54	0.36	0.66	0.38	0.85	0.50
BY4742(pBAT1-s)	1.36	0.94	1.84	2.08	0.28	0.27	0.49	0.27	0.63	0.41	0.76	0.47
BY4742(pBAT2-s)	2.51	2.07	2.97	4.26	0.82	0.66	1.06	0.67	1.42	0.93	1.74	1.04
BY4742Dbat2	1.16	0.85	1.78	1.93	0.37	0.20	0.44	0.30	0.47	0.32	0.62	0.42
VIN13	1.26	0.75	2.75	1.19	0.72	0.34	0.88	0.45	1.68	0.42	2.04	0.58
VIN13(pBAT1-s)	1.94	1.07	4.07	1.64	1.38	0.70	1.58	0.83	1.57	0.63	2.47	0.92
VIN13(pBAT2-s)	4.11	2.92	4.91	5.39	2.25	0.93	2.55	1.12	3.93	1.33	4.59	1.47

Table 5.7. Routine FOSS Analysis of the Colombard White Wine After Bottling and the Colombard Base Wine After Alcoholic Fermentation but Before Distillation.

parameters	Colombard white wine after bottling.			Colombard base wine after fermentation, just before distillation.		
	VIN13	VIN13 (pBAT1-s)	VIN13 (pBAT2-s)	VIN13	VIN13 (pBAT1-s)	VIN13 (pBAT2-s)
pH	3.42	3.44	3.43	3.74	3.70	3.77
volatile acid g/l	0.26	0.28	0.25	0.32	0.23	0.28
total acids g/l	5.38	5.41	5.35	5.20	5.48	5.27
malic acid g/l	2.82	2.92	2.86	2.99	3.47	3.21
lactic acid g/l	-0.18	-0.13	-0.16	-0.07	-0.13	0.02
glucose g/l	-0.16	-0.07	-0.06	-0.06	0.48	-0.10
fructose g/l	2.08	2.80	2.35	1.27	0.55	0.94
glycerol g/l	5.48	5.64	5.40	5.28	5.28	5.10
ethanol %v/v	11.49	11.68	11.58	11.96	12.22	12.63

Table 5.8. Yeast Strain Effect on the Concentration of Major Volatiles in Colombard White Wines, Colombard Base Wines and the Respective 70% Distillates. Values in Bold Differ Significantly from VIN13 on a 5% Significance Level.

component	concentration (mg/liter)								
	Colombard white wine after bottling			Colombard base wine after alcoholic fermentation			70% distillate after second distillation		
	VIN13	VIN13 (pBAT1-s)	VIN13 (pBAT2-s)	VIN13	VIN13 (pBAT1-s)	VIN13 (pBAT2-s)	VIN13	VIN13 (pBAT1-s)	VIN13 (pBAT2-s)
acetic acid	109.62	68.91	129.62	212.29	149.63	141.81	14.82	**1.27**	**3.56**
decanoic acid	3.18	2.44	4.02	5.37	6.81	6.33	41.98	45.55	45.97
hexanoic acid	6.40	**4.96**	**8.35**	7.55	8.43	8.08	34.35	48.56	48.15
i-butyric acid	1.06	0.90	**5.66**	0.62	**1.06**	**3.58**	1.00	1.35	**5.09**
n-butyric acid	0.69	0.52	0.82	1.77	1.71	1.72	1.48	1.09	0.70
i-valeric acid	0.69	0.66	**1.19**	0.00	0.00	0.00	0.00	0.00	0.00
n-valeric acid	0.53	**0.23**	0.49	0.70	0.51	0.57	0.00	0.00	0.00
octanoic aicd	9.63	7.27	12.46	11.40	12.96	12.42	49.54	48.06	52.38
propionic acid	0.79	**2.18**	**6.31**	31.01	27.24	32.72	0.00	0.00	0.00
acetate	0.00	0.00	0.00	0.00	0.00	0.00	89.54	69.58	70.44
2-phenylethyl acetate	0.39	**0.29**	0.44	0.55	0.51	0.48	2.66	2.53	2.57
diethyl succinate	0.16	0.22	**0.48**	1.14	**1.44**	**1.45**	2.55	2.79	**3.31**
ethyl acetate	71.77	**52.38**	83.75	95.54	89.32	88.14	250.10	222.19	234.39
ethyl butyrate	0.00	0.00	0.47	0.31	0.53	0.53	0.70	0.37	1.05
ethyl caproate C6	1.86	**1.40**	2.19	2.14	1.99	2.02	8.96	8.02	8.95
ethyl caprylate C8	1.63	1.24	2.03	2.11	2.18	2.16	13.71	15.43	16.47
ethyl caprate C10	1.60	**1.02**	1.63	1.42	1.39	1.41	29.63	36.96	36.93
ethyl lactate	1.34	1.00	**2.15**	0.00	0.16	0.20	2.25	2.35	**3.08**
hexyl acetate	1.17	**0.89**	1.40	1.35	1.24	1.17	6.51	5.67	5.74
iso-amyl acetate	8.52	**10.13**	**6.83**	10.01	**14.52**	**7.13**	39.17	51.41	27.93
acetoin	0.63	0.91	2.01	0.00	0.00	0.16	0.00	0.00	0.00
methanol	24.57	19.48	**34.20**	43.41	37.94	39.95	216.87	209.57	218.71
2-phenylethyl alcohol	9.62	**6.60**	**10.62**	13.67	12.17	**11.80**	8.50	6.98	7.44
propanol	32.88	**18.14**	36.80	41.66	**32.73**	41.23	256.69	215.24	268.22
iso-butanol	5.62	**11.33**	**81.63**	8.87	**15.06**	**85.85**	61.60	**111.00**	**607.84**
n-butanol	0.98	**0.22**	**0.48**	1.15	**0.38**	**0.48**	6.56	**2.36**	**2.73**
iso-amyl alcohol	129.57	**177.92**	106.71	121.54	**163.09**	115.95	716.02	**1008.68**	701.63
hexanol	1.62	**1.14**	**1.97**	2.07	1.96	2.05	12.34	12.21	12.42

