Engineering yeast for the production of optimal levels of volatile phenols in wine

by

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Sciences at Stellenbosch University.

December 2002

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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.

SUMMARY

Phenolic acids (principally ρ-coumaric and ferulic acids), which are generally esterified with tartaric acid, are natural constituents of grape must and wine, and can be released as free acids during the winemaking process by certain cinnamoyl esterase activities. Free phenolic acids can be metabolised into 4-vinyl and 4-ethyl derivatives by several microorganisms present in wine. These volatile phenols contribute to the aroma of the wine.

The *Brettanomyces* yeasts are well known for their ability to form volatile phenols in wine. However, these species are associated with the more unpleasant and odorous formation of the ethylphenols and the formation of high concentrations of volatile phenols. Other organisms, including some bacterial species, are responsible for the formation of volatile phenols at low concentrations, especially the 4-vinylphenols, and this enhances the organoleptic properties of the wine.

The enzymes responsible for the decarboxylation of phenolic acids are called phenolic acid decarboxylases; and several bacteria and fungi have been found to contain the genes encoding these enzymes. The following genes have been characterised: *PAD1* from *Saccharomyces cerevisiae*, *fdc* from *Bacillus pumilus, pdc* from *Lactobacillus plantarum* and *padc* from *Bacillus subtilis. PadA* from *Pediococcus pentosaceus* was also identified.

S. cerevisiae contains the *PAD1* (phenyl acrylic acid decarboxylase) gene, which is steadily transcribed in yeast. The activity of the *PAD1*-encoded enzyme is low. Phenolic acid decarboxylase from *B. subtilis,* as well as ρ-coumaric acid decarboxylase from *L. plantarum* displays substrate inducible decarboxylating activity with phenolic acids. Both the ρ-coumaric acid decarboxylase (*pdc*) and phenolic acid decarboxylase (*padc*) genes were cloned into $PGK1_{PT}$ expression cassette. The $PGK1_{PT}$ expression cassette consisted of the promoter ($PGK1_P$) and terminator ($PGK1_T$) sequence of the yeast phosphoglyceratekinase I gene (*PGK1*). Episomal and yeast integration plasmids were constructed for the *PAD1* gene under the control of the $PGK1_{PT}$ for overexpresion in yeast. Industrial strains with the *PAD1* gene disrupted were also made. Overexpression of ρ-coumaric acid decarboxylase (*pdc*) and phenolic acid decarboxylase (*padc*) in *S. cerevisiae* showed high enzyme activity in laboratory strains. The overexpressed *PAD1* gene did not show any higher enzyme activity than the control strain. Both bacterial genes, under the control of the $PGK1_{PT}$ cassette, were also cloned into a yeast-integrating plasmid, with the *SMR1* gene as selective marker. The cloning and transformation of *pdc* and *padc* into industrial wine yeast strains can therefore be used to detect the effect of phenolic acid decarboxylase genes in the winemaking process for the possible improvement of wine aroma. Wine was made with all three strains (the bacterial genes overexpressed and *PAD1* disrupted). The effect of these genes in wine was determined through GC analysis. The

results showed that the bacterial genes could effectively produce higher levels of volatile phenols in the wine. The manipulated strains also produced enzymes capable of producing large amounts of favourable monoterpenes in the wine.

This study paves the way for the development of wine yeast starter culture strains for the production of optimal levels of volatile phenols, thereby improving the sensorial quality of wine.

OPSOMMING

Die fenoliese sure (ρ-kumaarsuur en ferolsuur), wat as natuurlike komponente in mos en wyn voorkom, word gewoonlik as esterverbindings in wynsteensuur gevind. Seker esterase-aktiwiteite kan die fenoliese sure as vrye sure vrystel gedurende die wynmaakproses. Hierdie vrye fenoliese sure kan dan weer deur verskillende mikro-organismes na 4-viniel en 4-etiel derivate omgesit word. Hierdie derivate staan as vlugtige fenole bekend en kan tot die aroma van wyn bydra.

Die *Brettanomyces* giste is baie bekend vir hulle vermoeë om vlugtige fenole in wyn te vorm, maar dit is gewoonlik die formasie van hoë konsentrasies van vlugtige fenole, veral die 4-etiel derivate, wat met af geure geassosieer word. Ander organismes besit egter die vermoeë om vlugtige fenole teen lae konsentrasies te vorm, veral die 4-viniel derivate, wat 'n aanvullende effek op die wyn aroma kan hê. .

Die ensieme wat verantwoordelik is vir die dekarboksilasie van fenoliese sure staan as fenolsuurdekarboksilases bekend. Verskeie bakterieë en fungi bevat gene wat vir hiedie ensieme kodeer. Die volgende gene is reeds gekarakteriseer: *PAD1* van *Saccharomyces cerevisiae*, *fdc* van *Bacillus pumilus, pdc* van *Lactobacillus plantarum* en *padc* van *Bacillus subtilis. PadA* van *Pediococcus pentosaceus* is ook reeds geïdentifiseer.

S. cerevisiae bevat die *PAD1*- (fenielakrielsuurdekarboksilase) geen, wat teen 'n vaste tempo in gis getranskribeer word. Die aktiwiteit van hierdie ensiem is egter laag. Fenolsuurdekarboksilase van *B. subtilis,* sowel as ρ-kumaarsuurdekarboksilase van *L. plantarum,* vertoon 'n substraat-induseerbare dekarboksilerende aktiwiteit met fenoliese sure. Beide die ρ-kumaarsuur dekarboksilase en die fenolsuurdekarboksilase gene is in die $PGK1_{PT}$ ekspressie kasset gekloneer. Episomale en gisintegreringsplasmiede is vir die *PAD1*-geen onder beheer van die $PGK1_{PT}$ ekspressiekasset gekonstrueer vir die ooruitdrukking van hierdie geen in gis. Die $PGK1_{PT}$ ekspressiekasset het bestaan uit die promotor- ($PGK1_P$) en termineerdersekwense ($PGK1_T$) van die gisfosfogliseraatkinasegeen (*PGK1*). Industriële gisrasse is ontwikkel waarin die *PAD1*-geen onderbreek is. Ooruitdrukking van ρ-kumaarsuurdekarboksilase (*pdc*) en fenolsuurdekarboksilase (*padc*) in *S. cerevisiae* toon hoë ensiemaktiwiteit in laboratoriumgisrasse. Die ooruitdrukking van die *PAD1*-geen het nie hoër aktiwiteit as die kontroleras gewys nie. Albei die bakteriële gene, onder die beheer van die $PGK1_{PT}$ ekspressiekasset, is ook in 'n gisintegreringsplasmied met die *SMR1*-geen as selektiewe merker geplaas. Die klonering en transformasie van *pdc* en *padc* in industriële wyngiste kan dus gebruik word vir die bepaling van die effek van fenolsuur dekarboksilases in die wynmaakproses en die moontlike verbetering van wynaroma. Wyn is met al drie die industriële rasse (die ooruitgedrukte bakteriële gene en die ontwrigte *PAD1*-

geen) gemaak. Die effek van die teenwoordigheid van hierdie gene in die wynmaakproses is deur gaschromatografie bepaal. Die resultate het aangedui dat die bakteriële gene op 'n effektiewe wyse vlugtige fenole in die wyn kan produseer. Sekere monoterpene is ook in 'n verhoogde mate gedurende hierdie proses gevorm.

Hierdie studie baan die weg vir die ontwikkeling van reingisinentingskulture vir die produksie van optimale vlakke van vlugtige fenole om sodoende die sensoriese gehalte van die wyn te verbeter.

This thesis is dedicated to André R Smit.
Hierdie tesis is opgedra aan André R Smit.

BIOGRAPHICAL SKETCH

Annél Smit was born on the 17th of November 1976 in Paarl, South Africa. In 1994 she matriculated from Stellenberg High School in the Cape. She obtained her BSc (Microbiology and Biochemistry) degree in 1998 and completed her BScHons degree (Wine Biotechnology) in 1999. She is married to André Remeires Smit.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

The IWBT for the opportunity to study;

The National Research Foundation (NRF) and the South African wine industry (Winetech), for funding the project;

Prof IS Pretorius, for critical reading of this thesis and support;

Dr P van Rensburg for his supervision and support over a 3-year period;

Dr RR Cordero Otero, for his valuable contribution and insight to the project;

Dr MG Lambrechts, for critical reading of this thesis;

André R Smit and my family, for their support.

> "...strewe na diep en volledige insig sodat hulle God se geheimenis kan ken. Die geheimenis is Christus, en in Hom is al die verborge skatte van wysheid en kennis te vind."
>
> Kolossense 2:2-3

> "...so that they may have the full riches of complete understanding, in order that they may know the mystery of God, namely, Christ, in whom are hidden all the treasure of wisdom and knowledge."
>
> Colossians 2:2-3

PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal *Applied and Environmental Microbiology,* to which Chapter 3 will be submitted for publication.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Phenols and wine aroma and the influence of phenolic acid decarboxylases

Chapter 3 **Research results**
Manipulation of volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae*

Chapter 4 **Additional research results**
Monoterpene formation by recombinant yeast strains

Chapter 5 **General discussion and conclusions**

CONTENTS

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

CHAPTER 1

1.1 INTRODUCTION

The chemical composition of grapes and wine makes a significant contribution to the sensory evaluation of wine. Each component can contribute to the eventual flavour and aroma of the wine. Phenols have an important and specific function in wine. They contribute to the colour, antimicrobial action, antioxidative action, aroma and flavour of the wine. Phenols in wine originate in the grape berry itself and are abundant in various forms in the must. When analyzing the contribution of phenolic compounds to the aroma of wine, a large number of different compounds can be considered (Ribéreau-Gayon et al., 2000).

Before hydrolyzation, phenolic compounds, in the extracted form, contribute to a variety of characteristics of the wine. In their aggregation and reaction with other molecules, phenolic compounds form, among others, various flavour compounds. A discussion follows on phenolic compounds and their different contributions to the wine aroma.

Apart from the role that phenolic compounds play in wine, they are also responsible for the formation of a new set of substances that are present in the wine after hydrolysis. The decarboxylation of hydroxycinnamic acids (phenolic acids) produces volatile phenols that contribute specifically to the aroma of the produced wine. These volatile phenols are very important, as they can either improve the flavour of the wine or produce odours.

The formation of volatile phenols is due to the action of some of the microorganisms present in the wine. These actions also contribute to the concentration of volatile phenols in the wine. Various microorganisms, including some winemicroorganisms, are responsible for the decarboxylation of phenolic acids. Phenolic acids are usually first decarboxylated into 4-vinyl derivatives, and then reduced to 4-ethyl derivatives (Cavin et al., 1993). The enzymes responsible for the decarboxylation are called phenolic acid decarboxylases and several bacteria and fungi have been found to contain the genes encoding these enzymes. Clausen et al. (1994) characterized *PAD1* from *Saccharomyces cerevisiae*. Zago et al. (1995), Cavin et al. (1997b, 1998) characterized *fdc* from *Bacillus pumilus, pdc* from *Lactobacillus plantarum* and *padc* from *Bacillus subtilis* respectively. *PadA* from *Pediococcus pentosaceus* was identified and cloned by Barthelmebs et al. (2000). All these genes have been cloned and expressed in *E. coli* by the various authors.

This study will look at the role of phenols in wine and their contribution to the production of flavour and aroma compounds in the wine. A closer look will be taken at the production of the volatile phenols through phenolic acid decarboxylases and their contribution to wine aroma. For this project, the phenolic acid decarboxylase genes of *S. cerevisiae, L. plantarum* and *B. subtilis* were used.

1.1 PROJECT AIMS

Free phenolic acids can be metabolized into 4-vinyl derivatives and 4-ethyl derivatives in wine by different microorganisms (as previously mentioned). These derivatives can contribute to the improvement of wine aroma. *S. cerevisiae* contains the phenylacrylic acid decarboxylase gene, *PAD1* (Clausen et al., 1994). Pad1p exhibits very low activity with ferulic and ρ-coumaric acids and is therefore not efficient for the improvement of wine aroma. However, Cavin et al. (1997a, 1998) found that *L. plantarum* (*pdc*) and *B. subtilis* (*padc*) both contain a substrate-inducible phenolic acid decarboxylase gene that shows high activity against phenolic acids.

The aim of this project was to determine the effect of the overexpression of phenolic acid decarboxylase genes (*PAD1, pdc* and *padc*) in *S. cerevisiae,* as well as to understand more about the mode of operation of the enzymatic activity in yeast. A further aim was to determine the ability of these recombinant strains to influence the aroma and the bouquet of wine.

The specific aims of this study were:

i) The isolation of the *PAD1* gene through polymerase chain reaction (PCR) technique, from the *S. cerevisiae* WE372 strain. The two bacterial genes, *pdc* from *L. plantarum* and *padc* from *B. subtilis,* were obtained from Professor J-F Cavin (Cavin et al., 1998; Cavin et al., 1997a).

ii) The construction of yeast episomal plasmids containing *PAD1, pdc* and *padc* (YEpPADC, YEpPDC and YEpPAD1) under the control of the $PGK1_{PT}$ cassette in the YEp352 plasmid. The $PGK1_{PT}$ expression cassette consisted of the promoter ($PGK1_P$) and terminator ($PGK1_T$) sequence of the yeast phosphoglyceratekinase I gene (*PGK1*).

iii) The creation of integration vectors containing the three expression cassettes. The plasmids YPADC, YPDC and YPAD1 were constructed by cloning the expression cassettes, under the control of *PGK1*, into the yeast integration plasmid YIp5. For industrial yeast integrations, the *SMR1* gene was used for selection.

iv) The transformation of *S. cerevisiae* ∑1278b with the three YIp5 plasmids to obtain the lab strains ∑YPADC, ∑YPDC and ∑YPAD1.

v) The creation of recombinant industrial strains of *S. cerevisiae* by transforming VIN13 with the YSPADC and YSPDC plasmids. This resulted in the VIN13YSPDC and VIN13YSPADC strains. The native *PAD1* gene of *S. cerevisiae* VIN13 was also doubly disrupted to create the VIN13POF strain.

vi) Analyzing the activity of the recombinant strains through assays to detect the decrease in ρ-coumaric acid by the phenolic acid decarboxylases present in the recombinant laboratory strains.

vii) The execution of microvinifications with recombinant laboratory strains to test for the increase in volatile phenols.

viii) Performing small-scale industrial fermentations with the recombinant VIN13 strains to detect volatile phenol increase and possible monoterpene formation.

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CHAPTER 2

LITERATURE REVIEW

Phenols and wine aroma and the influence of phenolic acid decarboxylases

2. LITERATURE REVIEW

2.1 INTRODUCTION

Phenols play an important role in wine through their contribution to the colour, aroma and flavour of the wine, and by providing an antimicrobial and antioxidative effect. Phenols in wine originate in the grape berry itself and are abundant in the must in various forms. There are a large number of phenolic compounds that can contribute to the aroma of wine.

Before the action of microbes and other vinification processes on phenolic compounds, the phenolic molecules contribute to the formation of important wine components. In the aggregation and reaction of phenolic compounds with other molecules, they form various flavour compounds, among others (Amerine and Roessler, 1983).

Volatile phenols are synthesised by wineassociated microorganisms. The presence of the microorganisms has a significant effect on the concentration of volatile phenols present in the wine. This can influence the quality of the wine, keeping in mind how critical the specific concentration of the volatile phenols found in wine is. Volatile phenols can either improve or be detrimental to the flavour of wine. The decarboxylation of hydroxycinnamic acids (phenolic acids) results in the formation of volatile phenols. Various microorganisms, including some winemicroorganisms, contain enzymes responsible for the decarboxylation of phenolic acids. The function of these enzymes and the organisms that produce these enzymes will be discussed.

This review will look at the role of phenols in wine and their contribution to the production of flavour and aroma compounds. The microorganisms involved in the production of the volatile phenols will also be discussed.

2.2 GENERAL OVERVIEW OF AROMA IN WINE

2.2.1 WINE AROMA

Since 6000 BC, when wine was first made, winemaking has developed into the fine art of producing a wine with the ideal balance between colour, flavour, mouth feel and aroma (Unwin, 1996). Starting off merely as a preservable product, it grew into an industry with very high standards. For the wine industry, it is of great importance to produce wines that are in harmony with their specific style, and which fulfil the requirements of the consumer. Therefore, when producing wines, it is of the utmost importance to give special attention to the aromatic compounds that are formed and the way in which they contribute to the wine.

