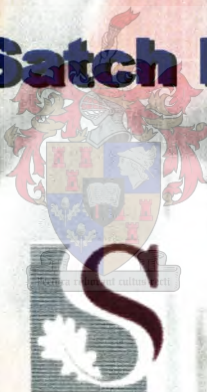
The deletion and overexpression of two esterase genes, *IAH1* and *TIP1*, in *Saccharomyces cerevisiae* to determine their effects on the aroma and flavour of wine and brandy

by

Jason Satch Hignett



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DECLARATION

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

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SUMMARY

No single chemical constituent can be accredited with giving wine and brandy their overall aroma and flavour. The aroma and flavour of wine and brandy are rather attributed to a number of chemical constituents reacting together and it is these reactions that give the beverage its character. Certain chemicals within wine and brandy do, however, make larger contributions to the flavour. These include the esters, terpenes and volatile acids, although others also exist.

Esters are a large group of volatile compounds with variable aroma and flavour characteristics, including banana-like (isoamyl acetate), apple-like (ethyl caproate) and chemical/solvent-like (ethyl acetate). Esters are produced as secondary metabolites during the conversion of sugar to ethanol and are formed when an alcohol binds with a fatty acid. Chemically, ester metabolism is well documented and understood; however, much work still needs to be done on a genetic level. The yeast strain used during fermentation is one of the most important factors contributing to the type and quantity of esters produced. This is due to differences in genetic makeup. The metabolism of esters is controlled largely on a genetic level, with numerous genes being involved. The alcohol acetyltransferase genes are involved in ester anabolism, whilst esterase genes are involved in ester catabolism. Esterases have a negative effect on the overall level of esters within an alcoholic beverage, as they are capable of reducing the number of esters and are thus capable of altering the beverage's aroma and flavour profile. The *IAH1* and the *TIP1* gene products are believed to encode for two such esterases.

The objective of this study was to investigate the contribution of the *IAH1* and *TIP1* genes to the level of esters in both wine and brandy. This was accomplished by using two approaches. Firstly, the above genes were disrupted using a polymerise chain reaction (PCR)-generated disruption cassette homologous to either the *IAH1* or the *TIP1* gene. These cassettes were integrated into the industrial wine yeast, *Saccharomyces cerevisiae* strain VIN13. The integrations were verified by Southern blot analysis to produce yeasts VIN13-ΔIAH1 and VIN13-ΔTIP1; however, only a single copy of each was disrupted. Secondly, the *IAH1* and the *TIP1* genes were cloned from *S. cerevisiae* using PCR into plasmid pJ between the phosphoglycerate kinase gene (*PGK1*) promoter and terminator, producing plasmids pJ-IOE1 and pJ-TOE1. The *PGK1* promoter has previously been shown to constitutively express genes at high levels. These new constructs were then used as template for PCR to produce two overexpression cassettes, one for *IAH1* and the other for *TIP1*. These cassettes were integrated into *S. cerevisiae* VIN13 and verified by Southern blot analysis to produce strains VIN13-IOE1 and VIN13-TOE1.

The above yeast strains including VIN13 were used for the production of wines and base wines from Colombard must. Reverse-transcriptase (RT-PCR) confirmed that the VIN13-IOE1 and VIN13-TOE1 strains overexpressed the appropriate gene at

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a higher level than the control VIN13 strain. The VIN13-ΔIAH1 disrupted strain showed no difference in expression level to that of the control strain, whilst VIN13-ΔTIP1 showed lower levels of expression than that of the control strain. VIN13-IOE1 behaved as expected, with a decrease of between 30% and 60% in the total ester level in the wine and base wine respectively, a 30% decrease in the total acid level and no change in the higher alcohol level. The VIN13-ΔIAH1 strain showed no difference to the control wine, most likely as this strain still expressed the *IAH1* gene at levels consistent with the control strain. VIN13-TOE1 behaved in an unexpected manner - instead of hydrolysing esters, it appeared to produce them. This increase in the total ester level was most noticeable during distillation, when a 20% increase took place. Another unexpected occurrence was a large decline in the total acid level, with acetic acid being the most significant contributor, decreasing by up to 78%. This is a very favourable finding, as acetic acid is a known spoilage molecule and is a cause of sluggish/stuck fermentations. VIN13-ΔTIP1 behaved in an opposite manner to VIN13-TOE1, with higher total acid levels and slightly decreased total ester levels, especially during distillation. Neither affected the total higher alcohol levels. Sensorially, the only significant difference in the wine samples was for the fruity flavour. A panel of judges distinguished that VIN13-TOE1 was fruitier than the other wines, with VIN13-ΔTIP1 being the least fruity.

This study again proves the significant impact that a single gene can have on the chemical makeup of wine and brandy. The relatively simple genetic alteration of an organism can drastically change and improve not only the organoleptic properties of the organism, but its viability as well. These alterations can produce more favourable organisms with more desirable characteristics for the fermenting beverage industry to produce products of higher quality and better suitability.

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OPSOMMING

Geen chemiese komponent kan uitgesonder word as die produseerder van aroma en geur in wyn of brandewyn nie. Die aroma en geur van wyn en brandewyn word eerder toegeskryf aan die interaksie tussen `n groot aantal chemiese komponente om aan die drank sy karakter te gee. Enkele van hierdie chemiese komponente sluit in esters, terpene en vlugtige sure, om maar `n paar te noem.

Esters is 'n groot groep van vlugtige verbindings wat beskik oor `n verskeidenheid van aroma- en geurkenmerke, soos piesangagtig (isoamielasetaat), appelagtig (etielkaproaat) en chemies/oplosmiddelagtig (etielasetaat). Esters word as sekondêre metaboliete geproduseer wanneer suikers na etanol omgeskakel word en word gevorm wanneer 'n alkohol met 'n vetsuur verbind. Estermetabolisme is chemies goed beskryf en verstaan, maar op 'n genetiese vlak is daar nog heelwat aspekte wat nagevors moet word. Die gisras betrokke gedurende fermentasie word beskou as een van die grootse bydraes tot die tipe en die hoeveelheid esters wat geproduseer word. Dit word toegeskryf aan verskille in die genetiese saamestelling van die gisras. Ester metabolisme word grootliks deur genetiese faktore beheer en verskeie gene is betrokke. Dit is hoofsaaklik die alkoholasetieltransferasegene wat vir esterkatabolisme verantwoordelik is, terwyl die esterasegene vir esteranabolisme verantwoordelik is. Esterases het `n negatiewe effek op die totale estervlak binne alkoholiese dranke deurdat hulle in staat is om die aantal esters drasties te verminder en sodoende die drank se aroma- en geurprofiel te verander. Daar is voorgestel dat die *IAH1*- en die *TIP1*-geen produkte is wat vir twee sulke esterases kodeer.

Die doel van hierdie studie was om die *IAH1*- en die *TIP1*-gene se bydrae tot die totale estervlak in wyn en brandewyn te ondersoek. Dit is deur twee benaderings uitgevoer. Eerstens is die bogenoemde gene d.m.v. disrupsiekassette wat homoloog aan die *IAH1*- of die *TIP1*-gene was, uitgeslaan. Die disrupsiekassette is deur die polimerasekettingreaksie (PKR) geproduseer. Hierdie kassette is in die industriële wyngis, *Saccharomyces cerevisiae* VIN13, geïntegreer. Die integrasies is deur Southernkladanalise bevestig en het die giste VIN13-ΔIAH1 en VIN13-ΔTIP1 gelewer. Net `n enkele kopie van elke geen is egter uitgeslaan. Tweedens is die *IAH1*- en *TIP1*-gene d.m.v. PKR vanaf *S. cerevisiae* binne in plasmied pJ gekloneer, tussen die fosfogliseraatkinasegeen (*PGK1*) se promotor en termineerder, om plasmiede pJ-IOE1 en pJ-TOE1 te produseer. Die *PGK1*-promotor is al tevore geïdentifiseer as 'n hoë-vlak konstitutiewe uitdrukker van gene. Hierdie twee nuwe konstrukte het vervolgens gedien as templaat vir PKR om twee ooruitdrukkingskassette, een vir *IAH1* en die ander vir *TIP1*, te produseer. Hierdie kassette is in *S. cerevisiae* VIN13 geïntegreer en bevestig deur Southernkladanalise. Hierdie integrasies het die giste VIN13-IOE1 en VIN13-TOE1 geproduseer.

All die nuwe gisrasse, tesame met VIN13, is gebruik vir die produksie van wyne sowel as rebatwyne vanaf Colombard-mos. Omgekeerde-transkriptase

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polimerasekettingreaksie (OT-PKR) het bewys dat die VIN13-IOE1 en VIN13-TOE1 rasse die geskikte geen ooruitgedruk het, met hoër vlakke as van die kontrole VIN13-ras. Dit het ook aangedui dat die VIN13-ΔIAH1-ras, waarvan die geen uitgeslaan was, geen verskil in uitdrukking gehad het in vergelyking met die kontroleras nie, terwyl VIN13-ΔTIP1 'n lae uitdrukkingsvlak getoon het. VIN13-IOE1 het teen verwagting opgetree, met 'n afname van tussen 30% en 60% in die totale estervlak in beide die wyne en rebatwyne. `n Afname van 30% in die totale suurvlak, asook geen waarneembare verskil in die hoër alkoholvlak, in vergelyking met die kontroleras, is ook opgemerk. Die VIN13-ΔIAH1-ras het glad nie van die kontroleras verskil nie, heel waarskynlik omdat hierdie ras die *IAH1*-geen teen dieselfde vlak as die kontroleras kon uitdruk. Die VIN13-TOE1-ras het teen verwagting opgetree deurdat dit esters geproduseer het i.p.v. om esters te hidroliseer. Hierdie toename in die totale estervlak is die meeste waarneembaar tydens distillasie, met tot 'n 20% toename. Nog 'n onverwagte effek was die groot afname in die totale suurvlak, met asynsuur wat die betekenisvolste bydrae gelewer het deurdat dit `n afname van tot 78% getoon het. Hierdie bevinding is baie voordelig, aangesien asynsuur, 'n bekende bederfmolekuul, veral vir slepende/gestaakte fermentasies verantwoordelik is. VIN13-ΔTIP1 het op die teenoorgestelde wyse opgetree as VIN13-TOE1, met 'n hoër totale suurvlak en 'n klein afname in die totale estervlak. Weereens is dit meer gedurende distillasie waargeneem. Beide rasse het egter geen effek op die hoër alkoholvlak gehad nie. Die proepaneel het, met betrekking tot die vrugtige geur, een betekenisvolle geurverskil tussen die wyne gevind. VIN13-TOE1 was meer vrugtig as al die ander wyne en VIN13-ΔTIP1 was die minste vrugtig.

Die studie het weereens bewys dat 'n enkele geen 'n betekenisvolle effek op die chemiese samestelling van wyn en brandewyn kan hê. Die relatief eenvoudige genetiese verandering van 'n organisme kan die organoleptiese eienskappe asook die lewensvatbaarheid van `n organisme, drasties verander en verbeter.

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This thesis is dedicated to Marché and Satch Hignett.
Hierdie tesis is aan Marché and Satch Hignett opgedra.

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BIOGRAPHICAL SKETCH

Jason Satch Hignett was born in Cape Town, South Africa on Monday the 28th of June 1976. He attended Boston Primary School, Settlers High School and matriculated at Table View High School in 1994. Jason enrolled at Stellenbosch University in 1995 and obtained a BSc degree in 1998, majoring in Biochemistry, Genetics and Microbiology. In 1999, Jason enrolled at the Institute for Wine Biotechnology and obtained an Honours degree in Wine Biotechnology in December of that year.

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PREFACE

This thesis is presented as a compilation of five chapters with each of the chapters being introduced separately. All chapters are written according to the style of the journal *Yeast*.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

Chapter 2 **LITERATURE REVIEW**

The genes involved in ester metabolism

Chapter 3 **RESEARCH RESULTS**

The disruption and overexpression of two esterase genes, *IAH1* and *TIP1*, in the *Saccharomyces cerevisiae* wine yeast strain VIN13 and their effects on wine and brandy

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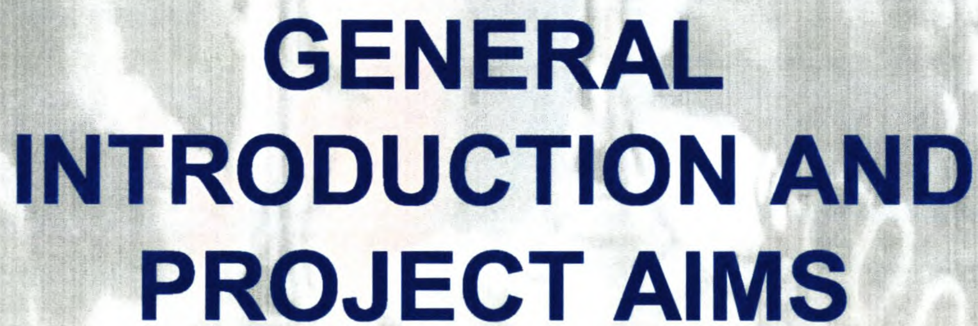
CHAPTER 1



GENERAL INTRODUCTION AND PROJECT AIMS

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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

For centuries, man has tried to modify the aroma and taste of alcoholic beverages, especially beer and wine, to improve the quality and allure of these products. Although progress has been made over the years, a good deal of research and development (R & D) still needs to be done. In today's modern lifestyle, the consumer is becoming more interested in tailor-made products that will suite their exact needs. The wine industry and, indeed, any food or beverage industry needs to look at new and improved methods to deal with the consumers' demands.

Wine fermentations originally occurred spontaneously. It was conducted with the native yeast flora located on the grapes. Over the years, this proved to be unreliable, as consistent products could not be obtained because the yeast flora would differ from year to year. Today, most fermentations are inoculated with an appropriate yeast strain and thus, regarding quality and aroma, wines are produced with more consistency and reliability (Dequin, 2001). The yeast strain chosen is of the utmost importance, as this yeast will have a large influence on the quality and overall flavour of the wine (Nykänen and Nykänen, 1977). Modern science has made it possible to modify specific traits of organisms, including their genetic makeup. With molecular research, it has become possible to study a variety of genes within an organism and the function of a number of these genes is becoming more evident. It has also become possible to manipulate these genes and thus adapt organisms on a genetic level (Dequin, 2001; Pretorius, 2000).

Over the years, wine has been associated with romance and mystery. This is most likely due to its endless variety and complexity, especially with regard to its aroma and taste. Taste or flavour is one of the most important and distinguishing characteristics of wine. Flavour, however, is a complex term, as it can describe numerous attributes within the wine, including aroma, taste, mouth feel and body. Many chemical compounds influence the flavour of wines, including esters, terpenes and volatile acids, to name but a few. One of the more important of these chemical groups is the esters (Peddie, 1990; Bardi *et al.,* 1993; Lambrechts and Pretorius, 2000). Ester production is influenced by a number of factors, with the yeast strain being one of the more important (Nykänen and Nykänen, 1977). This clearly indicates that the yeast synthesise esters as byproducts for detoxification (Peddie, 1990). Esters are a group of volatile compounds that are the product of an alcohol combining with an acid. This reaction can occur spontaneously, but on the whole occurs with the help of enzymes (Nordström, 1961). The enzymes produced by the yeasts involved in this reaction are known as alcohol acetyltransferases and include the genes *ATF1* (Fujii *et al.*, 1994), *ATF2* (Nagasawa *et al.,* 1998) and *EHT1* (Mason and Dufour, 2000). Although a fair amount of information is available for the *ATF1* gene, limited information is available on the remaining alcohol acetyltransferases.

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The above-mentioned ester-synthesising reaction is also reversible with the assistance of enzymes known as esterases (Suomalainen, 1981; Peddie, 1990).

Esterases are a class of enzymes related to the lipases, differing only in their inability to hydrolyse triacylglycerols or TAGs. Esterases hydrolyse esters to produce the corresponding alcohol and acid and so decrease the aroma associated with that specific ester. These esterase activities occur aerobically or anaerobically within the yeast *Saccharomyces cerevisiae* (Schermers *et al.*, 1976) and esterase activity has been located both within and on the outside of the plasma membrane (Suomalainen, 1981). Many organisms produce esterases, including humans, mice and yeast (Fukuda *et al.,* 1996), nevertheless very little information is available on these esterases and more detailed studies are required to fully establish the effects of these genes.

For the wine industry, the most important organism to study is the fermenting yeast *S. cerevisiae*, followed closely by the plant species from which the beverage is derived, such as, in the case of the wine industry, *Vitis vinifera*. Detailed studies of these organisms will hopefully help discover new genes involved in aroma and flavour formation.

1.2 PROJECT AIMS

The Institute for Wine Biotechnology has an extensive research programme aimed at improving the organoleptic qualities of South African wines and brandies. A large part of this project deals with aroma and flavour improvement, especially concerning the positive effects of esters, which play a large role in the aroma and flavour of all alcoholic beverages. A deeper look at the molecular control mechanisms within yeast, specifically the genes involved in ester anabolism and ester catabolism, would be advantageous. Studies like this will indicate which genes are involved and to what degree they will effect the level of esters in the final product.

The *IAH1* and *TIP1* gene products encode for two esterases (Kondo and Inouye, 1991; Fukuda *et al.*, 1996; Horsted *et al.*, 1998). These esterases are involved in the hydrolysis of esters into their principle components. The *IAH1* gene is relatively well documented and is known to play a pivotal role in the catabolism of isoamyl acetate and isobutyl acetate. Isoamyl acetate is a major ester found within wine and brandy and contributes significantly to their aroma and flavour. The *TIP1* gene is well studied on a molecular level, but less well documented with regard to its ability as an esterase. Horsted *et al.* (1998) have shown that Tip1p has esterase activity against a number of different esters, particularly the longer chain esters. A previous study at our institute (Lilly *et al.,* 2000), involving the *ATF1* gene, proved that the manipulation of genes could make a significant impact on the organoleptic quality of both wine and brandy. Based on this information, it was decided that studies of other genes involved in ester production would be advantageous.

The focus of this study was to individually investigate the contribution of the *IAH1*

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and *TIP1* genes to the level of esters in wine and brandy and to determine if these genes could contribute significantly to the overall sensory quality of these beverages. Any other secondary effects of these two genes would also be noted, analysed and documented.

The specific aims can be outlined as follows:

1. to clone the *IAH1* and *TIP1* genes from *S. cerevisiae* wine yeast strain VIN13;
2. to overexpress these genes in *S. cerevisiae* wine yeast strain VIN13 under the constitutive phosphoglycerate kinase I gene (*PGK1)* promoter using PCR-generated overexpression cassettes;
3. to disrupt these genes in the industrial wine yeast VIN13, thus preventing expression by means of a PCR-generated disruption cassette;
4. to evaluate these transformed strains for their ability to degrade esters in wine and brandy;
5. to evaluate to what extent the levels of esters are altered and which specific esters are affected the most;
6. to sensorially evaluate the individual contributions of the *IAH1* and the *TIP1* genes to the aroma and flavour of wine and brandy; and
7. to determine any other effects of the genes on the chemical composition and flavour of the final products.

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CHAPTER 2

LITERATURE REVIEW

The genes involved in ester metabolism

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2. LITERATURE REVIEW

2.1 INTRODUCTION

All alcoholic beverages can be classified and distinguished according to aroma and flavour profiles. A number of compounds in these beverages can be directly associated with aroma and flavour, with esters being one of the largest and the most important. Esters are a group of volatile compounds found in many alcoholic beverages produced by the yeast during alcoholic fermentation. Esters are produced during alcoholic fermentation by either (a) the esterification of acetic acid to an alcohol or (b) the enzyme-catalysed esterification of a fatty acid to an alcohol (Nordström, 1961). The most important esters produced are the acetate esters, such as isoamyl acetate (pear drops), ethyl acetate (solvent-like), isobutyl acetate (banana), 2-phenethyl acetate (honey, fruity and flowery) and ethyl hexanoate (apple-like) (Yoshioka and Hashimoto, 1983; Peddie, 1990). These ester molecules are an important source of both aroma and flavour, usually imparting a fruity character to the beverage or food. The sensory threshold values of esters are low, ranging from 0.2 ppm for isoamyl acetate to 20 ppm for ethyl acetate, and they are thus easily detectable (Peddie, 1990). Ester production is influenced by a number of factors, including the yeast strain (Nykänen and Nykänen, 1977), availability of acetyl-CoA (Yoshioka and Hashimoto, 1984a), presence of fatty acids (Nordström, 1964b, d), the presence of oxygen (Dufour and Malcorps, 1994), fermentation temperature (Engan, 1974), pH (Marais, 1978), and CO_2 levels (Nakatani *et al.*, 1991). Furthermore, the ester content of distilled beverages can be increased greatly if the yeast lees are present during distillation. This indicates that large portions of the volatile esters are found intracellularly and that they are unable to diffuse into the medium (Nykänen *et al.*, 1977).

Nordström (1961) suggested that the synthesis of esters could be controlled by genetic factors or, put more simply, by genes. He suggested that yeast strains with improved ester forming abilities could be obtained by hybridisation and mutation experiments. To date, a number of ester-forming genes as well as a number of esterase genes have been identified and cloned. The entire genome of *Saccharomyces cerevisiae* has been sequenced and is readily available on the internet (Cherry *et al.*, "*Saccharomyces* genome database"). This information can be used to search for new genes involved in ester metabolism. With current genetic manipulation techniques, it has become possible to manipulate and even control ester formation in the yeast *Saccharomyces cerevisiae*.

This review will discuss the production of esters and their importance and will take a closer look at the genes involved in ester and fatty acid metabolism.

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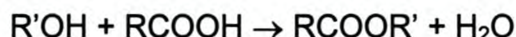
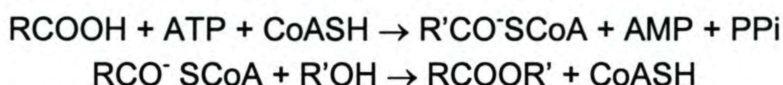
2.2 ESTERS: HOW ARE THEY FORMED?

It is believed that esters can be formed by two different chemical reactions. The first is by a direct chemical reaction involving an alcohol and a carboxylic acid. This is represented chemically as follows:



$$R'OH + RCOOH \rightarrow RCOOR' + H_2O$$

The final product is an ester and a water molecule (Engan, 1974; Peddie, 1990). The general kinetics of this reaction are extremely slow, indicating that this could not possibly be the only reaction responsible for the formation of the esters in alcoholic beverages and that some other reaction must be involved (Nordström, 1961). 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:

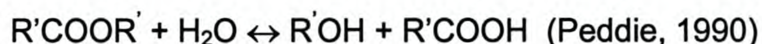


$$RCOOH + ATP + CoASH \rightarrow R'CO^{-}SCoA + AMP + PPi$$
$$RCO^{-}\ SCoA + R'OH \rightarrow RCOOR' + CoASH$$

The above-mentioned reaction requires an alcohol, a fatty acid, Co-enzyme A (CoA) and an ester-synthesising enzyme (Nordström, 1961; Peddie, 1990). Formic acid, propionic acid, *iso*butyric acid and *iso*valeric 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, 1962b).

The ester-synthesising enzyme is usually an alcohol acyltransferase (Howard and Anderson, 1976; Peddie, 1990). Acetyl-CoA plays an extremely important role in the formation of esters. This is clearly seen by the last reaction shown above and in **Figure 2.1**. This molecule is also involved in many other reactions within the yeast, including fatty acid, amino acid and lipid biosynthesis (Nordström, 1962a, b, 1963, 1964a, b, d). As all these reactions require acetyl-CoA, they will indirectly affect the rate and quantity of ester formation (Peddie, 1990).

The formation of esters is also affected by esterases, as they have the ability to break esters down. This reaction is represented as follows:



$$R'COOR' + H_2O \leftrightarrow R'OH + R'COOH \quad \text{(Peddie, 1990)}$$

Note that the reaction is reversible, as esterases can also produce esters and water from their principle components, but only at an extremely slow rate (Suomalainen, 1981).

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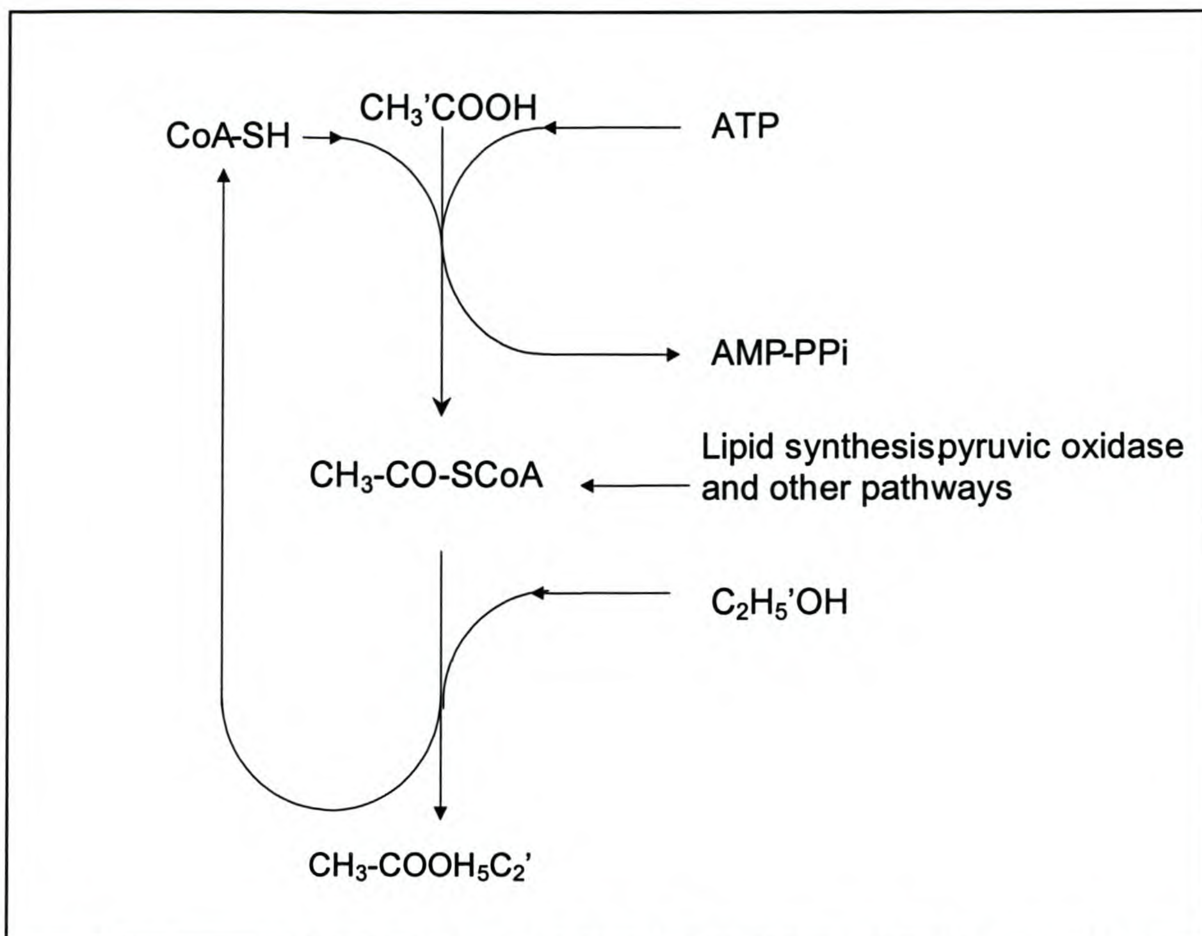

Figure 2.1. Proposed scheme for the formation of ethyl acetate (Adapted from Nordström, 1964b)

2.3 FORMATION OF ACETATE ESTERS

Acetate esters are formed during alcoholic fermentation and make significant contributions to the overall aroma and flavour. The predominant acetate ester found in wine is that of ethyl acetate (Lambrechts and Pretorius, 2000). In general, esters are amphiphatic in nature and have a polar head and a hydrocarbon tail (Conner *et al.*, 1994). Limited quantities are formed during anaerobic and fermentative conditions. The formation of ethyl acetate reaches its maximum rate close to days 3 and 4 during winemaking. It is not known if this is due to the lower pH or to the presence of metabolites required for ethyl acetate formation (Nordström, 1962a). The optimum pH for ethyl acetate formation is about 4.5, with the formation occurring intracellularly and not in the medium. This suggests the need for an enzyme that functions intracellularly and that is not secreted into the medium.