Table 5.9. Statistical Differences Between the Wines and Distillates Produced by the Control and Modified Strains with Respect to Certain Fermentation Bouquet Volatiles.

component	statistical difference					
	Colombard white wine after bottling		Colombard base wine after alcoholic fermentation		70% distillate after second distillation	
	VIN13 vs VIN13(pBAT1-s)	VIN13 vs VIN13(pBAT2-s)	VIN13 vs VIN13(pBAT1-s)	VIN13 vs VIN13(pBAT2-s)	VIN13 vs VIN13(pBAT1-s)	VIN13 vs VIN13(pBAT2-s)
acetic acid	1.00	1.00	1.00	1.00	0.01	0.01
isobutyric acid	1.00	0.02	0.04	0.01	1.00	0.01
n-butyric acid	1.00	1.00	1.00	1.00	1.00	1.00
isovaleric acid	1.00	0.01	nd	nd	nd	nd
n-valeric acid	0.01	1.00	1.00	1.00	nd	nd
propionic acid	1.00	1.00	1.00	1.00	1.00	1.00
2-phenylethyl acetate	0.01	1.00	1.00	1.00	1.00	1.00
diethyl succinate	1.00	0.05	0.03	0.02	1.00	0.04
ethyl acetate	0.01	0.07	1.00	1.00	1.00	1.00
ethyl caproate C6	0.05	0.62	1.00	1.00	1.00	1.00
ethyl caprate C10	0.01	1.00	1.00	1.00	1.00	1.00
ethyl lactate	0.13	0.01	1.00	0.32	1.00	0.01
hexyl acetate	0.04	0.19	1.00	1.00	1.00	1.00
isoamyl acetate	0.05	0.01	0.01	0.01	1.00	1.00
methanol	0.69	0.01	1.00	1.00	1.00	1.00
2-phenylethyl alcohol	0.01	0.05	0.27	0.05	1.00	1.00
propanol	0.01	0.70	0.04	1.00	0.38	1.00
isobutanol	0.01	0.01	0.01	0.01	0.01	0.01
n-butanol	0.01	0.01	0.01	0.01	0.01	0.01
isoamyl alcohol	0.01	0.07	0.01	1.00	0.01	1.00
hexanol	0.01	0.01	1.00	1.00	1.00	1.00

Chapter **6**

GENERAL DISCUSSION AND CONCLUSIONS

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

The end of the twentieth century was marked by major advances in life science technology, particularly in areas related to genetics and genomics. Considerable progress was made in the development of genetically modified (improved) yeast strains for the wine, brewing and baking industries. Recombinant DNA technology widened the possibilities for introducing new characteristics into the existing yeast strains and thereby improving their performance. Although public acceptance considerations remain the major obstacle to the commercialization of genetically modified industrial yeast strains, the number of potential applications is likely to increase in the coming years as our knowledge derived from genomic analyses increases. The successful commercialization of genetically modified industrial yeasts also depends on a multitude of scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues. Although genetically modified industrial strains are being constructed according to the general requirements for genetically modified organisms, particularly regarding the absence of resistance markers and the stability usually obtained by chromosomal integration, efforts to increase public awareness of the potential benefits of recombinant DNA technology need to be enhanced.