Acree (1980) defined flavour in terms of three components: odour, taste and texture. The most important factors, except for the grape, influencing the flavours in wine

are claimed to be the yeast and the fermentation conditions. The origin of specific flavour compounds is of importance to wine classification. Flavour can be classified as varietal, prefermentative, fermentative and postfermentative (Boulton et al., 1996; Rapp, 1998; Schreier, 1979), indicating the origin of the specific flavour compounds. This classification system stresses the importance of each of the steps during the winemaking process on the aroma and bouquet formation. Grape varieties that give wine a characteristic, recognisable aroma are used to produce the finest wines. The (unfermented) grape berry may or may not have the characteristic aroma, or the specific aroma may be present in too small an amount to be detected. During fermentation, the wine acquires its varietal aroma by enhancing the existing berry aroma. Odours derived from fermentation, processing or ageing are called the bouquet of the wine (Amerine and Roessler, 1983).

A large number of components contribute to the overall aroma of a wine. The bouquet of wine expresses the complex flavour compounds, whereas the aroma of the wine refers to the more volatile and odorous compounds. Robinson (1994) defines the measurable aspects of acidity, sweetness, alcoholic strength, fizziness, astringency and bitterness as the components of the sensory impression of wine flavour.

2.2.2 COMPOSITION OF WINE

The sensory evaluation of wine is influenced by the chemical composition of the wine. The main components of wine are: alcohols, aldehydes and ketones, phenols, polyphenolic compounds, sulfur-containing compounds, amines and amino acids, esters, sugars, acids and gases (Amerine and Ough, 1980; Amerine and Roessler, 1983).

Ethanol, second to water, is the most important component in wine and the most abundant alcohol. Ethanol has an influence on the perception of acidity on the wine and helps to determine the viscosity. Other than enhancing certain aromas in the wine, ethanol has a distinctive odour, taste and feel (Amerine and Ough, 1980; Amerine and Roessler, 1983). Methanol appears to be formed through the hydrolysis of pectin. The sensory impact of methanol is mainly through methyl ester formation. Aliphatic alcohols cause the "fusel oil" odour in wine. Among the other alcohols that contribute to the complexity in wine are the terpene alcohols, glycerol and sugar alcohols (Amerine and Ough, 1980; Amerine and Roessler, 1983).

Although aldehydes and ketones are present in grapes, most are produced during fermentation. Most ketones are found in wine. However, only a few aldehydes are present in wine, since they are reduced to alcohols during fermentation. Acetaldehyde is an important odour component in wine and is perceived as negative in wine, whereas in flor sherries it is essential for the specific character of the sherry (flor aroma). Some of the other aldehydes and ketones found in wine include vanillin, furfural, cinnamaldehyde, hexanal and lactones (Amerine and Ough, 1980; Amerine and Roessler, 1983). Diacetyl is also an important odourous compound found in wine and in some bacterially spoiled wines.

Compounds containing sulfur are common in wine. Yeast form dimethyl sulfide from cysteine, cystine and glutathione. Free sulfur, which is used for microbial control on grapes, forms hydrogen sulfide in wine and is easily detected.

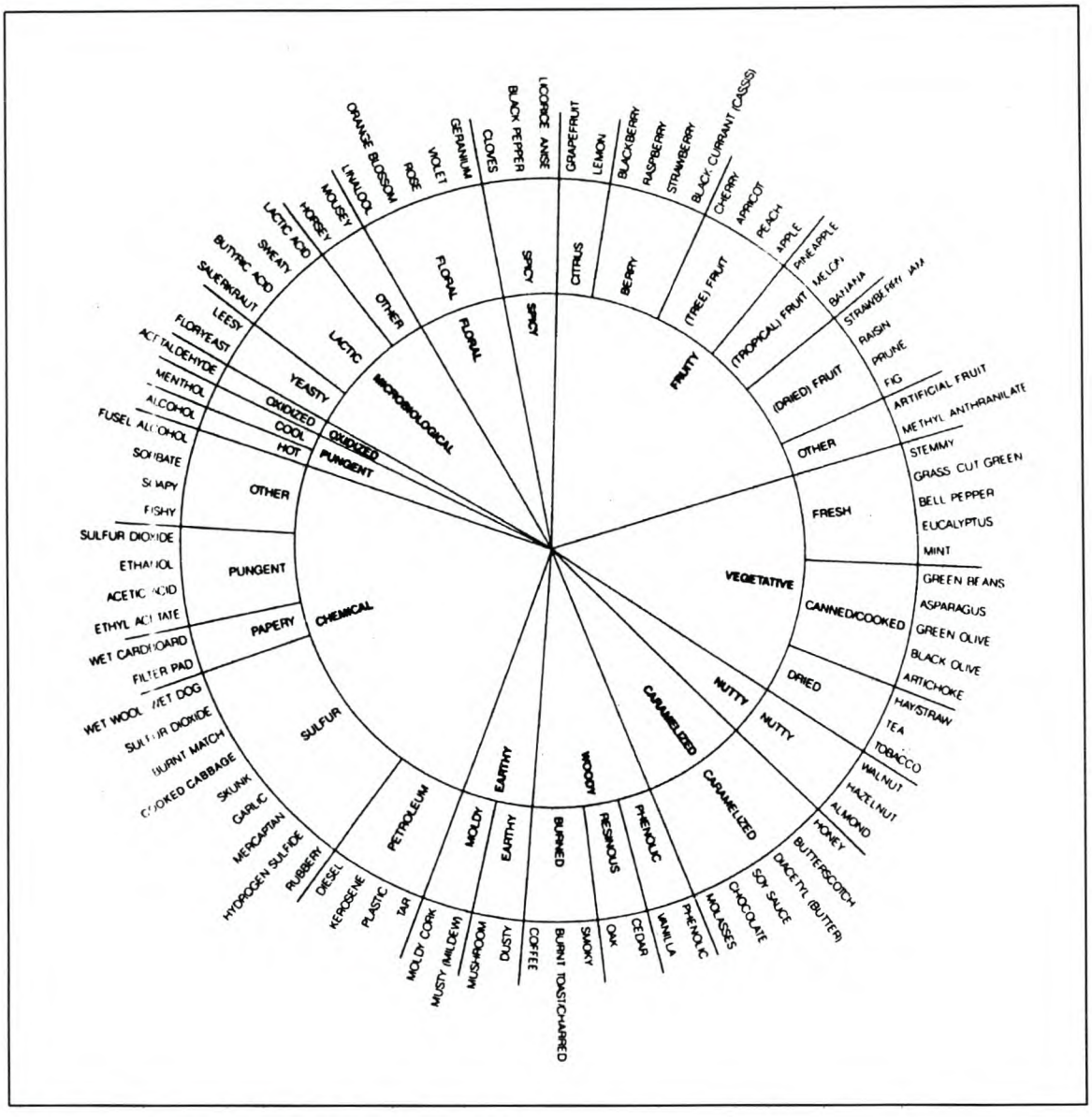


Figure 1. Aroma wheel (Zoecklein et al., 1995).

Volatile esters are partly responsible for the fruity character of wines. Esters are formed during ageing and fermentation and are also present in grapes. Amerine and Roessler (1983) indicate that Avakyants et al. (1981) defined the basic odour of wine as consisting of four esters, ethyl acetate, isoamyl acetate, ethyl caproate and ethyl caprylate, along with the two alcohols, isobutyl and isoamyl alcohol and finally acetaldehyde. Ethyl acetate has a pleasant aroma at low concentrations but gives a vinegary off-flavour above a certain threshold value (Van der Merwe and Van Wyk, 1981). The latter two

authors also found that a complex mixture of different ester odours improves the quality of the wine.

Glucose and fructose are the main contributors to sweetness in wine. The other components that play a role are glycerol and ethanol. Small amounts of other sugars may also be present in wine. When it comes to acids in wine, yeast and mostly lactic acid bacteria form lactic acid, which has a distinctive aroma. Few of the organic acids found in wine are volatile enough to be detected as aroma contributors. The aliphatic acids; acetic, butanoic and propanoic acid, form vinegary, spoiled butter and goaty odours respectively (Amerine and Ough, 1980; Amerine and Roessler, 1983). Carbon dioxide is present in all wines and in some wines it could contribute to a pleasant sensory effect, whereas it is highly undesirable in other wines.

After discussing all the components contributing to wine aroma, it is clear that each componant can have a specific influence on the final product. These specific characteristics are used to classify wine. **Figure 1** is an example of an aroma wheel. In the tasting and evaluation of a wine, wheels like these are used to describe the different aromas (Zoecklein et al., 1995).

Polyphenolic compounds and phenols also contribute to the composition of grapes and wine and play a role in aroma and flavour formation in wine. More detail will be provided regarding the role of phenols and the contribution they make to wine.

2.3 PHENOLS IN WINE

The contribution of phenolic compounds to wine is important and varied. In red wines, phenolic pigments are secondary products of the sugar metabolism of the grape and are responsible for the red colour of the wine (Ribéreau-Gayon et al., 2000a,b). Phenols also have the ability to act as antimicrobial agents, containing bacteriocidal properties (Ribéreau-Gayon et al., 2000b), and as antioxidants in the reduction of low density lipoprotein formation in human arteries. Lastly, phenols are responsible for the formation of aroma compounds and contribute to the astringent taste and bitterness of the wine.

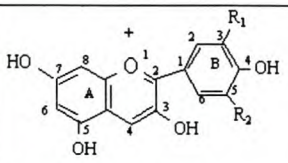
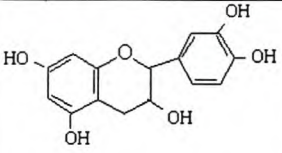
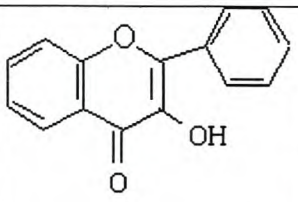
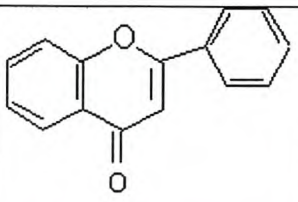
2.3.1 PHENOLIC COMPOUNDS IN WINE

Phenolic compounds in wine range from simple compounds, found in the grape, to complex substances extracted from the wood during ageing. The influence of these compounds on wine can vary from the production of odours, the cause of browning to meaningful contributions to the colour and taste complexities of the wine. Phenolic compounds are divided into two main groups: the flavonoid and nonflavonoid phenols.

Flavonoid phenols (**Table 1**) found in wine are the anthocyanins (pigments in red grapes), anthocyanogens, catechins, flavonols and flavanones (Ribéreau-Gayon et al., 2000b). Anthocyanogens and catechins are the main components of the condensable

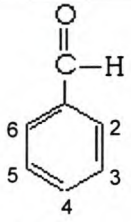
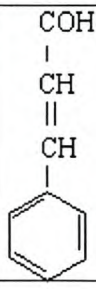
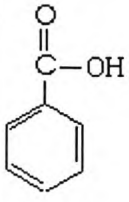
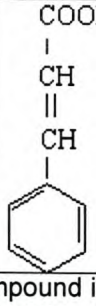
tannins and are found in the polymerized form. Flavonols are found in wine as glucosides. Phenolic components as anthocyanins, flavonols, flavonoids, anthocyanogenic tannins and catechin are the main contributors to the flavour of red wine (Amerine and Ough, 1980; Ribéreau-Gayon et al., 2000b).

Table 1. Flavonoid phenols found in wine (Amerine and Ough, 1980).

General name	Basic structure[a]	Specific common name	Additional structure -OH(-OCH_3)	Molecular weight[b]	Melting point[c] (°C)
Anthocyanogens (Leucocyanidins) Flavan-3,4-diols	 Flavonoid ring structure	Cyanidinol Delphinidinol Malvidinol Petunidinol	3', 4' 3', 4', 5' 4'(3', 5') 4', 5'(3')	306.28 322.28 350.28 336.28	
Catechins	 Catechin (+)	(+)-Catechin (2,3*H-trans*) (-)-Epicatechin (2,3*H-cis*) (+)-Gallocatechin (2,3*H-trans*) (-)-Epigallocatechin (2,3*H-ciss*)	3', 4' 3', 4' 3', 4', 5' 3', 4', 5'	290.28 290.28 306.28 306.28	174 236 245
Flavonols		Caempherol Quercitin Quercitin (-3-rhamnoside) Myricitrin (-3-rhamnoside) Rutin (-3-rhamnoglucoside)	4' 3', 4' 3', 4' 3', 4', 5' 3', 4'	285.24 302.24 448.37 463.37 610.51	277 314 168 198 215[d]
Flavanones		Naringenin Naringenin (-7-rhamnoglucoside) Hesperitin Hesperitin (-7-rhamnoglucoside)	4' 4' 3', (4') 3', (4')	272.25 580.53 302.27 610.55	251 82 251[d] 260

[a]The numbering system for all these ring structures is the same as that given for the anthocyanogens.
[b]Molecular weight data for flavonols are for the sugar-free molecules, except for rutin. For the flavonones, the molecular weights are for the sugar-free molecules.
[c]Melting point data for the flavonols are for the sugar-free molecules, except for rutin. The melting point of naringin with $8H_2O$ is given.
[d]Decomposes

Table 2. Nonflavonoid phenols found in wine (Amerine and Ough, 1980).

General name	General structure[a]	Specific common name	Additional structure -OH	Additional structure -OCH_3	Molecular weight	Melting point (°C)
Benzaldehyde		Vanillin	4	3	152.16	80
		Syringaldehyde	4	3, 5	182.18	113
Cinnamaldehyde		Coniferaldehyde	4	3	178.19	
		Sinapaldehyde	4	3, 5	208.22	84
Benzoic acid		Salcyclic acid	2		138.12	159
		ρ-Hydroxybenzoic acid	4		138.12	215
		Vanillic acid	4	3	168.16	214
		Gentesic acid	2, 5		154.12	205
		Syringic acid	4	3, 5	198.19	204
		Gallic acid	3, 4, 5		170.12	253[c]
		Protocatechuic acid	3, 4		154.12	201[c]
Cinnamic acid		ρ-coumaric acid	4		164.17	215[c]
		Ferulic acid	4	3	194.19	171
		Caffeic acid	3, 4		180.17	225[c]
		Chlorogenic acid[b]	3, 4			

[a]The parent compound is represented by the general structure and probably is not present in grapes or wine in significant amounts.
[b]Caffeoyl ester of 3-OH-quinic acid.
[c]Decomposes

Nonflavonoids phenols (**Table 2**) present in wine are classified as benzaldehyde, cinnamaldehyde, benzoic acid and cinnamic acid. The first two groups originate from wood extracts, whereas benzoic acid and cinnamic acid originate from the metabolic processes of the grapes (Amerine and Ough, 1980). These phenols contribute to ester formation and thus contribute to the aroma of the wine. In understanding how phenols contribute to the complexity and aroma of wine, it is important to have a closer look at how they are formed and in which ways they are utilised to produce other compounds.

2.3.2 PHENOLIC ACIDS AS SUBSTRATE IN GRAPE MUST AND WINE

Flavonoid and nonflavonoid phenols, along with their derivatives, are present in grapes and wine. These phenolic compounds are derived from the basic hydroxybenzene structure (Zoecklein et al., 1995; Ribéreau-Gayon et al., 2000b). Phenolic acids are colourless (except when oxidation takes place) and have no particular flavour or odour (Ribéreau-Gayon et al., 2000b). How phenols are formed and transformed is important to the overall understanding of the role they play in wine. What follows is an explanation of how the flavonoids, nonflavonoids and tannins are formed.

Wine contains a lower total phenol content than grapes. The extraction of phenolic compounds from the fruit occurs during destemming and crushing of the grapes and during the fermentation process. These processes result in a maximum of 60% extraction (Zoecklein et al., 1995). Microbial activity and the use of oak for fermentation or storage, however, can increase the percentage of extraction.

Figure 2 illustrates how the primary structure of all the phenolics in wine is formed. Ribéreau-Gayon et al. (2000a) proved that a single benzene ring is created through the condensation of erythrose-4-phosphate. Erythrose-4-phosphate is an intermediary product of the pentose phosphate cycle. The pathway responsible for the formation of benzoic and cinnamic acid from erythrose-4-phosphate is known as the shikimic acid pathway (**Figure 2**).