Nordström (1962b) showed that the addition of ethanol to the medium causes an immediate increase in the formation of ethyl acetate and a decrease in acetic acid concentration. This addition also results in the immediate production of this ester. The addition of acetaldehyde has no affect on ethyl acetate production. The level of ethyl acetate in the medium prior to fermentation is critical in determining its production, as higher initial levels inhibit the formation of ethyl acetate and thus decrease the final concentration (Nordström, 1962a). Acetyl-CoA plays a central role

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in ethyl acetate formation. It acts as an energy-transferring agent and, during its accumulation through the lag phase of the yeast, acetate esters are formed very slowly (Yoshioka and Hashimoto, 1984a, b). Molecules that inhibit acetyl-CoA formation, such as sodium arsenate, also inhibit acetate ester formation (Nordström, 1962b). It must also be mentioned that some of the inhibitors tested also inhibited ester-forming enzymes, including the alcohol acetyltransferases (to be discussed later). The addition of pantothenic acid increases ester synthesis, as pantothenic acid has an important metabolic function as a component of co-enzyme A and makes acetate available for other metabolic functions (Alvarez *et al.*, 1958; Nordström, 1962c).

It is a well-known fact that alcohols compete with each other to bind to fatty acids during ester formation (Nordström, 1963). As ethanol is the dominant alcohol formed and acetic acid the dominant acid formed during yeast fermentations, it is logical to conclude that most of the volatile esters formed during fermentations will be either ethyl or acetate esters. The chain length of alcohols plays a crucial role in ester formation, with higher alcohols being involved in esterification at lower frequencies than ethanol (Nordström, 1963). This is clearly seen by the high formation of ethyl and isoamyl acetate esters and the low formation of heptyl acetate esters.

2.4 THE ROLE OF ACETYL CO-ENZYME A

As discussed earlier, acetyl-CoA plays an central role in yeast metabolism. Acetyl-CoA provides energy via the thio-ester linkage of the co-substrate. Its availability is essential not only for survival, but also for the formation of esters and other metabolic products. It is a key substrate for ester formation and also for lipid, protein and nucleic acid synthesis, as indicated in **Figure 2.2**.

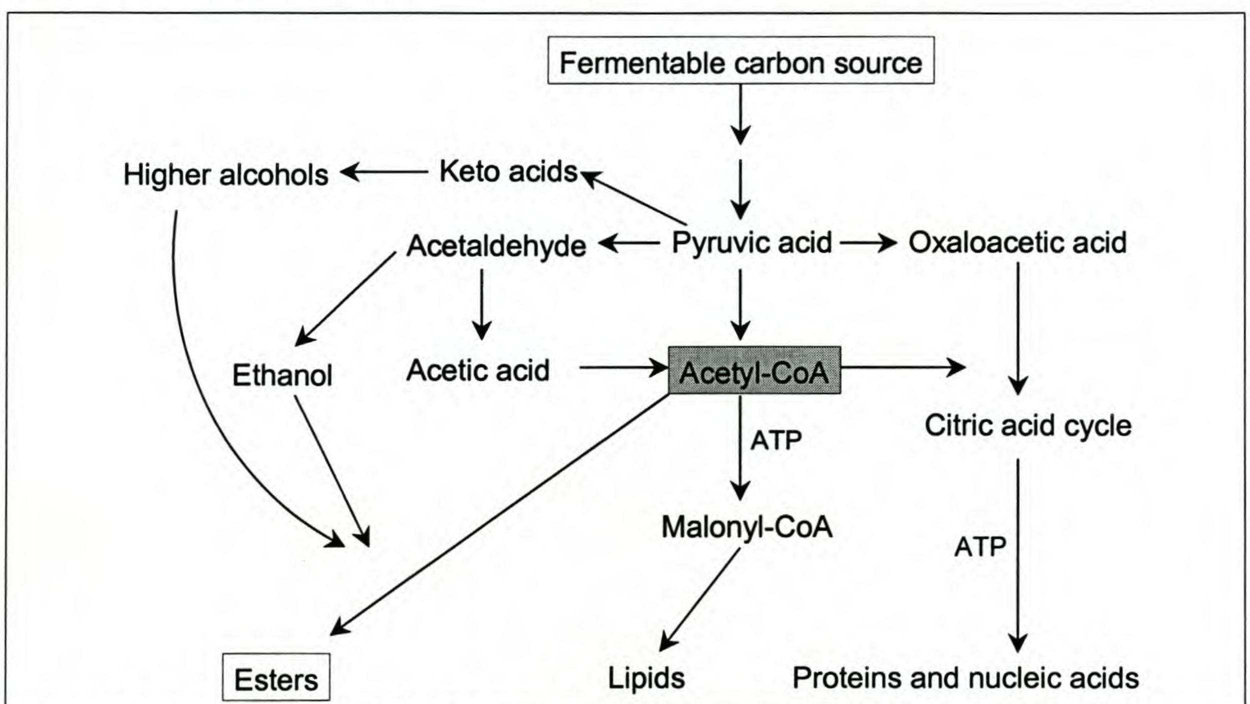

Figure 2.2. The central role of acetyl-CoA in yeast metabolism (adapted from Nordström, 1963)

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A possible way to increase the level of ester synthesis would be by inhibiting or in some way affecting the other metabolic routes that utilise acetyl-CoA. This can be achieved via a number of methods. One method is the addition of malonic acid, resulting in the inhibition of succinic acid dehydrogenase and thus inhibiting the citric acid cycle. This leads to a decrease in the usage of acetyl-CoA by the citric acid cycle and thus a higher availability of this substrate (Nordström, 1963). A second method is the genetic manipulation of the succinic acid dehydrogenase gene. By targeting this gene, or more specifically, reducing its level of expression, higher yields of esters would be obtained (Nordström, 1963).

A biotin deficiency can cause reduced production of oxaloacetic acid and also disturb the citric acid cycle, leading to an increase in available acetyl-CoA. Yet another method is illustrated by a hypothesis of Nordström (1963). Cell growth requires ATP and acetyl-CoA. The synthesis of acetyl-CoA does not require ATP. The addition of dinitrophenol (DNP) causes a reduction in the formation of ATP and hence a reduction in energy. This energy is now no longer available for certain metabolic processes. Thus, a large quantity of acetyl-CoA is not utilised and becomes available for other metabolic reactions that do not require energy (**Figure 2.2**). Ester formation is one such reaction. This brings us to the next section, in which the genes involved in ester formation will be discussed.

2.5 REASONS FOR ESTER SYNTHESIS

First and foremost, esters could be formed as byproducts from sugar metabolism with no advantage for the yeast cell. This is extremely unlikely, as there are too many tightly regulated genes involved in ester metabolism. The simple fact that fatty acid and ester synthesis share many control mechanisms would also suggest otherwise. Secondly, fatty acids are toxic to the yeast. This is especially true for those fatty acids with carbon chains of between 8 and 14. These fatty acids also demonstrate anti-microbial activity, especially if unsaturated (Peddie, 1990; Lie Ken Jie and Syed-Rahmatullah, 1995). Nordström (1962a, b, 1964a) proposed that esters might be produced to remove these toxic fatty acids from the cell and the medium and thus assist the cell to maintain a more suitable habitat. A third reason could be to maintain a balance between acetyl-CoA and CoASH (Peddie, 1990).

2.6 ESTER-SYNTHESISING GENES

The synthesis of acetate esters in yeast is catalysed by alcohol acetyltransferases (AATases) and utilises available alcohols and acetyl-CoA (Nordström, 1961, 1962a, b, 1963; Yoshioka and Hashimoto, 1981, 1984b; Malcorps and Dufour, 1992; Mauricio *et al.*, 1993). Thus far, three different AATase genes have been cloned, namely *ATF1*, *ATF2,* and *LgATF1*, and a fourth (*EHT1*) has also been identified (Fujii *et al.*, 1994; Nagasawa *et al.*, 1998; Yoshimoto *et al.*, 1998; Lilly *et al.*, 2000; Mason and Dufour, 2000). **Table 2.1** summarises certain properties of these genes.

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These genes are present in different yeast genera, including *S. cerevisiae*, *Saccharomyces uvarum* and *Hansenula mrakii* (Inoue *et al.*, 1997). Other kinds of AATase have been described, including isoamyl alcohol acetyltransferase (IATase) (Minetoki *et al.*, 1993) and ethanol acetyltransferase (EATase) (Malcorps and Dufour, 1992).

Table 2.1. A comparison of the different ester-forming genes

Gene name	Chromosome	ORF	Size (Da)	Codon Bias	pI	Repression	Promoter Sequences
ATF1	XV (*S. cerevisiae*)	YOR377w	61036	0.044	6.94	Unsaturated fatty acids (UFAs) O_2	WRLICLP Rox1p Rap1p ORE
LgATF1	850-kb (*S. pastorianus*)	N/A	63000		7.68	UFAs O_2	WRLICLP Rox1p
ATF2	VII (*S. cerevisiae*)	YGR177c	61898	0.133	5.77	O_2	WRLICLP Rox1p
EHT1	II (*S. cerevisiae*)	YBR177c	51255	0.110	7.83	Unknown	Unknown

2.6.1 ALCOHOL ACETYLTRANSFERASE I

The *ATF1* gene has been cloned from *S. cerevisiae, Saccharomyces pastorianus* (formerly *Saccharomyces carlsbergensis)* and *S. uvarum* (Fujii *et al.*, 1994; Yoshimoto *et al.*, 1998; Lilly *et al.*, 2000). In *S. cerevisiae*, it is located on chromosome XV in an open reading frame (ORF) designation YOR377w (Mason and Dufour, 2000), with only one copy occurring per haploid genome. The structural gene consists of a 1575 bp ORF encoding for 525 amino acids, giving the protein a calculated molecular mass of 61036 Da (Fujii *et al.*, 1994) and a pI of 6.94 (Nagasawa *et al.*, 1998). Sequence homology indicates a 99,4% similarity to the *S. pastorianus ATF1* gene, with a difference of just three amino acids, an 81% identity with *LgATF1* (Mason and Dufour, 2000) and a 35.9% identity with the *ATF2* gene (Nagasawa *et al.*, 1998).

The *ATF1* protein accounts for 80% of the total AATases activity in yeasts during fermentative conditions (Mason and Dufour, 2000) and is involved in the production of both short and medium-chain aliphatic esters from ethanol or isoamyl alcohol and acetyl-CoA (Malcorps and Dufour, 1992). It plays a pivotal role in isoamyl acetate production and has a lesser role in ethyl acetate production (Yoshimoto *et al.*, 1998). The *ATF1* protein can be described as an acetyl-CoA: *O*-alcohol acetyltransferase as it is capable of utilising acetyl-CoA as substrate (Malcorps and Dufour, 1992). It has been recognised as a membrane-bound protein (Yoshioka and Hashimoto, 1981; Malcorps and Dufour, 1992), but recent evidence indicates otherwise. Fujii *et al.* (1994) showed that the protein did not have a significantly hydrophobic domain for it to be membrane bound. A mean index of -0.38 was calculated, showing that the protein is more hydrophilic; however, short sections of hydrophobic residues are

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present, suggesting that it is membrane/lipid associated (Mason and Dufour, 2000). Inoue *et al.* (1997) isolated an AATase from the yeast *H. mrakii.* This AATase is different from that of *S. cerevisiae*, as it is heat stable, has no processable N-terminal sequence to act as a potential signal sequence for secretion and is membrane bound (Inoue *et al.*, 1997).

ATF1 has a codon bias index equal to 0.07 (Fujii *et al.*, 1994), suggesting a low natural expression level. Another interesting feature is the presence of 14 cysteine residues in the deduced 525 amino acid sequence. This is more than in any other known acetyltransferase or acyltransferase and this could be responsible for the heat lability of the *S. cerevisiae* enzyme (Fujii *et al.*, 1994).

A closer inspection of *S. cerevisiae's* Atf1p C-terminal domain revealed a short five amino acid sequence that is homologous to the *HEM0* and *HEM1* genes found in chickens, humans and mice. The *HEM0* and *HEM1* genes catalyse the synthesis of aminolevulinate from glycine and succinyl co-enzyme A. It is interesting to note that the *ATF1*, *HEM0* and *HEM1* genes (1) possess a large number of cysteine residues, (2) are inhibited by sulfhydryl compounds, and (3) are membrane bound (Fujii *et al.*, 1994). The *ATF1* protein is also inhibited by zinc (Cauet *et al.*, 1999) and magnesium ions (Howard and Anderson, 1976).

Expression is repressed by both oxygen and unsaturated fatty acids (UFAs). Anderson and Kirsop (1975) reported a lowering of the isoamyl acetate and ethyl acetate levels by the addition of oxygen to wort fermentations. They speculated that changes in yeast metabolism were responsible for this. These factors have no inhibitory effect on the enzyme itself, but rather control the level of gene expression (Mason and Dufour, 2000). Fusion of the *ATF1* gene to the *LacZ* gene by Yoshimoto *et al.* (1998) showed that the gene was repressed by conditions of aeration and induced by anaerobic conditions. Fujiwara *et al.* (1998) showed that aeration caused repression within 30 minutes. It was not clear, however, if oxygen acted as the repressing agent or if the shift to aerobic conditions resulted in an increase in UFAs, which then caused the repression.

Inhibition of *ATF1* transcription is caused by a number of UFAs, as indicated in **Figure 2.3**. These include linolenic (18:3), linoleic (18:2), oleic (18:1) and palmitoleic (16:1) acids, of which only palmitoleic and oleic acids occur naturally in yeast (Mason and Dufour, 2000). This introduces a new gene that could be used to control ester synthesis, namely *OLE1*. This gene encodes for a Δ9-fatty acid desaturase, which catalyses the formation of a double bond in the Δ-9 position of the UFAs. UFAs with a double bond in position Δ-9 repress transcription the most, but other UFAs also cause repression. This indicates that the position of the double bond does not determine repression (Fujiwara *et al.*, 1998), but rather intervenes with the level of transcription. Other unsaturated fatty acids, including the 18:1 Δ-15 UFA, have very little influence on the level of transcription. A close relationship has been found between the repression of *ATF1* expression and the melting point of the fatty acids in the medium. Another interesting finding by Fujiwara *et al.* (1998) was that the type of

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growth medium used also influenced transcription levels. In a minimal medium, such as SD, which contains glucose and yeast nitrogen base (YNB), repression by the UFAs is easier to detect. However, in a rich medium containing yeast extract, the repression by UFAs was inhibited and therefore more difficult to detect. There are two possible hypotheses to explain this phenomenon. Firstly, an unknown molecule in the yeast extract could inhibit this repression or, secondly, the high levels of nitrogen in the medium could activate transcription of *ATF1* and thereby overcome the repression by the UFAs.

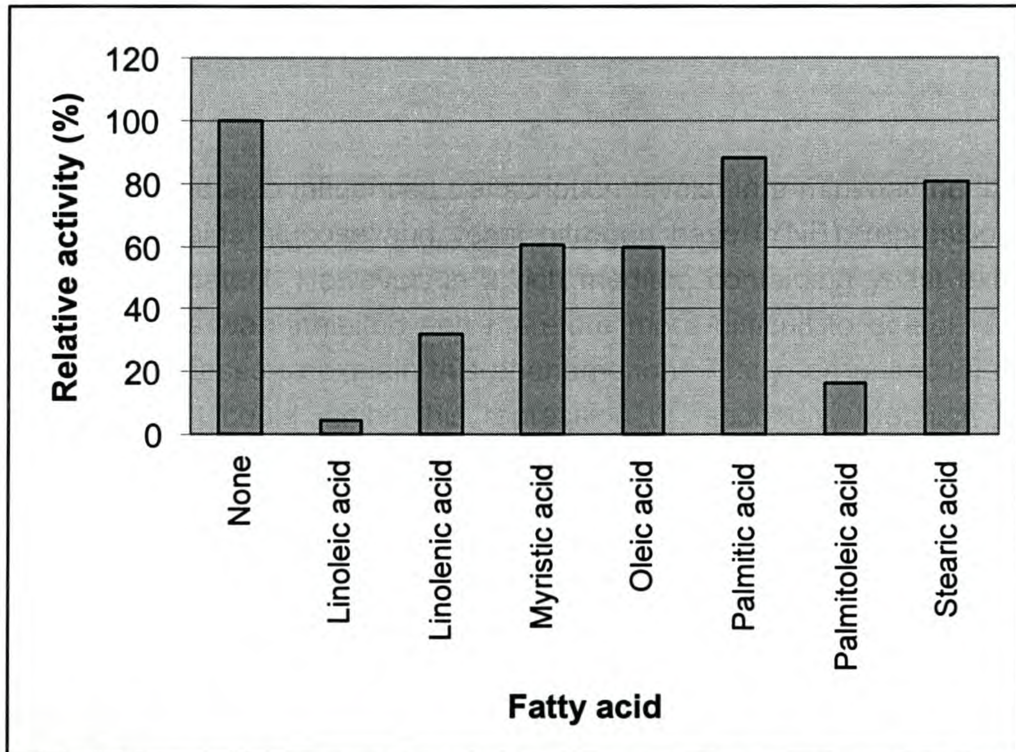

Figure 2.3. The effect of different fatty acids on the activity of AATase1 (Minetoki *et al.*, 1993)

The *FAA1-4* genes are involved in the activation of fatty acids to form acyl-CoAs. These genes encode for acyl-CoA synthetases. Fatty acids must be converted to acyl-CoA before they can form membrane or storage lipids. The *ATF1* transcript should not be repressed if these genes are unavailable to perform their tasks, as UFAs would not be produced. A disruption of either *FAA1* or *FAA4* resulted in a 30% greater expression of *ATF1* under anaerobic conditions. A double disruptant of both genes also showed increased expression levels of *ATF1*, but repression was regained by the addition of oxygen (Fujiwara *et al.*, 1998). The addition of UFAs did not cause repression in this double disruptant. This indicates that repression by oxygen must have occurred via a different mechanism to that of UFA.

A number of genes in yeast are expressed/induced during times of stress. These include genes involved in heat shock, oxidative stress, nitrogen starvation and osmotic stress. They all contain the DNA motif CCCCT (C_4T), which is a known stress response element. The *ATF1* sequence also has this element located in the

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promoter region of the gene. This stress response element (STRE) is induced during glucose depletion and decreased levels of cAMP (Mason and Dufour, 2000). A possible reason for the induction of this gene during times of stress could be due to its ability to utilise acetyl-CoA without the need for energy, thus preventing acetyl-CoA from being utilised in other energy-requiring reactions.

The *ATF1* promoter has a Rox1p-binding domain found between –120 and -108 bp upstream of the *ATF1* gene. The *ROX1* protein has been found to bind to this *ATF1* sequence (Fujiwara *et al.,* 1998). This sequence may be involved in UFA-mediated regulation of the gene and oxygen repression. Oxygen repression is brought about by the Rox1p-Tup1p-Ssn6p hypoxic repressor complex (Yoshimoto *et al.*, 2001). Further analysis by Yoshimoto *et al.* (2001) revealed an 18 bp element with the consensus sequence for the binding of the transcription activator Rap1p. This factor is essential for transcriptional activation and repression by UFAs. Another sequence was located upstream of the gene known as an oleate response element (ORE), which is targeted by the transcription factors Oaf1p and Pip2p. These factors are induced when yeast is grown on fatty acids as sole carbon source and could be negative regulators and thus prevent transcription (Mason and Dufour, 2000). These proteins, however, do not regulate the expression of the *ATF1* gene by induction in the presence of oleate (Karpichev and Small, 1998). This ORE sequence is also present in 39 other genes, including *FAA2* (discussed above) and *TES1* (to be discussed later). *TES1* is believed to be involved in peroxisome proliferation and fatty acid metabolism (Karpichev and Small, 1998).

By controlling the expression level of this gene, it is possible to produce higher levels of isoamyl acetate and ethyl acetate. Copy number, level of expression and source of the *ATF1* gene (yeast strain) are important for activity. Under the *PGK1* promoter, high levels of expression of the *ATF1* gene can be obtained, resulting in a 3.8- to 27-fold increase in the production levels of isoamyl acetate (Fujii *et al.*, 1994; Lilly *et al.*, 2000). These discrepancies are yeast strain dependent. Lilly *et al.* (2000) noted that by overexpressing this gene in fermenting yeast, lower levels of acetic acid were produced in the final fermented product. This is of immense importance in the wine industry, as acetic acid has been known to induce sluggish/stuck fermentations by inhibiting the yeast, as well as contributing to the volatile acidity (VA) of the wines. The legal limit for acetic acid is 1.2 g/l in wine. Wines with higher levels of this acid are regarded as spoiled.

From the above-mentioned data it can be concluded that *ATF1* possibly is involved in both fatty acid metabolism and the regulatory mechanisms for the ester synthesis housing complex.

2.6.2 ALCOHOL ACETYLTRANSFERASE I LIKE GENE (*LGATF1*)

Very similar to the *ATF1* gene is the *LgATF1*, for Like Gene *ATF1*, which was isolated and cloned by Fujii *et al.* (1994). It was discovered while probing for the *ATF1* gene in yeasts other than *S. cerevisiae.* With a 76% similarity to *ATF1*, it is not

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surprising that it was stumbled upon accidentally. It has been located in the bottom fermenting yeast *S. pastorianus* and the larger yeast *S. uvarum* (Fujii *et al.*, 1994; Yoshimoto *et al.*, 1998).

Like *ATF1, LgATF1* is an AATase involved in the production of isoamyl acetate and, to a lesser extent, ethyl acetate. Its activity is somewhat lower than that of *ATF1*, with overexpression resulting in a six-fold increase in isoamyl acetate production (Yoshimoto *et al.*, 1998). Possible reasons for the differences in the activities of the two genes could be variable copy numbers of the overexpressing plasmid and differences in substrate specificity, heat liability and temperature optimum for the respective enzymes. The gene is located on the 850 kb chromosome of *S. pastorianus*, with ORF designation Yscatf1b (Yoshimoto *et al.*, 1998). It has a predicted size of 63000 Da, with a pI of 7.68 (Nagasawa *et al.*, 1998).

Comparisons between the promoter regions of the *ATF1* and *LgATF1* genes by Yoshimoto *et al.* (1998) showed significant similarities. Both have the hypoxic consensus sequence TCTATTGTTTTT for the Rox1p. This sequence causes transcriptional repression by aeration. The Rox1p consensus sequence is found in the –65 to –54 region of the *LgATF1* promoter. The upstream sequence of *LgATF1* from –141 to –1 is very homologous to the *ATF1* upstream sequence from –196 to –60, indicating highly conserved domains between the two genes (Yoshimoto *et al.*, 1998). The transcription of this protein is also repressed by UFAs. The N-terminal extension includes 20 amino acids identical to that of the *ATF1* gene. This indicates that this protein is membrane associated (Mason and Dufour, 2000). *LgATF1* is fully functional when expressed in *S. cerevisiae* and the theory suggests that both *ATF1* and *LgATF1* may have diverged from the same ancestral gene (Fujii *et al.*, 1994; Yoshimoto *et al.*, 1998). It is as yet unknown if a null mutant is viable, but this would seem likely as the genes are so similar and an *ATF1* null mutant is viable.

2.6.3 ALCOHOL ACETYLTRANSFERASE II

The *ATF2* gene encodes for alcohol acetyltransferase II (AATaseII). This gene has been cloned by Nagasawa *et al.* (1998) from *S. cerevisiae sake* yeast. The protein is 36.9% identical to the *ATF1* protein and 36.6% identical to the *LgATF1* protein. The ORF encodes for a protein containing 535 amino acids. It has a molecular weight of 61898 Da, making it similar to both *ATF1* and *LgATF1*. The Atf2p has a pI of 5.77. This is somewhat lower than both the Atf1p and the LgAtf1p. The enzyme is capable of using short chain acyl-CoA esters, but cannot use long chain acyl-CoA esters. The *ATF2* gene is found in all *S. cerevisiae* strains, but is not found in *Saccharomyces bayanus. ATF2* is found as a single copy per haploid genome (Mason and Dufour, 2000). Its location is believed to be on chromosome VII (91% certainty) in *S. cerevisiae* on ORF designation YGR177c (Nagasawa *et al.*, 1998).

The protein is believed to be hydrophilic rather than hydrophobic, implying that it is membrane associated and not membrane bound. Limited quantities of the *ATF2* protein are found within the yeast cell. The C-terminal region does not have

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sequence homology to the *HEM0* and *HEM1* genes as the *ATF1* gene does.

Overexpression of the *ATF2* gene in *S. cerevisiae* increased the activity of the Atf2p 2.4-fold compared to that of the control strain. This activity increase is only 50% of an *ATF1* overexpression strain. The purified protein has a greater K_m than that of *ATF1* for isoamyl acetate. After the disruption of the *ATF1* gene, about 20% AATase activity is still left in *S. cerevisiae.* This is most likely due to the *ATF2* (Nagasawa *et al.*, 1998) and the *EHT1* protein products. Yoshimoto *et al.* (1999) reported that *ATF2* was repressed by aeration, but activated by the addition of 1 mM oleic acid. The *ATF2* gene is unaffected by UFAs at either the transcriptional or post-transcriptional level. The promoter region contains no STRE or ORE elements, as do *ATF1* and *LgATF1*. Within the promoter sequence located at position –139 and –128 upstream of the ATG is a sequence similar to that of the Rox1p consensus sequence responsible for repression by aeration (Yoshimoto *et al.*, 1999). One sequence common to AATases (*ATF1, LgATF1* and *ATF2*) is a heptapeptide protein sequence, WRLICLP. This sequence is located only within these three genes in yeast (Cauet *et al.*, 1999).

A possible biological function of the *ATF2* gene is as a detoxifying agent. It acts together with plasma membrane pumps to rid the cell of toxic 3-β-hydroxysteroids, which reduce cell growth (Cauet *et al.*, 1999). A null mutant indicates that the gene is not essential as long as the yeast is not grown with pregnenolone. Pregnenolone is toxic to the yeast, but its esterification by the *ATF2* protein reduces the toxicity in the cell. The *ATF2* protein has a high affinity for pregnenolone and other steroids, with a K_m of 0.5 μM for pregnenolone (Cauet *et al.*, 1999). Another possible biological function of the *ATF2* gene is in the detoxification of flavanoids that come from plants. Yeasts in their natural environment would come into contact with such flavanoids and would require some form of defence against these compounds (Cauet *et al.*, 1999).

2.6.4 ETHANOL HEXANOYL TRANSFERASE I

Ethanol hexanoyltransferase I or *EHT1* is a fourth ester-synthesising gene. It produces ethyl hexanoate from ethanol and hexanoyl-CoA and is thought to be an alcohol acetyltransferase. This gene has not been studied in much detail. It is located on chromosome II, with ORF designation YBR177C, and it encodes for a protein containing 451 amino acids. It has only a 17% identity to the *ATF1* and *ATF2* genes at the amino acid level. The *EHT1* protein does not contain the conserved heptapeptide sequence, WRLICLP, which is found in the other three AATases. The *EHT1* protein's substrate specificity is extremely broad and it even possesses some ethanol esterification activity (Mason and Dufour, 2000). The deletion of this gene results in an altered phospholipid pattern. The cellular levels of phosphatidylinositol and phosphatidic acid increase, with a resulting decrease in the cellular levels of phosphatidylethanolamine and phosphatidyldimethylethanolamine (Athenstaedt *et al.*, 1999). It also produces higher levels of ergosteryl esters, but a smaller amount of fecosterol. Steryl esters are essential for cell growth, important for membrane

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formation and are found in protein-enclosed microdroplets within the cell (Keesler *et al.,* 1992a, b). It is possible that the *EHT1* gene is involved in yeast lipid metabolism where it associates with lipid particles (Athenstaedt *et al.*, 1999). The protein itself has a predicted molecular weight of 51255 Da and a pI of 7.83, making it a very neutral protein (Athenstaedt *et al.*, 1999).

2.7 ESTERASES

Esterases are enzymes capable of hydrolysing aliphatic and aromatic esters into their principle components, namely an alcohol and a carboxylic acid. Esterases therefore limit the final level of esters formed during fermentation. They are different from lipases in that they are unable to hydrolyse triacylglycerols or TAGs. In *S. cerevisiae*, esterases usually break down esters, but Suomalainen (1981) proposed that they could also produce esters by the reverse reaction of the esterase in the absence of acetyl-CoA. However, this reaction is extremely slow. Schermers *et al.* (1976) found a positive correlation between esterase activity and the level of acetate esters in *S. cerevisiae.* They are of substantial interest in the fermented beverage industry because they affect flavour development during fermentation (Bardi *et al.,* 1993). By controlling esterase transcription, it might be possible to extensively improve the flavour and aroma profiles of alcoholic beverages. There is a comparatively limited amount of information regarding yeast esterases, with their genetic and physiological roles being poorly understood (Degrassi *et al.*, 1999). Wohrmann and Lange (1979), using gel electrophoresis, showed that there are four loci for esterases in wine yeast, but six esterases are known to date (Kondo and Inouye, 1991; Abraham *et al.*, 1992; Fukuda *et al.*, 1996; Degrassi *et al.*, 1999; Jones *et al.*, 1999; Wu *et al.*, 2000).