Yeasts play a central role during the production of wine, brandy and other fermented alcoholic beverages. The yeasts, mainly of the species *S. cerevisiae*, are responsible for the conversion of grape sugars to ethanol and, traditionally, this function was performed spontaneously by the microorganisms present in the grape must after pressing. However, because of the economic risks and poor reproducibility associated with this practice, commercial active dried wine yeast strains with reliable fermentation characteristics were developed. In addition to the fermentation property, these yeasts also contribute to the sensorial quality of the alcoholic beverage. Several hundred volatile compounds contribute to the aroma of wine and brandy. With respect to their origin, these aroma compounds are divided into four categories: (i) the primary aroma compounds that originate from the grapes; (ii) the precursors from the grapes that are transformed into aroma compounds during fermentation; (iii) the secondary aroma compounds produced by the yeast and other microorganisms during the primary fermentation; and (iv) the tertiary aroma compounds that are formed as a result of transformations during aging. The fruitiness of wine and brandy has been shown to be largely attributed to esters produced by the yeast during fermentation (Engan, 1974).

Esters that impart fruity aromas to alcoholic beverages are the most important contributors to the fermentation bouquet. The fruity odors are due to a mixture of ethyl acetate (fruity/solvent-like aroma), isoamyl acetate and isobutyl acetate (banana flavor), ethyl caproate and ethyl caprylate (apple aroma), and hexyl acetate and 2-phenylethyl acetate (fruity, flowery flavor with a honey note) (Piendl and Geiger, 1980). The synthesis of these acetate esters in yeast is catalyzed by alcohol acetyltransferases and utilizes available alcohols and acetyl-CoA (Nordström, 1961). Thus far, four different alcohol acetyltransferases have

been cloned, namely *ATF1* (Fujii *et al.*, 1994), *ATF2* (Nagasawa *et al.*, 1998), *LgATF1* (Yoshimoto *et al.*, 1998) and *EHT1* (Mason and Dufour, 2000).

By using genetic manipulation techniques, it has become possible to drastically alter the aroma and flavor of an alcoholic beverage. Lilly *et al.* (2000) investigated the effect of increased alcohol acetyltransferase activity on the sensory quality of Chenin blanc wines and distillates from Colombard base wines by the overexpression of the *ATF1* gene in the commercial wine yeast strain of *S. cerevisiae*, VIN13. The levels of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate in the wines increased three- to 10-fold, 3.8- to 12-fold and two- to 10-fold respectively. The concentrations of ethyl caprate, ethyl caprylate and hexyl acetate only showed minor changes, whereas the acetic acid concentration decreased by more then half of that of the control fermentation. These changes had a pronounced effect on the solvent or chemical aroma (associated with ethyl acetate and isoamyl acetate) and the herbaceous and heads-associated aromas of the final distillate, and on the solvent or chemical and fruity or flowery characters of the Chenin blanc wines. This study clearly demonstrated that, by manipulating a single yeast gene, one could alter the ester production profile significantly during wine fermentation, thereby adjusting the aroma profile of wine and brandy considerably.

In this study we therefore overexpressed the other two alcohol acetyltransferases genes, *ATF2* and *EHT2*, in the commercial wine yeast strain VIN13. We found that the overexpression of the Atf2p caused a 1.3-, 1.5, and 1.8-fold increase in ethyl acetate, isoamyl acetate and 2-phenylethyl acetate concentrations, respectively, and also slightly increased ethyl caproate, ethyl caprate and hexyl acetate concentrations, but had no effect on ethyl caprylate production. The smaller increase in ethyl acetate and isoamyl acetate in the beverages produced with VIN13(pATF2-s) compared to those produced with VIN13(pATF1-s) might be advantageous to the wine and brandy industry, since it resulted in other fruity aromas being more prominent. The Colombard white wines fermented with VIN13(pATF2-s) had an intense mixture of apple, banana and guava aromas, while a more apricot, apple, peachy flavor was detected in the distillates. The overexpression of the *EHT1* gene caused the levels of all the esters that were evaluated to increase slightly, but the most significant were the 1.4- and 1.5-fold increase in ethyl caprate and ethyl caprylate concentrations, respectively. The wines also presented the definite apple aroma that is usually associated with these esters.