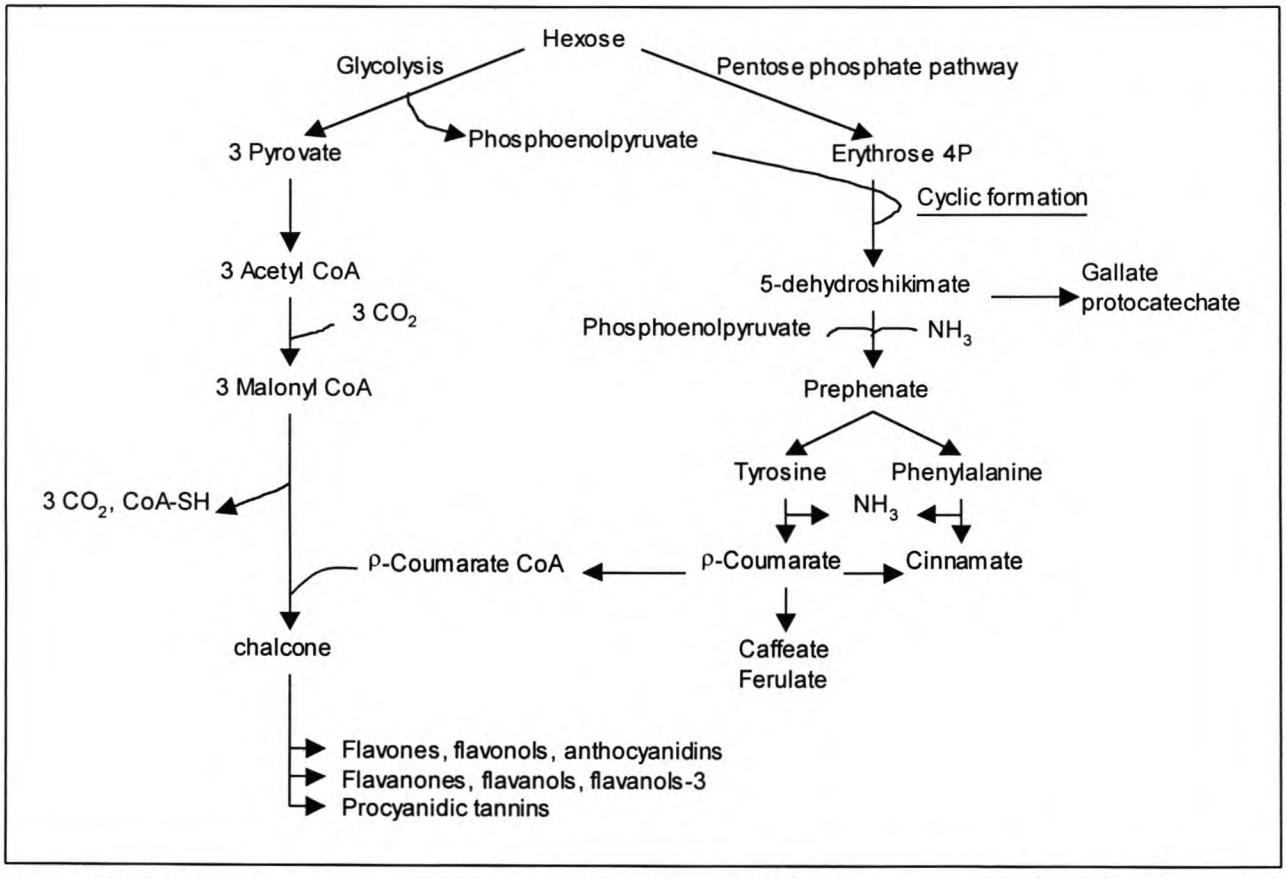


Figure 2. Biosynthesis pathways of phenolic compounds (Ribéreau-Gayon et al., 2000a).

The Krebs cycle leads to the production of another benzene ring from three acetyl coenzyme A molecules. Condensation of a cinnamic acid molecule with a benzene ring leads to the formation of a flavonoid molecule. The flavonoid then leads to the formation of other wine phenols (Ribéreau-Gayon et al., 2000a).

Hydroxycinnamic and hydroxybenzoic acid derivatives are the primary nonflavonoid phenols found in wine. Most of these derivatives are esterified to alcohols, organic acids or sugars (Ribéreau-Gayon et al., 2000b). The concentration of benzoic and cinnamic acids in wine is in the region of 100-200 mg/L for red wine and 10-20 mg/L for white wine. The nonflavonoid components present in wine originate primarily from juice extraction. Nonflavonoid phenols are easily extracted from the grape pulp and are predominantly the hydrolysis products of anthocyanins and hydroxycinnamic acyl groups. Slow hydrolysis of the extracted derivatives takes place during fermentation. Phenolic acids are formed due to the free acid and specific forms of esters present after hydrolysis (**Figure 3**) (Ribéreau-Gayon et al., 2000b).

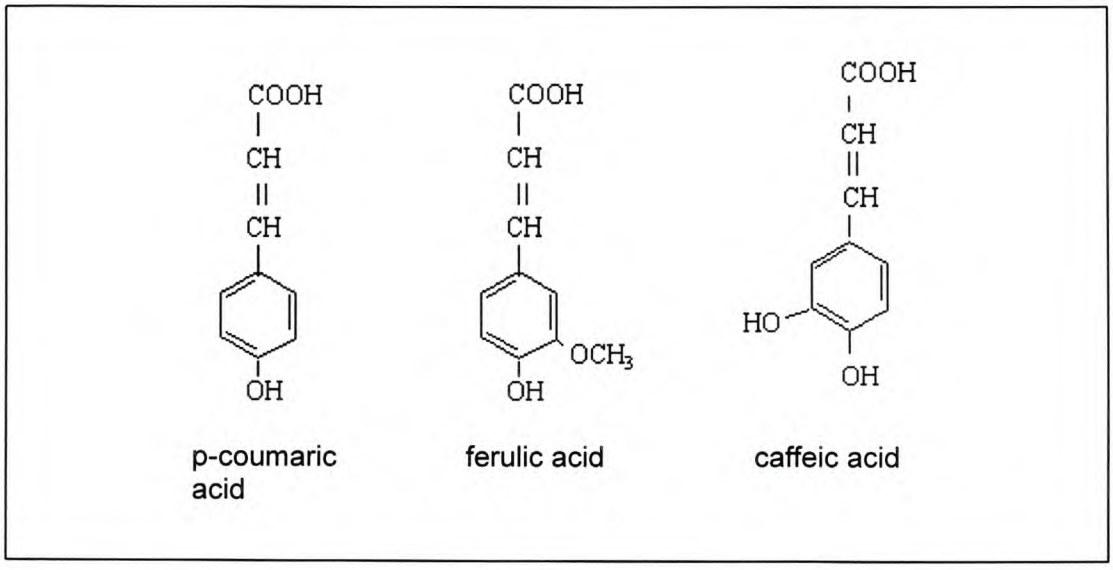


Figure 3. Substituted cinnamic acids.

With the help of microbial actions, these free phenolic acids can be transformed into volatile phenols, which contribute to the sensory character of the end product. Oak extraction leads to an increase in the concentration of nonflavonoid phenols in the hydrolyzable form. The main examples of these are vanillin, sinapaldehyde, coniferaldehyde and syringaldehyde (Zoecklein et al., 1995).

Flavonoid phenols consist of two aromatic rings joined to a pyran ring (**Table 1**). Different members of the group depend on different molecules associated with the carbon groups. Flavonoids can exist in a polymerized form or as free molecules. When esterified to nonflavonoids or sugars, they are called acyl and glycoside derivatives respectively.

Anthocyanogens, catechins and flavonols are present in the grape, while flavanones are found in the grape seeds (Amerine and Ough, 1980; Ribéreau-Gayon et al., 2000b).

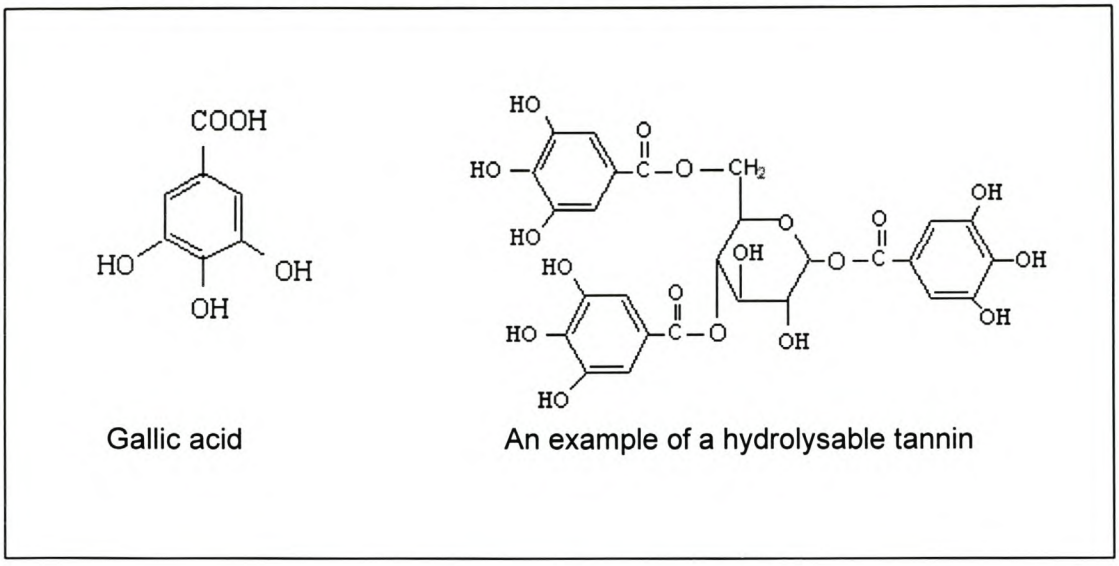


Figure 4. Hydrolysable tannins (Ribéreau-Gayon et al., 2000b).

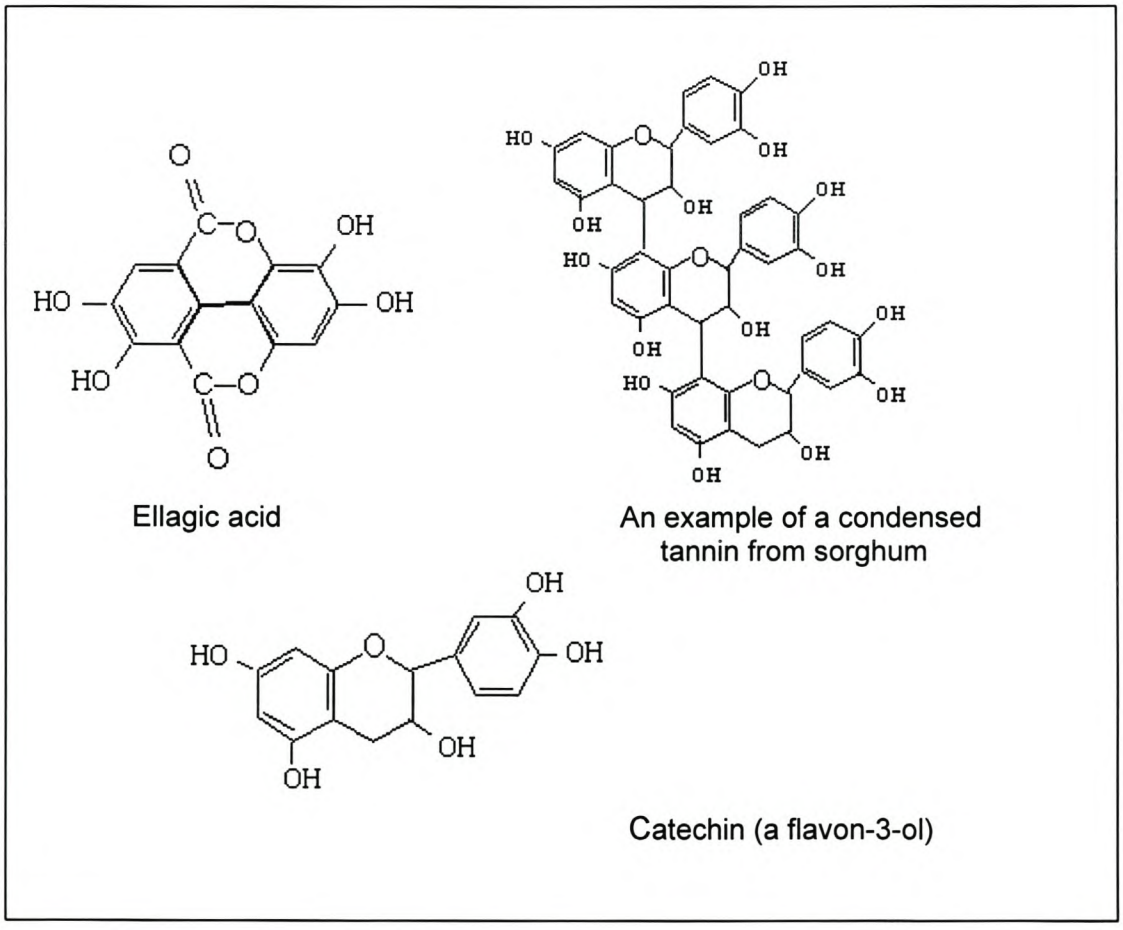


Figure 5. Condensed tannins (Ribéreau-Gayon et al., 2000b).

The polymerization of certain flavonoids and nonflavonoids leads to tannin formation. Tannins are categorized as condensed or hydrolyzable. Hydrolyzable tannins (**Figure 4**) consist of nonflavonoids in the form of esters, and can thus be degraded. Procyanidins are formed after the polymerization of the flavonoid catechin and leucoanthocyanidin. These procyanidins are known as the condensed tannins and do not hydrolyze easily. Most tannins in wine are of the condensed form (**Figure 5**).

On the base of this general understanding of phenols and their contribution to wine, we will now proceed to the next aspect, which focuses on the formation of volatile phenols.

2.4 VOLATILE PHENOLS AND PRODUCT FORMATION

2.4.1 VOLATILE PHENOLS IN WINE

Volatile phenols are valuable intermediates in the biotechnological production of new flavour and fragrance chemicals. However, due to their characteristic aroma and their low threshold detection, they are also regarded as sources of phenolic off-flavours in many beers and wines (Chatonnet et al., 1997). The two main groups of volatile phenols present in wine are the ethyl and the vinylphenols.

Volatile phenols have a relatively low threshold value and are easily detected if present in high concentrations. Although they possess the ability to make a favourable contribution to the aroma of a specific wine, they can easily be the cause of off-flavours. The ethylphenols are known to produce a "barnyard" or "stable" smell if present in high concentrations (Ribéreau-Gayon et al., 2000b). The vinylphenols can be responsible for a "pharmaceutical" odour, particularly in white wines.

These volatile phenols are formed from the hydroxycinnamic acids present in the grape must. The vinylphenols formed are 4-vinylguaiacol (ρ-coumaric acid) and 4-vinylphenol (ferulic acid). The ethylphenols that are formed as a consequence are 4-ethylguaiacol and 4-ethylphenol (**Figure 6**). We will now take a closer look at the formation of the volatile phenols.

2.4.2 GENERAL SOURCES OF VOLATILE PHENOLS IN WINE

Although grape must contains only trace amounts of volatile phenols, they occur in wines after the vinification process. During fermentation and the winemaking processes, volatile phenols are formed by yeast, thus resulting in an increase in their concentrations in wine (Lambrechts and Pretorius, 2000). Yeast uses enzymatic reactions to catalyse the formation of volatile phenols in the wine.

The nonflavonoid hydroxycinnamic acids act as the main substrate for the formation of volatile phenols. *S. cerevisiae* has the ability to decarboxylate ρ-coumaric and ferulic

acid through a non-oxidative action (Chatonnet et al., 1993). These nonflavonoids act as the main precursors for vinyl and ethylphenol production. Chatonnet et al. (1993) indicated that ρ-coumaric acid is responsible for vinylguaiacol production and that ferulic acid forms 4-vinylphenol. The *Brettanomyces* yeasts are also well known for their ability to form volatile phenols in wine (**Figure 7**) (Chatonnet et al., 1995). However, these species are associated with the more unpleasant odorous formation of the ethylphenols and are present in cellars as spoilage organisms. Phenolic acid is decarboxylated to the corresponding vinylphenol via a carboxylase enzyme. Thereafter, through the action of an oxido-reductase enzyme, the vinylphenols are converted to the corresponding ethylphenols (Chatonnet et al., 1993). These activities are not inhibited by other grape phenolics, resulting in a high transformation of the vinylphenols into ethylphenols.