One way to classify esterases is according to their substrate specificity. One class of esterases, the carboxyl esterases, prefer short-chain fatty acid esters as their substrates, particularly those containing six carbons. They have a broad substrate range and are also called unspecific esterases (Parkkinen and Suomalainen, 1982). They can be subdivided into a number of classes, such as phenolic acid esterases, which act on esterified phenolic acids, and acetyl esterases, which are involved in cell wall degradation. There are also other types of esterases, including acetylcholin esterases, cholesterol esterases and thio-esterases (Kroon *et al.*, 1997).

Most esterases contain a serine active site. This serine functions with a histadine on the carboxylic acid (Blow *et al.*, 1969). Esterase activity increases rapidly after the start of fermentation, after which a marked decrease is noted (Guldfeldt *et al.,* 1998). This could be due to an increase in UFAs in the cell. Intracellular esterase activity is possibly related to the cell cycle, as the synthesis of esters increases towards the end of the growth phase (Guldfeldt *et al.*, 1998). **Table 2.2** summarises the known esterase genes in *S. cerevisiae.*

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Table 2.2. A comparison of the different esterase genes of *S. cerevisiae*

Gene	Chromosome	ORF	Size (Da)	Codon bias	pI	Null mutant	Inhibited by
IAH1	XV	YOR126c	27346	0.007	5.48	Viable	DFP, PMSF
TIP1	II	YBR067c	20727-80000	0.577	4.02	Viable	DFP
YJL068c	X	YJL068c	33934	2.300	6.47	Viable	Unknown
PPE1	VIII	YHR075c	44887	-0.007	7.05	Viable	Unknown
***TES1* (*PTE1*)**	X	YJR019c	40259	0.097	9.58	Viable	Unknown
TGL1	XI	YKL140w	62978	0.099	6.83	Viable	Unknown

2.7.1 ISOAMYL ACETATE HYDROLASE

Formally known as *EST2*, the isoamyl acetate-hydrolysing gene (*IAH1*) encodes for the major isoamyl acetate-hydrolysing esterase (Fukuda *et al.*, 1996). It is a carboxyl esterase and has been cloned from *S. cerevisiae*. This gene, along with the *ATF1* gene, is in control of the formation of isoamyl acetate during anaerobic fermentation (Fukuda *et al.*, 1998a). The gene has an ORF of 714 bp, which accounts for 238 amino acid residues. It is located on chromosome XV, as is *ATF1,* with ORF designation YOR126c (Wohrmann and Lange, 1979). Genomic Southern blot analysis has shown it to be present as a single copy in haploid strains. The molecular weight calculated from the amino acid sequence is 27346 Da. Fukuda *et al.* (2000) purified the enzyme and, using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), showed it to have a size of 28000 Da while it was found to have a size of 52000 Da by gel filtration. This indicates that the *IAH1* protein occurs naturally as a homodimer.

Iah1p is thought to be a serine-type esterase. It lacks the consensus sequence Gly-Xaa-Ser-Xaa-Gly, which is found in these types of esterases or lipases (Fukuda *et al.*, 1996). However, a similar sequence is present (Ala-Cys-Ser-Ala-Gly). **Table 2.3** shows the active site sequence of esterases and lipases from different organisms. Serine-type esterases are inhibited by phenylmethylsulfonyl fluoride (PMSF) and di-isopropylfluorophosphate (DFP) (Hoshino *et al.*, 1992; Shimada *et al.*, 1993). Fukuda *et al.* (2000) indicated that the *IAH1* protein is slightly inhibited by PMSF, but completely inhibited by DFP. Sulfhydryl-modifying chemicals, including iodoacetamide, and $HgCl_2$ also inhibit this enzyme, which signifies an active cysteine residue. The only cysteine residue is located adjacent to the serine residue, indicating that this area is the active site of the enzyme. By having cysteine as an active site, the enzyme can also be classified as an arylesterase (Fukuda *et al.*, 2000). A "TATA"-like sequence is located upstream of the gene and the

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polyadenylation consensus sequence AATAAA is found 190 bp downstream of the stop codon (Fukuda *et al.*, 1996).

Table 2.3. A comparison of the active sites of esterases and lipases (Fukuda *et al.*, 1996)

Origin	Motif sequence
S. cerevisiae (*IAH1*, esterase)	85-Ala-Cys-Ser-Ala-Gly-89
C. cylindracea (lipase)	207-Gly-Glu-Ser-Ala-Gly-211
G. candidum (lipase)	215-Gly-Glu-Ser-Ala-Gly-219
Rat liver (esterase)	219-Gly-Glu-Ser-Ala-Gly-223
Rabbit liver (esterase)	199-Gly-Glu-Ser-Ala-Gly-203
Hamster liver (esterase)	198-Gly-Val-Ser-Ala-Gly-202
Mouse liver (esterase)	213-Gly-Asn-Ser-Ala-Gly-217
Human liver (esterase)	106-Gly-Glu-Ser-Ala-Gly-110
Human serum (esterase)	196-Gly-Glu-Ser-Ala-Gly-200
Torpede californica (esterase)	632-Gly-Glu-Ser-Ala-Gly-636
Bacillus subtilis (lipase)	106-Ala-His-Ser-Met-Gly-110
P. fluorescens (lipase)	204-Gly-His-Ser-Leu-Gly-208
Pseudomonas sp. KWI-56 (esterase)	90-Gly-His-Ser-Cys-Gly-94
A. calcoaceticus (esterase)	129-Gly-Asp-Ser-Cys-Gly-133
A. calcoaceticus (esterase)	199-Gly-Asp-Ser-Ala-Gly-203
Consensus	Gly-Xaa-Ser-Xaa-Gly

Mutants deficient in Iah1p have been used in brewing trials to determine the effect of the gene on ester formation. These mutants accumulated higher amounts of isoamyl acetate and isobutyl acetate in comparison to their parent strains (Fukuda *et al.*, 1996). Fukuda *et al.* (1998b) also disrupted the *IAH1/EST2* gene in industrial *sake* yeast. This strain produces 1.8-fold more isoamyl acetate than the wild type strain. Its brewing ability was also evaluated. Fermentation speed was measured by CO_2 evolution and this indicated that the protein had no effect on the speed of fermentation in the industrial strain, although the fermentation rate was reduced in the laboratory strains. Even after disruption, trace amounts of esterase activity were still detected. This is most likely due to another esterase that is also capable of hydrolysing isoamyl acetate. Overexpression of the *IAH1* gene resulted in a decrease in isoamyl acetate and isobutyl acetate production, clearly indicating the esterase activity of the gene. When overexpressing, copy number plays an important role (Fukuda *et al.*, 1998a). These studies showed that AATase from the *ATF1* gene is the most important enzyme for isoamyl acetate production and that the balance between the activities of the *ATF1* enzyme and the *IAH1* enzyme is further responsible for its formation.

When isoamyl monofluoroacetate is hydrolysed by esterases, it forms monofluoracetic acid. This substance is toxic to the yeast, as it is a strong inhibitor of aconitase. Aconitase is involved in the Krebs cycle and thus the energy supply. Yeasts with low esterase activities, especially esterases acting on isoamyl acetate, should be resistant to monofluoroacetate, as they cannot break it down into its toxic

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form. Watanabe *et al.* (1993) isolated a number of yeasts that are resistant to isoamyl monofluoroacetate, with some having lower esterase activities.

The activity of the *IAH1* enzyme is more dependent on the length of the main chain of the aliphatic group than on the structure with a carbon number of 3 being the best. The *IAH1* enzyme has a K_m value of 40.3 mM for isoamyl acetate and 15.3 mM for isobutyl acetate. This indicates that the enzyme is more reactive against isobutyl acetate. This is clearly indicated in **Figure 2.4**. Enzyme activity is best at 25°C and stable below 30°C. Activity is lost quickly above this temperature. The enzyme has an optimum pH at 7.5, but is stable between pH 5 and 7.5 (Fukuda *et al.*, 2000).

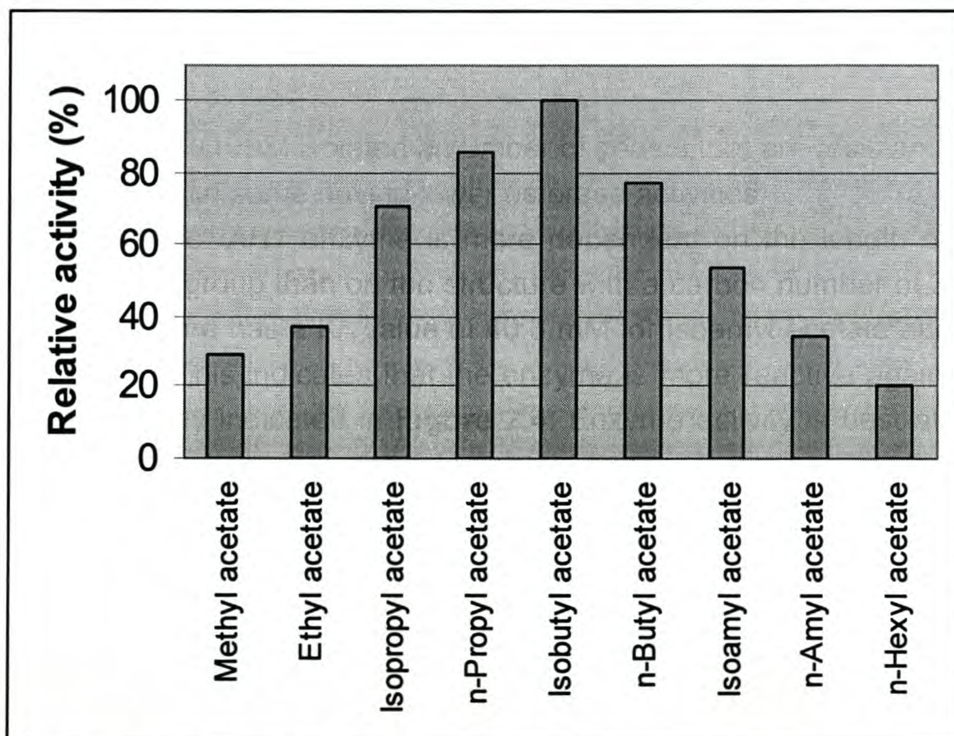

Figure 2.4. Substrate specificity of Iah1p for acetate esters (Fukuda *et al.*, 2000)

2.7.2 *TIP1*

The *TIP1* protein encodes for an extracellular serine-type esterase isolated from brewer's yeast. Kondo and Inouye (1991) originally cloned the gene from *S. cerevisiae*. Horsted *et al.* (1998) only recently discovered that it functions as an esterase. It was the first gene ever to be discovered that was both cold and heat shock-inducible and hence its name *TIP1* or **T**emperature shock **i**nduced **p**rotein (Kondo and Inouye, 1991). It can also be classified as a mannoprotein due to high glycosylation. The gene is located on the left arm of chromosome II and has an ORF consisting of 630 bp encoding for 210 amino acids. Its chromosomal location is YBR067c (Horsted *et al.*, 1998). The gene is found as a single copy in a haploid genome (Kondo and Inouye, 1991). Southern hybridisation analysis indicated that there are at least four homologous genes in the yeast genome, including the *SRP1* gene. The *TIP1* protein has a molecular weight of between 20727 and 80000 Da due

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to high levels of *O*-mannosylation and it contains 20% alanine and 23.3% serine residues (Kondo and Inouye, 1991). The protein is acidic in nature, with a pI of 4.02 (Kondo and Inouye, 1991).

The *TIP1* protein is processed by a signal peptidase (Kowalski *et al.*, 1995). The N-terminal signal sequence suggests that it is imported into the endoplasmic reticulum. The carboxyl-terminal end is serine rich, implying it to be highly glycosylated. The predicted protein sequence is consistent with high *O*-mannosylation (Kowalski *et al.*, 1995). The *TIP1* protein is also extremely hydrophobic, suggesting that it is anchored to the plasma membrane facing towards the outside of the cell. Van der Vaart *et al.* (1995, 1997) and Kowalski *et al.* (1995) suggested it to have a putative GPI or glycosyl-phosphatidyl inositol attachment signal, further indicating it to be membrane bound. The *TIP1* gene's putative N-terminal and C-terminal sequence suggests it to be secreted to the plasma membrane via the endoplasmic reticulum Golgi secretion pathway.

Located immediately upstream from the start codon is a sequence that contains only one mismatch to the consensus sequence A/TAA/CAA/CA<u>ATG</u>TCC, which is found before many highly expressed yeast genes (Hamilton *et al.*, 1987). Another interesting fact is that the gene has a codon bias of 0.577, further suggesting high levels of expression. There are two possible TATA boxes, one at position –68 and the other at –142 in the promoter region, but it is most likely that the –142 TATA sequence is the one for *TIP1* expression (Kondo and Inouye, 1991). A transcription terminator is located 59 bp after the TAA stop codon and has the sequence TATG…TAGT…TTT.

Horsted *et al.* (1998) isolated the *TIP1* protein from the brewing yeast *S. carlsbergensis*. Its molecular mass was determined by mass spectrometry as 16.9 kDa, which is slightly smaller than that of the *S. cerevisiae TIP1* protein. Using ρ-nitrophenol laurate as substrate, the pH optimum was determined to be between 4 and 5, making it an acidic esterase. This correlates well with its pI of 4.02. The enzyme was stable over a large pH range (pH 3 – 5), but pH levels above 7 resulted in a dramatic decrease in enzyme activity. The pH optimum varied depending on the substrate used. For ρ-nitrophenol laurate it was at pH 4.5 with a hydrolysis rate of 0.46 nmol/min per μg, whilst for triolein the optimum was at pH 6 with a hydrolysis rate of 0.10 nmol/min per μg (Horsted *et al.*, 1998). The hydrolysis rate of the enzyme was tested against esters having different chain lengths. Hydrolysis occurred for substances with four to 16 carbons in the fatty acid chain, but esters with a fatty acid chain of 12 carbons were hydrolysed best. The enzyme also has lipolytical activity and is capable of hydrolysing olive oil (triolein, C-18:1). The addition of this esterase to beer further proved its preference for esters with longer fatty acid chains. Only ethyl hexanoate and ethyl octanoate were hydrolysed, whilst ethyl acetate, isobutyl acetate and isoamyl acetate were not.

A *TIP1* null mutant is viable and has no noticeably different phenotype from that of a wild type strain. A disruptant also shows no difference in growth rate to that of a

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wild type strain (Kondo and Inouye, 1991). Esterase activity was assayed in the null mutant and no activity was found at pH 4.5, but activity was still present at pH 7.5 (Horsted *et al.*, 1998). This activity could be attributed to the *IAH1* gene, which has its optimum pH at 7.5. Induction by heat or cold shock is regulated by the temperature difference as well as by the initial temperature used. The mRNA levels show very little increase between the temperatures 21°C and 37°C (16°C difference), but a large increase in expression occurs with a change in temperature from 21°C to 15°C, a difference of 6°C (Kondo and Inouye, 1991). *TIP1* gene expression is also enhanced under anaerobic conditions, making it a hypoxic gene (Donzeau *et al.*, 1996). Another significant finding is that this gene is highly expressed during the G1 cell cycle phase. During this time, the mRNA levels increase dramatically.

As the amino acid sequence contained a large number of serine residues, inhibition tests were performed with DFP. The enzyme is inhibited by DFP, implying that the *TIP1* protein is a serine-type hydrolase (Horsted *et al.*, 1998). As this gene is one of a family of genes, it is possible that the remainder of the genes could also function as esterases. The ability of the *SRP1* gene to function as an esterase has not yet been researched. It remains possible that *SRP1* may have no esterase abilities at all.

2.7.3 YJL068C

YJL068c is an intracellular carboxylic esterase found in *S. cerevisiae*. Information on this novel esterase is very limited, as it has not been assigned a name and is known only by its chromosome location. This enzyme was first purified in 1999 (Degrassi *et al.,* 1999). The gene is located on chromosome X in the ORF designation YJL068c. The gene encodes for a protein with 299 amino acids in its deduced nucleotide sequence (Vandenbol and Portetelle, 1999). YJL068c preferentially hydrolyses aliphatic and aromatic short-chain fatty acid esters. It can also hydrolyse S-formylglutathione that is produced by the glutathione-dependent formaldehyde dehydrogenase. The protein has a molecular weight of 33934 Da, as determined by SDS-PAGE, and a pI of 6.47, making it almost a neutral protein. YJL068c is believed to occur naturally as a monomer. A null mutant is viable under a variety of growing conditions, with no noticeable phenotype occurring (Vandenbol and Portetelle, 1999). A codon bias of 2.3 implies that the gene has a low expression level under normal conditions (Vandenbol and Portetelle, 1999).

This enzyme's esterase activity was assayed at a pH of 7 and a temperature of 50°C. From these assays, it was found that the enzyme could utilise a number of substrates, including α-naphthyl acetate, 4-methyl-umbelliferyl acetate, ρ-nitrophenol acetate, carboxyfluorescein diacetate and S-formylglutathione, with K_m values of 0.86, 0.07, 0.35, 0.05 and 0.88 respectively. Its role in the hydrolysis of S-formylglutathione suggests that the protein is involved in formaldehyde detoxification (Degrassi *et al.*, 1999).

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2.7.4 *PPE1*

This is a newly discovered gene in *S. cerevisiae* (Wu *et al.*, 2000). Located on chromosome VIII in ORF designation YHR075c, the gene encodes for a carboxyl methyl esterase weighing 44887 Da and comprising 400 amino acids with a pI of 7.05. The *PPE1* protein is known to promote the activity of the phosphoprotein phosphatase 2A methyltransferase (*PPM1*) gene. The overexpression of the *PPE1* gene results in the yeast becoming supersensitive to benomyl, while its disruption results in enhanced methylation of *PP2A* (phosphoprotein phosphatase 2A). The disrupted strain grows in a normal fashion at all temperatures on both fermentable and non-fermentable carbon sources (Wu *et al.*, 2000). No other information is currently available on this gene.

2.7.5 *TES1*

TES1 is also a newly discovered esterase located on ORF designation YJR019c in *S. cerevisiae*. It is classified as a thioesterase, with the gene product being an abundant peroxisomal membrane-associated acetyl-CoA thioesterase (Jones *et al.*, 1999). The protein has a predicted molecular weight of 40259 Da and contains 349 amino acids. Acyl-CoA thioesterases catalyse the hydrolysis of acyl-CoA molecules to free fatty acids and CoA. Previous thioesterases were found to associate with fatty acid synthetase (FAS), where they assisted in fatty acid synthesis. That makes this esterase particularly interesting, as no FAS is located in the peroxisomes, which means that this esterase must perform a different function. Jones *et al.* (1999) believe that this enzyme is involved in growth on fatty acid media, resulting in fatty acid breakdown. The gene is induced by fatty acids and mildly repressed by growth on ethanol. When grown in a medium containing glycerol-oleate, the gene is transcribed at a 10-fold higher level (Karpichev and Small, 1998).

The *TES1* promoter contains the consensus sequence $CGGN_2TNAN_{(6\text{-}13)}C/GCG$, which is an upstream activating sequence known as the oleate response element or ORE. This ORE is responsible for activating expression in response to fatty acids such as oleate. Karpichev and Small (1998) discovered that two proteins, designated Oaf1p and Pip1p, are required for the activation of certain genes on oleate. *TES1* requires these proteins for this induction. As discussed earlier, this response element is also located before the *ATF1* gene. The gene shows a 26.3% homology to the human acyl-CoA thioesterase *hTE*.

Disruption of the *TES1* gene produces a strain with considerable growth defects. This strain is only capable of growing up to 53% of the final density of the wild type on a medium containing oleate. This indicates that the gene is required for growth on fatty acids and is possibly involved in fatty acid oxidation. A loss of 80% in the total *n*-decanoyl-CoA esterase activity occurred as a result of its disruption in yeast cells (Jones *et al.*, 1999). Jones *et al.* (1999) have suggested a name change from *TES1* to *PTE1*, for **P**eroxisomal **t**hio**e**sterase.

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2.7.6 *TGL1*

Triglyceride **l**ipase-cholesterol esterase, or *TGL1,* is another known esterase gene in *S. cerevisiae*. It is located on ORF designation YKL140w on chromosome XI. Information regarding this gene is extremely limited, with only two papers available currently (Abraham *et al.*, 1992; Athenstaedt *et al.*, 1999). The gene is somehow linked to the previously discussed *EHT1* gene, being involved in lipid metabolism, although via secondary effects. When disrupted, the yeast strain produces higher amounts of ergosteryl esters than its wild type.

It also exhibits different phospholipid and neutral lipid patterns to that of the wild type strain. The protein has a predicted molecular weight of 62978 Da, according to its deduced amino acid sequence, and its pl is 6.83. The *TGL1* gene has a codon bias of approximately 0.2, indicating that it is expressed at low levels under normal conditions (Abraham *et al.*, 1992). Surprisingly, the gene's transcript is 700 bp longer than the proposed coding region, with the extra fragment located on the tail side of the protein. This tail region is located on the ORF designation YKL5A and it overlaps the 3'-end of the *TGL1*-encoding sequence. This added sequence has no initiation (ATG) codon and its function is as yet unknown (Abraham *et al.*, 1992).

2.7.7 OTHER YEAST ESTERASES

Lee *et al.* (1987) found an extracellular esterase in the red yeast *Rhodotorula mucilaginosa*. The enzyme is relatively specific for substrates containing an *O*-acetyl group and the remainder of the molecule can contain one of the following: a phenol, a monosaccharide, a polysaccharide or an aliphatic alcohol. It shows highest specificity to acetylxylan and glucose β-D-penta-acetate and can even hydrolyse ρ-nitrophenol. It has an optimum pH of 9 and a pH range of between 5.5 and 10. This characteristic would make the enzyme unsuitable for use during winemaking.

Lipases are enzymes that act preferentially on lipids such as triacylglycerols. They hydrolyse the ester bonds in these molecules to form free fatty acids and glycerols (Brahimi-Horn *et al.*, 1990). Most lipases are also able to cleave simple esters into their principle components. Some lipases have been known to catalyse the reverse reaction and thus synthesise esters (Deetz and Rozzell, 1988). Brahimi-Horn *et al.* (1989, 1990) showed that a commercial preparation of lipases from the yeast *Candida cylindracea* had esterase activity towards short-chained and long-chained ρ-nitrophenol esters and other esters. The enzymes are also capable of hydrolysing long-chain N-heterocyclic fatty esters (Lie Ken Jie and Syed-Rahmatullah, 1995). Other enzymes, including cellulases, hemicellulases and pectinases, have also been shown to possess esterase activities (Dugelay *et al.*, 1993).

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2.8 FATTY ACID SYNTHESIS

Fatty acids are an integral part of esters and are needed for the formation of esters. Yeast are capable of producing different types of fatty acids. The fatty acid content of the cell membrane affects the level of esters and ester synthesis (Fujiwara *et al.*, 1998). Lipids themselves play an important role in cell survival and are basic components of the cell membrane. Altering the cell membrane composition can drastically affect the cell's tolerance to different compounds, including ethanol. Lipids also provide the cell with valuable energy through β-oxydation (McHale *et al.*, 1996). Fatty acid synthetase (FAS) is an enzyme involved in fatty acid synthesis and is capable of producing long-chain fatty acids that can utilise acetyl-CoA or malonyl-CoA as starting materials (Tanaka *et al.,* 2000). The deletion or inhibition of FAS could possibly increase the production of esters; however, this will have a negative effect on the growth of the cells. Saturated and unsaturated fatty acids are vital molecules that are important for controlling the expression of many lipid biosynthetic genes and even ester metabolism. It is vital for the yeast to maintain a balanced ratio of unsaturated and saturated fatty acids in its membrane. This assists the cell in its ability to survive a number of different physiological conditions. Ester and fatty acid synthesis share many common regulatory mechanisms (Mason and Dufour, 2000) and a closer look at fatty acid synthesis is required. In *S. cerevisiae*, the end products of fatty acid synthesis are fatty acyl-CoA esters, which are the main aroma compounds found in fermented beverages (Bardi *et al.,* 1999).

2.8.1 Δ-9 FATTY ACID DESATURASE

The Δ-9 fatty acid desaturase is an enzyme found in the yeast that catalyses the formation of double bonds between carbons 9 and 10 of saturated fatty acyl substrates, palmitoyl- and stearoyl-CoA (Stukey *et al.*, 1989). It accounts for all the *de novo* UFA production in *S. cerevisiae*. In *S. cerevisiae*, the *OLE1* gene encodes for this enzyme. The *OLE1* gene sequence is 1530 bp long and produces a protein that contains 510 amino acids with a molecular weight of 57.4 kDa (Stukey *et al.*, 1990). A total of 25.7% of the amino acid residues are charged, with 10% being acidic and 15.7% being alkaline. The protein has a high degree of hydrophobicity, with 251 amino acids (49.2%) being hydrophobic, and thus it suggests that the enzyme is membrane bound. The Δ-9 fatty acid desaturase has extensive similarity to the rat liver stearoyl-CoA (36%). The gene sequence contains no sites for N-glycosylation and there is no cleavable N-terminal signal sequence (Stukey *et al.*, 1990).

There are two possible TATA consensus sequences to the 5' side of the gene. They are located at position –30 and –156 relative to the ORF, but it is thought that the TATA sequence at –156 is the most important of the two. In frame to the first start codon are an additional three ATG (start codon) codons, located at positions 56, 61 and 116 respectively. The ATG at position 1 has been proven experimentally to be

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the one used for gene translation (Stukey *et al.*, 1990).

Numerous control studies have been performed on this gene (McDonough *et al.*, 1992; Choi *et al.*, 1996; Gonzalez and Martin, 1996) and it is known that the *OLE1* gene expression is regulated by a number of mechanisms. The most obvious of these mechanisms are by (1) saturated fatty acids causing up to a 1.6-fold increase in expression, (2) UFAs causing up to a 60-fold repression, and (3) oxygen causing repression (Choi *et al.*, 1996). Previously, a Rox1p binding site has been found in the promoter region (Zitomer and Lowery, 1992; Fujiwara *et al.*, 1998) of the *OLE1* gene, but this sequence does not control gene repression by oxygen. Nakagawa *et al.* (2001) deduced from deletion experiments of the appropriate sequence that the repression of the *OLE1* gene is mediated by the Rox1p-independent pathway. They identified a new 50 bp sequence between position –356 and –307. The sequence was designated O2R or the O_2-regulated element. This sequence is considered to be an anaerobic upstream activation site. Anaerobic derepression was decreased from 4.4 to 1.4-fold if the sequence was deleted. This sequence contains no homology to the Rox1p consensus sequence. Through a number of deletion and mutation experiments, they found that there was a GATAA sequence within this O2R sequence. This sequence is a binding site for the GATA family of transcriptional activation factors, including Gln3p and Gat1p. Mutation analysis of this sequence resulted in a decrease of 7.3 to 3.3-fold for anaerobic derepression. This shows that the sequence is important for O_2 repression.

The transcription level is also controlled by UFAs and by the saturated fatty acid level (McDonough *et al.*, 1992; Choi *et al.*, 1996). Deletion experiments in the promoter region have also found a fatty acid regulated region or FAR (Choi *et al.*, 1996). It is a 111 bp sequence found 580 bp upstream from the start codon. The deletion of an 88 bp region within this FAR element resulted in the complete loss of UFA regulation of the gene and a 27-fold decrease in transcription. This indicates that this site is essential for gene expression. Within the FAR element is a 9-bp sequence responsible for fatty acid-mediated repression and, to some extent, expression of the gene. A series of GC-rich sequences is found within this FAR element. Two of these were of particular interest, as they were also found in the *ERG11* gene. This gene encodes for the sterol biosynthetic gene that is also involved in fatty acid regulation. The FAR element is recognised by a number of transcription activators, including the *HAP1* protein. According to Choi *et al.* (1996), the *HAP1* protein is responsible for more than half of the *OLE1* transcription activity during fermentative growth conditions. Hap1p is not essential for activation of the *OLE1* gene, indicating that other proteins are also responsible for its activation (Choi *et al.*, 1996). In the absence of the *HAP1* protein, sufficient levels of mRNA are produced to maintain the normal membrane fatty acyl lipid composition.

It is now known that the Δ-9 fatty acid desaturase gene is repressed by both oxygen and UFAs, but not exactly which UFAs are responsible for this repression. McDonough *et al.* (1992), through fusion of the *OLE1* promoter to the *lacZ* gene,

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concluded that only certain UFAs were responsible. Mono-unsaturated and polyunsaturated fatty acids containing a double bond at the Δ-9 position were strong repressors of gene transcription, but mono-unsaturated fatty acids with double bonds in positions Δ-5, Δ-10 and Δ-11 showed no repression. This indicates that the *OLE1* gene transcripts are repressed by their own products and introduces a new level of control. A recent study by McHale *et al.* (1996) demonstrated a new class of genes involved in the control of the *OLE1* gene. These genes were designated *FRM1-6*, for **F**atty acid **R**epression **M**utants. These mutant strains did not repress *OLE1* transcription under normal repression conditions and resulted in constitutive expression.