Esters can also be synthesized by esterases, although esterases function mainly by hydrolyzing esters (Peddie, 1990). Fukuda *et al.* (1996) isolated and characterized the *IAH1* gene and concluded that the Iah1p is likely to play a role in isoamyl acetate hydrolysis. In this study, we overexpressed the *IAH1* gene in VIN13 and found that it had a drastic effect on ester hydrolysis. In all of the wines and distillates, hexyl acetate was completely hydrolyzed and an 11.4-fold decrease in isoamyl acetate concentration was observed. 2-Phenylethyl acetate and ethyl acetate concentrations were also decreased, by 3.3- and 1.6-fold, respectively. The overexpression of Iah1p also caused a slight decrease in ethyl caprate, ethyl caprylate and ethyl caproate concentrations. The sensory evaluation of the wines and distillates produced with VIN13(pIAH1-s) still showed strong fruity aromas for the products,

which again illustrates the complexity of flavor chemistry and perception. Horsted *et al.* (1998) isolated the *TIP1* gene and concluded that it is the structural gene for an esterase. However, the overexpression of the *TIP1* gene in the wine yeast strain VIN13 resulted in only a slight decrease in ethyl acetate, 2-phenylethyl acetate, ethyl caproate, ethyl caprylate and ethyl caprate. This could suggest that Tip1p is either inactive under the conditions used, or has a different substrate affinity to that of Iah1p.

Higher alcohols are quantitatively the largest group of aroma compounds in alcoholic beverages and are recognized by a strong, pungent smell and taste. They have a significant effect on the organoleptic quality and character of wine and brandy (Rapp and Mandery, 1986). Higher alcohols are also secondary products produced by *S. cerevisiae* during fermentation. Branched-chain amino acid transferases catalyze the last step of the biosynthesis and the initial step of the degradation of branched-chain amino acids in the higher alcohol metabolic pathway. The *BAT1* and *BAT2* genes encode the mitochondrial and cytosolic branched-chain amino transferases, respectively (Eden *et al.*, 1996, 2001; Kispal *et al.*, 1996). In this study, we also overexpressed the *S. cerevisiae BAT1* and *BAT2* genes in VIN13 and laboratory strain BY4742 and assessed the effect on aroma compound production during fermentation, as well as the effect of different amino acid concentrations on aroma compound production. The VIN13(pBAT1-s) strain resulted in a significant increase in isoamyl acetate, isoamyl alcohol, isobutyric acid and isobutanol concentrations. The wines produced with VIN13(pBAT2-s) showed an increase in isobutanol, isobutyric acid, propanol, propionic acid and isovaleric acid concentrations, but a decrease in isoamyl acetate and isoamyl alcohol. The sensory evaluations also showed that the wines and distillates had a fruitier peach and apricot aroma than the control strain. We also found that, when valine was added to the media, the concentrations of isobutanol, isobutyric acid, propanol and propionic acid increased significantly. When leucine was added to the media, the concentrations of isoamyl alcohol and 2-phenylethyl alcohol increased. On the basis of all of these results, we concluded that the Bat1p is mainly responsible for the production of isoamyl alcohol from leucine and that the Bat2p is mainly responsible for the production of isobutanol and isobutyric acid from valine and, to a lesser extent, the production of propanol, propionic acid and isovaleric acid.

The data show that the targeted modification of specific enzymes in wine yeast strains can result in new strains with specifically modified aroma compound production capacity without affecting the other enologically important characteristics of the parental wine yeast strains. These strains could be used by winemakers and distillers as a powerful tool with which to adjust the flavor profiles of their products in order to satisfy the different sensory preferences of consumers. These strains could also be used in studies to further unravel the complex ester and higher alcohol metabolism of *S. cerevisiae*, thereby supplying further knowledge to manipulate aroma compound production during wine fermentations. Therefore, from a practical point of view, this study lays the foundation for further investigations of how the expression of the abovementioned genes could be optimized in wine yeast for the enhancement of the aroma of wine.