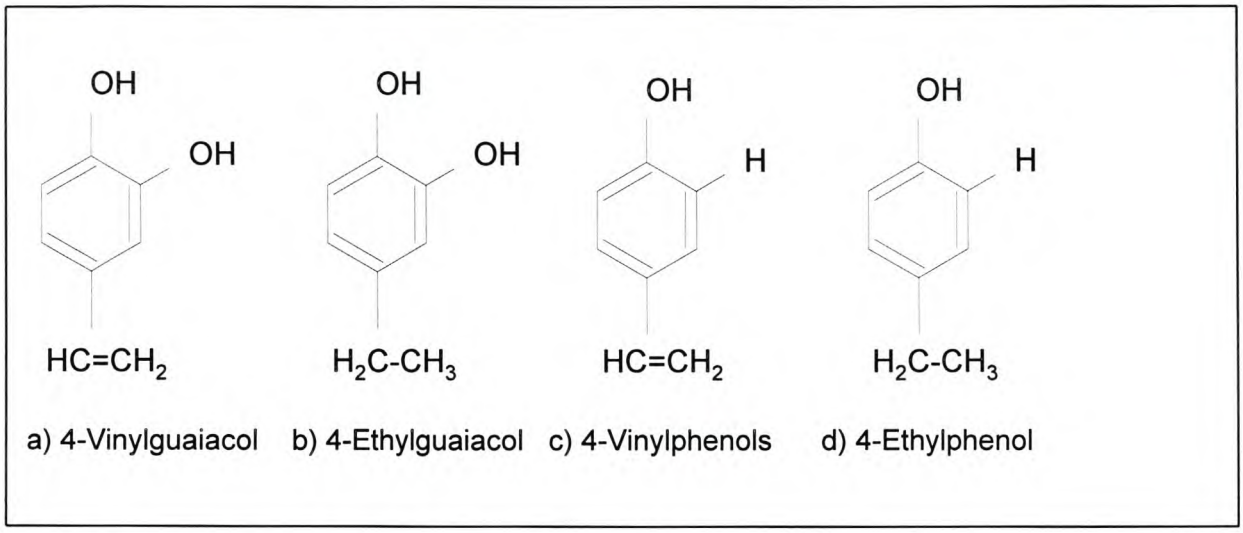


Figure 6. Volatile phenols (Ribéreau-Gayon et al., 2000b).

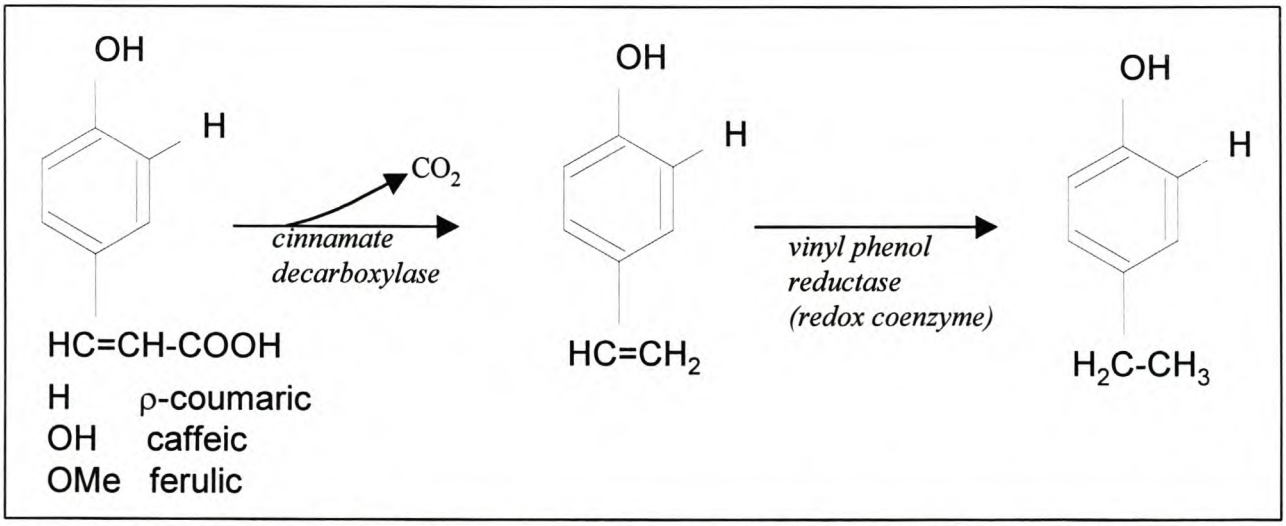


Figure 7. *Brettanomyces* formation of vinyl and ethyl phenols of hydroxycinnamic acids (Boulton et al., 1996).

Bacteria, especially the lactic acid bacteria present in the winemaking process, are also responsible for the formation of volatile phenols. It was found that lactic acid bacteria, as well as acetic acid bacteria, are responsible for 4-vinylphenol formation. It is however debatable, however, whether these lactic acid bacteria play a role in the formation of the ethyl phenols (Chatonnet et al., 1992). Whiting and Carr (1959) showed that certain bacteria also have the ability to decarboxylate cinnamic acid to its correspondent ethyl phenol.

Oak maturation can also increase the number of volatile phenols in wine. 4-Ethylguaiacol and the 4-ethylphenol concentrations show a marked increase during oak maturation (Pollnitz et al., 2000).

2.4.3 THE IDEAL CONCENTRATION OF VOLATILE PHENOLS IN WINE

The concentration of volatile phenol components in wine (as mentioned previously) plays a large role in the effect they have on the aroma of wine (Lambrechts and Pretorius, 2000). Etiévant et al. (1989) and Chatonnet et al. (1992) found that volatile phenols present at a concentration higher than 4 mg/L can have a negative effect on the organoleptic qualities of a wine. Etiévant et al., (1989) showed that the ideal concentration for ethylphenol was 2.2 mg/L and that this concentration increased the pleasantness of the aromatic perception. The concentration of volatile phenols naturally present in wine varies dramatically. All winemaking practices influence the concentration of volatile phenols, which differs between red and white wine. **Table 3** indicates the different concentrations of volatile phenols found in wines. It is clear that white wines contain vinylphenols and red wines contain ethylphenols.

Table 3. Some vinyl and ethylphenols produced by yeast and their concentrations, threshold values and odours in wine (Chatonnet *et al.*, 1992, 1993; Rapp, 1998).

Compound	Conc. in wine (μg/L)	Threshold value white wine (μg/L)	Threshold value red wine (μg/L)	Odour
4-Vinylphenol	0-1150	770		Pharmaceutical, elastoplast, gouache
4-Vinylguiacol	0-496	440		Smoky, vanilla, clovelike
4-Vinylguiacol + 4-Vinylphenol [1:1]		752		
4-Ethylphenol	0-6047		605	Wet horse
4-Ethylguiacol	0-1561		110	Smoky, vanilla, clovelike
4-Ethylphenol + 4-ethylguiacol [10:1]			369	

2.5 HYDROXYCINNAMIC ACID DEGRADATION BY DIFFERENT ORGANISMS

Plant cell walls are degraded by a large number of microorganisms for various reasons. Due to the fact that plant cell walls consist of lignin polymers, hemicellulases are present in bacteria and fungi to degrade the plant cell walls. The degradation of the lignin polymers, consisting of cellulose and hemicellulose, leads to the release of hydroxycinnamic acids (Chesson, 1988). To understand the reason for hydroxycinnamic acid degradation, it is important to observe the role that phenolic acids play in microorganisms. The catabolic reaction of yeast and the role it plays in the winemaking process may be better understood by observing other microorganisms and their metabolism in relation to the same molecules.

2.5.1 THE ROLE OF HYDROXYCINNAMIC ACID DEGRADATION IN DIFFERENT ORGANISMS

The biological importance of the hydroxycinnamic acids is diverse. It is known that microorganisms present in the rumen of ruminant animals are able to defend themselves against the toxic effect of phenolic acids by degrading phenolic acids (Chesson, 1988). For *Agrobacterium tumefaciens,* phenolic acids act as signals. Lee et al. (1995) showed how these molecules act to induce the *vir* gene expression through a two-component system consisting of VirA and VirG proteins. Kalogeraki et al. (1999) indicated that ferulic acid could be o-demethylated into caffeic acid by the VirH2 protein in order to turn off *vir* gene expression.

Phenolic acids play an essential role in the biodegradation of plant wastes. Several bacteria find it possible to grow on these compounds as their sole carbon source. This was shown by Venturi et al. (1998) for *Pseudomonas putida* WCS358, by Segura et al. (1999) for *Acinetobacter* and by Narbad and Gasson (1998) for *Pseudomonas florescens*. In all these organisms, ρ-coumaric acid and ferulic acid are converted into ρ-hydrobenzoic and vanillic acids respectively. These acids are then transformed into protocatechuic acid and integrated into the tricarboxylic acid cycle via the β-ketoadipate pathway. It is also possible for ferulic acid to be degraded into vanillin by a two-step process. Gasson (1998) found that *Pseudomonas florescens* involves a coenzyme A (CoA) ligase, which is followed by side chain cleavage. Overhage (1999) found the same for *Pseudomonas* sp. strain HR199. In the white-rot fungus *Pycnoporus cinnabarinus,* this same two-step conversion takes place via a propionic acid side chain cleavage followed by a reductase (Bernard et al., 1999; Lomascolo et al., 1999).

It is clear that a wide spectrum of microorganisms have developed a system to deal with the presence of phenolic acids in their environment. Whether it is to protect themselves from the toxic effect of these acids, or to utilize them, the degradation of

phenolic acids is found in a variety of microorganisms. One such degradation method is the decarboxylation of phenolic acids. Many microorganisms contain a specific gene that expresses a peptide with the ability to decarboxylate hydroxycinnamic acid. These peptides are commonly referred to as phenolic acid decarboxylases.

2.5.2 DECARBOXYLATION OF PHENOLIC ACIDS TO VOLATILE PHENOLS AND THE ORGANISMS THAT PLAY A ROLE IN THIS PROCESS

Phenolic acids can be decarboxylated into volatile phenols. They are usually decarboxylated into 4-vinyl derivatives and then reduced to 4-ethyl derivatives (Cavin et al., 1993). The enzymes responsible for the decarboxylation are called phenolic acid decarboxylases and several bacteria and fungi have been found to contain the genes encoding these enzymes. Clausen et al. (1994) characterized *PAD1* from *S. cerevisiae*. Zago et al. (1995), Cavin et al. (1997a) and Cavin et al. (1998) characterized *fdc* from *Bacillus pumilus, pdc* from *L. plantarum* and *padc* from *B. subtilis* respectively. *PadA* from *Pediococcus pentosaceus* was identified and cloned by Barthelmebs et al. (2000b). All these genes have been cloned and expressed in *E. coli*.

Resent studies have showen that various other organisms also contain genes similar to the *S. cerevi*siae gene that encodes the Pad1p enzyme. These genes were discovered through genome sequencing, but corresponding enzymatic activity has not been demonstrated yet. Whiting and Carr (1959) have proposed a second pathway for *Lactobacillus pastorianus.* In this proposal, ρ-coumaric acid and caffeic acid are reduced into substituted phenyl priopionic acids and then decarboxylated into 4-ethyl derivatives.

Phenolic acids are toxic to many microorganisms and the microorganisms contain some method of avoiding the effect that these acids might have on their survival. Weak acids, including phenolic acids, can be highly detrimental to some microorganisms, because of the ability of the phenolic acids to decrease the internal cellular pH at low environmental pHs (Lambert et al., 1997). At low pHs, phenolic acids are mostly protonated and are able to enter a bacterial cell through diffusion. *B. pumilus* is an example of a ruminant microorganism that uses its phenolic acid decarboxylase enzyme to reduce the toxic effect of phenolic acids. It is important to note the effect that phenolic acids have on microorganisms and the different ways in which microorganisms deal with them. Although many microorganisms contain a phenolic acid decarboxylase homologue, it may not be the sole defence mechanism against phenolic acids.

Due to the fact that microorganisms play an intricate role in wine production, it is interesting to note the effect that these genes have on the fermentation process. Research has shown that wine microorganisms producing the decarboxylase enzyme influence the aromas formed from the must. A more detailed analysis of the microorganisms expressing these decarboxylase genes will follow. *S. cerevisiae* will be used as an example, as this yeast is capable of the mentioned action. For the bacterial

phenolic acid decarboxylases, *L. plantarum, B. subtilis, B. pumilus and Pediococcus pentosaceus* will be discussed.

2.5.2.1 Phenolic acid decarboxylase enzymes expressed by yeast

Phenolic off-flavours have presented problems in the brewing industry for many years. The production of these off-flavours has many origins, including the decarboxylating effect that some microorganisms have on phenolic acids (Shinohara et al., 2000). In the selection process for brewing strains of *S. cerevisiae* it was found that all selected strains have something in common. Meaden and Taylor (1991) found that *S. cerevisiae* strains that are capable of producing off-flavours in beer contain the *POF1* gene (Hwang, 1992). They went on to show that brewing strains without a functional *pof*$^+$ phenotype, thus lacking the ability to decarboxylate phenolic acids, lack the ability to produce off-flavours. Clausen et al. (1994) found that the *S. cerevisiae* gene *PAD1* encodes for the enzyme phenylacrylic acid decarboxylase. This enzyme confers resistance to cinnamic acids, and it was shown through sequence comparisons that *POF1* and *PAD1* are the same gene.

S. cerevisiae is an example of a microorganism that produces a phenolic acid decarboxylase, but does not use this enzyme as its sole defense against phenolic acid toxicity. Barthelmebs et al. (2000a) speculate that this might result in the low activity of its Pad1p enzyme. Chambel et al. (1999) found that the different cinnamic acids act as activators of the H^+-ATPase action in *S. cerevisiae*. The H^+-ATPase acts as a proton pump to restore the internal ΔpH. Carmelo et al. (1997) also found, as previously mentioned for bacteria, that the rapid activation of *S. cerevisiae's* H^+-ATPase is not due to the drop in pH. In fact, the presence of a weak organic acid is the cause of induction.

Phenylacrylic acids are decarboxylated into styrenes by phenylacrilic acid decarboxylase in *S. cerevisiae* (Goodey and Tubb, 1982; Huang et al., 1993; Meaden and Taylor, 1991). ρ-Coumaric acid and ferulic acid are decarboxylated to 4-vinylphenol (4-OH styrene) and 4-vinylguaiacol (4-OH, 3-OCH^3 styrene) respectively. Chatonnet et al. (1993) indicates that the enzyme shows a greater affinity to ρ-coumaric acid than to ferulic acid.

Clausen et al. (1994) found that *PAD1* is a single copy gene and that it is situated on chromosome IV. Furthermore, it was shown that the gene is not essential for the viability of *S. cerevisiae*. When Pad1p was investigated, it appeared to be localized in the cytoplasma and that the enzyme activity is regulated post-transcriptionally. Pad1p is a peptide of 242 amino acids. Clausen et al. (1994) also showed that *PAD1* mRNA is present at steady state levels, despite the low Pad1p activity.

Chatonnet et al. (1993) indicated that tannins inhibit the cinnamate decarboxylase enzyme of *S. cerevisiae*. This may be a factor in red wines, in which tannins are abundant. Pad1p does not commonly cause odour formation in wine, whereas odour formation is very common in beer. This might be due to the lack of inhibitors in beer. It was not shown whether tannins inhibit the enzymes in the case of the bacterial phenolic acid decarboxylases.

2.5.2.2 Phenolic acid decarboxylase enzymes expressed by bacteria

2.5.2.2.1 *Lactobacillus plantarum*

L. plantarum can be seen as a model for ubiquitous lactic acid bacteria (Barthelmebs et al., 2000a). *L. plantarum* is also used in various fermentation processes. The wine industry uses this bacterium as a malolactic starter in the production of some wines and for vegetable fermentations that contain phenolic acids.

An investigation of different *Lactobacillus* species found that, except for *L. plantarum*, six other lactobacilli also showed phenolic acid decarboxylase activity. Van Beek and Priest (2000) screened *Lactobacillus* species found in malt fermentations. The following microorganisms were found to contain a gene with high homology to the *pdc* gene of *L. plantarum: L. brevis, L. crispatus, L. fermentum, L. hilgardii, L. paracasei* and *L. pentosus* (**Table 4**). Except for *L. hilgardii,* all the other strains showed decarboxylase activity on ρ-coumaric acid and/or ferulic acid. As has been shown by other studies, Van Beek and Priest (2000) also detected substrate inducible activity on the phenolic acids and found that most *Lactobacillus* species seem to utilize ρ-coumaric acid better than the other phenolic acids (**Figure 8**).

Table 4. Hydroxycinnamic acid decarboxylation and reduction activities of several *Lactobacillus* species (Van Beek and Priest, 2000).