The mRNA half-life of the *OLE1* gene is also affected by the presence of UFAs in the media (Gonzalez and Martin, 1996). When the yeast is grown in a medium without exogenous fatty acids, the mRNA transcript is moderately stable and has a half-life of about 10 minutes (± 90 seconds). The addition of UFAs to the medium drastically shortens the mRNA stability, resulting in a half-life of approximately 150 seconds. Although the addition of saturated fatty acids has been shown to activate transcription of the *OLE1* gene (Choi *et al.*, 1996), they have no effect on the stability of the mRNA. The reason for this induction of mRNA stability can be attributed to the 5' untranslated region of the *OLE1* mRNA. The replacement of this region with the *GAL1* sequence stabilised the mRNA, even in the presence of UFAs. Another gene, *XRN1*, encodes for a 5' – 3' ribonuclease. The disruption of this gene results in a four-fold increase in the mRNA half-life of the *OLE1* gene in the absence of fatty acids. Its stability, however, is reduced by the addition of UFAs. This indicates that the *XRN1* gene is required for the degradation of the *OLE1* mRNA transcript, but is not required for the destabilisation of the transcript in the presence of UFAs (Gonzales and Martin, 1996).

The acyl-CoA-binding protein (ACBP) transcribed by the *ACB1* gene also plays a role in the activation of the *OLE1* gene. It is a 10 kDa protein that binds to the FAR element and causes repression of the expression. It also binds medium and long-chain saturated and unsaturated fatty acid acyl-CoA esters (Schjerling *et al.*, 1996). The disruption of the ACBP gene results in a three- to five-fold activation of transcription of the *OLE1* gene and drastically increases the mRNA levels of this product (Choi *et al.*, 1996; Schjerling *et al.*, 1996). The disruption of this gene does not affect the growth rate of the yeast on glucose, but the growth rate is reduced if it is grown on ethanol. The ACBP gene is believed to be involved in the transport of newly synthesised acyl-CoA esters from the fatty acid synthetase (FAS) to acetyl-CoA consuming processes (Schjerling *et al.*, 1996).

Previously, the *FAA1* and *FAA4* genes and their role in the repression of the *ATF1* gene were described. These genes are also involved in repressing the *OLE1* gene. They are essential for the repression of the *OLE1* gene mediated by UFAs via the FAR regulatory element located upstream of the gene (Choi *et al.*, 1996). Disruption of either of the *FAA* genes resulted in lowered levels of repression via

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UFAs, but regulation by UFAs is completely lost if both genes are disrupted.

On a tertiary level, the *OLE1* enzyme has two hydrophobic regions and Stukey *et al.* (1990) have proposed that they form two membrane-traversing loops. Neither the N-terminal nor the C-terminal domains of the enzyme sequence are very hydrophobic. This suggests that they do not add to the integral membrane domains of the enzyme (Stukey *et al.*, 1990). Most of the enzyme is located on the cytosolic side of the endoplasmic reticulum, with two segments looping between the membrane of the endoplasmic reticulum. Amino acids 116 through 159 and 257 through 305 are thought to make these trans-membrane loops. There are at least three shorter hydrophobic regions of less than seven amino acids. These regions probably associate with the endoplasmic reticulum on the cytosolic side of the membrane. These sequences are extremely homologous to the sequences found in the rat liver Δ-9 enzyme (Stukey *et al.*, 1990).

The *OLE1* gene itself has been both disrupted and overexpressed (Stukey *et al.*, 1989; Kajiwara *et al.*, 2000). The null mutant is viable, but requires the presence of unsaturated fatty acids in the medium to sustain growth. Stukey *et al.* (1989) disrupted the gene in *S. cerevisiae*, producing a non-reverting UFA-requiring strain. The membrane lipids of this strain contain only UFAs that were taken up from the medium in which it was grown. Removal of UFAs from the medium resulted in normal growth of the disrupted strain for about three generations, after which the cell's growth rate decreased rapidly. During this time, the UFA content of the cells decreased from 63 to 7.3 mol% of the total fatty acid content. Kajiwara and his co-workers (2000) overexpressed this gene in *S. cerevisiae* using the powerful *PGK1* promoter. This strain could produce between 1.8- and 2.5-fold more ethanol during aerobic fermentation and the strain also grew more rapidly. During anaerobic fermentation, this strain produced 1.3-fold more ethanol and it was also more tolerant to stressful conditions. The fatty acid content of both the wild type and the overexpression strain were examined. The overexpression strain contained approximately 9% more UFAs than the wild type strain, with this value approaching 87%. Kajiwara *et al.* (2000), however, did not look at mRNA stability in their experiments. With these high levels of UFAs in the cell, the mRNA must have become extremely unstable. It could be possible to even further improve these overexpression experiments by altering the 5' sequence of the transcript. This would improve the mRNA's stability, resulting in an increase in enzyme production. The *OLE1* gene can be functionally replaced with the rat stearoyl-CoA desaturase gene (Stukey *et al.*, 1990). This results in the yeast regaining an almost normal phenotype.

It is clear from the above text that fatty acid metabolism in yeast is extremely complicated. Manipulation of fatty acid synthesis could provide the brewer and wine maker with new methods for altering the ester concentration in alcoholic beverages. Manipulation of all these genes would be extremely difficult and getting the correct expression levels and ester formation could be even more troublesome.

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2.9 CONCLUSION

The regulation of ester synthesis shares many features with other metabolic pathways, particularly with those involved in fatty acid metabolism. It is clear that acetyl-CoA plays a crucial role in both ester and fatty acid metabolism and that acetyl-CoA is essential to both. To gain a clear understanding of ester metabolism, one needs to take a closer look at the individual genes involved, their functions and their regulatory mechanisms. Clearly, there are a large number of known genes that can alter the aroma and flavour of alcoholic beverages. There are also a number of regulatory genes acting on these AATases or esterases to ensure the correct level of expression at all times. These regulatory genes should also be studied and their effects on aroma and flavour determined. For the alcoholic beverage industry, a look at all these genes and the way in which they affect the aroma and flavour profiles of the final product could provide new solutions for the improvement of these products. If we consider that today's consumer is better educated and becoming more health conscious than ever before, it is clear that there is a need to produce healthier, better quality products for this user. The market is also becoming saturated with new products and the need for a competitive edge to ensure the manufacturer's survival is essential.

The regulation and functional understanding of these genes is not only essential for the alcoholic beverage industry, but also for molecular scientists. These genes could shed some light on the overall functioning of the cell and teach us new means of cell signalling, genetic control and regulation. The yeast is a model organism for the understanding of eukaryotic cell signalling and general metabolism. If we can gain an understanding of how all the genes function on their own and in conjunction with others, we could eventually discover how the cell functions as a unit. Perhaps then this information can be applied in the understanding of more complex organisms, such as animals and even humans. As a long-term goal, the understanding of more simple systems will make it possible to understand more complex systems and this will provide us with new approaches for the treatment of diseases and infections, and hopefully lead to an improvement in the quality of life.

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CHAPTER 3

RESEARCH RESULTS

The disruption and overexpression of two esterase genes, *IAH1* and *TIP1*, in the *Saccharomyces cerevisiae* wine yeast strain VIN13 and their effects on wine and brandy

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3. RESEARCH RESULTS

THE DISRUPTION AND OVEREXPRESSION OF TWO ESTERASE GENES, *IAH1* AND *TIP1*, IN THE *SACCHAROMYCES CEREVISIAE* WINE YEAST STRAIN VIN13 AND THEIR EFFECTS ON WINE AND BRANDY.

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A group of volatile compounds known as esters impart fruity aromas and flavours to alcoholic beverages. Esters are produced when an alcohol and a carboxylic acid combine. There are a number of genes involved in ester metabolism, including alcohol acetyltransferases and esterases, and it is the balance between these genes that is responsible for ester formation. Esterases, such as those encoded the *IAH1* and the *TIP1* genes, are involved in the catabolism of esters into their principal components. The *IAH1* and *TIP1* genes were amplified from the widely used *Saccharomyces cerevisiae* wine yeast strain VIN13 using the polymerase chain reaction (PCR) technique and sub-cloned under the control of the phosphoglycerate kinase gene (*PGK1*) promoter and terminator. These plasmids were used as a template to generate overexpression cassettes by means of PCR. The disruption cassettes were produced using plasmid pEG6 as template for PCR. These cassettes were transformed into VIN13 and verified by Southern blot hybridisation. Reverse Transcriptase-PCR (RT-PCR) verified the expression of these genes during normal growth conditions, with differences in expression between the parental, disrupted and overexpressing strains. These new strains underwent fermentation trials for the production of wine and brandy. The *IAH1* overexpressing strain performed as expected, decreasing the level of isoamyl acetate and ethyl acetate, while the single disrupted strain had no significant effect. The *TIP1* overexpressing strain showed no significant changes to the level of esters; however, the total acid level was decreased dramatically, especially the acetic acid levels. The *TIP1* disrupted strain showed an increase in the level of acetic acid. During the sensorial evaluation, the wine and brandy showed only small differences with regard to their aroma and flavour profiles. This study has shown that single gene conversions can make significant contributions to the overall organoleptic properties of an alcoholic beverage.

3.1 INTRODUCTION

Alcoholic beverages can be classified according to their aroma and flavour profiles. A number of factors are capable of altering these aroma and flavour profiles, with the yeast strain and fermentation conditions having the strongest influences (Engan, 1974; Nykänen and Nykänen, 1977). A large group of volatile compounds, known as esters, are of particular importance due to their fruity characteristics and low sensory thresholds (Peddie, 1990). These esters include ethyl acetate (solvent-like), isoamyl acetate (pear drops), isobutyl acetate (banana), and ethyl hexanoate (apple-like)

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(Yoshioka and Hashimoto, 1983). Most esters present in wine and brandy are secondary products produced by the yeast during alcoholic fermentation (Lambrechts and Pretorius, 2000). An ester is formed when an alcohol reacts with a carboxylic acid. This can occur spontaneously or through an enzyme-catalysed reaction (Nordström, 1961; Peddie 1990). Alternatively, the reverse reaction can also occur with the assistance of enzymes. Esterases are the class of enzymes involved in the latter reaction. They are capable of limiting the final number of esters produced and should thus be able to alter the aroma and flavour profiles. Two such esterase genes are the *IAH1* and *TIP1* genes (Fukuda *et al.*, 1996; Horsted *et al.*, 1998).

The *IAH1* gene encodes for an isoamyl acetate hydrolase that preferentially acts on isoamyl acetate and isobutyl acetate (Fukuda *et al.*, 1996, 2000). It was formerly known as the *EST2* gene and produces a serine-type carboxyl esterase. As acetate esters are the largest group of esters affecting aroma in alcoholic beverages, the overexpression and disruption of this gene in industrial yeast would provide useful information about its contribution to the aroma and flavour of wine and brandy. The *IAH1* gene is located on chromosome XV, with an open reading frame of 714 bp, and it encodes for a protein with a molecular weight of 28 kDa. This protein occurs naturally as a homodimer and has a molecular weight of 52 kDa (Fukuda *et al.*, 2000).

The *TIP1* gene encodes for an extracellular serine-type esterase (Horsted *et al.*, 1998) and the gene locus is situated on the left arm of chromosome II. The *TIP1* protein has a molecular weight between 20 kDa and 80 kDa and contains 20% alanine and 23.3% serine residues (Kondo and Inouye, 1991). This was the first gene to be discovered that was induced by both heat and cold shock (Kondo and Inouye, 1991). The *TIP1* protein has a putative glycosyl-phosphatidyl inositol (GPI) attachment signal, indicating that it is membrane bound (Kowalski *et al.* 1995; Van der Vaart *et al.*, 1995, 1997). The *TIP1* gene is a hypoxic gene, as it is expressed under anaerobic conditions (Donzeau *et al.*, 1996). The *TIP1* protein operates in a pH range of between 3 and 5, being most active at a pH of 4 (Horsted *et al.*, 1998).

Even though the consumers are better informed in today's competitive market, they are overwhelmed with choice. It would be advantageous for the alcoholic beverage industry to be able to produce tailor-made beverages to suit the needs and desires of consumers. This would give a producer a competitive edge over other producers.

The focus of this study was to both disrupt and overexpress the *IAH1* and the *TIP1* genes in an industrial wine yeast to gain a better understanding of the ability of these genes to alter the aroma and flavour profiles, which would provide a possible tool to improve the quality of both wine and brandy. It will be necessary to overexpress the *IAH1* and *TIP1* genes under a strong constitutive promoter, as the natural promoters of these genes are affected by too many variables, including the presence of oxygen and unsaturated fatty acids.

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3.2 MATERIALS AND METHODS

3.2.1 STRAINS AND GROWTH CONDITIONS

All strains used in this study are listed in **Table 3.1.** The bacteria *Escherichia coli* DH5α was used as host for all plasmid constructions and amplifications. For selective growth, the bacteria were grown in Luria-Bertani (LB) media (Biolab) containing 100 mg/l ampicillin (Roche, Germany) and incubated at 37°C overnight. The *S. cerevisiae* wine yeast strain VIN13 (Anchor Yeast) was used for all genetic manipulations. Yeast strains were grown at 30°C on yeast peptone dextrose (YPD) plates containing yeast extract (1% w/v), peptone (2% w/v), glucose (2% w/v) and bacteriological agar (2% w/v) (Biolab). For yeast transformations, all the cells were grown aerobically in 50 ml of YPD broth at 30°C and then harvested for transformation. After transformation, the yeast cells were either plated onto YPD agar containing 400 mg/l geneticin (Sigma), when screening for the *KanMX* marker gene, or onto SCD media containing glucose (2% w/v), yeast nitrogen base (0.67% w/v) without amino acids (Difco), bacteriological agar (2% w/v) (Biolab) and 60 mg/l sulfometuron methyl (SMM) (Dupont), when screening for the *SMR1-410* marker. For the recovery of the loxP-*KanMX*-loxP cassette, yeast cells were grown in 2 ml of yeast peptone galactose (YPG) for 4 hours and then plated onto YPG agar plates containing yeast extract (1% w/v), peptone (2% w/v), galactose (2% w/v) and bacteriological agar (2% w/v).

3.2.2 PLASMID CONSTRUCTIONS

Standard procedures for the isolation and manipulation of DNA were used throughout this study (Sambrook *et al.*, 1989). Restriction enzymes (Roche) and T4 DNA-ligase (Promega) were used for the enzymatic manipulation of DNA. Expand Hi-Fidelity DNA polymerase (Sigma) was used for all polymerase chain reaction (PCR) experiments. All enzymes were used according to the supplier's specifications. All plasmids used in or constructed for this study are listed in **Table 3.1**.

3.2.2.1 Construction of disruption plasmids

Plasmid pUG6 was digested with *Xba*I, subjected to Klenow and finally digested with *Nco*I to remove the *TEF* promoter located before the *KanMX* gene. It was replaced with a *Sma*I–*Nco*I *ENO2* fragment from plasmid pG-ENO2p to produce plasmid pEG6. The *TEF* promoter is originally from the filamentous fungus *Ashbya gossypii*.

For the construction of plasmid pSHeSMR, the *ENO2* promoter region and the *SMR* gene were amplified by PCR from VIN13 genomic DNA and plasmid pWX509 (Casey *et al.*, 1988) respectively. A 0.6 kb *ENO2* fragment and a 2 kb *SMR1-410* fragment were digested with *Pst*I. The two fragments were ligated together and this entire fragment was then amplified using PCR to generate a 2.6 kb *ENO2-SMR* fragment. This was ligated into pGEMT-easy, producing plasmid pG-eSMR. Plasmid

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pG-eSMR was digested with *Nco*I and *Nsi*I and the 2.6 kb *ENO2-SMR* fragment was isolated. Plasmid pSH47 was finally digested with *Nco*I and *Nsi*I and the major fragment was retained and ligated to the 2.6 kb *ENO2-SMR* fragment. This produced plasmid pSHeSMR. The 0.6 kb *ENO2* fragment was ligated into plasmid pGEMT-easy to produce plasmid pG-ENO2.

Table 3.1. Microbial strains and plasmids used in this study

Plasmids and strains	Constructs	Source
Escherichia coli DH5α	*Sup*E44 ρ*lac*U169 (φ80*lac*ZρM15) *hsd*R7 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	GIBCO-BRL/Life Technologies
Saccharomyces cerevisiae strain VIN13	Commercial wine yeast strain	Anchor Yeast, South Africa
Transformants		
VIN13-ΔIAH1	loxP *KanMX* loxP δ-*IAH1*	This study
VIN13-ΔTIP1	loxP *KanMX* loxP δ-*TIP1*	This study
VIN13-IOE1	loxP *KanMX* loxP *PGK1*$_P$-*IAH1*-*PGK1*$_T$	This study
VIN13-TOE1	loxP *KanMX* loxP *PGK1*$_P$-*TIP1*-*PGK1*$_T$	This study
Plasmids		
pUG6	*bla* loxP *KanMX* loxP	Güldener *et al.*, 1996
pEG6	*bla* loxP *ENO2*$_P$-*KanMX* loxP	This study
pGEMT-easy	*bla LacZ*	Promega
pG-IAH1	*bla LacZ-IAH1*	This study
pG-TIP1	*bla LacZ-TIP1*	This study
pG-ENO2p	*bla LacZ-ENO2*$_P$	This study
pJCP	*bla* loxP *TEF1*$_P$-*KanMX* loxP *PGK1*$_P$-*PGK1*$_T$	Justin Corrans
pJ	*bla* loxP *ENO2*$_P$-*KanMX* loxP *PGK1*$_P$-*PGK1*$_T$	This study
pJ-IOE1	*bla* loxP *ENO2*$_P$-*KanMX* loxP *PGK1*$_P$-*IAH1*-*PGK1*$_T$	This study
pJ-TOE1	*bla* loxP *ENO2*$_P$-*KanMX* loxP *PGK1*$_P$-*TIP1*-*PGK1*$_T$	This study
pSH47	*bla URA3*	Güldener *et al.*, 1996
pWX509	*bla SMR1-410*	Casey *et al.*, 1988
pG-eSMR	*bla LacZ-ENO2*$_P$-*SMR1-410*	This study
pSHeSMR	*bla SMR1-410 Cre*-recombinase	This study

3.2.2.2 Construction of overexpression plasmids

The *IAH1* and *TIP1* genes and the enolase 2 (*ENO2*) promoter region were PCR-generated from *S. cerevisiae* strain VIN13 and cloned into the pGEMT-easy PCR cloning kit (Promega), producing plasmids pG-IAH1, pG-TIP1 and pG-ENO2p respectively. Plasmid pJ is a modification of plasmid pJCP (Justin Corrans, personal communication), which is a descendent of plasmid pUG6 (Güldener *et al.*, 1996) and has the phosphoglycerate kinase 1 (*PGK1*) promoter and terminator sequence with a multiple cloning site (*Bgl*II, *Eco*RI and *Xho*I) in between. Plasmid pJCP was digested

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with *Xba*I, the overhangs were filled in with Klenow and finally digested with *Nco*I. This generated a 3616 bp fragment used for further experimentation. Plasmid pG-ENO2p was digested with *Sma*I and *Nco*I, generating a 601 bp *ENO2* promoter fragment. This was cloned into the *Xba*I (blunt)/*Nco*I site of plasmid pJCP, producing plasmid pJ with the *KanMX* gene under the yeast *ENO2* promoter. Plasmid pG-TIP1 was digested with *Bgl*II and *Xho*I and yielded a 2 kb *TIP1* gene fragment. Plasmid pJ was linearised with enzymes *Bgl*II and XhoI within a multiple cloning site located between a *PGK1* promoter and terminator. The 2 kb *Bgl*II-*Xho*I *TIP1* fragment was directionally cloned into this site, producing plasmid pJ-TOE1. Plasmid pG-IAH1 was digested with *Bgl*II and *EcoR*I, yielding a 0.7 kb *IAH1* gene fragment. This fragment was directionally cloned into pJ linearised with *Bgl*II and *EcoR*I, producing plasmid pJ-IOE1 (See **Figure 3.1**).

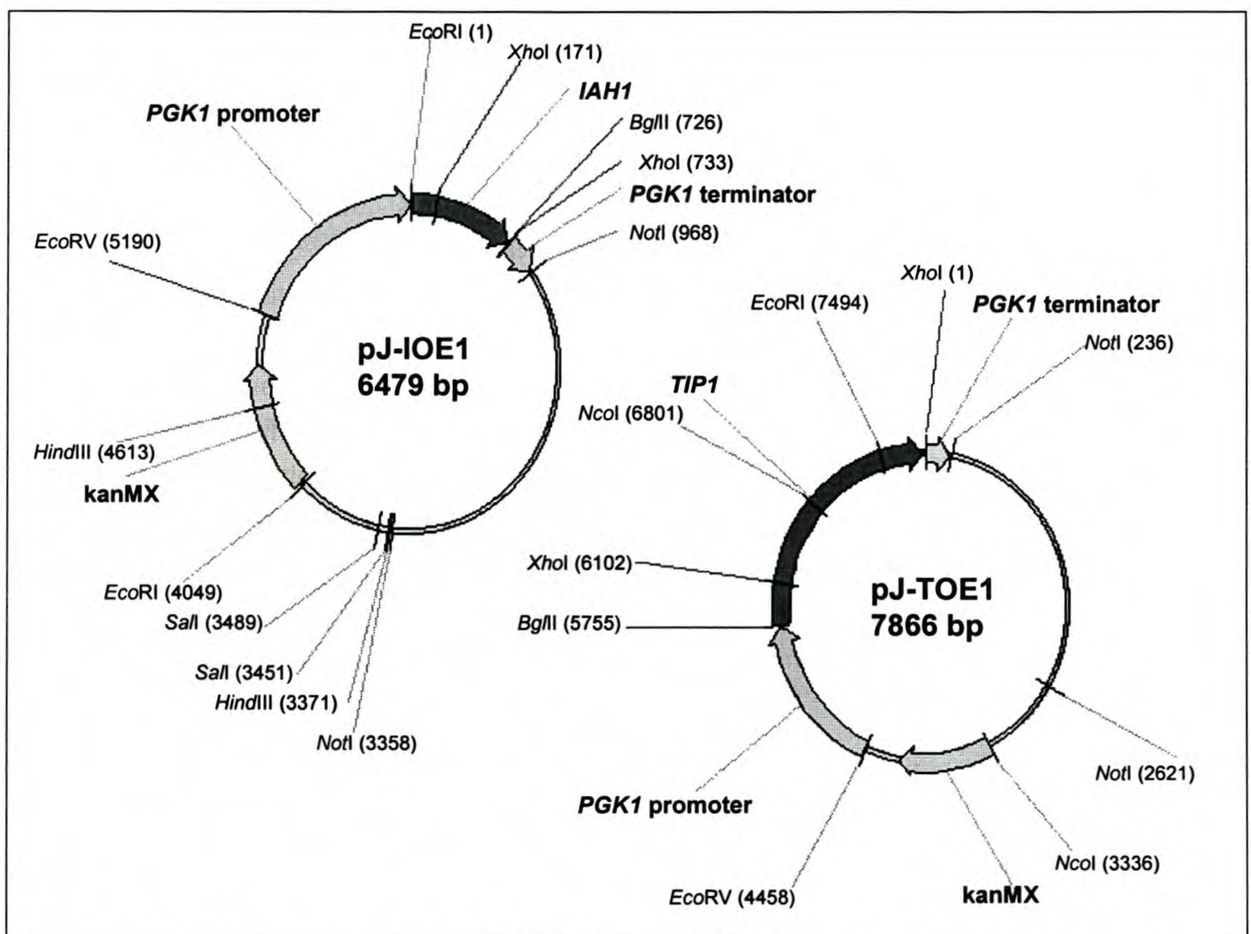

Figure 3.1. Restriction maps of the constructed overexpression plasmids pJ-IOE1 and pJ-TOE1

3.2.3 PCR AMPLIFICATIONS

A PCR-Biometra TRIO-Thermoblock machine was used for all PCR reactions. For all PCR reactions, 2 μl of DMSO, 4 μl of dNTP mix (Roche), 7 μl of a 25mM $MgCl_2$ (supplied with Expand) and 5 μl of a 2.5 μM primer stock solution were added to a total volume of 50 μl, unless otherwise stated. All primers used in this study are listed in **Table 3.2.** The genome of *S. cerevisiae* strain VIN13 was used as template for the amplification of the yeast *IAH1* and *TIP1* genes. The PCR conditions were: 52°C for

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1 min, 72°C for 2 min, 94°C for 20 sec (34 cycles), 52°C for 5 min and 68°C for 5 min.

Table 3.2. Primers used in this study. Underlined sequence corresponds to restriction site

Disruption and overexpression primers			
Primer name	**Sequence (5'-3')**	**Template**	**Primer breakdown**
IAH1'F 72bp	TGCAGAAGCTGAGACCTCATCAGTATGGCCATTATCAATCACAGGTGTGGCTTCGTACGCTGCAGGTCGAC	PUG6, pEG6	First 50 bp homologous to *IAH1* gene, last 22 bp homologous to pEG6
IAH1'R 72bp	TGCACCTAATTCGCCAGCTTGATCTACACGAATAACACGGTCCAAAAATGCCACTGGTGGATCTGATATCA	PUG6, pEG6	First 50 bp homologous to *IAH1* gene, last 22 bp homologous to pEG6
Sigma'F 72bp	ACCGGAGTGTCTTGACAATCCTAATATAAACAGTCTTAGGGAAGTAACCATTCGTACGCTGCAGGTCGAC	pJ	First 50 bp homologous to Sigma sequence, last 22 bp homologous to pJ
Sigma'R 72bp	GAGATATGTCAGTATGACAATACGTCACCCTGAACGTTCATAAAACACATCGGCCGCATAGGCCACTAGT	pJ	First 50 bp homologous to Sigma sequence, last 22 bp homologous to pJ
TIP1'F 72bp	TATATTAAGTTATTGTTTATAAGCATAGTCACAAGTGCATAAAAACTATGACTTCGTACGCTGCAGGTCGAC	pUG6, pEG6	First 50 bp homologous to *TIP1* gene, last 22 bp homologous to pEG6
TIP1'R 72bp	CCTAATACTCGTCTTGTTGATTTTTTTCCTTCTCTTTTTTTTACGAGCTATCCACTAGTGGATCTGATATCA	pUG6, pEG6	First 50 bp homologous to *TIP1* gene, last 22 bp homologous to pEG6
Primers for probe production			
Primer name	**Sequence (5'-3')**	**Template**	**Recognition sequence**
IAH1'P'F	CCCTGTCTATAGGAGCTTGGA	VIN13	5'-side of *IAH1* gene
IAH1'P'R	CCCAGAAAAAACATTTAGCAGAC	VIN13	3'-side of *IAH1* gene
Prob1	CAATCAGGTGCGACAATCTA	*KanMX*	Within *KanMX* gene
Prob2	CGAGCATCAAATGAAACTGC	*KanMX*	Within *KanMX* gene
TIP1'P'F	GAGCCCCATTCGCCTATCTGT	VIN13	5'-side of *TIP1* gene
TIP1'P'R	ACAATTTTTAGGATTCATCTACTA	VIN13	3'-side of *TIP1* gene
Gene primers			
Primer name	**Sequence (5'-3')**	**Template**	**Recognition sequence**
ENO2-PstI	TGCACTGCAGCATTATTATTGTATGTTATAGTA	VIN13	*Pst*I
SCIAH1'F	AATTGAATTCATGGATTACGAGAAGTTTCT	VIN13	*EcoR*I
SCIAH1'R	GATCAGATCTATTCAAGACATTATGTTATA	VIN13	*Bgl*II
SCTIP1'F	GATCAGATCTATGAACGGTATTGATGATCT	VIN13	*Bgl*II
SCTIP1'R	TCGACTCGAGCTATAATATGTTACCATATA	VIN13	*Xho*I
SMR-Pst1	TGCACTGCAGATGATCAGACAATCTACGCT	pWX509	*Pst*I
SMR-NsiI	TGCAATGCATTCAGTGCTTACCGCCTGTAC	pWX509, *SMR1-410*	*Nsi*I

Plasmid pEG6 was used as template for the production of disruption cassettes.

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Primers contain 50 bp overhangs homologous to the 5' and 3' sides of the appropriate gene. The PCR conditions were: 58°C for 1 min, 72°C for 1 min 30 sec, 94°C for 20 sec (34 cycles), 58°C for 5 min and 68°C for 5 min. The PCR-generated disruption cassettes had a size of 1.7 kb. These cassettes were then purified using a PCR clean up kit (Sigma) and the DNA was concentrated to 0.5 μg/μl. A total of 10 μl of this DNA was used per transformation.