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(2.4) met 'n skoon kantruimte van minstens 2 cm breed aan die linkerkant en 1 cm aan die ander drie kante.

(3) Kopieer en Sorteer van meesterswerkstukke, tesisse en doktorale proefskrifte

(3.1) Die fotokopieermasjien doen die sortering outomaties. Kandidate moet egter toesien dat die volgorde korrek is en hulle tevrede is met die kopiëring van die teks. Indien dit vir die kandidaat onmoontlik is om self te sorteer, moet die kandidaat die Drukkery voorsien van die naam en kontaknommer van 'n persoon wat dit namens hom/haar sal behartig. Die US Drukkery sal met die aangewese persoon in verbinding tree sodra die kopiëring afgehandel is.

(3.2) Werkstukke, tesisse en proefskrifte wat elders gekopieer is, moet steeds by die US Drukkery ingehandig word voor die sluitingsdatums vir inhandiging in Februarie en November.

(3.3) Kleurafdrukke word tydens die sorteringsproses ingevoeg.

(3.4) Die outomatiese kopieermasjien kan nie ingeplakte materiaal soos foto's hanteer nie. Foto's moet dus verwyder word wanneer die manuskrip vir kopiëring en bind ingehandig word. Hierdie foto's word weer ingevoeg nadat bindwerk voltooi is.

(4) Indien daar van adres verander word, moet die Drukkery sonder verwyl daarvan verwittig word.

PRYSE AANGAANDE MEESTERSWERKSTUKKE, -TESISSE EN DOKTORALE PROEFSKRIFTE

	Prys per eenheid	Hoeveelheid kopieë	R
DRUKWERK			
Fotokopieë (A4)	0.22		
Rug aan rug (A4)	0.40		
BINDWERK			
Hardeband (gouddruk)	75.00		
Sagteband (skoon omslag)	5.00		
MICROFICHE			
Die prys word deur die aantal bladsye bepaal. Slegs een mikrofiche word gemaak. (Prys is op aanvraag by die US Drukkery verkrygbaar)			
Subtotaal			
BTW (14%)			
TOTAAL			

Sodra die werkstuk, tesis of proefskrif meer as 6 ingeplakte foto's (A4) of enige aantal kaarte (A3) bevat, moet die rugkant van die uiteindelike dokument opgebou word. Dit behels 'n addisionele koste van R10.00 per eksemplaar.

Onmiddellike betaling in kontant, per tjek of departementele rekwisisie word vereis wanneer manuskrip vir die fotokopiëring en bindwerk ingehandig word. Kredietkaarte word ook aanvaar. Alle tjeks moet aan die *Universiteit Stellenbosch* uitgemaak word.

MEESTERS EN DOKTORALE KANDIDATE – PERSOONLIKE GEGEWENS

1. Gebruik hoofletters.

2. Handig voltooide vorm met die oorspronklike weergawe van die werkstuk, tesis of proefskrif vroegtydig in, voor of op die sluitingsdatum vir die gradeplegtighede in Maart en Desember. Raadpleeg die Algemene Jaarboek vir nadere besonderhede. (Sluitingsdatums is gewoonlik in Februarie en November.)

[Let Wel: Om verwarring te voorkom, maak seker dat die voorletters op die vorm ooreenstem met dié op die studentekaart.]

VAN: | L | I | L | L | Y | | | | | | | | | | |

VOORLETTERS: | M | | |

TITEL: Mnr | Me | Dr | Prof | Ds

STUDENTENOMMER: | 1 | 1 | 5 | 7 | 9 | 7 | 3 | 0 |

POSADRES: Mooizicht singel no. 6
Uitzicht
Durbanville

KODE: 7550

TELEFOONNOMMER MET KODE:

(h) | 0 | 2 | 1 | 9 | 7 | 6 | 7 | 7 | 1 | 7 |

(w) | | | | | | | | | | |

(sel) | 0 | 8 | 2 | 8 | 5 | 9 | 4 | 7 | 6 | 8 |

E-POS ADRES: 24006l551@ctech.ac.za

DEPARTEMENTELE ADRES: _____

KODE: _____

DEPARTEMENTELE TELEFOONNOMMER: | | | | | | | | | | |

STUDIELEIERS/PROMOTORS:

1. Naam: Prof. I.S. Pretorius Departement: Australian Wine Research Institute

2. Naam: Prof. F.F. Bauer Departement: Wynbiotegnologie

Prof. M.G. Lambrechts Distell

blaai om

BESTELVORM

JS GERICKE BIBLIOTEEK: 1 hardeband en 1 sagteband (verpligtend)
STUDIELEIERS/PROMOTORS/EKSAMINATORE: hardeband (verpligtend)
KANDIDAAT (opsioneel) hardeband

	2
	7
	2

Totale aantal bladsye [125]   Laaste bladsynommer [106]   Aantal kleurafdrukke (A4) [ ]

AANTAL HARDEBANDE (Nagemaakte leer met gouddruk):   TOTAAL: [10]

KLEUR: | Rooi (X) | Groen | Blou | Swart |

AANTAL SAGTEBANDE (skoon omslag / buiteblad)   TOTAAL: [1]

KLEUR: | Geel | Groen (X) | Blou | Pienk | Buff |

RUGTITEL (Beperk tot 55 letters, spasies ingesluit)

(HOOFLETTERS) Die 55 blokkies hieronder is vir u voorletters, van en die titel van die teks. Wanneer die blokkies ingevul word, moet die gebruiklike spasies gelaat word. Om die titel in te pas, kan dit afgekort word. As dit nie sinvol is nie, kan die titel weggelaat word, in die geval waarvan net die naam en van voldoende is.

M		L	I	L	L	Y												

MIKROFICHE TITEL (Beperk tot 50 letters, spasies ingesluit):

(HOOFLETTERS) Die 50 blokkies hieronder is vir die titel van die teks. Wanneer die blokkies ingevul word, moet die gebruiklike spasies gelaat word. Om die titel in te pas, kan dit afgekort word.

D	E	V	E	L	O	P	M	E	N	T		O	F		Y	E	A	S	T
F	O	R		O	P	T	I	M	A	L		A	R	O	M	A		C	O
M	P	O	U	N	D		P	R	O	D	U	C	T	I	O	N			

SES SLEUTELWOORDE (In volgorde van belangrikheid)

1. yeast
2. flavor-active
3. esters
4. higher alcohols
5. wine
6. distillates

SORTERING EN BINDWERK

Voordat die teks gebind kan word, moet al die gesorteerde fotokopieë nagegaan word om te verseker dat elke stel akkuraat is. Die kandidaat is self hiervoor verantwoordelik. Indien dit nie vir die kandidaat moontlik is nie, moet reëlings getref word dat iemand anders dit namens hom/haar doen. Hierdie persoon se besonderhede moet aan die US Drukkery verskaf word.

Naam: _____   Kontaknommer: _____

BINDWERK VAN DIE OORSPRONKLIKE MANUSKRIP

NEE [ ]   JA: Stuur aan JS Gericke Biblioteek [ ]

INGEHANDIG DEUR: _____

VERKLARING DEUR STUDIELEIER/PROMOTOR

Hiermee word gesertifiseer:

(i) dat die meegaande ongebonde eksemplaar van die meesterswerkstuk, -tesis en doktorale proefskrif van

(voorletters en van) _____ M. Lilly _____

[Om verwarring te voorkom - maak asseblief gebruik van die amptelike formaat]

vir die graad _____ Ph. D. Agric (Wine Biotechnology) _____

in die finale vorm is en gereed is om by die US Drukkery ingehandig te word vir die duplisering en bind van die voorgeskrewe aantal eksemplare en

(2) dat die voorgeskrewe verklaring (soos in die Jaarboek uiteengesit) behoorlik deur die kandidaat onderteken is.

MERK GEPASTE BLOKKIE MET 'n KRUISIE:

☐	Meesterswerkstuk (verteenwoordig **minder as 25%** van die finale punt)
☐	Meesterswerkstuk (verteenwoordig **25% - 49%** van die finale punt)
☐	Meesterstesis (verteenwoordig **50% of meer** van die finale punt)
☒	Doktorale proefskrif

_____   27/10/04
Handtekening van Studieleier/Promotor   Datum

VERTROULIKHEID VAN DIE WERKSTUK, TESIS OF PROEFSKRIF

Hiermee word gesertifiseer dat hierdie eksemplaar as vertroulik geklassifiseer moet word.

_____   _____
Handtekening van Studieleier/Promotor   Datum