Strain	Origin	*pdc* GenBank accession no.	% Identity with reference *pdc*[a]		Decarboxylation activity[b]					Reduction activity[b]	
					UV spectroscopy		HPLC				
			N	P	PCA[c]	FA[d]	PCA[c]	FA[d]	PCA and FA[e]	4-VP	4-VG
L. brevis 113	Scotland	AF257164	81	89	+	-	ND	ND	ND	ND	ND
L. crispatus H8	Japan	AF257159	99	92	+	-	+	+	++	+	-
L. fermentum 70	Scotland	AF257162	100	100	+	+	-	-	-	-	-
L. hilgardii 84	Scotland	AF257158	80	88	-	-	ND	ND	ND	ND	ND
L. paracasei 69	Scotland	AF257160	100	100	-	+	-	-	+	-	-
L. pentosus 128	Scotland	AF257161	99	100	++	+	++	+	++	-	-
L. plantarum 72	Scotland	AF257163	100	100	++	+	++	+	++	++	-

[a] The reference *pdc* gene was from *L. plantarum* (accession no. U63827). N, nucleotide sequence; P, protein sequence.
[b] ++, high quantity of products (35 to 95 µg/mL) detected by HPLC, high level of change in UV spectroscopy; +, medium quantity (5 to 35 µg/mL) of products detected by HPLC and some level of change in UV spectroscopy; , low quantity (0 to 5 µg/mL) of products detected by HPLC and no change in level of UV spectroscopy. ND, not determined.
[c] Cultures were exposed to 100 µg of PCA per mL.
[d] Cultures were exposed to 100 µg of FA per mL.
[e] Cultures were exposed to PCA and FA, each at 50 µg/mL.

Barthelmebs et al. (2000a) speculated that the conversion of ρ-coumaric acid to 4-vinyl phenol is a good method for the *L. plantarum* bacteria to defend itself against the toxic effect of the phenolic acid. They also showed that the Pdcp activity confers a selective advantage for growth in media containing ρ-coumaric acid. Along with the fact that 4-vinyl

phenols are less toxic to *L. plantarum,* it is proposed that the synthesis of Pdcp might be a stress response.

Cavin et al. (1997b) found that *L. plantarum* shows substrate-inducible decarboxylase activities on ferulic and ρ-coumaric acids of 0.01 and 0.6 μmol min^{-1} mg^{-1} respectively (**Figure 8**). Activity was not detectable in uninduced cells. A gene for ρ-coumaric acid decarboxylase was isolated from *L. plantarum.* The *pdc* gene has an open reading frame of 519 bp (Cavin et al., 1997b). Corresponding with the results obtained by Cavin et al. (1997a), Cavin et al. (1997b) found that in mRNA from *pdc* or Pdcp activity could not be detected in uninduced cells, indicating that the expression is transcriptionally regulated by ρ-coumaric acid. No cofactors or metal ions were required for activity.

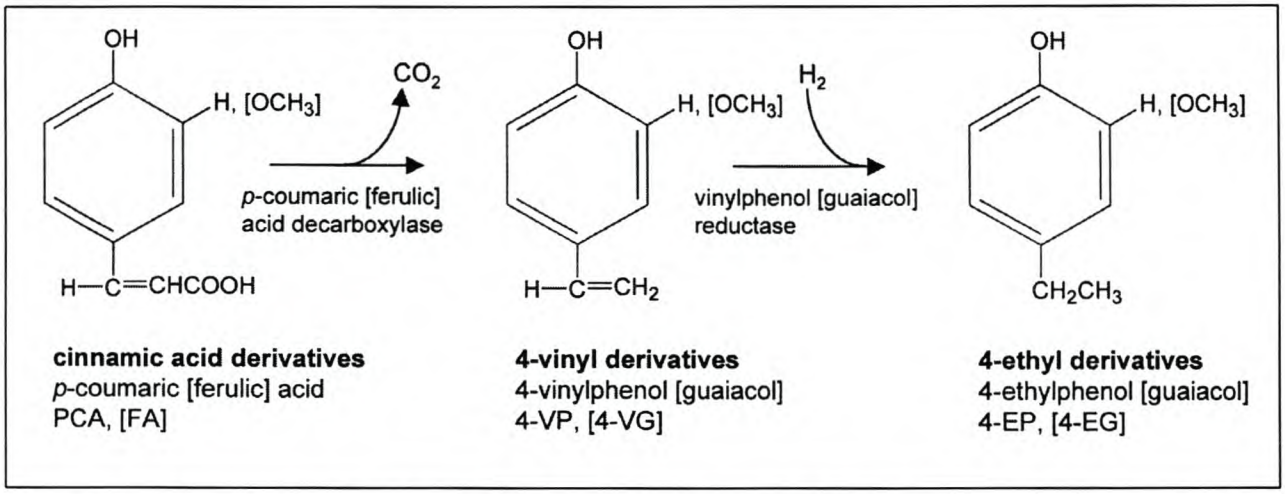


Figure 8. Pathway for the biotransformation of cinnamic acid derivatives by *L. plantarum* (Van Beek and Priest, 2000).

Cavin et al. (1997b) also indicated that Pdcp activity correlates with a homotetramer with a molecular mass of 93 kDa. Barthelmebs et al. (2000a) revealed the existence of two other inducible enzymatic activities for the degradation of phenolic acids (**Figure 9**). This can perhaps explain the 0.01 μmol. min^{-1}. mg^{-1} substrate-inducible decarboxylase activity of *L. plantarum* found by Cavin et al. (1997a). ρ-Coumaric acid decarboxylase (Pdcp), however, does not show detectable activities with ferulic acid *in vivo*. *In vitro* PDC appeared to decarboxylate ferulic acid in the presence of ammonium sulfate, with a specific activity of 10 nmol min^{-1} mg^{-1} (Barthelmebs et al., 2000a).

2.5.2.2.2 *Bacillus subtilis*

B. subtilis is a bacterium that has the ability to decarboxylate ferulic, ρ-coumaric and caffeic acids (Cavin et al., 1998). The *padc* gene in *B. subtilis* codes for the Padcp enzyme. For activity to be detected, the induction of the cells is necessary and this can be done by ferulic or ρ-coumaric acid and, to a lesser extent, by caffeic acid (Cavin et al.,

1998). Due to the fact that uninduced cells do not show activity, *B. subtilis* must have a substrate- inducible decarboxylase action.

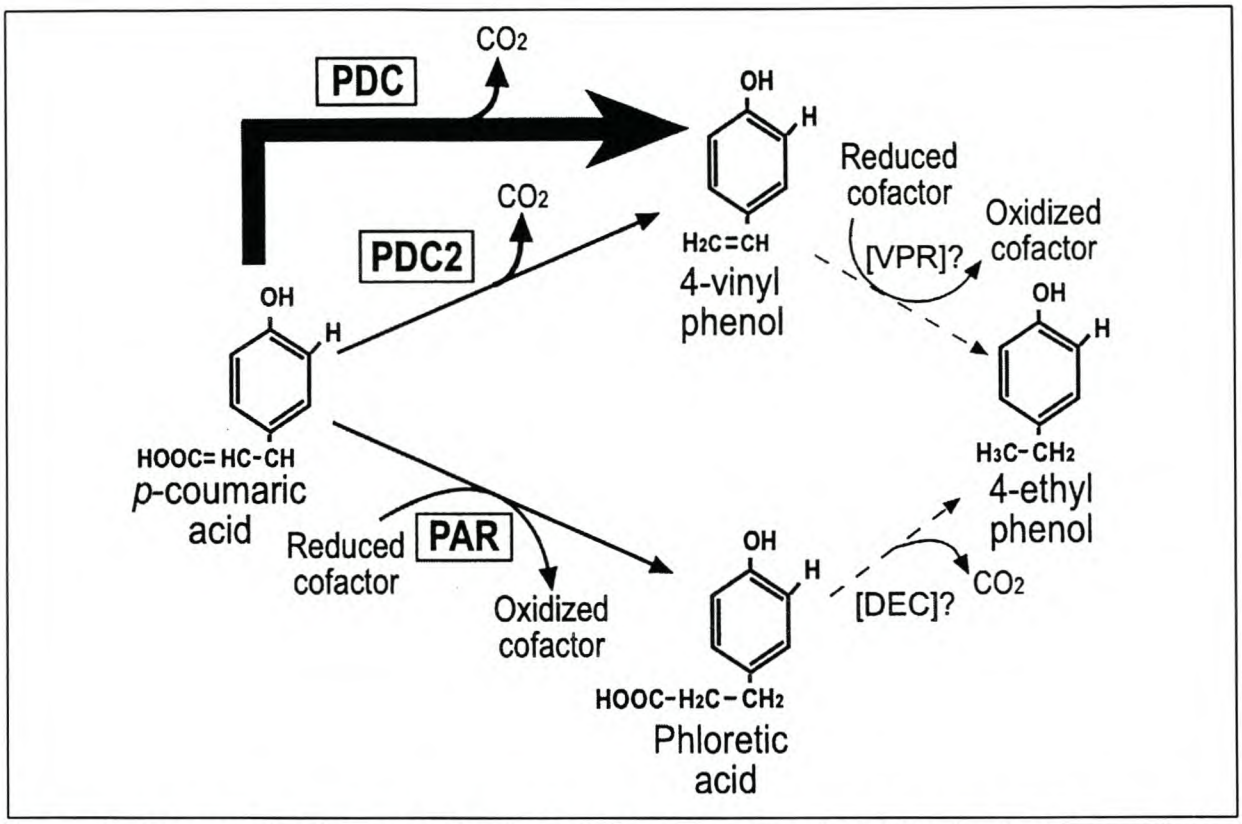


Figure 9. Proposed pathway for the degradation of ρ-coumaric acid in *L. plantarum*. The arrow thickness represents the relative intensity of enzymatic activity. PDC, ρ-coumaric acid decarboxylase; PDC2, phenolic acid decarboxylase; PAR, phenolic acid reductase; VPR, putative 4-vinyl phenol reductase; DEC, putative phloretic acid decarboxylase. (Barthelmebs et al., 2000a).

Cavin et al. (1998) found that the *padc* gene has an open reading frame of 483 bp and codes for a 161-amino acid protein of 45 kDa. A 45 kDa homodimer with two 22 kDa subunits was found in the recombinant Padcp. No co-factor is necessary for activity and Padcp shows a high level of stability, even when expressed in *E. coli* (Cavin et al., 1998).

2.5.2.2.3 *Bacillus pumilus*

Graminaceous plants represent the majority of plants found in the diets of ruminant animals. ρ-Coumaric and ferulic acids in their *trans* configuration are commonly found in these plants (Zago et al., 1995). Ruminant microorganisms have developed a defence agent against these phenolic acids. *B. pumilus,* previously isolated from the rumen of a cow (Susmel et al., 1989), was tested by Zago et al. (1995) for its ability to decarboxylate ρ-coumaric acid and ferulic acid when expressed in *E. coli*. In this ruminant organism, it is of the utmost importance to eliminate the toxic effect of the phenolic acids present in its environment.

Fdc encoding for a ferulic acid decarboxylase was identified, isolated and characterized by Degrassi et al. (1995). A homodimer of 45 kDa was identified as the Fdcp enzyme. Degrassi et al. (1995) indicated that ρ-coumaric acid and/or ferulic acid induce the *fdc* gene of *B. pumilus*. Zago et al. (1995) also indicated that *fdc* is substrate inducible. According to Degrassi et al. (1995), both 4-vinylphenol and 4-vinylguaiacol were produced, and they also found that the Fdcp enzyme is specific for ρ-coumaric acid and ferulic acid.

2.5.2.2.4 *Pediococcus pentosaceus*

P. pentosaceus is classified as a lactic acid bacteria, the same as *L. plantarum.* The lactic acid bacteria are responsible for the malolactic fermentation in the winemaking process. They function to convert L-malic acid to L-lactic acid and CO_2 (Barthelmebs et al., 2000b).

Cavin et al. (1993) indicated that *P. pentosaceus* has the ability to decarboxylate phenolic acids into the 4-vinyl derivatives that could then be reduced to 4-ethyl derivatives. By using the *pdc* gene from *L. plantarum,* Barthelmebs et al. (2000b) screened the genomic library of *P. pentosaceus* and found a phenolic acid decarboxylase gene. The gene was called *padA* and consisted of an open reading frame (ORF) of 534 bp. The ORF coded for a 178-amino-acid protein, which showed 81.5% identity with the *L. plantarum* Pdcp enzyme (Barthelmebs et al., 2000b). *P. pentosaceus* showed substrate-inducible activity to ρ-coumaric acid.

2.6 CONCLUSION

Phenols contribute to the colour, aroma and flavour of wine. Apart from the basic contributions of phenols to wine, they also act as antimicrobial and antioxidative agents. Phenolic compounds play a role in wine before degradation and after hydrolysis. The actions of microbes on phenolic compounds contribute to the formation of important wine components (Amerine and Roessler, 1983). The decarboxylation of phenolic acids produces volatile phenols. The importance of these volatile phenols is varied in that they can either improve or be detrimental to the flavour.

Volatile phenols are synthesised by wine-associated microorganisms. The presence of these microorganisms has a significant effect on the concentration of volatile phenols present in the wine (Shinohara et al., 2000). Various microorganisms, including some wine microorganisms, contain enzymes that are responsible for the decarboxylation of phenolic acids. A closer examination of the five peptides discussed (Pad1p, Pdcp, Padcp, Fdcp and PadAp) clearly shows that some characteristics are present in all of them. All the genes have an ORF of approximately 500 bp.

Barthelmebs et al. (2001) found that ρ-coumaric acid seems to be the main substrate for these phenolic acid decarboxylases. They compared the bacterial enzymes and found that the *L. plantarum* and *P. pentosaceus* phenolic acid decarboxylase peptides

show 87% sequence similarity, whereas the *B. pumilus* and *B. subtilis* peptides showed 89% similarity. The amino acid sequence identity between all four these bacterial phenolic acid decarboxylases was 61%. The *L. plantarum* and *P. pentosaceus* phenolic acid decarboxylases showed high activity on ρ-coumaric acid, but a 500-fold lower utilization of ferulic acid. The two *Bacillus* species showed the same affinity for ρ-coumaric, ferulic and caffeic acid (Barthelmebs et al., 2001). There is a degree of substrate specificity towards the different phenolic acids by the different enzymes.

To some degree, all the microorganisms containing phenolic acid decarboxylases use them in defence against the toxic effect of phenolic acids. Although, as in *S. cerevisiae,* this may not be the main defence mechanism, it can reduce the toxicity of these organic acids towards the cells.

The Pad1p enzyme that is found in *S. cerevisiae* shows low activity (Clausen et al., 1994). The production of volatile phenols by yeast during the winemaking process is limited. Due to the fact that certain concentrations of volatile phenols that are present in wine can enhance the organoleptic quality of the wine (Etiévant et al., 1989), an increase in volatile phenol formation might improve the wine aroma. When considering that the Pad1p activity in yeast is low and that the bacterial enzymes show high activity, it is plausible to express the bacterial genes in *S. cerevisiae*. This might aid in determining the effect of the over-expression of bacterial phenolic acid decarboxylase genes in *S. cerevisiae,* as well as providing greater understanding of the functioning of this enzymatic activity in yeast. The ability of these recombinant strains to influence the aroma bouquet of wine can then be determined.

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CHAPTER 3

RESEARCH RESULTS

Manipulation of volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae*

A modified version of this chapter will be submitted for publication in
Applied and Environmental Microbiology

CHAPTER 3

Manipulation of volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae*

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ABSTRACT

Free phenolic acids can be metabolized by different microorganisms into 4-vinyl and 4-ethyl derivates in wine. These volatile phenols contribute to the aroma of the wine. *Saccharomyces cerevisiae* contains the gene for phenyl acrylic acid decarboxylase (*PAD1*). Pad1p shows low activity, despite the fact that the gene is steadily transcribed in yeast. Phenolic acid decarboxylase (PADC) from *Bacillus subtilis,* as well as ρ-coumaric acid decarboxylase (PDC) from *Lactobacillus plantarum,* displays substrate-inducible decarboxylating activity with phenolic acids. The *padc, pdc* and *PAD1* genes were placed under the control of the $PGK1_{PT}$ (the promoter and terminator sequences from the yeast phosphoglyceratekinase I gene). These genes, under the control of the $PGK1_{PT}$ cassette were integrated into the *URA3* site of *S. cerevisiae* ∑1278b. The overexpression of *padc* and *pdc* in *S. cerevisiae* showed high enzyme activity. This was however not the case for *PADC*. The *padc* and *pdc* genes were integrated into *S. cerevisiae* VIN13 along with the *SMR1* gene for selection. The *PAD1* gene of VIN13 was also disrupted. Microvinifications were done with the three recombinant ∑1278b strains, which showed an increase in volatile phenol formation for the strains containing the bacterial genes. The three recombinant VIN13 strains were used to perform small-scale vinifications. Vinification showed that the Padcp and Pdcp enzymes increased the production of volatile phenol compounds in the wine. The ability of recombinant *S. cerevisiae* strains to decarboxylate phenolic acids offers prospects for the possible improvement of wine aroma.