For the overexpression cassettes, the primers contained 50 bp overhangs homologous to the yeast's sigma sequence and plasmid pJ was used as template. The sigma nucleotide sequence is available on Genbank/EMBL Data Bank using accession number J03500 (Clark *et al.,* 1988). The *IAH1* overexpression cassette was produced using plasmid pJ-IOE1 as template and a 4.1 kb PCR fragment was generated that was capable of integration into the yeast's sigma sequences. Plasmid pJ-TOE1 was used as template for the *TIP1* overexpression cassette. The amplified PCR product was 5.2 kb long. The PCR conditions for both overexpression cassettes were: 52°C for 1 min, 68°C for 3 min, 94°C for 30 sec (34 cycles), 52°C for 5 min, 68°C for 6 min. Both of these fragments were purified using a PCR clean up kit (Sigma). These DNA products were then concentrated to 0.5 μg/μl and 10 μl was used for each transformation.

The probe for Southern blot analysis was generated using the following PCR conditions: 48°C for 1 min, 72°C for 1 min 30 sec, 94°C for 30 sec (30 cycles), 48°C for 5 min, 72°C for 5 min. Standard dNTPs were replaced with the DIG-dNTP mix (Roche) for the production of the probe. This DNA was quantified on a gel and used at a concentration of 20 μg/μl for Southern blot analysis.

The volume of $MgCl_2$ was adjusted to 5 μl for the production of the *ENO2* promoter. The PCR conditions were: 53°C for 1 min, 72°C for 1 min 20 sec, 94°C for 30 sec (30 cycles), 53°C for 5 min, 68°C for 5 min. This produced a 620 bp fragment, which was purified and used further.

The *SMR1-410* gene was amplified from plasmid pWX509 using the following PCR conditions: 52°C for 1 min, 72°C for 2 min 30 sec, 94°C for 30 sec (34 cycles), 52°C for 5 min and 68°C for 5 min. The DNA was then purified and used further.

The *ENO2-SMR* ligation fragment was amplified using the following PCR conditions: 58°C for 1 min, 68°C for 3 min, 94°C for 30 sec (34 cycles), 52°C for 5 min and 68°C for 5 min.

3.2.4 YEAST TRANSFORMATION

S. cerevisiae wine yeast strain VIN13 was transformed using the lithium acetate transformation protocol of Gietz and Schiestl (1995). A minimum of 1 μg of linear DNA was used per transformation in a total volume of 10 μl or less. After the final heat shock, cells were first centrifuged in a bench top centrifuge at 12500 rpm for 30 sec, resuspended in 2 ml of YPD broth and shaken for 2-4 hours at 30°C before being plated onto YPD agar containing 400 mg/l geneticin. The plates were incubated at 30°C for 3-5 days until colonies appeared. Positive colonies were

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restreaked onto YPD agar plates containing 400 mg/l geneticin and allowed to grow for a further three days. A single colony was then selected and restreaked onto YPD agar geneticin plates for a second time, allowed to grow for three days and finally plated onto YPD agar. The reason for the restreaking is to eliminate false positives.

When transforming with plasmid pSHeSMR, the same transformation protocol was utilised (Gietz and Schiestl, 1995), with the following modifications: after the final heat shock, the cells were plated onto SD media containing 60 mg/l SMM as selection medium and the plates were covered with tinfoil, as SMM is light sensitive. Positive single colonies were transferred to YPG broth and incubated for 2-4 hours at 30°C before being plated onto YPG agar and grown at 30°C for 2-3 days.

These plates were replicated onto YPD with geneticin to determine if the *KanMX* marker was recovered. Any colonies that did not grow on geneticin-containing plates were selected for further analysis.

3.2.5 SOUTHERN BLOT ANALYSIS

Genomic DNA was isolated from the control yeast strain VIN13 as well as from the transformed strains (VIN13-1ΔIAH1, VIN13-1ΔTIP1, VIN13-IOE1, VIN13-TOE1), using the standard method from Sambrook *et al.* (1989). The DNA of the overexpressed strains was digested overnight with *Sal*I and *Xho*I. This digestion yielded a 1.7 kb internal fragment. The VIN13-ΔIAH1 DNA was digested with *Hpa*I and *Xba*I, yielding a 1.5 kb fragment. The VIN13-ΔTIP1 was digested with *Cla*I and *Sfu*I, producing a 3.4 kb fragment. The DNA was separated in a 1% agarose gel by electrophoresis, quantified and transferred to a Hybond-N nylon membrane (Amersham).

All probes for Southern blot analysis were generated by means of PCR. For the overexpressed strains, a 600 bp DNA fragment was obtained that was homologous to the *KanMX* gene. For the VIN13-ΔIAH1 disruption, a 394 bp fragment was generated homologous to a region upstream of the initiation codon of the *IAH1* gene. For the VIN13-ΔTIP1 disruption strain, a 554 bp probe was generated homologous to the 5' side of the *TIP1* gene. All DNA probes were labelled with the DIG-PCR-dNTP labelling mix (Roche) according to Van Miltonburg *et al.* (1995). The primers used to generate the probes are listed in **Table 3.2.** The membrane was hybridised and washed according to Van Miltonburg *et al.* (1995). These membranes were then placed with an X-ray film and incubated for 1 hour at room temperature. The X-ray films were then developed using standard developing techniques.

3.2.6 RT-PCR VERIFICATION OF DISRUPTION AND OVEREXPRESSION STRAINS

Total RNA was isolated from the yeast cells grown overnight on YPD using the Fast-RNA® Kit-Red product (BIO 101). The following strains were used for verification: VIN13-1ΔIAH1, VIN13-1ΔTIP1, VIN13-IOE1, VIN13-TOE1 and VIN13 as control. This RNA was subjected to reverse-transcriptase PCR (RT-PCR) using the

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C. therm. Polymerase One-step RT-PCR System (Roche, Germany). These reactions were performed in duplicate. The RNA was first quantified with a spectrophotometer at an absorbance of 260 nm and equal concentrations of RNA were added to each reaction. Primers SCTIP1'F and SCTIP1'R were used for the verification of the *TIP1* gene expression. For the verification of the *IAH1* gene expression, primers SCIAH1'F and SCIAH1'R were used. The kit was used according to the supplier's specifications. The following PCR programme was used for verification: 95°C for 2 min, 55°C for 30 min, followed by 94°C for 30 sec, 50°C for 1 min and 72°C for 3 min, repeated for 30 cycles. The PCR products were analysed on a 1% agarose gel.

3.2.7 YEAST STRAIN VERIFICATION

Total chromosomal DNA was isolated according to the embedded-agarose procedure of Carle and Olson (1985). The intact chromosomal DNA was separated by means of a contour clamped homogenous electric field (CHEF) electrophoresis. The transformed VIN13 strains were electrophoresed with a known VIN13 strain as a control. The CHEF separations were carried out according to Van der Westhuizen *et al.* (1992), using a CHEF-MAPPER from Bio-Rad Laboratories, Richmond, USA.

3.2.8 FERMENTATIONS AND SMALL-SCALE DISTILLATION

3.2.8.1 White wine production

The pre-inoculum was prepared by growing the wine yeast strains VIN13, VIN13-TOE1, VIN13-ΔTIP1, VIN13-IOE1 and VIN13-ΔIAH1 in 150 ml of YPD media at 30°C overnight. The yeast cells were centrifuged for 5 min at 3000 rpm and the supernatant was discarded. Yeast cells were resuspended in 10 ml of Colombard grape juice. This was inoculated into 4.5 l of Colombard grape juice and fermented at 15°C until dry. The wines were analysed for sugar concentration by means of a clini test to ensure the completion of fermentation. The wines were then cold stabilised at -4°C, filtered and bottled as with standard winemaking practices. All fermentations were performed in duplicate.

3.2.8.2 Base wine production and distillations

The pre-inoculum was prepared by growing the yeast strains VIN13, VIN13-TOE1, VIN13-ΔTIP1, VIN13-IOE1 and VIN13-ΔIAH1 in 450 ml of YPD media overnight at 30°C. The yeast cells were centrifuged and resuspended in 20 ml of Colombard grape juice. This was used to inoculate 15 l of Colombard grape juice to which no SO_2 had been added and allowed to ferment at 15°C until dry. The base wines were fermented in duplicate. The wines were analysed for sugar concentration by means of a clini test to ensure the completion of fermentation. Distillations were done according to the method described by Lilly *et al.* (2000).

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3.2.9 GC ANALYSIS

Ten ml of each of the Colombard wines and base wines was measured off and added to 15 ml of dimethyl ether and 800 μl of the internal standard 4-methyl-2-pentanol. These flasks were then rotated on a rotary mixer (Labinco) for 30 min at 60 rpm. The top layer was isolated and used for analysis.

The wine samples were taken immediately before bottling, while base wine samples were taken prior to distillation. Samples of the distillates were taken on completion of the first and second distillations. Analyses were performed on a Hewlett Packard Model 6890 series II gas chromatograph with a Lab Alliance capillary column of 60 m x 0.32 μm with a film width of 0.5 μm. This is the same method used by Lilly *et al.* (2000).

3.2.10 SENSORY EVALUATIONS

The Colombard wines and brandies were sensorially evaluated for aroma and flavour differences by a panel of nine (wine) and eight (brandy) trained and experienced judges. In total five wine samples including VIN13-ΔIAH1, VIN13-TOE1, VIN13-IOE1, VIN13-ΔTIP1 and VIN13 and four brandy samples including VIN13, VIN13-TOE1, VIN13-IOE1 and VIN13-ΔTIP1 were tasted. For the wines, judges were to identify the following flavours: fruity, herbaceous, nutty, chemical, pungent and flowery, and then to allocate a score of between 1 and 5 for each of these flavours. The higher the value, the better the presence of that flavour. For the brandy, the following flavours were to be identified: fruity, flowery, chemical, herbaceous, sweet and oily. Each of these flavours was allocated a score of between 1 and 5. The data can be found in the Appendix (**Table 5.1** and **Table 5.2**). This data was then further analysed to establish whether significant could be detected differences between the different samples. STATISTICA (data analysis software system) version 6 was used for the analysis (StatSoft, Inc. 2001).

3.3 RESULTS

3.3.1 CLONING AND CONSTITUTIVE EXPRESSION OF THE *IAH1* AND *TIP1* GENES IN INDUSTRIAL WINE YEAST STRAIN VIN13

Both the *IAH1* and *TIP1* genes were cloned from the widely used commercial wine yeast strain *S. cerevisiae* VIN13 and plasmids pJ-TOE1 and pJ-IOE1 were generated. These plasmids were used to PCR generate overexpression cassettes. The *IAH1* and the *TIP1* overexpression cassettes were transformed into *S. cerevisiae* strain VIN13 and positive colonies were screened by their ability to grow on 400 mg/l of the antibiotic geneticin on YPD agar media. Positive strains were verified with Southern blot hybridisation (**Figure 3.2**) and the following strains were produced: VIN13-IOE1 and VIN13-TOE1. These strains were used further for the production of wine and brandy.

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RT-PCR verified higher levels of expression of the *IAH1* and *TIP1* genes under the control of the PGK_P than of the native promoters when grown in YPD. This can be seen in **Figure 3.3**. For the *IAH1* and *TIP1* gene products, band sizes of ± 700 bp and ± 2000 bp respectively were expected.

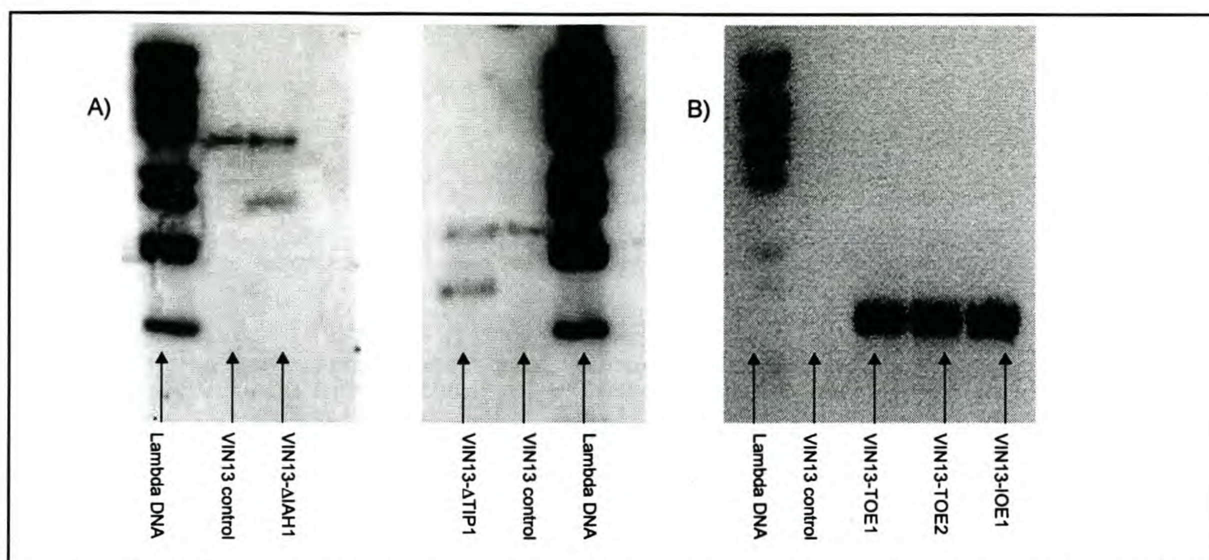

Figure 3.2. Verification of A) single disrupted mutants and B) integration of overexpression cassettes by means of Southern blots. From left to right, A) shows the VIN13 control strain next to the VIN13-ΔIAH1 strain and VIN13-ΔTIP next to VIN13 and B) shows the VIN13 control with no band next to the overexpressing strains VIN13-TOE1, VIN13-TOE2 and VIN13-IOE1 with expected band sizes of 1.7 kb

3.3.2 THE DISRUPTION OF THE *IAH1* AND *TIP1* GENES IN INDUSTRIAL YEAST STRAINS

The disruption cassettes removed the entire coding sequence for each gene. Gene disruptions were verified by Southern blot hybridisation (**Figure 3.2**). Only one copy of the *IAH1* and *TIP1* gene was disrupted in each yeast strain. The use of the loxP/*Cre*-recombinase system was chosen for marker recovery. In the *IAH1* gene disruption experiment, the *KanMX* gene was effectively excised using plasmid pSHeSMR containing the *Cre*-recombinase. This was verified by Southern hybridisation. Attempts to disrupt the second copy of the gene proved difficult and further attempts were aborted. The disruptions produced the following yeast strains: VIN13-ΔIAH1 and VIN13-ΔTIP1. These strains were used to produce wine and brandy. RT-PCR indicated no significant difference in expression levels of the VIN13-ΔIAH1 strain and the control strain. For this reason, this strain was not used for base wine production. The VIN13-ΔTIP1 strain showed a significant decrease in expression compared to the control strain during RT-PCR analysis. These results are indicated in **Figure 3.3**. This strain was used for brandy production.

All yeast strains were positively identified as *S. cerevisiae* by means of a CHEF with VIN13 as positive control (data not shown).

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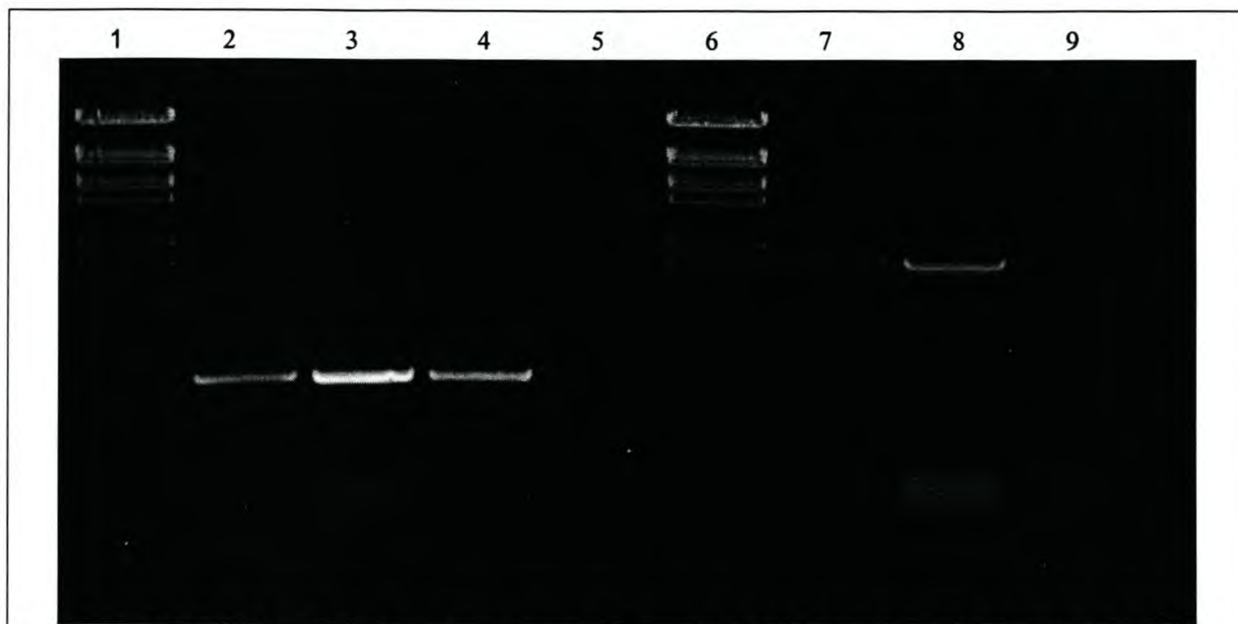

Figure 3.3. RT-PCR verification of expression with gel loaded as follows: Lane 1, *Bst*II-digested lambda DNA; lane 2, the *IAH1* gene under the native promoter from VIN13; lane 3, the overexpressed *IAH1* gene under the PGK_P; lane 4, PCR product of the single disrupted *IAH1* strain; lane 5 is empty; lane 6, *Bst*II-digested lambda DNA; lane 7, the *TIP1* gene under the native promoter; lane 8, the *TIP1* gene under PGK_P; lane 9, PCR product of the single disrupted *TIP1* strain

3.3.3 FERMENTATION TRIALS

Strains VIN13-ΔIAH1, VIN13-ΔTIP1, VIN13-IOE1, VIN13-TOE1 and the control VIN13 strain completed fermentations within 14 days, indicating no change in the viability of these strains. All strains fermented the must to dryness with less than 1 g/l of residual sugar remaining in both the wines and the base wines. No noticeable off-odours were detected before bottling. The wine and base wines showed no discolouration.

3.3.4 GC ANALYSIS OF WINES AND DISTILLATES

The concentration of certain acids, esters and higher alcohols was determined for all the wines and distillates. The results are indicated in **Table 3.3** and **Table 3.4**. No more than a 10% variance occurred between the duplicate samples.

The overexpression of the *TIP1* gene showed no significant changes in the higher alcohol concentrations and a minor increase in the total ester level. However, a drastic decrease in the overall acid concentration occurred in both the wine and the base wine. With respect to the total acids, acetic acid levels were affected significantly, with the transformed *TIP1* overexpression strain showing a 78% and 61% reduction in acetic acid levels in the wine and base wine respectively. The small increase in total esters can be attributed to an increase in ethyl acetate, ethyl caprylate and isoamyl acetate, with an increase of 18%, 20% and 20% in the base wines respectively. In the 30% and 70% distillates, large increases of between 31% and 23% occurred in the overall level of esters. This can be attributed primarily to ethyl acetate and isoamyl acetate increases of about 30% and 27% respectively. The

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total acid level was again decreased, as in the wine and rebate wine.

Table 3.3. GC analysis of the Colombard wines and the base wines

Component	Concentration (mg/l)								
	Colombard wines					Colombard base wines			
	VIN13	VIN13-IOE1	VIN13-ΔIAH1	VIN13-TOE1	VIN13-ΔTIP1	VIN13	VIN13-IOE1	VIN13-TOE1	VIN13-ΔTIP1
Acetic acid	267.5	184.6	310	57.2	328.2	231.7	180.9	88.1	414.4
Propionic acid	39.5	1.6	17.9	31.4	16.0	14.2	1.8	2.3	4.3
Isobutyric acid	0.4	0.7	0.6	0.4	0.5	0.6	0.6	1.7	1.3
n-Valeric acid	0.7	0.7	1.2	1.0	0.8	0.8	1.3	0.6	0.7
2-Phenylethyl acid	0.6	0.6	0.5	0.6	0.6	0.5	0.5	0.6	0.6
Hexanoic acid	6.4	5.6	5.6	6.4	4.8	5.9	5.2	6.4	4.6
Octanoic acid	5.6	9.6	4.9	5.8	4.3	5.5	8.1	6.3	4.6
Decanoic acid	5.1	2.7	4.0	4.6	4.2	4.4	4.2	5.6	4.6
Ethyl acetate	103.5	79.1	98.8	113.7	107.6	105.0	72.3	124.8	108.7
Ethyl butyrate	0.5	0.5	0.6	0.6	0.6	0.6	0.6	0.7	0.6
Isoamyl acetate	23.0	12.1	22.1	25.1	20.6	22.9	12.4	27.6	19.7
Ethyl caproate	3.0	2.6	3.2	3.1	2.6	3.2	1.3	3.7	2.7
Hexyl acetate	2.9	1.6	3.0	2.8	2.8	2.9	1.3	3.0	3.0
Ethyl caprylate	4.8	1.5	4.3	5.1	3.7	4.7	1.8	5.9	3.8
Ethyl caprate	3.0	1.1	3.2	3.2	3.1	3.6	2.4	4.6	3.7
Diethyl succinate	1.0	1.4	1.2	0.9	1.0	0.9	1.0	0.9	0.8
n-Butanol	1.8	1.7	1.5	2.1	1.6	1.8	1.7	2.3	1.6
Isoamyl alcohol	98.5	84.4	96.1	96.1	87.3	97.1	81.3	95.1	87.0
n-Propanol	61.2	80.1	76.1	68.1	66.5	62.3	92.6	68.6	76.5
Isobutanol	9.5	9.1	9.7	9.2	10.9	8.9	8.5	9.2	10.0
Hexanol	1.4	1.3	1.0	1.2	1.1	1.2	1.1	1.1	1.1
2-Phenyl ethanol	7.9	8.9	6.9	6.5	6.8	6.9	7.2	6.8	6.7
Total acids	325.4	205.5	344.2	106.8	358.8	259.2	202.1	111.2	434.6
Total esters	142.3	100.5	136.9	155.1	142.6	144.3	93.6	172.1	143.6
Total higher alcohols	180.3	185.5	191.3	183.2	174.2	178.2	192.4	183.0	182.9

The VIN13-ΔTIP1 single disrupted strain behaved in virtually the opposite manner. It showed an increase in the overall acids, but no significant changes in both ester and higher alcohol levels during wine and base wine production. Acetic acid was again affected the most, showing an increase of between 22% and 78%. With respect to the esters, isoamyl acetate and ethyl caprylate showed small decreases of 12% and 23% respectively. The 30% and 70% distillates again showed significant differences in the overall ester level, with a decrease of 40%. Ethyl acetate and

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isoamyl acetate were again affected the most, with a decrease of 30% and 50% respectively.

Table 3.4. GC analysis of the 30% and 70% distillates

Component	Concentration (mg/l)							
	30% Distillates				70% Distillates			
	VIN13	VIN13-IOE1	VIN13-TOE1	VIN13-ΔTIP1	VIN13	VIN13-IOE1	VIN13-TOE1	VIN13-ΔTIP1
Acetic acid	110.1	95.2	39.0	131.2	25.7	22.2	9.3	31.6
Propionic acid	1.5	1.5	1.1	0.7	-	-	-	-
Isobutyric acid	0.5	1.1	0.5	0.5	-	0.9	-	-
2-Phenylethyl acid	1.2	1.3	1.5	1.2	2.0	3.0	2.2	2.3
Hexanoic acid	11.7	13.7	14.2	15.1	9.4	11.5	9.8	8.2
Octanoic acid	24.1	33.0	27.4	21.3	35.8	41.7	35.5	32.9
Decanoic acid	17.1	22.8	18.8	14.2	34.2	40.9	34.0	36.5
Acetate	45.3	31.2	56.4	26.6	123.5	81.0	110.9	33.2
Ethyl acetate	177.0	128.6	242.1	121.5	231.7	153.5	299.3	156.3
Ethyl butyrate	1.1	1.0	1.5	-	2.1	-	2.7	-
Isoamyl acetate	41.4	47.1	55.3	21.1	73.1	43.1	90.1	31.5
Isoamyl alcohol	209.1	215.6	210.6	185.3	722.8	723.9	737.5	664.3
Ethyl caproate	5.1	6.2	6.5	-	6.5	4.1	7.3	2.5
Hexyl acetate	6.1	6.0	6.3	2.5	7.2	5.4	7.6	3.8
Ethyl caprylate	8.9	13.5	10.5	6.2	14.6	9.5	16.3	6.0
Ethyl caprate	31.1	47.8	34.8	14.5	44.5	32.2	44.2	25.9
Diethyl succinate	1.4	2.8	1.6	1.3	1.1	1.8	1.1	1.1
n-Propanol	190.2	275.3	223.6	245.6	334.1	442.8	373.4	408.8
Isobutanol	20.9	22.8	24.2	20.2	49.9	54.1	54.8	54.8
2-Phenyl ethanol	11.6	13.1	11.1	10.1	4.6	5.1	4.4	4.2
n-Butanol	4.3	4.3	6.1	4.1	9.3	8.0	12.3	9.5
Hexanol	2.7	3.2	2.7	2.8	7.4	7.5	7.1	7.5
Total acids	165.0	168.6	102.5	182.4	107.1	120.2	90.8	111.5
Total esters	272.1	253.0	358.6	167.1	380.8	249.6	468.6	227.1
Total higher alcohols	438.8	531.8	478.3	468.1	1128.1	1241.4	1189.5	1149.1

The single disrupted strain of VIN13-ΔIAH1 showed a small increase in the total ester and higher alcohol levels, but this phenomenon cannot be attributed to a single chemical compound. Acetic acid levels were again affected, with a minor increase

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taking place. As this strain showed no significant difference than the control strain, it was not used for the production of base wine and brandy. RT-PCR showed that this strain did not express this disrupted gene at lower levels to that of the control strain and the GC analysis further supported these findings, with only minor differences occurring.

The VIN13-IOE1 strain behaved as expected. The total ester level and the total acid level decreased. The base wine production showed the largest decrease in these levels. The ester concentration decreased by a substantial 40% and the total acids decreased by nearly 24%. This effect can be attributed to the following esters: ethyl acetate, isoamyl acetate, ethyl caproate, hexyl acetate, ethyl caprylate and ethyl caprate, which show decreases of 31%, 45%, 62%, 55%, 44% and 33% respectively. With regard to the acids, acetic and propionic acids displayed marked decreases, while octanoic acid increased. Although the overall higher alcohol level did not change significantly, two alcohols did show changes. N-propanol increased moderately, while the isoamyl alcohol levels decreased to some extent.

3.3.5 SENSORY EVALUATIONS

The data was analysed by means of a cluster analysis, a factor analysis and a non-parametric Kruskal-Wallis analysis of variance. The cluster analysis was performed to find differences/similarities in taste between the different wine or brandy samples produced by the different yeasts. Euclidian distances were used for the clustering. The cluster analysis for the wine samples is represented in **Figure 3.4** and for the brandy samples in **Figure 3.5.** As can be seen in **Figure 3.4**, on the basis of the five variables, the wine produced by VIN13 was found to be most similar to VIN13-ΔTIP1, followed by VIN13-ΔIAH1.

VIN13-TOE1 was noticeably the most different from VIN13, followed closely by VIN13-IOE1.

With regard to the four brandy samples, **Figure 3.5** shows clearly that VIN13-ΔTIP1 and VIN13-TOE1 formed a cluster, with VIN13-IOE1 some distance away. VIN13 was the most different of the samples.

A factor analysis was performed on the different taste components for both the wine and brandy data and is represented in **Figure 3.6** and **3.7** respectively. The factor analysis was done using a principle component extraction. It is clear from the wine tasting data that the tastes of fruity, nutty, herbaceous and floral seem to be closely related, with pungent and chemical being seperate. No comparisons of flavours could be noted for the brandy data (**Figure 3.7**). This is most likely due to the stronger taste of the brandy, making it more difficult to taste the individual flavours over the strong ethanol flavour.