3.1 INTRODUCTION

The growth and metabolism of the indigenous yeast and bacterial flora are responsible for changes in the organoleptic properties of wine during the process of vinification, ageing

and storage (Davis et al., 1986). Phenolic acids (principally ρ-coumaric, caffeic and ferulic acids) are generally esterified with tartaric acid or bind the complex lignin polymer to cellulose and hemicellulose in plants (Cavin et al., 1998). These phenolic acids are natural constituents of the grape must and wine and can be released as free acids by certain cinnamoyl esterase activities during winemaking (Dugelay et al., 1993).

Free phenolic acids can be metabolized by different microorganisms into 4-vinyl derivatives and then reduced to 4-ethyl derivatives in wine. These derivatives can contribute to the improvement of wine aroma (Chatonnet et al., 1993). Etiévant et al. (1989) and Chatonnet et al. (1992) found that volatile phenols that are present in a concentration higher than 4 mg/L can negatively affect the organoleptic qualities of a wine. Etiévant et al. (1989) showed that the ideal concentration for 4-ethylphenol is 2.2 mg/L and that this concentration increased the pleasantness of the aromatic perception. *Saccharomyces cerevisiae* contains the phenylacrylic acid decarboxylase gene, *PAD1* (Clausen et al., 1994). Pad1p exhibits very low activity with ferulic- and ρ-coumaric acids and is therefore not efficient enough for the improvement of the aroma of wine.

The enzymes responsible for the decarboxylation form part of a peptide group called phenolic acid decarboxylases and several bacteria and fungi have been found to contain the genes for these enzymes. Phenolic acids play an essential role in the biodegradation of plant wastes. Several bacteria are able to grow on these compounds as the sole carbon source. This was shown by Venturi et al. (1998) for *Pseudomonas putida* WCS358, by Segura et al. (1999) for *Acinetobacter* and by Narbad and Gasson (1998) for *Pseudomonas florescens*. In all of these organisms, ρ-coumaric and ferulic acid are converted to ρ-hydrobenzoic and vanillic acids respectively. These acids are then transformed into protocatechuic acid and integrated into the tricarboxylic acid cycle via the β–ketoadipate pathway.

The *Brettanomyces* yeasts are also well known for their ability to form volatile phenols in wine (Chatonnet et al., 1995). These species, however, are associated with the more unpleasant odorous formation of the ethylphenols and are present in cellars as spoilage organisms. Phenolic acid is decarboxylated to the corresponding vinylphenol via a carboxylase enzyme. Then, with the action of an oxido-reductase enzyme, the vinylphenols are converted to the corresponding ethylphenols (Chatonnet et al., 1993). These activities are not inhibited by other grape phenolics, resulting in a high transformation of the vinylphenols to ethylphenols.

Certain microorganisms contain phenolic acid decarboxylase enzymes. These enzyme decarboxylates phenolic acids to substituted phenyl priopionic acids and then the microorganism forms 4-vinyl derivatives, which can be reduced to 4-ethyl derivatives. Zago et al. (1995), Cavin et al. (1997b) and Cavin et al. (1998) characterized *fdc* from *Bacillus pumilus, pdc* from *Lactobacillus plantarum* (**Figure 1**) and *padc* from *Bacillus subtilis* respectively. All these genes have been cloned and expressed in *E. coli*. The bacterial enzymes mentioned here use the same metabolic pathway for the decarboxylation of phenolic acid as that of the Pad1p of *S. cerevisiae*.

The strategy used in this study was to sub-clone *padc* (*B. subtilis*), *pdc* (*L. plantarum*) and *PAD1* (*S. cerevisiae*) into the shuttle vector YEp352 with the $PGK1_{PT}$ cassette. Through the construction of intergrational plasmids (YIp5), all three genes were overexpressed in *S. cerevisiae* ∑1278b. The bacterial genes were overexpressed in *S. cerevisiae* VIN13. The *S. cerevisiae* VIN13 *PAD1* gene was disrupted. The ∑1278b strains were used to test for activity through enzymatic assays. Using the recombinant strains, wine was made and the chemical and aroma profiles were studied.

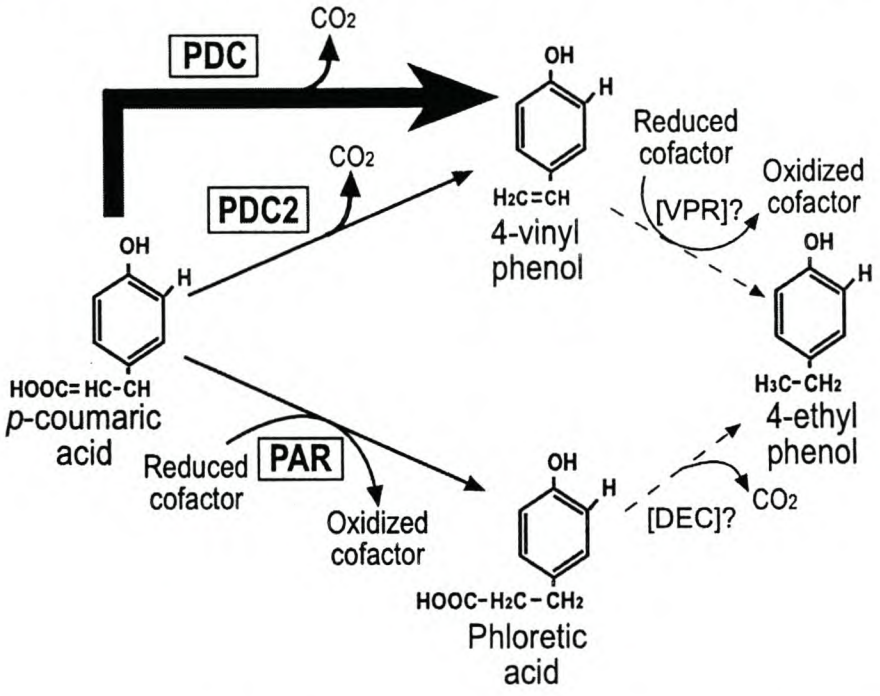

Figure 1. Proposed pathway for the degradation of ρ-coumaric acid in *L. plantarum*. The arrow thickness represents the relative intensity of enzymatic activity. PDC, ρ-coumaric acid decarboxylase; PDC2, phenolic acid decarboxylase; PAR phenolic acid reductase; VPR, putative 4-vinyl phenol reductase; DEC, putative phloretic acid decarboxylase (Barthelmebs et al., 2000).

The long-term aim is to determine the effect of the overexpression of phenolic acid decarboxylase genes in *S. cerevisiae,* as well as to gain greater understanding of the functioning of this enzymatic activity in yeast. A further aim is to determine the ability of these recombinant strains to influence the aroma bouquet of wine.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS AND PLASMIDS

The sources of yeast and bacterial strains and the plasmids used in this study are shown in **Table 1**.

Table 1. Microbial strains and plasmids used in this study.

Strains and plasmids	Genotypes	Source/reference
***S. cerevisiae* strains**		
Σ1278b	*MATα, ura3*	Liu et al., (1993)
WE 372	commercial diploid strain	Anchor Yeast Technologies (SA)
VIN13	commercial diploid strain	Anchor Yeast Technologies (SA)
ΣYPADC	*MATα, ura3* :: $PGK1_{PT}$ *padc*	This study
ΣYPDC	*MATα, ura3* :: $PGK1_{PT}$ *pdc*	This study
ΣYPAD1	*MATα, ura3* :: $PGK1_{PT}$ *PAD1*	This study
VIN13YSPADC	*MATα, ura3:: padc*	This study
VIN13YSPDC	*MATα, ura3:: pdc*	This study
VIN13POF	*MATα, URA3, PAD1*	This study
***Escherichia coli* strain**		
DHα	*sup*E44 *ρlac*U169 (*ϕ80lacZ*ρM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*-1 *rel*A1	[a]GIBCO/Bethesda Research Laboratories
Plasmids		
pHPAD	Em^R *ΔlacZ PADC*	Cavin et al. (1998)
pJPDC1	Em^R *ΔlacZ PDC*	Cavin et al. (1997b)
YEp352	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$	This laboratory
YIp5	Ap^R Tc^R *URA3*	This laboratory
pSH47	Ap^R *URA3* $GAL1_P$ $CYC1_T$ *CRE*	Güldner et al. (1996)
pEG6	Ap^R kan^R	This laboratory
pSHeSMR	Ap^R *URA3 SMR1* $GAL1_P$ *CRE* $CYC1_T$	This laboratory
pDLG31	Ap^R Tc^R *URA3 SMR1*	Gundllapalli Moses et al. (2001)
YEpPADC	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *padc*	This study
YEpPDC	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *pdc*	This study
YEpPAD1	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *PAD1*	This study
YPADC	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *padc*	This study
YPDC	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *pdc*	This study
YPAD1	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *PAD1*	This study
YSPADC	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *padc SMR1*	This study
YSPDC	Ap^R Tc^R *URA3* $PGK1_P$ $PGK1_T$ *pdc SMR1*	This study

[a]GIBCO/Bethesda Research Laboratories, Life Technologies Ltd., 3 Fountain Drive, Ichinnan Business Park, Paisley PA4 9RF.

3.2.2 MEDIA

The *E. coli* DH5α cells were cultured in Luria Bertani medium (0.5% yeast extract, 1% tryptone, 1% sodium chloride). 2% agar was added in the preparation of solid media and, in both cases, ampicillin (100 μg/mL) was added as selective pressure for recombinant transformant selection. The cells were incubated at 37°C for bacterial growth; yeast cells were cultured in Yeast Peptone Dextrose medium (1% yeast extract, 2% peptone, 2% glucose). For the selection of transformants, SC^{-Ura} medium (0.67% yeast nitrogen base without amino acids, 2% glucose and 0.83% of the growth factors: Leu, His, Trp) was used to provide auxotrophic pressure. 2% Agar was added for the preparation of solid media. All yeast cells were incubated at 30°C for growth.

3.2.3 DNA AMPLIFICATION

Polymerase chain reaction (PCR) was used for the isolation of the *padc, pdc* and *PAD1* genes. Primers (**Table 2**) were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241). *Padc* and *pdc* were amplified from pHPAD and pPDC1 respectively. The genomic DNA from *S. cerevisiae* WE 372 was used as template for *PAD1* amplification. The following PCR program was followed to amplify the *padc*, *pdc* and *PAD1* genes: template denaturation, 94°C for 2 min; primer annealing, 50°C for 1 min; primer extension, 72°C for 1 min; denaturation, 94°C for 30 seconds (this cycle was repeated 30 times); final annealing, 50°C for 5 min; final elongation, 72°C for 5 min.

Expand High Fidelity enzyme and Expand buffer without $MgCl_2$ were used (Boehringer Mannheim). The reaction mixture that was used contained: 1.25 mM dNTPs, 2 ng/μL template, 2.5 ng/μL of each primer and 0 μL, 2.5 μL, and 5 μL of Mg^{2+}. The PCR products were purified and underwent restriction digests for preparation for the cloning process. TRIO-Thermoblock (Biometra) was used for the reactions.

Tabel 2. Primers designed for PCR.

$pPDC_L$	5'-AGTCGAATTCACATAAGGAAGGTAATTCTA-3'	*Eco*RI
$pPDC_R$	5'-AGTCCTCGAGAGACCAGAATGTTTCACGTG-3'	*Xho*I
$pPADC_L$	5'-AGTCAGATCTGATTACGTTCTACTAGACAT-3'	*Bgl*II
$pPADC_R$	5'-AGTCCTCGAGGATGGTTCCCGCATACTCAG-3'	*Xho*I
$pPAD1_L$	5'-GAATTCATGCTCCTATTTCCA-3'	*Eco*RI
$pPAD1_R$	5'-GATCCTCGAGTTCAATTAGAATGATAT-3'	*Xho*I
$pPOF_R$	5'-ATCAACTCTATTAGTAGTTGAGTAACGTAATAAAATGCT CAGAAAAAGTCCAACTAGTGGATCTGATATCA-3'	
$pPOF_L$	5'-TGATTTCAATCTACGGAGTCCAACGCATTGAGCAGCTT CAATTGAGTAGATCTTCGTACGCTGCAGGTCGAC-3'	

The plasmid pEG6 (a modified version of pUG6) was used as a template for creating a disruption cassette for the *S. cerevisiae* VIN13 *PAD1* gene. The primers POF_R and POF_L were designed with tail ends that are homologous to the areas just outside the *PAD1* gene on the VIN13 genome. For the PCR reaction, the primers were designed to anneal to the pEG6 plasmid outside the two loxP sites. After amplification, a product containing the loxP sites along with the kan^R gene was obtained with two flanking regions with homology outside the *PAD1* gene (**Figure 2**). The same program and reaction mixtures that were used for the amplification of *padc*, *pdc* and *PAD1* were used again. However, the annealing step was done at 58°C.

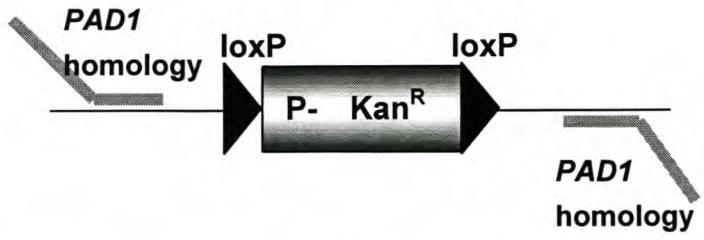

Figure 2. PCR product of pEG6, with POF_L and POF_R used as primers.

3.2.4 CLONING AND SEQUENCING

Standard methods were used for the DNA restriction digests, DNA purification, DNA ligation, DNA isolation, plasmid transformation into *E. coli* and agarose-gel electrophoresis (Sambrook et al., 1989). T4 DNA ligase and all the restriction endonucleases were purchased from Boeringer Mannheim (Pinelands, RSA).

After PCR amplification, *PAD1* and *pdc* was digested with *Eco*RI and *Xho*I for ligation with YEp352 into the $PGK1_{PT}$ cassette. *Padc* was digested with *Bgl*II and *Xho*I for ligation with YEp352 into the $PGK1_{PT}$ cassette. YEp352 with the $PGK1_{PT}$ cassette were also digested with *Eco*RI and *Xho*I, and *Bgl*II and *Xho*I respectively. The following plasmids were obtained after ligation: YEpPADC, YEpPDC and YEpPAD1.

All three $PGK1_{PT}$ cassettes containing the three different genes were cut out of YEpPADC, YEpPDC and YEpPAD1 with *Hin*dIII and run through a gel for DNA isolations to be performed (**Figure 3**). The three cassettes were ligated into the YIp5 yeast integrational plasmid after *Hin*dIII linearization. The following plasmids were obtained: YPADC, YPDC and YPAD1.

The *SMR1-410* gene of *S. cerevisiae,* which codes for sulfometuron methyl resistance, was cut out of pDLG31 with *Bam*HI. Plasmids YPADC and YPDC were also linearized with *Bam*HI and *SMR1* was ligated into both these plasmids. The following plasmids were obtained: YSPADC and YSPDC.

The analysis of the sequences was carried out using ABI PRISM® Big Dye™ Terminator cycle sequencing ready reaction kits with an ABI PRISM™ 377 DNA sequencer (PE/Applied Biosyctems). Both the coding and non-coding strands were sequenced to ensure the reliable identification of all constructs.

3.2.5 TRANSFORMATION INTO *S. CEREVISIAE* Σ1278B AND VIN13

Yeast transformations were done as stipulated by the protocol described in Sambrook et al. (1989). The three respective plasmids, YPADC, YPDC and YPAD1 were cut with *Nco*I (in the *URA3* site) restriction endonuclease to linearize the plasmids with two flanking *URA3* ends. These fragments were then used to recombine with the Σ1278b *URA3* site and SC^{-Ura} plates were used for the selection of putative transformants.

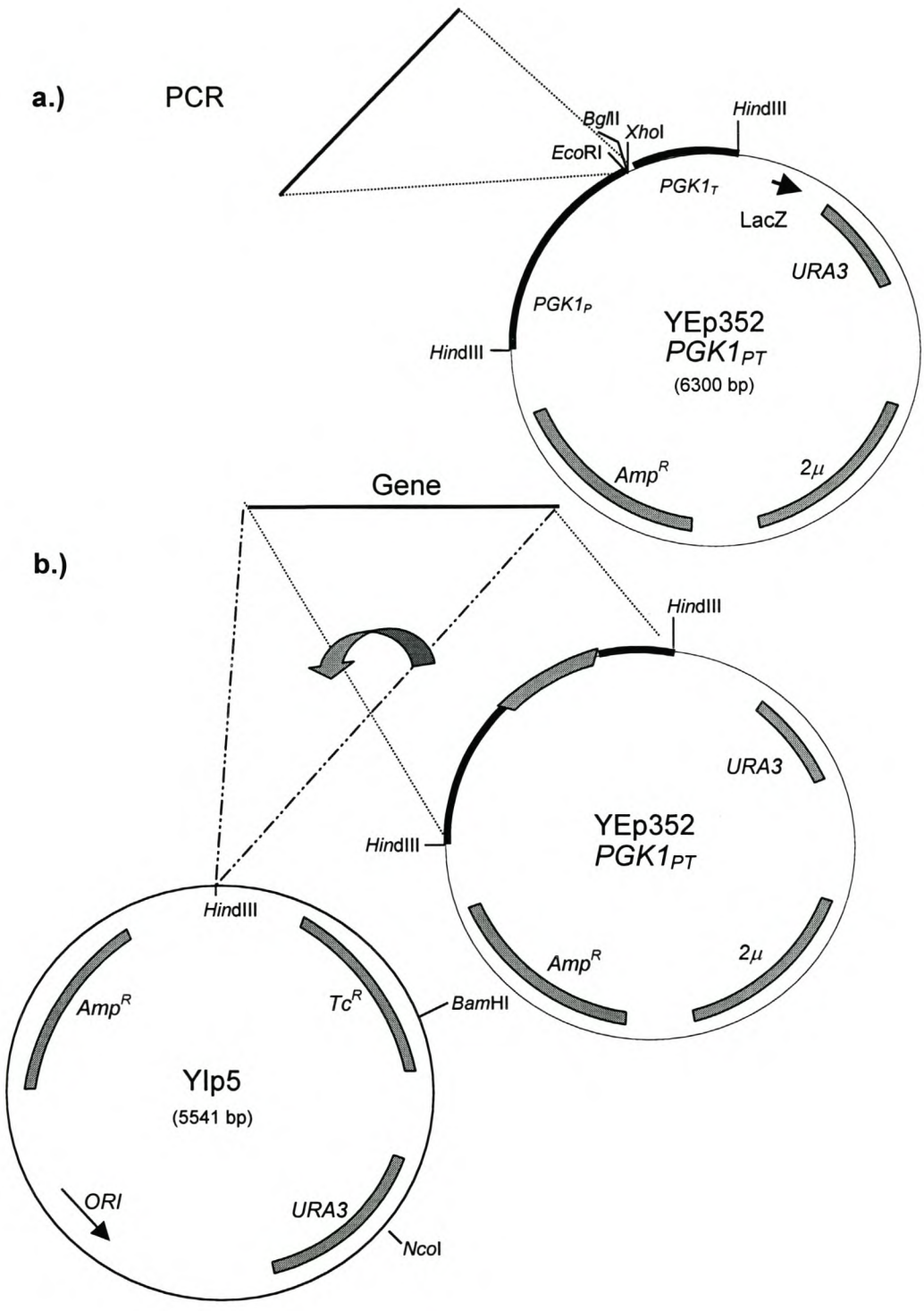

Figure 3. Cloning strategy for YEpPADC, YEpPDC, YEpPAD1, YPADC, YPDC and YEpPAD1 construction. a.) The PCR products of all three genes were cloned into the YEp352 plasmid. b.) The *PGK1*$_{PT}$ cassettes, containing the respective genes, were isolated with *Hin*dIII restriction digests and cloned into the *Hin*dIII site of the Ylp5 plasmid.

The same restrictions were performed for the YSPADC and YSPDC plasmids. These fragments were then used to recombine with the VIN13 *URA3* site, and SC plates containing 80 μg/mL SMM (sulfometuron methyl) were used for the selection of the putative transformants.

The POF PCR product was used to transform VIN13 and the recombination of the *PAD1* flanking regions with the regions just outside the *PAD1* gene in VIN13 caused a single disruption to occur (**Figure 2**). Geneticin (400 mg/L) was added to the YPD media to select for positive transformants. pSH47 was transformed into a single disrupted VIN13 strain and grown in galactose broth for 6 h to induce the cre-recombinase gene. The cre-recombinase causes the loxP sites to recombine and this leads to the excision of the *kanR* marker. Selection was done on SC plates containing 80 μg/L SMM and confirmed by testing on YPD media containing 400 mg/L geneticin. The POF PCR product was retransformed to disrup the second copy of *PAD1*.

3.2.6 SOUTHERN BLOTTING

Standard methods were used for yeast genomic DNA isolations (Sambrook et al., 1989). The digoxigenin nonradioactive nucleic acid labeling and detection system was used to perform the Southern hybridisation for the detection of integration in the different ∑1278b and VIN13 strains. The DIG Labeling Kit from Roche Biochemical Products (South Africa) was used.

3.2.7 ENZYMATIC ASSAYS

Protein extractions were performed on cells grown to an OD_{600} of 6.5 in 100 ml of SC^{-URA} broth. Uracil was added to the broth for the growth of the control strain. Cells were harvested and dissolved in 50 mM Tris (pH 7.5) / 10 mM NaCl. After 0.1 g of 0.2 mm glass beads were added, the mixture was vortexed for 3 min and spun down at 6000 rpm for 2 minutes. The supernatant was collected and stored at 4°C. The assay was performed in a 25 mM phosphate buffer (pH 6) and 0.3 mM ρ-coumaric acid was added with the extracted proteins. After 24 h, the reactions were stopped by diluting them 16-fold with 20 mM of Tris-HCl - 0.3% Sodium dodecyl sulfate, pH 6. The detection of enzyme activity, through the spectophotometer, was done as described in Cavin et al. (1997a;1993).

ρ-Coumaric acid decarboxylation can be detected on the spectophotometer due to the loss of the double bonds. This leads to a hypochrome UV spectrum displacement (Cavin et al., 1993). ρ-Coumaric acid shows a peak at 285-nm and the vinyl derivatives show a peak at 255-nm (Cavin et al., 1997a). The detection of the dissapearance of the 285-nm peak and the formation of a 255-nm peak indicate the degradation of ρ-coumaric acid and the formation of the corresponding vinyl derivatives.

3.2.8 MICROVINIFICATIONS AND INDUSTRIAL FERMENTATIONS

Weisser Riesling juice was used to perform microvinifications with laboratory strains. The juice had a sugar content of 23°B and a pH of 3.4. Half the juice was complemented with 0.04 mM of ρ-coumaric acid, ferulic acid, and caffeic acid and half was used without complementation. A total volume of 150 mL was used for each microvinification. The strains ∑YPADC, ∑YPDC and ∑YPAD1 were inoculated to 3 X 10^6 cells/mL after the overnight culture in YPD was washed with dH_2O. All microvinifications were repeated three times, along with a Sigma strain as control. Fermentations were done over a period of two weeks, after which cells were collected and samples were taken for GC analysis.

For industrial vinifications, the same Weisser Riesling juice was used, along with Chardonnay juice with a sugar concentration of 22°B and a pH of 3.3. The VIN13YSPADC, VIN13YSPDC and VIN13POF strains were used. Each strain was inoculated into one liter of Chardonnay juice and one liter of Riesling juice. Untransformed VIN13 was used as a control strain. All samples were fermented in duplicate at 15°C. The juice was fermented until dry and sampled for GC analysis.

3.2.9 GAS CHROMATOGRAPHY

The following apparatus and reagents was used for the detection of volatile phenols:

Reagents: The pure chromatographic standards of analytical quality were obtained from Fluka and Aldrich. Synthetic wine solutions: a semi-sweet Chenin Blanc (ethanol 10%) was used as background matrix in which to do calibrations. This was spiked with each of the four volatile phenols, so that their concentrations were 1 mg/L each. *1,1,2-Trichloro-1,2,2-trifluoroethane* (Freon113) of HPLC quality was obtained from Aldrich.

Apparatus: Volumetric material: Hamilton syringes with volumes of 10 and 100 μL, calibrated previously. Extraction tubes: centrifuge glass tubes with a conical bottom, 12 cm x 17 o.d., with a glass stopper. Mechanical shaker: Labinco rotary mixer. HP 6890 series gas chromatograph, fitted with an MSD, fitted with a splitless injector, and an automatic sampler 7673 were used. Column: Supelco HP-5MS (HP 19091S-433), 30 m x 0.25 mm i.d., 0.25 μm film thickness, initial flow 1.7 mL/min, average velocity 48 cm/sec, worked at constant flow. Chromatographic conditions: carrier gas, He; head pressure, 99.5 kPa; total flow, 11.8 mL/min; purge flow, 7.4 mL/min; purge time, 0.5 min; injector (splitless) and detector temperature, 250°C; initial column temperature, 50°C, held for 2 min and then raised to 150°C at 10°C/min, then to 160 at 5°C/min and then to 220°C at 10°C/min and held for 10 min.; injected volume, 2 μL. Data were recorded and processed by Hewlett Packard Chemstation software.

Thermal auxiliary: MSD transfer temperature at 280°C.

Tune file and acquisition mode: stune.u in scan.

MS information: Solvent delay, 3.9 min.

The following method was used: 10 mL of wine was introduced into the extraction tube. 200 μL of Freon 113 was added as extracting agent, as well as 2 μL of a solution of 2,6-dimethylheptenol (400 mg/L in ethanol as internal standard). 1,2 g NaCl was added. The tubes were capped and shaken for 30 min in the automatic shaker at maximium speed. The tubes were centrifuged (3000 rpm for 5 min) and the organic phase recovered with a pasteur pipette, transferred over 50 mg of Na_2SO_4 into a HP 2 mL vial with a 200 μL glass insert, and analyzed under the chromatogprahic conditions given above. After chromatographic analysis, the relative areas or heights of the calibrated peaks were interpolated from calibration graphs created with synthetic wine solutions having an alcohol content similar to that of the analyzed wine.

Calibration Graph: five calibration points were prepared by diluting the synthetic wine solution above: 1:100, 1:50, 1:20, 1:10 and 1:2. They were extracted following the proposed method and the calibration graphs were created with the chromatographic results.

The target ions used to calibrate each compound were as follows:

4-ethylphenol	107, 122, 77
4-vinylphenol	120, 91, 65
4-ethylguaiacol	137, 152
4-vinylguaiacol	150, 135, 107

Quantitative Analysis: A plot representing chromatographic height vs concentration in wine was made for each compound, using the five calibration points.

3.3 RESULTS

3.3.1 EXPRESSION OF *PAD1*, *padc* AND *pdc* IN LABORATORY AND INDUSTRIAL YEAST STRAINS

With the aim to study the effect of reduced and enhanced levels of 4-ethyl and 4-vinylphenols the *padc* (*B. subtilis*), *pdc* (*L. plantarum*) and *PAD1* (*S. cerevisiae*) genes were cloned for the production of overexpression cassettes. A disruption casette for the disruption of the *PAD1* gene was also constructed.

Padc, pdc and *PAD1* showed the expected size fragments after amplification, corresponding with the sizes on their database sequences. Ligations were performed. After transformation into *E. coli* and plasmid isolations, all three clones were cut out of YEp352 with the same restriction enzymes used to insert them. All three constructs showed fragments of the correct sizes. Conformation digests were performed and the fragments that were obtained again showed the correct, expected sizes. Positive clones were obtained for the ligation of the three respective cassettes into YIp5, along with

positive confirmations. The plasmids YEpPADC, YEpPDC and YEpPAD1 were sent for sequencing, which confirmed that their sequences were correct.

All three YIp5 plasmids were successfully transformed into *S. cerevisiae* ∑1278b (∑YPADC, ∑YPDC and ∑YPAD1) and the positive transformants were isolated from the SC^{-Ura} medium for Southern blotting.

The PCR product obtained for the disruption of VIN13 *PAD1* (**Figure 2**) was transformed successfully into VIN13, resulting in a single disruption. The Geneticin marker was removed by means of the pSH47 cre-recombinase action. A second transformation of the disruption cassette led to the double disruption of the *PAD1* gene in VIN13. This was confirmed by PCR.

∑YPADC, ∑YPDC and ∑YPAD1 were confirmed to contain the *padc*, *pdc* and *PAD1* genes respectively (results not shown). VIN13YSPADC and VIN13YSPDC were also confirmed to contain the *padc* and *pdc* genes respectively. The integration of the VIN13POF disruption was confirmed through Southern blotting.

3.3.2 ENZYMATIC ASSAYS

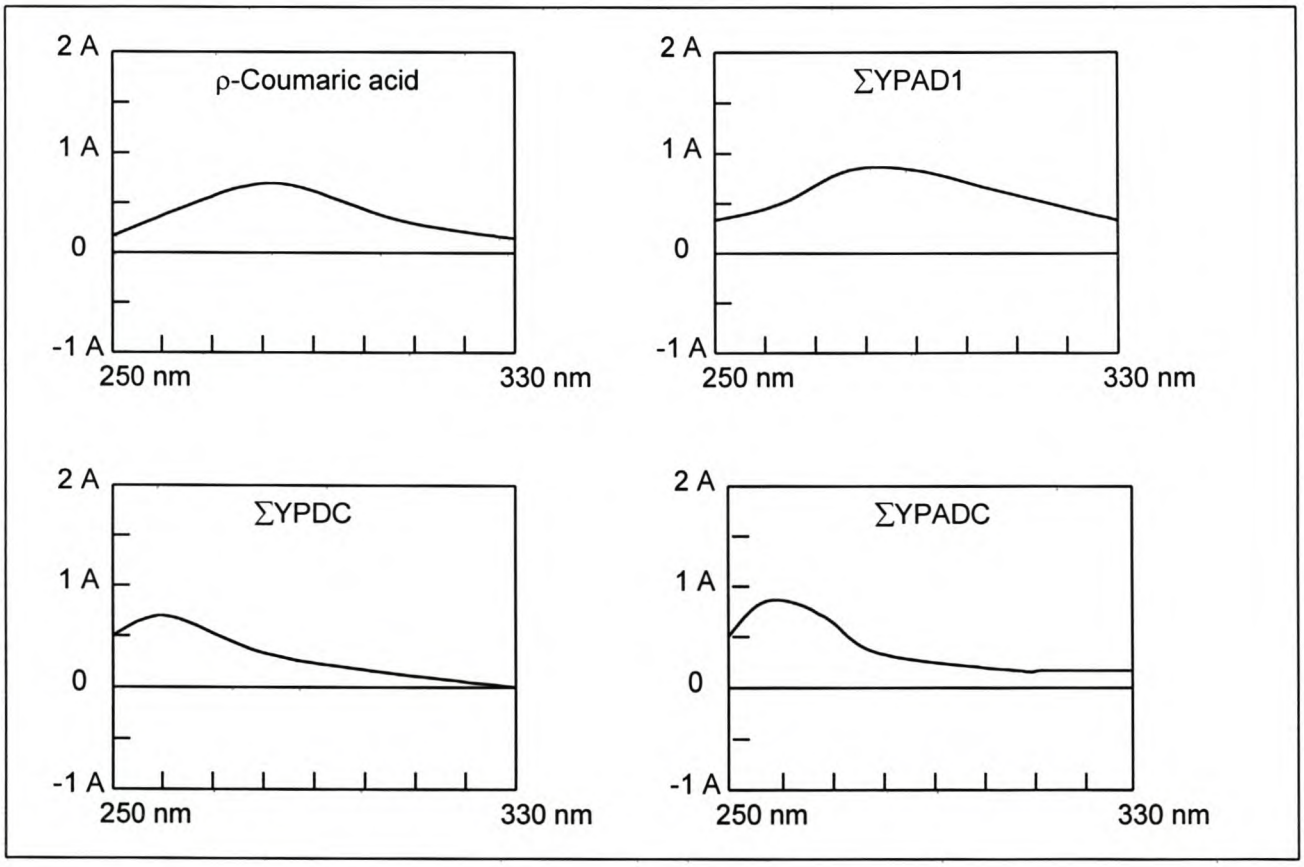

Figure 4. The absorption peaks for ρ-coumaric acid degradation by the ∑YPAD1, ∑YPDC and ∑YPADC strains (255 nm = volatile phenol derivatives; 285 nm = ρ-coumaric acid).

The extracted proteins of the ΣYPADC and ΣYPDC strains showed enzymatic activity responcible for a steady decrease in ρ-coumaric acid. Although the Σ1278b control and the ΣYPAD1 strains also showed a decrease in ρ-coumaric acid, the decrease was much smaller than that shown for the overexpressed bacterial enzymes. The readings from the spectrophotometer are shown in **Figure 4**.

3.3.3 MICROVINIFICATIONS AND INDUSTRIAL FERMENTATIONS

The GC analysis of the microvinifications detects a 1.7-fold increase in the production of 4-vinylphenol by the ΣYPADC and ΣYPDC strains, in comparison to the control and the ΣYPAD1 strain (**Table 3**). For the production of 4-vinylguaiacol a 1.6-fold increase was observed with the ΣYPADC strain and a 2.6-fold increase with the ΣYPDC strain.

Table 3. The GC analysis of the microvinifications by laboratory strains.