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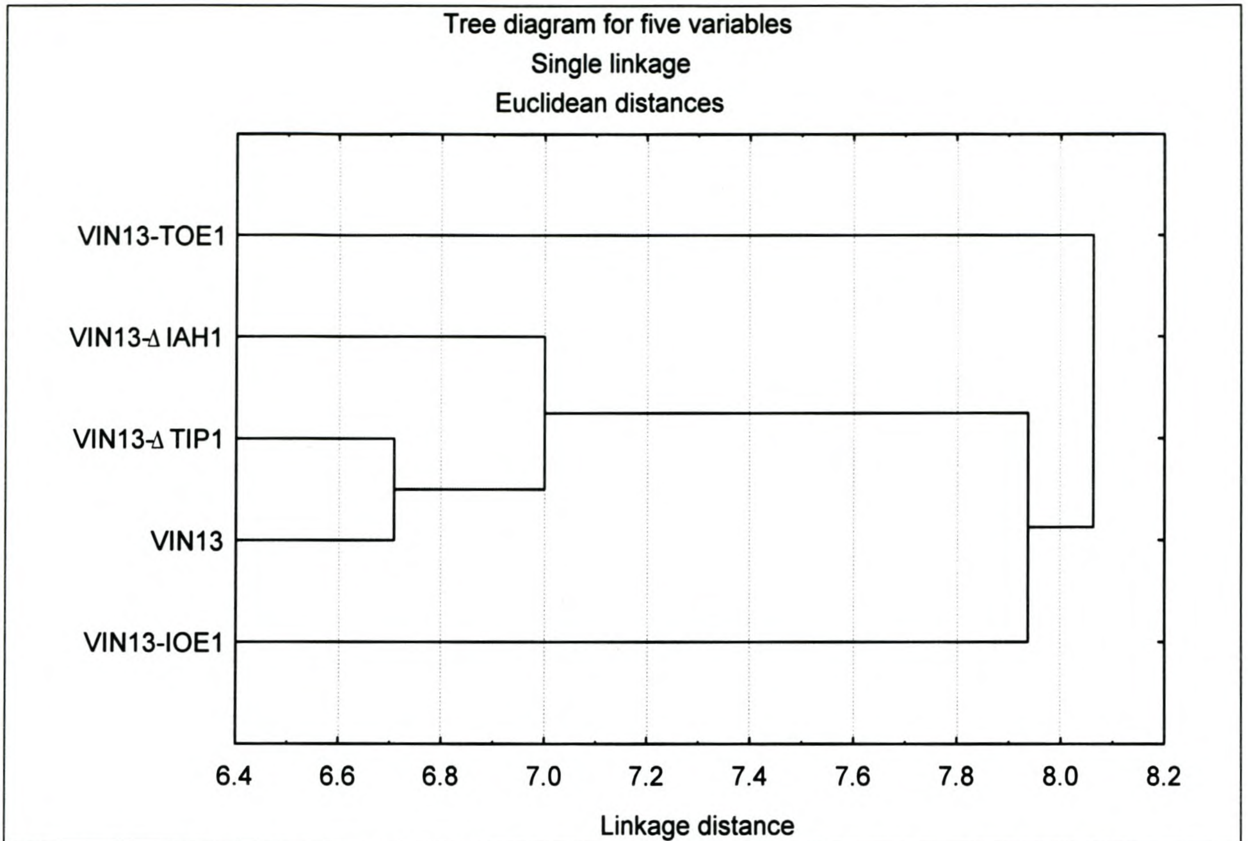

Figure 3.4. Joining (tree-cluster) results for the five variable wine samples showing the Euclidean linkage distances

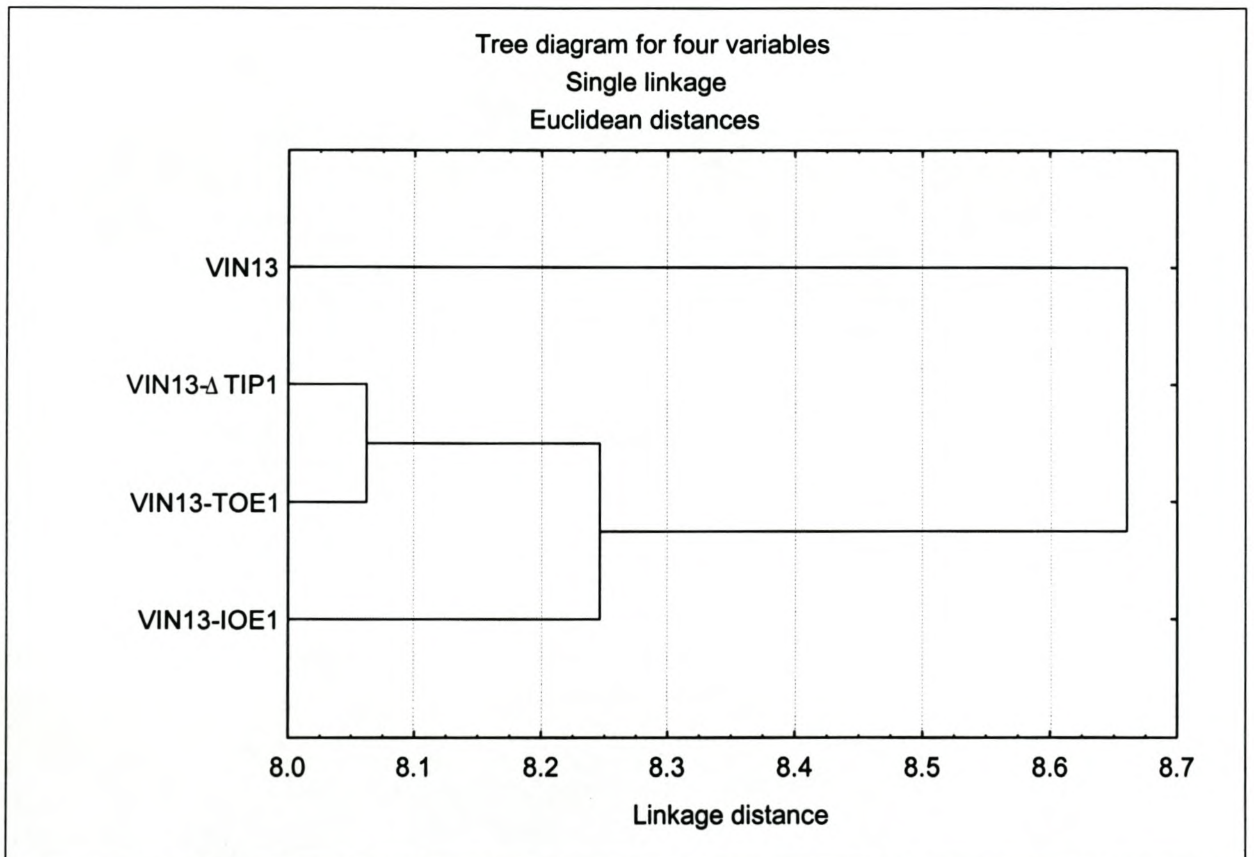

Figure 3.5. Joining (tree-cluster) analysis results for the four variable brandy samples VIN13, VIN13-ΔTIP1, VIN13-TOE1 and VIN13-IOE1, showing the Euclidean linkage distances

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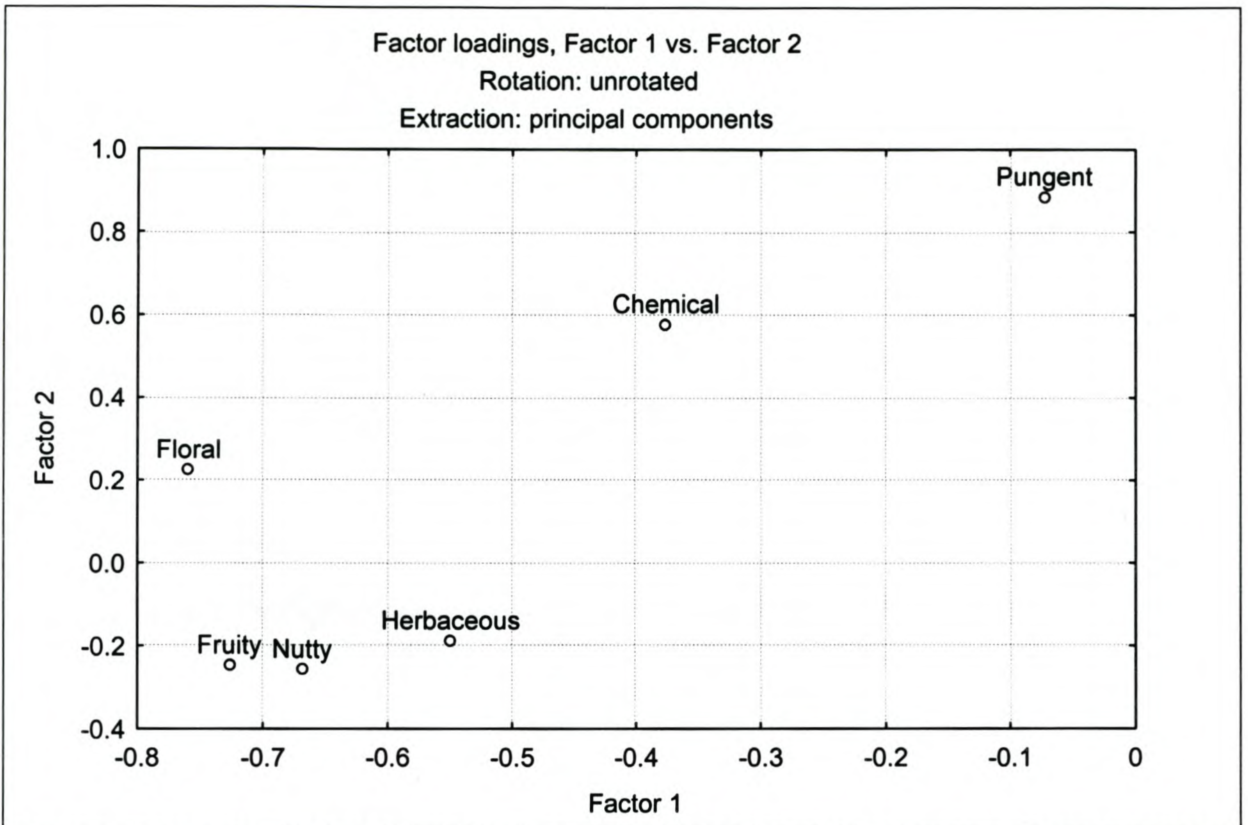

Figure 3.6. A principle component analysis of the wine tasting data graphically showing the different flavour components and their relatedness as detected by the tasters.

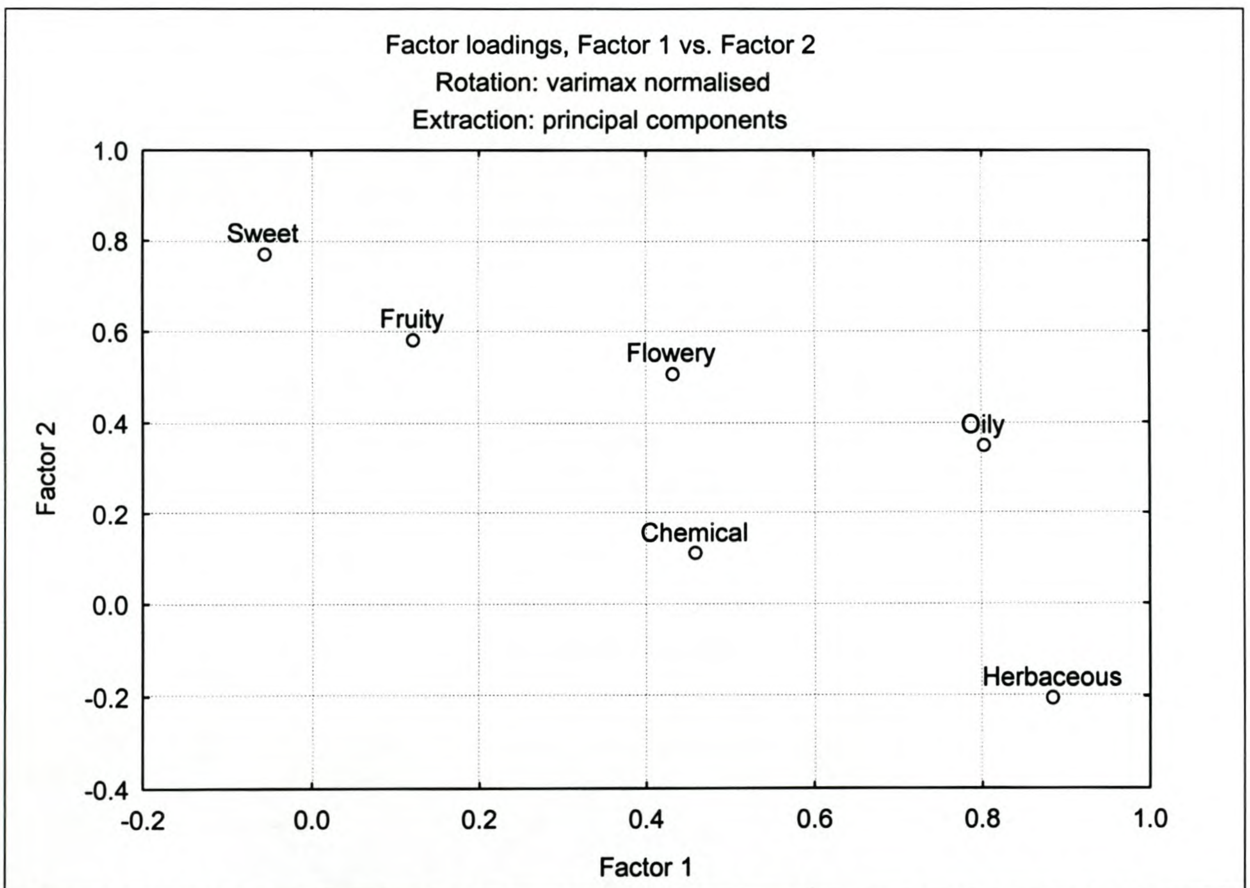

Figure 3.7. A principle component analysis of the brandy tasting data graphically showing the different flavour components and their relatedness as detected by the tasters

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The last method of analysis, namely the non-parametric Kruskal-Wallis analysis of variance, was performed to determine if there were significant changes in the flavours of the wines and brandies due to the different yeasts. Each flavour was tested individually for each of the different wines and brandies, with the null hypothesis stating no differences between the yeasts. Only one significant result was found, and that was in the comparison of the flavour fruity in the wine samples. The Kruskal-Wallis test gave a p-value of 0.01, which indicates a significant difference on a 5% level ($p<0.05$). **Table 3.5** shows the Cruskal-Wallis ANOVA scores for fruity. The categorised histogram for fruity can be seen in **Figure 3.8** and, from this, it is clear that sample VIN13-ΔTIP1 scored the worst, with the least amount of fruitiness, while VIN13-TOE1 had the highest fruitiness. This is an interesting finding, as it indicates that the *TIP1* gene may affect fruitiness by means of a mechanism other than ester manipulation. The remaining Kruskal-Wallis tests for both the wine and brandy samples (all flavours) are located in the Appendix.

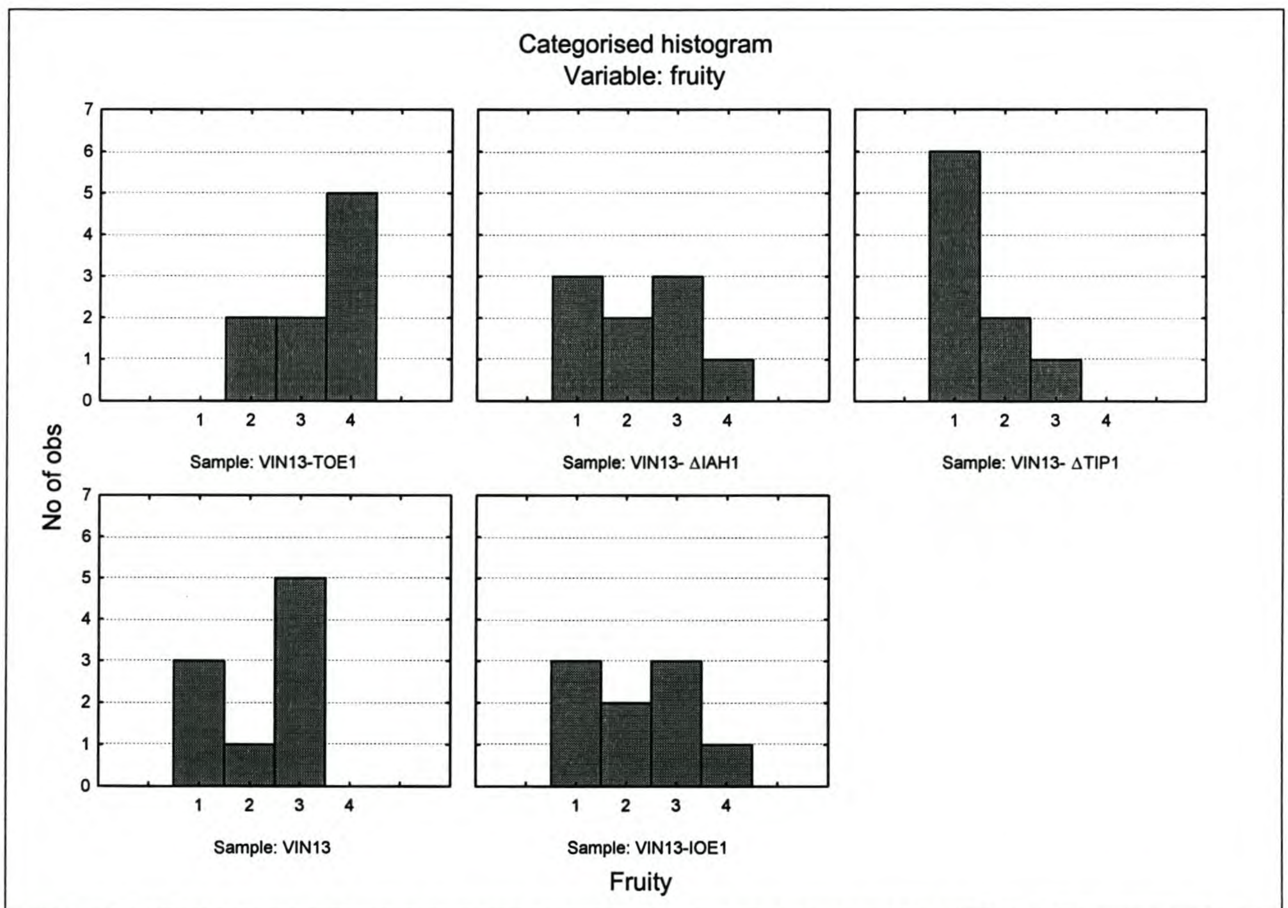

Figure 3.8. Categorised histograms for the variable fruity when looking at the different wine samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

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Table 3.5. Kruskal-Wallis ANOVA by ranks of fruity for the wine samples

Dependent: Fruity	Independent (grouping) variable: Sample Cruskal-Wallis test: H (4, N=45) =13.28517 p=0.010		
	Code	Valid N	Sum of ranks
VIN13-TOE1	101	9	313.00
VIN13-ΔIAH1	102	9	200.50
VIN13ΔTIP1	103	9	119.50
VIN13	104	9	201.50
VIN13-IOE1	105	9	200.50

3.4 DISCUSSION

3.4.1 GENE REGULATION

The regulatory elements of the *IAH1* gene are not yet understood well and little information is available. The *TIP1* gene's regulatory elements are affected by a number of different mechanisms, including heat and cold shock (Kondo and Inouye, 1991). For this reason, the constitutively expressed promoter and terminator sequence of the phosphoglycerate kinase gene (*PGK1*), which is found in yeast, was chosen as the regulatory sequence to obtain high levels of expression for this study. RT-PCR verified higher levels of expression of the *IAH1* and *TIP1* genes under the $PGK1_P$ than under their native promoters. This is clearly indicated in **Figure 3.3**, with the control strain consistently expressing the *IAH1* and *TIP1* genes at lower levels than that of the VIN13-IOE1 and VIN13-TOE1 strains. GC analysis further supports that the level of expression of these genes has been altered.

3.4.2 PCR GENERATION OF CASSETTES

The use of PCR has the advantage of fast DNA production, with cassettes being available within a short period of time. The integration site can easily be selected, as primers are designed to share homology with the area of interest. This eliminates the need to construct new template DNA, as the same integration cassette can be re-used. This also makes it possible to simultaneously disrupt one gene and overexpress another gene at the same locus position. In this study, the sigma sequences, which are located throughout the yeast genome, were chosen as integrating sites for gene overexpression (Clark *et al.,* 1988; Sandmeyer *et al.,* 1988). This minimised the expenditure on primers, as both overexpression cassettes can be produced from one set of primers.

3.4.3 GENE DISRUPTION

Limited numbers of dominant selectable markers are available and, for this reason, it

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was decided to use marker recovery. This limits the number of dominant selectable markers required. The *KanMX* gene was recovered with the *IAH1* disruption. Attempts to integrate another *IAH1* disruption cassette into the remaining *IAH1* gene proved ineffective. The cassette appeared to reintegrate into the already disrupted sequence, with no cassettes integrating into the remaining *IAH1* gene. A possible reason for this was that the disruption cassette shared more homology with the already disrupted gene. For this reason, it was decided to continue the research with the single disrupted strains, as these should give an indication of the effect that lower expression levels of the *IAH1* and *TIP1* genes would have on the level of esters. The *IAH1* single disrupted strain showed no significant variation to that of the control strain. A possible reason for this could be the *IAH1* gene's unknown regulatory mechanisms, allowing for the up-regulation of this gene to produce significant product for normal functioning. RT-PCR indicated no difference in the level of expression of the single disrupted strain and the parent strain (**Figure 3.3**). However, Southern hybridisation results indicate that a single copy of this gene was disrupted (**Figure 3.2**). The *TIP1* single disruption, however, showed significant differences. RT-PCR showed that the *TIP1* gene in the VIN13-ΔTIP1 strain was expressed at very low levels when compared to the parent VIN13 strain and the VIN13-TOE1 overexpressing strain. This correlates well with the results of the GC analysis, as the VIN13-ΔTIP1 strain performed differently during fermentation than the control and overexpressing strains.

3.4.4 THE ESTERASE ABILITIES OF THE GENES

The *IAH1* enzyme behaved as described previously (Fukuda *et al.,* 1996, 1998). It showed affinity towards isoamyl acetate, ethyl acetate, ethyl caproate, hexyl acetate, ethyl caprylate and ethyl caprate. These esters can contribute to the sensory perception and are associated mostly with fruity characters. For this reason, the creation of a null mutant would be of interest, as higher levels of these esters should be obtained.

The *TIP1* gene showed no esterase activity and in some cases, it actually produced esters. This possibly implies that this gene is involved in ester synthesis. This effect was most noticeable during distillation. This is most likely due to the yeast lees being present during distillation, allowing for esters trapped within the organism to be released. This is contrary to the findings of Horsted *et al.* (1998), who found this gene to have esterase activities towards longer chained esters. The gene has a significant effect on acetic acid production. Acetic acid contributes to the overall volatile acidity (VA) of both wine and brandy. Acetic acid levels in wine are limited to 1.2 g/l. If the level of acetic acid is above this level, the beverage is regarded as spoilt. High levels of acetic acid have also been associated with sluggish and stuck fermentations. This is caused as the acetic acid has an inhibitory effect on the yeast. This is a favourable finding, as both a decrease in the total acid level and a minor increase in the total ester level took place. The disrupted *TIP1* strain (VIN13-ΔTIP1)

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produced higher levels of acetic acid, in contrast to the effect achieved by the *TIP1* overexpressing strain. It is clear that this gene's metabolic function is still unknown, as contradictory results are now available. This gene needs further studies to fully evaluate and understand its function and where it fits in metabolism.

3.4.5 SENSORY EVALUATION

The only noticeable taste variation for the wines was found between VIN13-TOE1 and VIN13-ΔTIP1 when comparing the fruity flavour. From the GC results it is clear that the level of esters was affected only to a small degree and it is unlikely that these small changes would have such a drastic affect. VIN13-TOE1 proved to be fruitier, which is contrary to what was expected, as an esterase should break down esters and hence make the beverage less fruity. It is possible that the decrease in acetic acid may assist in making the wine appear fruitier. This is a positive finding, as it implies that the *TIP1* gene can be used to reduce or increase the level of acetic acid in a fermented beverage without the development of any off-odours. A fruity flavour is usually regarded as favourable. **Figure 3.4** clearly shows that VIN13-TOE1 and VIN13-ΔTIP1 taste somewhat different, as the largest linkage distance separates them. VIN13-ΔTIP1 showed very little difference to VIN13, showing that single disruption had very little effect on flavour and that the slight increase in acetic acid went unnoticed by the judges.

When comparing the overexpression and disruption of the *IAH1* gene, it becomes apparent that the level of esters needs to be altered to a greater extent to make any impact on the flavour of an alcoholic beverage such as wine or brandy. It also proves that ester synthesising rather than the esterase genes is of more importance in the formation of esters and the overall flavour of an alcoholic beverage. This also shows that certain esters are more responsible for flavour, as they have lower sensory thresholds than other esters. In this study, it appears that the overexpression of a gene has more of an impact on the overall aroma than a single disruption of a gene.

From the data, it is clear that brandy samples are far more difficult to evaluate, as no significant findings were made. This is most likely due to the overpowering taste of the alcohol, making it more difficult to detect any of the flavours.

3.5 ACKNOWLEDGEMENTS

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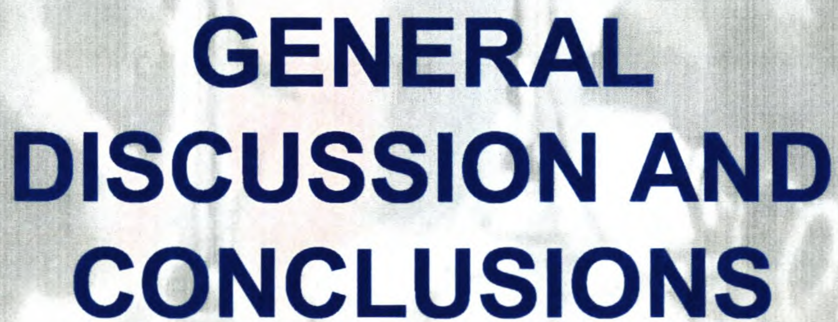
CHAPTER 4



GENERAL DISCUSSION AND CONCLUSIONS

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4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION

Yeasts, especially *Saccharomyces cerevisiae*, are important organisms involved in the production of many alcoholic beverages and their primary function is the conversion of sugars and other fermentable compounds into ethanol. Yeasts also produce a number of secondary metabolites during such fermentations and it is these metabolites that give an alcoholic beverage its principal character (Schreier, 1979; Lambrechts and Pretorius, 2000). The reasons for the production of secondary metabolites are numerous and include detoxification, cell wall production and redox balancing (Nordström, 1962a, b, 1964; Peddie, 1990; Lambrechts and Pretorius, 2000). An important class of volatile compounds that are produced as secondary metabolites are the esters. They impart fruity aromas to the end product or, in this case, the wine and brandy (Engan, 1974). Esters are associated with a number of different aromas, including ethyl acetate (fruity/solvent-like), ethyl caproate and ethyl caprylate (apple-like), isoamyl acetate and isobutyl acetate (banana-like) (Peddie, 1990; Yoshioka and Hashimoto, 1983).

The direct manipulation of genes within an organism is not a novel idea per se. The techniques involved have been around for many years. However, improvements to these techniques are being made on a daily basis. There are an abundance of genes involved in the production of esters, including *ATF1*, *ATF2*, *IAH1* and *TIP1* (Fujii *et al.*, 1994; Fukuda *et al.*, 1996; Nagasawa *et al.*, 1998; Horsted *et al.*, 1998; Lilly *et al.*, 2000; Yoshimoto *et al.*, 2001). The *ATF1* and *ATF2* genes are alcohol acetyltransferases involved in the production of esters, while the *IAH1* and *TIP1* genes are esterases implicated in ester catabolism. Thus, the balances of activities between alcohol acetyltransferases and esterases are responsible for the available levels of esters found within alcoholic beverages (Fukuda *et al.*, 1998a)

The aim of this study was to individually investigate the contributions of the *IAH1* and *TIP1* genes to the overall level of esters within wine and brandy. Colombard must was chosen as the substrate for fermentation, as this must can be used for both wine and brandy production. The approach used to achieve this goal was to both disrupt and overexpress each gene in the industrial *S. cerevisiae* wine yeast strain VIN13 and then to ferment with these new transformed strains. At the end of fermentation, GC analysis would be performed on the wines and brandies and the effect of the genes on the level of esters would be determined. For the overexpression, both genes were cloned within the constitutive *PGK1* promoter and terminator, thus eliminating the unknown effects of the *IAH1* promoter region and the heat and cold shock-inducible promoter of the *TIP1* gene (Kondo and Inouye, 1991). These constructs were then transformed into VIN13 and verified by Northern blot hybridisation. The Northern blot showed that both genes were expressed at high levels throughout fermentation compared to the control strain.

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Industrial wine yeasts are often diploid or even polyploid, having two or more copies of a gene within their genome. This complicated the disruption experiments, as, after the first round of disruptions, the strategy used seemed to show that the cassettes were more homologous to the disrupted region. The results obtained suggested that the disruption cassette reintegrated into the already disrupted locus with a higher frequency than into the non-disrupted locus. For this reason, only a single copy of each gene was disrupted. This is not the ideal situation, as these genes can therefore still be expressed; however, the reduction in copy number should reduce the effect of these genes. RT-PCR confirmed a decrease in the level of expression of these two genes. However, only the *TIP1* disrupted strain showed differences to the control strain when comparing the results of the GC analysis.

With regard to the overexpressed strains, some fears arose that the high levels of esterase activity could produce toxic long-chained fatty acids and thus hamper the rate of fermentation. This was not the case, as these genes in no way affected the fermentation rates and all the strains completed fermentations within 14 days.

The *TIP1* overexpressing strain did not show any esterase activity, but rather showed ester synthesising activity with increased ester levels in the wine and brandy. Previously, Horsted *et al.* (1998) implicated the *TIP1* gene in esters hydrolysis, especially of esters with between four and 16 carbons in the fatty acid chain, but not ethyl acetate and isoamyl acetate. If this was the case, lower levels of esters should have been obtained in comparison to the control strain. The findings of this study have shown that the levels of ethyl acetate, isoamyl acetate, ethyl caprate, ethyl caproate and ethyl caprylate all increased, although only to a small degree. One significant effect of the overexpression was the reduction in acetic acid levels in both the wine and brandy. A reduction of up to five-fold was noticed. This can be advantageous, as wines have a legal limit of 1.2 g/l for acetic acid and are regarded as spoiled above this level. Many countries have regulations relating to volatile acidity (VA) and high levels of acetic acid. These high levels of VA are often associated with wines that show sluggish/stuck fermentation. The above-mentioned result is of great significance to the wine industry. This wine yeast, or even other wine yeast strains overexpressing this gene, could help alleviate this problem. From a sensorial point of view, this wine was favoured above all the other wines, including the control strain. This wine was regarded as the subtlest and the easiest to drink. From this study it has become clear that the *TIP1* gene requires further analysis, as contradictory results have been obtained. The gene needs to be linked to a metabolic function and detailed protein studies would assist in its placement.

The *TIP1* single disrupted strain had a small effect on the acetic acid level and thus on the total acid concentration of the wine and brandy. The quantity of acetic acid increased even though the strain still had another copy of this gene to buffer this effect. No other acid tested for could be associated with this increase in overall acid level. This finding corresponds well with the *TIP1* overexpressing strain, as it had the opposite effect. The production of a null mutant would be of interest in determining

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the contribution of the remaining copy to the acetic acid level.

The *IAH1* overexpressing strain behaved as expected. The gene made a significant contribution to the catabolism of the following esters: ethyl acetate, isoamyl acetate, ethyl caproate, hexyl acetate, ethyl caprylate and ethyl caprate, showing decreases of 31%, 45%, 62%, 55%, 44% and 33% respectively. This correlates well with the findings of Fukuda *et al.* (1996, 1998a, 2000) and further confirms that this gene is an isoamyl acetate-hydrolysing esterase. Acetic acid levels decreased to some extent, although not to the same degree as the *TIP1* overexpressing strain. As discussed earlier, this is extremely favourable and often desirable. Although the overall higher alcohol level did not change significantly, two alcohols showed changes. N-propanol increased moderately, while isoamyl alcohol levels decreased moderately. The overall higher alcohol level therefore did not change due to a balancing out between the two components.

The *IAH1* single disrupted strain showed no significant variability from the control strain, other than an increase in the acetic acid level and overall acid concentration. Fukuda *et al.* (1996, 1998b) found higher levels of isoamyl acetate and isobutyl acetate in the final product after the disruption of this gene (null mutant). Possible reasons for these discrepancies could be that the remaining copy of the *IAH1* gene in the single disrupted strain from this study may be able to express enough protein to revert to an almost normal phenotype. Sensorially, this wine was not very different from the control strain wine. As this strain made no real significant changes to the wine (sensory or GC), it was not used for the production of base wine and brandy.

Ester production by yeast is influenced by a number of factors. The level of SO_2 was thought to play a role in ester production, but Daudt and Ough (1973) proved that this was not the case. This is also confirmed by this study, as the base wine fermentations, with no SO_2, showed no significant differences to the yeast fermented in the must to which SO_2 had been added. The presence of the yeast lees during distillation has previously been shown to increase the total level of esters (Nykänen *et al.*, 1977). This correlates well with the findings of this study, as the level of esters increased dramatically during distillation, particularly during the first distillation.

4.2 CONCLUSIONS

From the above-mentioned data it can clearly be seen that yeast can make a contribution to the overall sensory quality of an alcoholic beverage. By using relatively simple genetic manipulation techniques, it has become possible to drastically alter the aroma, flavour and indeed any other property of an alcoholic beverage, as long as this property can be related to a gene or group of genes (Dequin, 2001; Pretorius, 2000). Much work must still be done if we are to gain a full understanding of the functioning of yeast. As we unravel the mystery of life, we can use these newly discovered genes as tools to produce products with desirable outcomes. Unfortunately, it is currently illegal to use genetically modified organisms

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for the production of wine and brandy, even though certain modifications are beneficial to the final product. Today's genetic manipulation techniques are precise and relatively fast, and very specific traits can be altered using these improved methods. It seems very strange that these precise alterations are unacceptable and that the classical methods, such as yeast mating, which are very unspecific and even unpredictable, are acceptable. In the future, laws will change and the consumer will realise that the genetic manipulation of organisms is safe and beneficial to mankind. Tailor-made products, catering for specific niche markets, will become a reality and these products also will have incredible health benefits, lower costs and a better overall quality. The genetically modified organisms will also benefit companies by allowing them to produce these niche market products and thus gaining a competitive edge over other producers. We are entering the age of biotechnology and, in this age, the processes of life will be used to improve life.

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CHAPTER 5

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5. APPENDIX

5.1 STATISTICAL DATA

Table 5.1. Wine tasting data as scored by the panel of nine judges

Sample	Taster	Fruity	Herbaceous	Nutty	Chemical	Pungent	Floral
VIN13-TOE1	1	3	1	1	1	1	1
VIN13-ΔIAH1	1	1	1	1	1	1	1
VIN13-ΔTIP1	1	1	1	1	1	1	1
VIN13	1	1	1	1	1	1	1
VIN13-IOE1	1	3	1	2	1	1	1
VIN13-TOE1	2	4	1	1	1	3	4
VIN13-ΔIAH1	2	3	1	1	1	4	4
VIN13-ΔTIP1	2	1	1	2	2	3	3
VIN13	2	2	2	1	1	2	2
VIN13-IOE1	2	3	2	1	4	4	2
VIN13-TOE1	3	3	1	3	1	1	1
VIN13-ΔIAH1	3	1	1	1	5	5	1
VIN13-ΔTIP1	3	1	3	1	5	1	1
VIN13	3	1	1	1	3	1	1
VIN13-IOE1	3	3	3	1	1	1	1
VIN13-TOE1	4	4	2	1	1	1	2
VIN13-ΔIAH1	4	2	1	1	1	1	2
VIN13-ΔTIP1	4	3	2	1	1	1	2
VIN13	4	3	1	1	1	1	1
VIN13-IOE1	4	1	1	1	1	3	1
VIN13-TOE1	5	4	1	1	4	1	2
VIN13-ΔIAH1	5	1	1	1	1	1	1
VIN13-ΔTIP1	5	2	1	1	3	1	2
VIN13	5	3	2	1	1	1	2
VIN13-IOE1	5	2	1	1	1	1	2
VIN13-TOE1	6	2	1	1	3		1
VIN13-ΔIAH1	6	3	1	3	1	1	1
VIN13-ΔTIP1	6	1	1	1	1	1	1
VIN13	6	3	1	1	1	1	1
VIN13-IOE1	6	1	3	1	1	1	1
VIN13-TOE1	7	2	1	1	1	1	2
VIN13-ΔIAH1	7	3	2	1	1	1	3
VIN13-ΔTIP1	7	1	1	1	4	1	2
VIN13	7	3	2	1	1	1	2
VIN13-IOE1	7	1	2	1	1	1	1
VIN13-TOE1	8	4	2	3	3	1	4
VIN13-ΔIAH1	8	4	3	3	3	1	3
VIN13-ΔTIP1	8	2	3	3	3	1	2
VIN13	8	3	3	2	3	1	2
VIN13-IOE1	8	4	2	3	3	1	4
VIN13-TOE1	9	4	1	1	1	1	2
VIN13-ΔIAH1	9	2	1	1	2	1	1
VIN13-ΔTIP1	9	1	1	1	2	1	2
VIN13	9	1	1	2	1	1	1
VIN13-IOE1	9	2	1	1	1	1	1

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Table 5.2. Brandy tasting data as tasted by the panel of eight judges

Sample	Taster	Fruity	Flowery	Sweet	Chemical	Oily	Herbaceous
VIN13	1	3	1	3	1	1	1
VIN13-ΔTIP1	1	1	1	1	3	1	3
VIN13-IOE1	1	3	3	5	1	3	1
VIN13-TOE1	1	3	3	3	3	1	3
VIN13	2	3	3	1	2	3	4
VIN13-ΔTIP1	2	4	4	3	2	3	3
VIN13-IOE1	2	1	1	2	2	2	3
VIN13-TOE1	2	3	3	3	2	3	4
VIN13	3	3	5	3	1	1	1
VIN13-ΔTIP1	3	3	1	1	1	1	3
VIN13-IOE1	3	3	3	1	1	1	1
VIN13-TOE1	3	3	3	1	1	1	3
VIN13	4	4	1	1	1	1	1
VIN13-ΔTIP1	4	4	1	1	1	1	1
VIN13-IOE1	4	3	3	1	1	1	1
VIN13-TOE1	4	1	2	1	1	1	1
VIN13	5	4	3	4	4	2	2
VIN13-ΔTIP1	5	4	4	3	4	2	2
VIN13-IOE1	5	3	3	2	3	3	2
VIN13-TOE1	5	3	3	3	3	3	3
VIN13	6	3	1	4	2	1	1
VIN13-ΔTIP1	6	4	2	2	4	1	1
VIN13-IOE1	6	1	2	1	4	1	1
VIN13-TOE1	6	3	2	2	1	1	1
VIN13	7	2	3	1	1	1	1
VIN13-ΔTIP1	7	2	3	1	3	1	1
VIN13-IOE1	7	3	2	1	2	1	1
VIN13-TOE1	7	2	1	4	1	1	1
VIN13	8	1	3	5	1	1	1
VIN13-ΔTIP1	8	3	3	3	3	1	1
VIN13-IOE1	8	3	1	3	1	1	1
VIN13-TOE1	8	3	1	3	3	1	1

5.2 STATISTICAL ANALYSIS DATA FOR THE WINE SAMPLES

Table 5.3. Kruskal-Wallis ANOVA by Ranks for herbaceous for the wine samples

Dependent: Herbaceous	Independent (grouping) variable: Sample Cruskal-Wallis test: H (4, N=45) =3.257916 p=0.5156		
	Code	Valid N	Sum of Ranks
VIN13-TOE1	101	9	174.00
VIN13-ΔIAH1	102	9	182.00
VIN13ΔTIP1	103	9	209.50
VIN13	104	9	221.00
VIN13-IOE1	105	9	248.50

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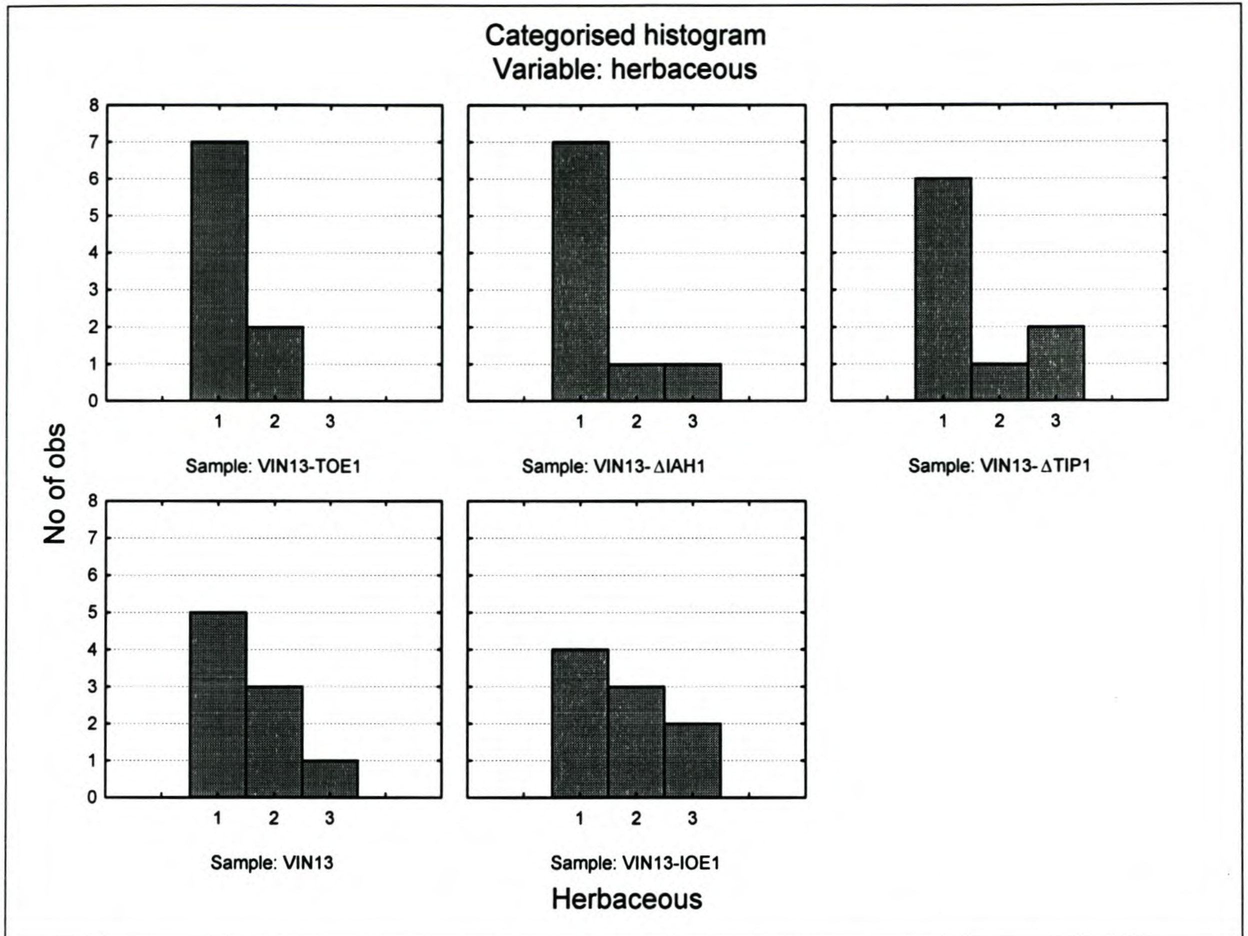

Figure 5.1. Categorised histograms for the variable herbaceous for the different wine samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.4. Kruskal-Wallis ANOVA by Ranks for nutty for the wine samples

Dependent: Nutty	Independent (grouping) variable: Sample Cruskal-Wallis test: H (4, N=45) =0.0856091 p=0.9991		
	Code	Valid N	Sum of Ranks
VIN13-TOE1	101	9	211.00
VIN13-ΔIAH1	102	9	211.00
VIN13ΔTIP1	103	9	206.00
VIN13	104	9	201.00
VIN13-IOE1	105	9	206.00

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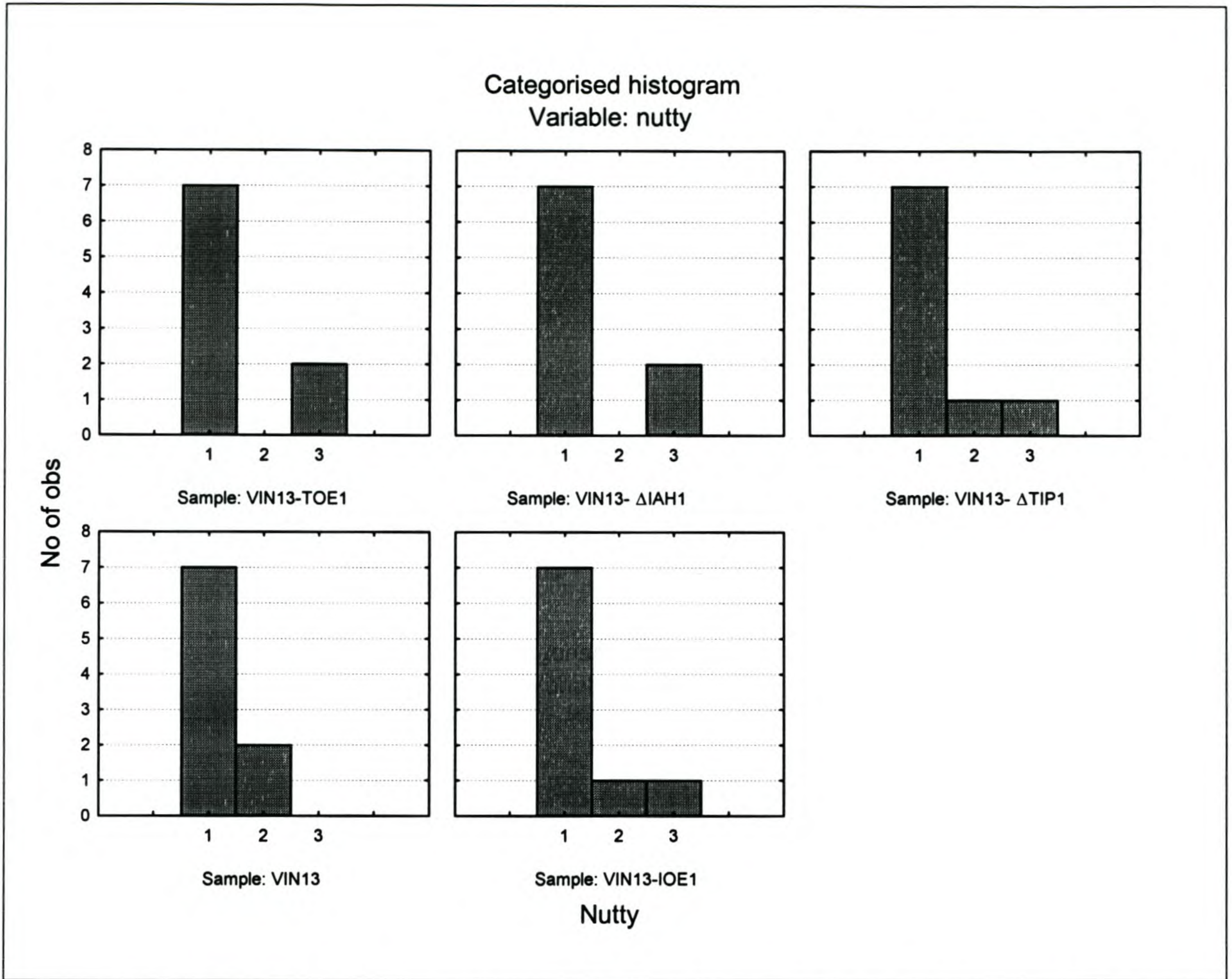

Figure 5.2. Categorised histograms for the variable nutty for the different wine samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.5. Kruskal-Wallis ANOVA by Ranks for chemical for the wine samples

Dependent: Chemical	Independent (grouping) variable: Sample Cruskal-Wallis test: H (4, N=45) =4.400310 p=0.3545		
	Code	Valid N	Sum of Ranks
VIN13-TOE1	101	9	205.00
VIN13-ΔIAH1	102	9	202.00
VIN13ΔTIP1	103	9	266.50
VIN13	104	9	178.00
VIN13-IOE1	105	9	183.50

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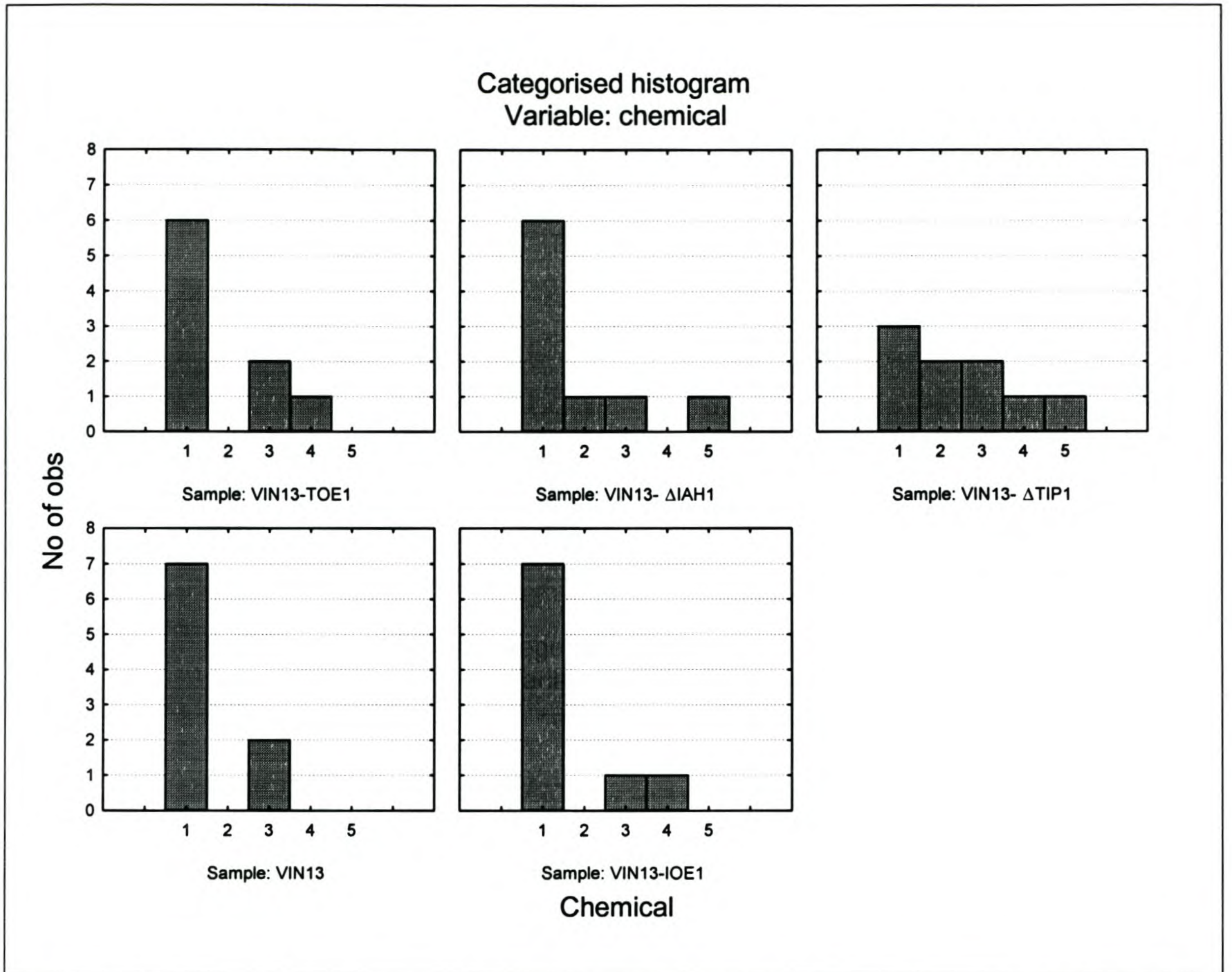

Figure 5.3. Categorised histograms for the variable fruity for the different wine samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.6. Kruskal-Wallis ANOVA by Ranks for pungent for the wine samples

Dependent: Pungent	Independent (grouping) variable: Sample Cruskal-Wallis test: H (4, N=44) =1.295834 p=0.8621		
	Code	Valid N	Sum of Ranks
VIN13-TOE1	101	8	173.00
VIN13-ΔIAH1	102	9	219.50
VIN13ΔTIP1	103	9	192.00
VIN13	104	9	190.00
VIN13-IOE1	105	9	215.50

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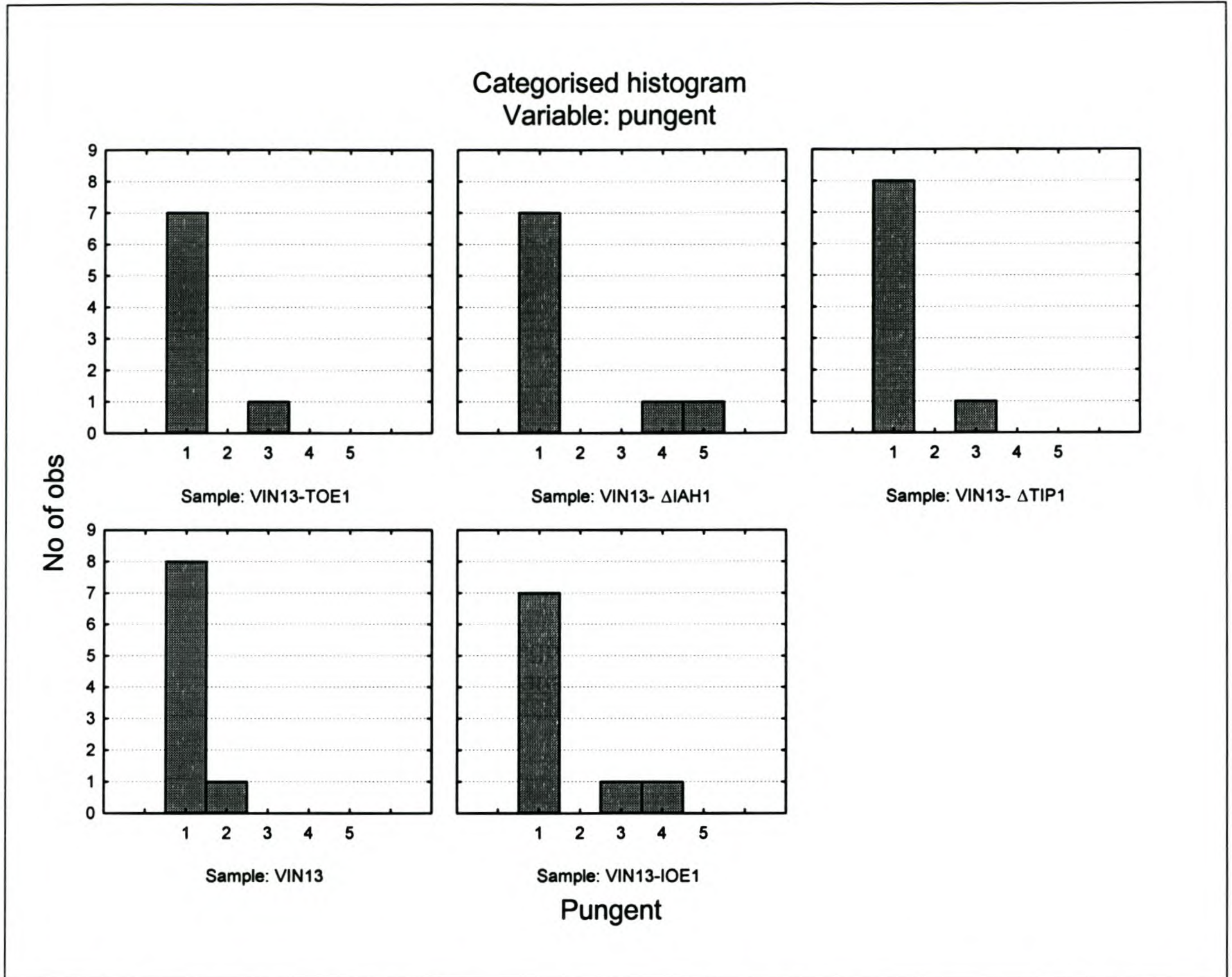

Figure 5.4. Categorised histograms for the variable pungent for the different wine samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.7. Kruskal-Wallis ANOVA by Ranks for floral for the wine samples

Dependent: Floral	Independent (grouping) variable: Sample Cruskal-Wallis test: H (4, N=45) =2.792223 p=0.5932		
	Code	Valid N	Sum of Ranks
VIN13-TOE1	101	9	243.50
VIN13-ΔIAH1	102	9	211.50
VIN13ΔTIP1	103	9	227.00
VIN13	104	9	179.50
VIN13-IOE1	105	9	173.50