	Uncomplemented Riesling juice	**Complemented juice (0.04 mM)**	**Uncomplemented Riesling juice**	**Complemented juice (0.04 mM)**
μg/L	4-vinylphenol	4-vinylphenol	4-vinylguaiacol	4-vinylguaiacol
Σ1278b (control)	81	1795	52	1683
ΣYPADC	142	2869	98	2526
ΣYPDC	149	2951	154	2643
ΣYPAD1	93	2355	68	1636

The vinification done by the industrial yeast, made from the recombinant *S. cerevisiae* VIN13 strains, with the uncomplemented Chardonnay and Riesling juice, did not show as high an increase in volatile phenols as the microvinifications (laboratory strains) when compared to the controls. **Table 4** indicates that there was a slight increase in 4-ethylphenol, 4-vinylphenol and 4-vinylguaiacol in most of the Chardonnay and Riesling wines.

Table 4. GC analysis results of volatile phenols formed by industrial transformants (in μg/L).

Wine	**Strain**	**4-vinylphenol**	**4-ethylphenol**	**4-vinylguaiacol**
Riesling	VIN13	3400.21	1089.49	51.11
	VIN13YSPADC	3879.84	1139.55	80.28
	VIN13YSPDC	3462.72	1107.74	68.02
	VIN13POF	2912.47	1131.34	ND
Chardonnay	VIN13	5712.72	1194.76	61.22
	VIN13YSPADC	6795.29	1235.03	60.81
	VIN13YSPDC	5970.56	1167.65	60.63
	VIN13POF	5500.22	1117.47	53.02

This was shown for VIN13YSPADC and VIN13YSPDC, in comparison to *S. cerevisiae* VIN13. There was a slight decrease for the most of the VIN13POF wines. The production of 4-vinylphenol was quite extensive with all the strains. No 4-ethylguaiacol was detected with any of the strains.

3.4 DISCUSSION

It is clear that the use of the recombinant strains of *S. cerevisiae* shows a definite increase in the utilization of phenolic acids when compared to the same strains without the *padc* and *pdc* genes. However, the overexpressed *PAD1* gene does not extensively improve the functioning of the Pad1p enzyme. This might indicate, as previously found by Clausen et al. (1994), that post-transcriptional regulation might be critical for the functioning of Pad1p. The enzymatic assays illustrate the decarboxylation of ρ-coumaric acid and the formation of volatile phenols. **Figure 4** indicates the formation of a peak at 285-nm for ρ-coumaric acid. The second graph in **Figure 4** shows the same peak for the assay done on ρ-coumaric acid decarboxylation in the presence of the Pad1p enzyme expressed by ∑1278b. No peak was detected at 255-nm. This indicates that ρ-coumaric acid was not degraded in the presence of Pad1p. The third and fourth graphs in **Figure 4** indicate the decarboxylation of ρ-coumaric acid in the presence of Pdcp and Padcp, the two bacterial decarboxylase enzymes expressed in yeast. These graphs indicate the formation of peaks at 255-nm, which represents the vinyl derivatives. This is a clear indication that the two bacterial genes expressed in yeast can lead to the effective decarboxylation of ρ-coumaric acid to its corresponding vinyl derivatives.

When considering the wines made by the recombinant strains, it was clear that the presence of the bacterial genes caused an increase in the formation of volatile phenols. The microvinifications clearly indicate a higher concentration of volatile phenols formed by the recombinant strains. In comparison to the control and the ∑YPAD1 strain, there is a 1.7-fold increase for 4-vinylphenol by the ∑YPADC and ∑YPDC strains and, for 4-vinylguaiacol a 1.6-fold increase by the ∑YPADC strain and a 2.6-fold increase by the ∑YPDC strain. This correlates with the results of the enzymatic assays.

The results from the industrial yeast fermentation analysis are not as clear, however. The production of 4-vinylphenol was extensive in the Chardonnay and Riesling wines. For all the recombinant strains as well as the control strains, the concentration of 4-vinylphenol for the Riesling wines was between 3 and 4 mg/L and for the Chardonnay wines between 5 and 7 mg/L. As the aroma can be perceived negatively at 4 mg/L and the ideal concentration is about 2.2 mg/L, the over production of 4-vinylphenol might be too extensive for aroma improvement (Chatonnet et al., 1992; Etiévant et al., 1989). The reason for this high production of volatile phenols (including by the control strains) might be due to the concentration of phenolic acids available for decarboxylation in the grape juice.

When considering all the results, it is clear that the laboratory strains are indicative of the functioning of the different phenolic acid decarboxylase genes in yeast. The assays and the wine analyses of the laboratory strains can be used to indicate that the *padc* and *pdc* genes are functional in *S. cerevisiae*. It also indicates that the *PAD1* gene is not as effective for the decarboxylation of phenolic acids to their derivatives. Compared to the laboratory strains, the industrial strain fermentation analysis shows a slight increase in the formation of volatile phenols. The results for the strain with the disrupted *PAD1* (VIN13POF) indicate that there is Pad1p functioning in the control VIN13 strain. To have a clearer picture of the decarboxylation enzymes active in the industrial strains, it would be recommendable to measure the amount of phenolic acids (the precursors) present in the grape juice. This could help to establish whether the amount of precursors present in the juice is responsible for the amount of volatile phenols formed, or whether it is the enzyme activity, that is responsible. Because we did not complement the juice used for fermentations by the industrial strains, the limiting effect of the substrate could not be tested. We suggest that further research should be done on this.

In conclusion, this study paves the way for the development of wine yeast starter cultures for the production of optimal levels of volatile phenols, thereby improving the sensorial quality of wine.

3.5 ACKNOWLEDGMENTS

Our sincere gratitude to Méchelle Gey van Pittius for the GC analysis, J-F Cavin for providing us with pHPAD and pPDC1, and the National Research Foundation (NRF) and the South African wine industry (Winetech) for financial support.

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CHAPTER 4

ADDITIONAL RESEARCH RESULTS

Monoterpene formation by recombinant yeast strains

CHAPTER 4

4.1 INTRODUCTION

Terpenols are molecules that contribute to the aroma and flavour profile of wine. Terpenols are known especially for their contribution to the character of the muscat varieties (Marais, 1983; Rapp and Mandery, 1986). Terpenols can occur as flavourless, non-volatile glycosidic complexes (Van Rensburg and Pretorius, 2000). These precursors can be hydrolysed during the fermentation process. The hydrolysis of these precursors occurs when the glycosidic linkages are cleaved. After the cleaving, β-glucosidases produce monoterpenes. The monoterpenes are shown in **Table 1** and are the volatile and odourous molecules responsible for enhancing the aroma of wine.

The occurrence of monoterpenes in wine can have a favourable effect on the aroma profile of the wine. The characteristic aromas formed by the monoterpenes are stipulated in **Table 1**. It is for this reason that β-glucosidase activity is artificially introduced into fermentation processes (Van Rensburg and Pretorius, 2000).

Table 1. Properties of monoterpenes – aroma and sensory threshold data in water (adapted from King and Dickinson, 2000).

Compound	Aroma	Sensory threshold (μg/L)
Geraniol	Floral, rose-like, citrus	132
Citronellol	Sweet, rose-like, citrus	100
Linalool	Floral, fresh, coriander	100
Nerol	Floral, fresh, green	400
α-Terpineol	Lilac	460

4.2 MATERIALS AND METHODS

The apparatus used for the GC analysis was as follows: An HP 6890 series gas chromatograph, fitted with an FID, fitted with a split-splitless injector and an automatic sampler 7683 for the volumetric material. A supelco SPB5, 60 m X 0.32 mm i.d., with a 0.25 μm film thickness was used for the column. The chromatographic conditions were as follows: He as carrier gas; head pressure of 140 kPa; flow of 12.5 mL/min; purge flow of 7.0 ml/min; injector and detector temperature of 250°C; initial column temperature of 50°C, held for 2 min and then raised to 150°C at 10°C/min, then to 160°C at 5°C/min and then to 220°C at 10°C/min and held for 10 min; make-up gas N_2 at 30 mL/min; detector FID, H_2 at 40 mL/min; air, 450 mL/min; injection volume of 2 μL.

The method used for the detection of monoterpenes was as follows: 10 ml of wine was introduced into the extraction tube. 200 ∑ of Freon 113 (1,1,2-Trichloro-1,2,2-

trifluoroethane, obtained from Aldrich) was added as extraction agent, as well as 2 μL of a solution of 2,6-dimethylheptenol (400 mg/L in ethanol as internal standard) and 1.3 g of NaCl. The tubes were capped and shaken for 30 min in an automatic shaker at maximum speed. The tubes were centrifuged (5 min at 3000 rpm), the organic phase was recovered with a pasteur pipette, then transfered over 50 mg of Na_2SO_4 into an HP 2 mL vial with a 200 μL glass insert, and analyzed under the chromatographic analysis. The relative areas or heights of the calibrated peaks are interpolated from calibration graphs created using synthetic wine solutions (ethanol 12% for white wine, 16% for red wine v/v; tartaric acid 6 g/L; pH 3.2) with an alcohol content similar to that of the analyzed wine.

4.3 RESULTS AND DISCUSSION

Table 2. GC analysis results on monoterpenes formed for industrial vinification.

Juice	μg/L	Linalool-oxide	Linalool	Terpineol	Citronellol	Nerol	Geraniol
Riesling	VIN13	1.655	111.512	48.650	11.904	2.309	12.648
	VIN13YSPADC	1.652	117.801	57.590		4.578	14.385
	VIN13YSPDC	4.240	113.341	56.579		11.811	8.127
	VIN13POF	4.312	115.872	64.3045		15.279	10.483
Chardonnay	VIN13	2.223	9.680	28.267			5.997
	VIN13YSPADC	1.863	10.969	33.312		21.793	
	VIN13YSPDC	2.261	10.896	33.853	18.453		4.822
	VIN13POF	1.900	10.026	31.293	9.995		3.764

The production of monoterpenes is indicated in **Table 2**. The Riesling wines display no significant difference in the production of linalool, geraniol and terpineol. VIN13YPDC and VIN13POF, however, show a 2.6-fold increase for linalooloxide. The increase in production of nerol was 2.0-fold, 5.1-fold and 6.6-fold higher for VIN13YPADC, VIN13YPDC and VIN13POF respectively. The GC results for the Chardonnay wines do not show a clear enough difference in any of the tested samples. More research needs to be done on the production of monoterpenes by the recombinant strains VIN13YPADC, VIN13YPDC and VIN13POF. The production of these aroma-enhancing molecules under these conditions can occur for various reasons. Seeing that the disrupted strain shows an increase in monoterpenes, it is necessary to establish whether it is the presence or absence of these peptides that might cause the increase. The enzyme activity of these

peptides might also play a role in the production of monoterpenes. However, no conclusions can be drawn without further research.

4.4 REFERENCES

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

CHAPTER 5

5.1 CONCLUSION

Phenols are abundant in grape must and in wine. The different forms of phenolic compounds present in wine fulfil a variety of different roles. Except for adding to the complexity and flavour profiles of wine, phenols are known to act as antimicrobial and antioxidative agents (Ribéreau-Gayon et al., 2000).

The microbial transformation of phenolic compounds leads to the formation of new aroma-contributing molecules. Yeast and bacteria can convert phenolic acids to volatile phenols. The volatile phenols, specifically 4-vinyl- and 4-ethylphenol, can be detected in wine at very low threshold values (Cavin et al., 1993). The contribution of volatile phenols can either enhance the aroma profile or create odours.

Various ways exist in which microorganisms can convert phenolic acids into volatile phenols. One such process is by decarboxylating the phenolic acid into phenyl propionic acid derivatives and then transforming these derivatives into volatile phenols. The microorganisms capable of using this method are: *S. cerevisiae, L. plantarum*, *L. brevis, L. crispatus, L. fermentum, L. paracasei, L. pentosus, B. subtillis, B. pumilus* and *Pediococcus pentosaceus* (Barthelmebs et al., 2000b; Cavin et al., 1997a; Cavin et al., 1998; Clausen et al., 1994; Van Beek and Priest, 2000; Zago et al., 1995). All these microorganisms contain a phenolic acid decarboxylase gene. This gene codes for a phenolic acid decarboxylase enzyme, which is responsible for the hydrolysis of the phenolic acids.

The reason why microorganisms decarboxylate phenolic acids may differ from organism to organism. To some degree, all the microorganisms containing phenolic acid decarboxylases use them as a defence against the toxic effect of phenolic acids (Lambert et al., 1997). Although, as in *S. cerevisiae*, this may not be the main defence mechanism, it does reduce the toxicity of these organic acids towards the cells.

In *S. cerevisiae,* the overexpressed *PAD1* gene does not, however, improve the functioning of the Pad1p enzyme. This indicates , as previously found by Clausen et al. (1994), that post-transcriptional regulation might be present with the functioning of Pad1p. To investigate the possible improvement of wine aroma by decarboxylating phenolic acids into volatile phenols, the *L. plantarum* (*pdc*) and *B. subtillis* (*padc*) genes were overexpressed in *S. cerevisiae*.

It is clear that the use of the recombinant strains of *S. cerevisiae* shows a definite increase in the utilization of phenolic acids when compared to the same strains without the *padc* and *pdc* genes. This was shown by means of enzymatic assays. The spectrophotometer clearly indicated the disappearance of the peak for ρ-coumaric acid and the formation of a peak for the derivatives for the laboratory strains containing the

bacterial genes. The control strain and the strain containing the overexpressed *PAD1* gene showed only the peak for ρ-coumaric acid.

To test for the possible improvement of wine aroma, the wine made by the *S. cerevisiae* ∑1278b strains containing *PAD1, padc* and *pdc* and by the *S. cerevisiae* VIN13 strains containing *padc*, *pdc* and a disrupted *PAD1* were analyzed. The microvinifications by the laboratory strains clearly indicate a higher concentration of volatile phenols formed by the recombinant strains containing the bacterial genes. When compared to the control and the overexpressed *PAD1* strain, a 1.7-fold increase for 4-vinylphenol by the bacterial strains, for 4-vinylguaiacol, a 1.6-fold increase by the strain containing *padc* and a 2.6-fold increase by the strain containing *pdc* are observed. This correlates with the results of the enzymatic assays. It is clear that the bacterial genes are functional in yeast and that the yeast's metabolism is capable of transforming the decarboxylated phenolic acids into volatile phenols.

The results from the industrial fermentation analysis are not as clear, however. The production of 4-vinylphenol in the Chardonnay and Riesling wines was extensive. In all the recombinant strains as well as in the control strains the concentration of 4-vinylphenol was between 3 and 4 mg/L for the Riesling wines and between 5 and 7 mg/L for the Chardonnay wines. Seeing than the aroma perception can be negative at 4 mg/L and that the ideal concentration is about 2.2 mg/L, the production of the 4-vinylphenol might be too extensive for aroma improvement (Chatonnet et al., 1993 and Etiévant et al., 1989). The influence of the amount of precursors was not measured for the VIN13 strains. The juice should be complemented with the phenolic acids to measure whether the amount of substrate is responsible for a limitation in the amount of product formed. An experimental fermentation system was used for the vinification done by the industrial strains. The system allowed excessive air contact, which could lead to a loss in the volatile aroma components.

An increase in monoterpene production was found by using the recombinant *S. cerevisiae* VIN13 strains. The production of Riesling wines shows no significant difference in the production of linalool, geraniol and terpineol. However, the VIN13 strain containing the *pdc* gene and the VIN13 strain with the disrupted *PAD1* gene show a 2.6-fold increase for linalooloxide. The increase in the production of nerol was 2.0-fold, 5.1-fold and 6.6-fold higher for VIN13 containing *padc, pdc* and a disrupted *PAD1* respectively. The GC results for the Chardonnay wines do not show a sufficiently clear difference in any of the samples tested. More research needs to be done on the production of monoterpenes by the recombinant VIN13 strains. The production of these aroma-enhancing molecules under these conditions can occur for various reasons. Because the disrupted strain shows an increase in monoterpenes, it is necessary to establish whether it is the presence or absence of these peptides that might cause the increase. The enzyme activity of these peptides might also play a role in the production of monoterpenes.

It might be possible to enhance the production of volatile phenols during the winemaking process. By using recombinant wine strains containing the bacterial phenolic acid decarboxylase genes, the production of 4-vinylphenols can be increased to a certain concentration. The optimization of the expression levels is essential, however, and this whole process will also depend on the phenolic acids present in the particular grape juice used. Chatonnet et al. (1993) indicated that tannins inhibit the cinnamate decarboxylase enzyme of *S. cerevisiae*. It would be recommended to use the recombinant strains containing the bacterial genes to produce red wine. This could indicate the effect of tannins on the functioning of the bacterial enzymes.

5.2 REFERENCES

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