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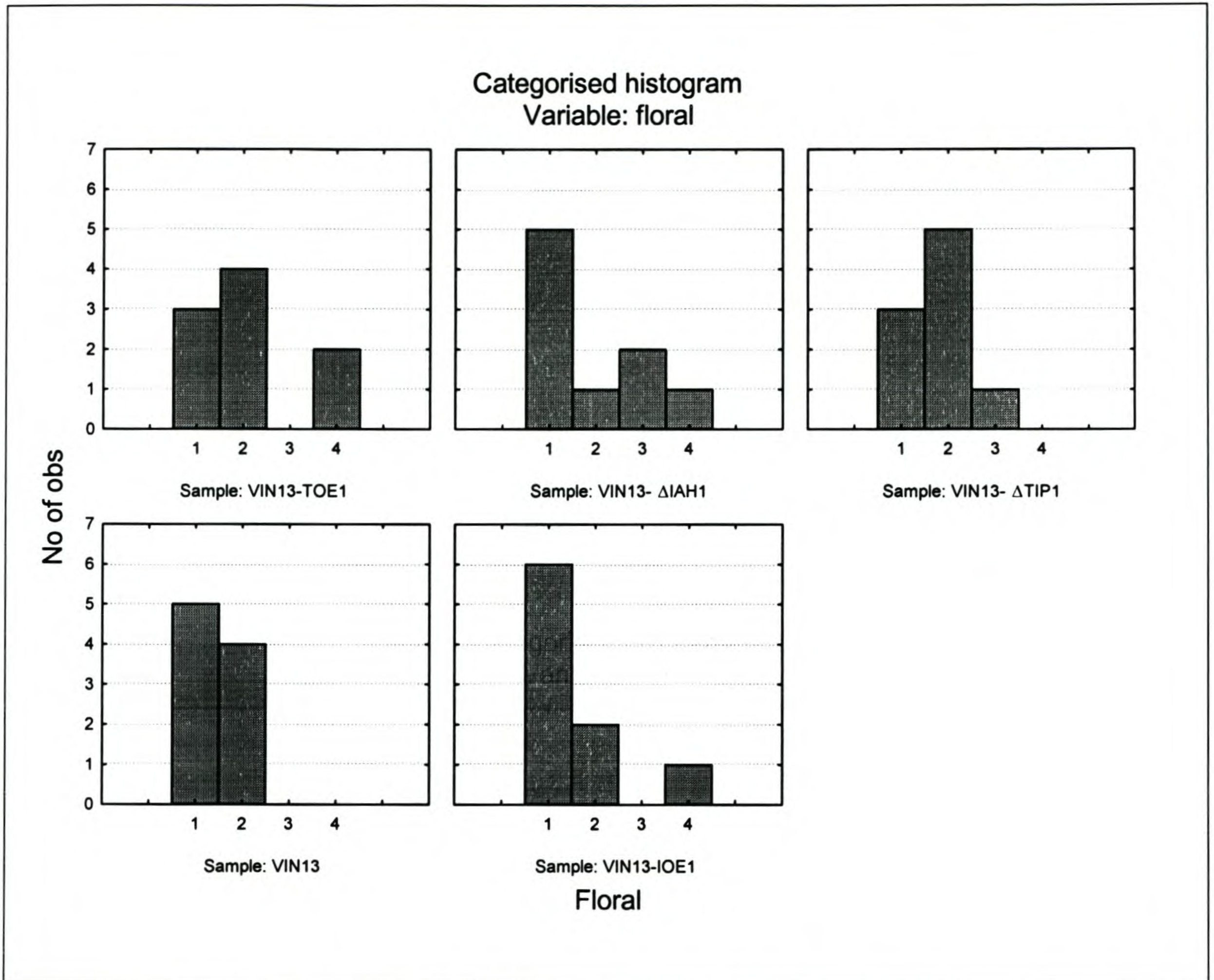

Figure 5.5. Categorised histograms for the variable floral for the different wine samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

5.3 STATISTICAL ANALYSIS DATA FOR THE BRANDY SAMPLES

Table 5.8. Kruskal-Wallis ANOVA by Ranks for fruity for the brandy samples

Dependent: Fruity	Independent (grouping) variable: Sample Cruskal-Wallis test: H (3, N=33) =3.045393 p=0.3847		
	Code	Valid N	Sum of Ranks
VIN13	101	8	139.00
VIN13ΔTIP1	102	8	163.00
VIN13-IOE1	103	8	111.00
VIN13-TOE1	104	8	115.00

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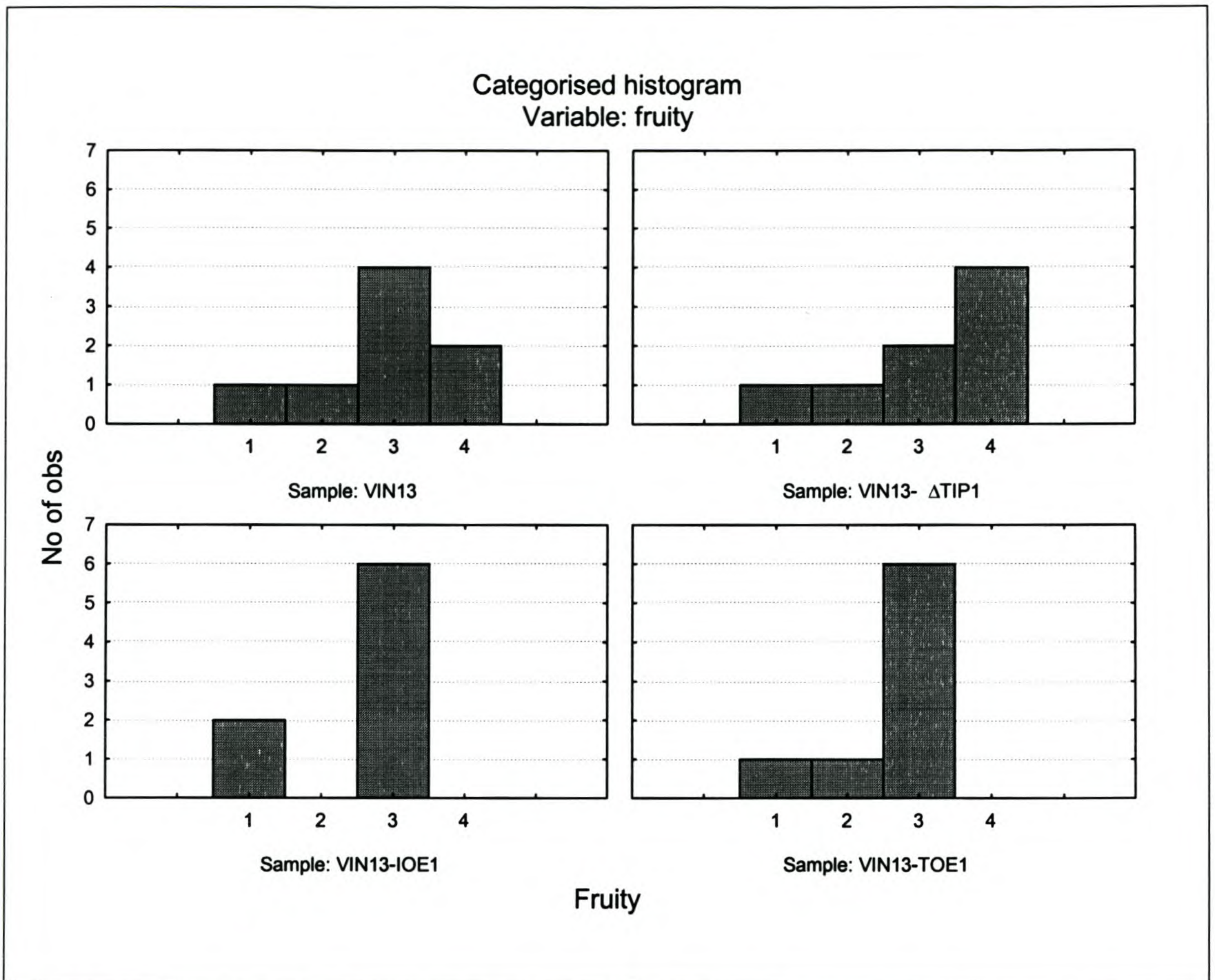

Figure 5.6. Categorised histograms for the variable fruity for the different brandy samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.9. Kruskal-Wallis ANOVA by Ranks for flowery for the brandy samples

Dependent: Flowery	Independent (grouping) variable: Sample Cruskal-Wallis test: H (3, N=32) =0.1681983 p=0.9826		
	Code	Valid N	Sum of Ranks
VIN13	101	8	138.50
VIN13ΔTIP1	102	8	135.50
VIN13-IOE1	103	8	127.00
VIN13-TOE1	104	8	127.00

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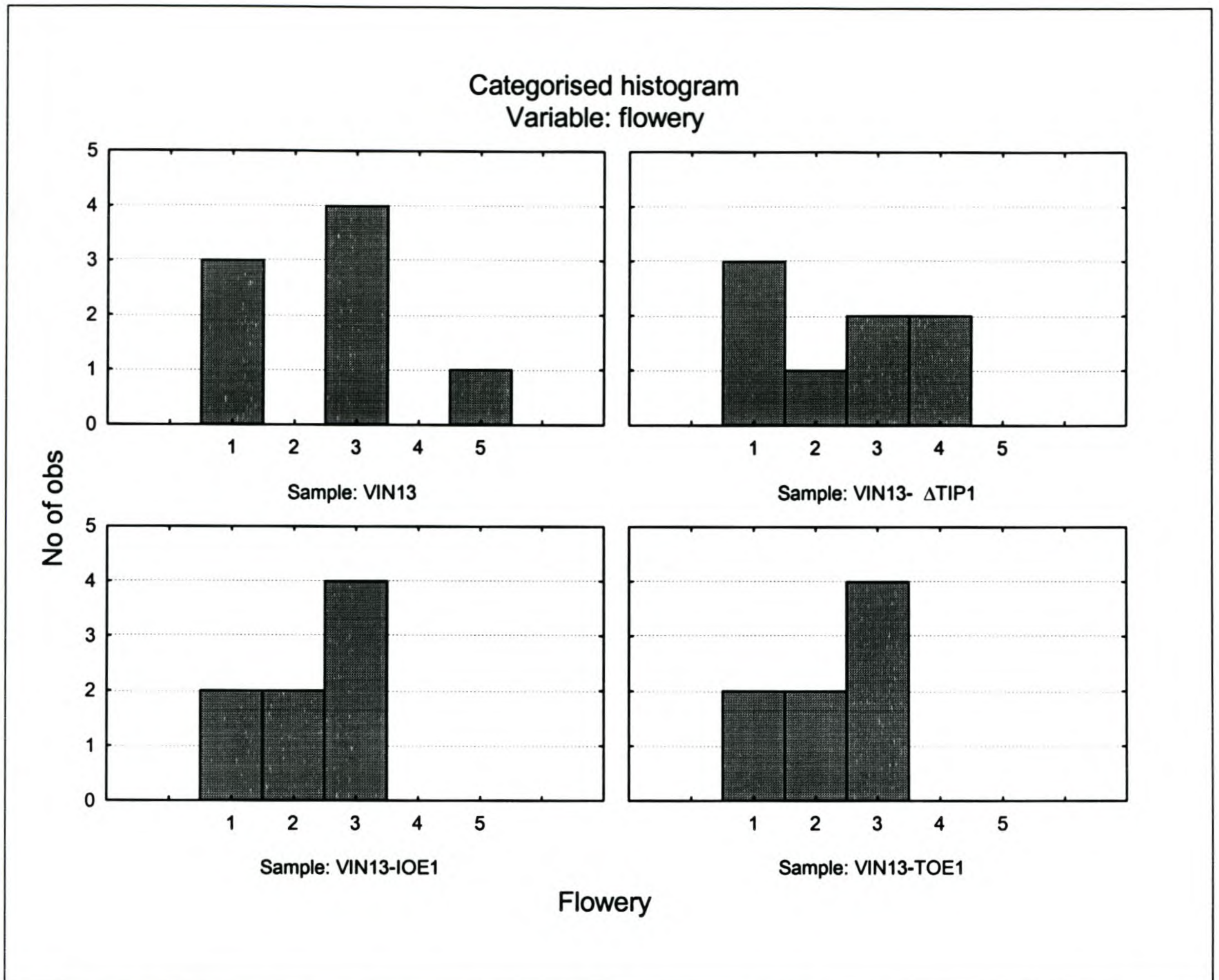

Figure 5.7. Categorised histograms for the variable flowery for the different brandy samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.10. Kruskal-Wallis ANOVA by Ranks for sweet for the brandy samples

Dependent: Sweet	Independent (grouping) variable: Sample Cruskal-Wallis test: H (3, N=32) =2.566241 p=0.4634		
	Code	Valid N	Sum of Ranks
VIN13	101	8	139.00
VIN13ΔTIP1	102	8	163.00
VIN13-IOE1	103	8	111.00
VIN13-TOE1	104	8	115.00

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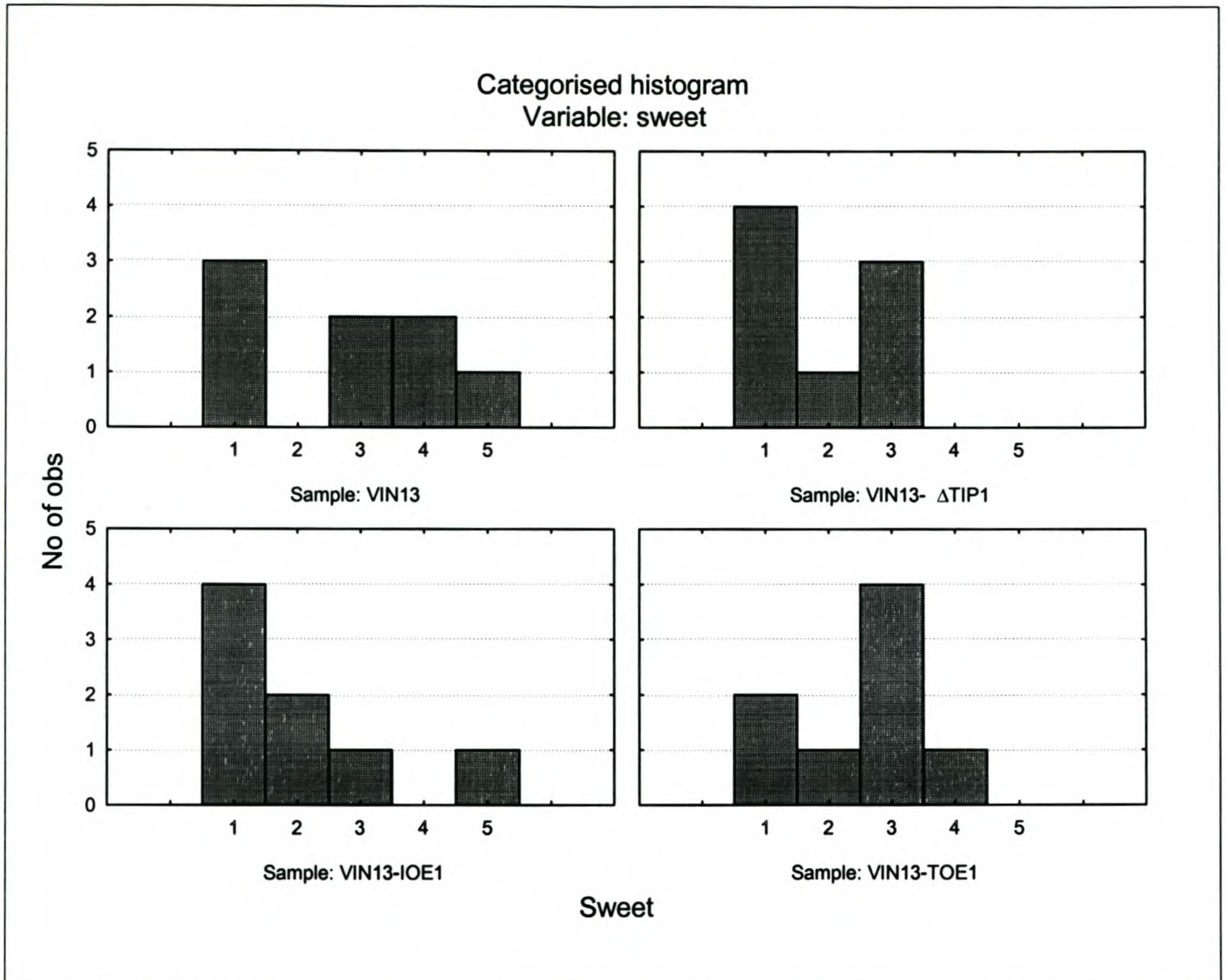

Figure 5.8. Categorised histograms for the variable sweet for the different brandy samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.11. Kruskal-Wallis ANOVA by Ranks for chemical for the brandy samples

Dependent: Chemical	Independent (grouping) variable: Sample Cruskal-Wallis test: H (3, N=32) =3.525078 p=0.3175		
	Code	Valid N	Sum of Ranks
VIN13	101	8	107.50
VIN13ΔTIP1	102	8	170.50
VIN13-IOE1	103	8	124.50
VIN13-TOE1	104	8	125.50

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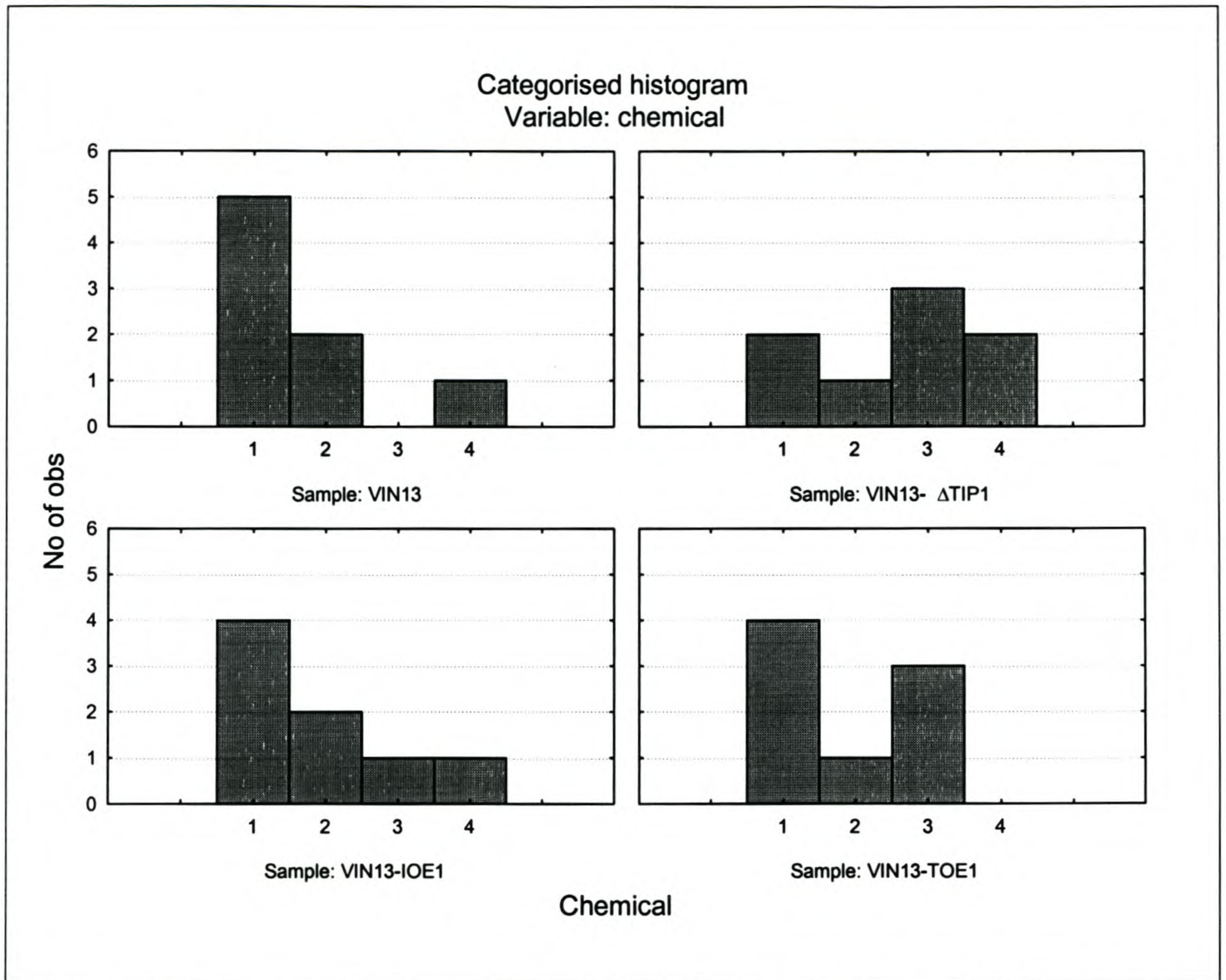

Figure 5.9. Categorised histograms for the variable chemical for the different brandy samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.12. Kruskal-Wallis ANOVA by Ranks for oily for the brandy samples

Dependent: Oily	Independent (grouping) variable: Sample Cruskal-Wallis test: H (3, N=32) =0.4693855 p=0.9256		
	Code	Valid N	Sum of Ranks
VIN13	101	8	126.50
VIN13ΔTIP1	102	8	126.50
VIN13-IOE1	103	8	144.00
VIN13-TOE1	104	8	131.00

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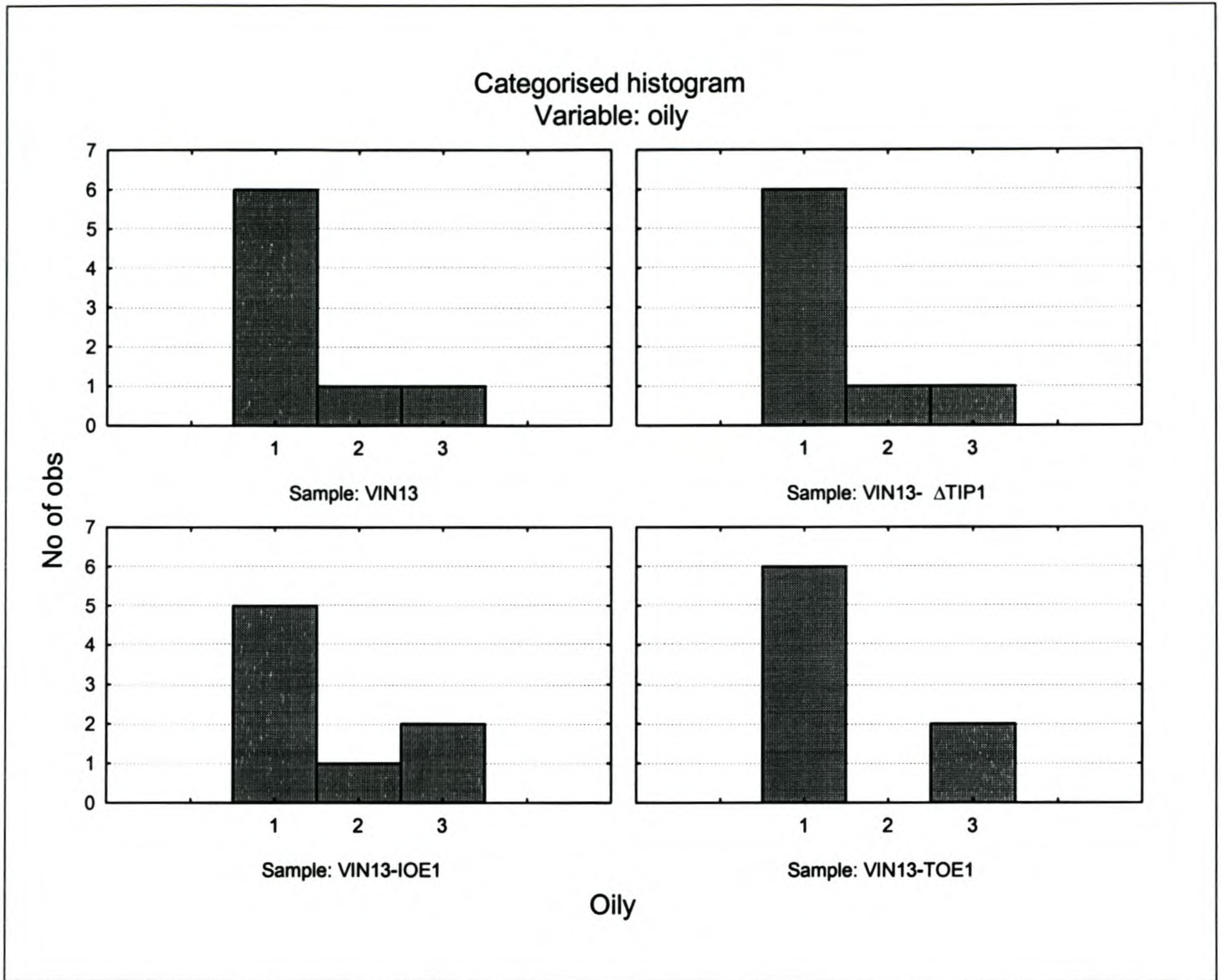

Figure 5.10. Categorised histograms for the variable oily for the different brandy samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred

Table 5.13. Kruskal-Wallis ANOVA by Ranks for herbaceous for the brandy samples

Dependent: Herbaceous	Independent (grouping) variable: Sample Cruskal-Wallis test: H (3, N=32) =2.508026 p=0.4738		
	Code	Valid N	Sum of Ranks
VIN13	101	8	116.50
VIN13ΔTIP1	102	8	145.00
VIN13-IOE1	103	8	112.00
VIN13-TOE1	104	8	154.50

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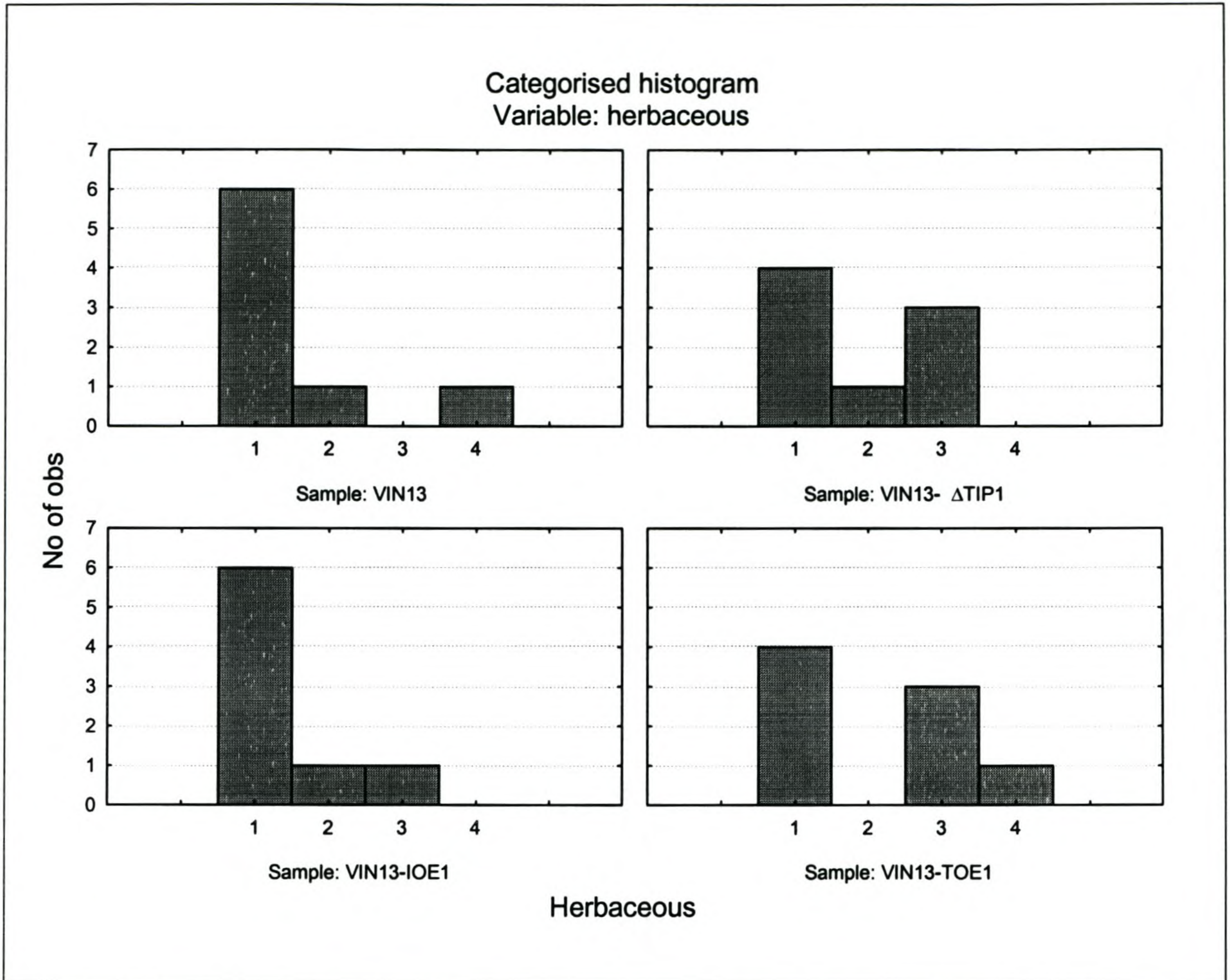

Figure 5.11. Categorised histograms for the variable herbaceous for the different brandy samples. The X-axis represents the score given by the tasters and the Y-axis is the number of times that score occurred