JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Enhancing Volatile Phenol Concentrations in Wine by Expressing Various Phenolic Acid Decarboxylase Genes in *Saccharomyces cerevisiae*

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Phenolic acids, which are generally esterified with tartaric acid, are natural constituents of grape must and wine and can be released as free acids (principally *p*-coumaric, caffeic, and ferulic acids) by certain cinnamoyl esterase activities during the wine-making process. Some of the microorganisms present in grape can metabolize the free phenolic acids into 4-vinyl and 4-ethyl derivatives. These volatile phenols contribute to the aroma of wine. The *Saccharomyces cerevisiae* phenyl acrylic acid decarboxylase gene (*PAD1*) is steadily transcribed, but its encoded product, Pad1p, shows low activity. In contrast, the phenolic acid decarboxylase (PADC) from *Bacillus subtilis* and the *p*-coumaric acid decarboxylase (PDC) from *Lactobacillus plantarum* display substrate-inducible decarboxylating activity in the presence of phenolic acids. In an attempt to develop wine yeasts with optimized decarboxylation activity on phenolic acids, the *padc*, *pdc*, and *PAD1* genes were cloned under the control of *S. cerevisiae*'s constitutive phosphoglyceratekinase I gene promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences. These gene constructs were integrated into the *URA3* locus of a laboratory strain of *S. cerevisiae*, Σ1278b. The overexpression of the two bacterial genes, *padc* and *pdc*, in *S. cerevisiae* showed high enzyme activity. However, this was not the case for *PAD1*. The *padc* and *pdc* genes were also integrated into an industrial wine yeast strain, *S. cerevisiae* VIN13. As an additional control, both alleles of *PAD1* were disrupted in the VIN13 strain. In microvinification trials, all of the laboratory and industrial yeast transformants carrying the *padc* and *pdc* gene constructs showed an increase in volatile phenol formation as compared to the untransformed host strains (Σ1278b and VIN13). This study offers prospects for the development of wine yeast starter strains with optimized decarboxylation activity on phenolic acids and the improvement of wine aroma in the future.

**KEYWORDS: Phenolic acid decarboxylation; volatile phenols; wine yeast; wine aroma**

## INTRODUCTION

The metabolism of the indigenous yeast and bacterial flora are responsible for changes in the organoleptic properties of wine during the process of spontaneous fermentation, aging, and storage (*1*). Phenolic acids (mainly *p*-coumaric, caffeic, and ferulic acids) are generally esterified with tartaric acid, or they bind the complex lignin polymer to cellulose and hemicellulose in plants (*2*). 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 wine-making (*3*).

Free phenolic acids can be metabolized by different microorganisms to form 4-vinyl derivatives, which can be reduced to 4-ethyl derivatives in wine. These derivatives can have a significant influence on wine aroma (*4*). It has been reported that the ideal concentration for 4-ethylphenol is 2.2 mg $L^{-1}$ and that this concentration increased the pleasantness of the aromatic perception (*5*). It was also found that volatile phenols present in concentrations >4 mg $L^{-1}$ could negatively affect the organoleptic qualities of a wine (*5*, *6*). *Saccharomyces cerevisiae* contains a phenylacrylic acid decarboxylase gene, *PAD1* (*7*), which codes for a protein (Pad1p) with very low activity against ferulic acid and *p*-coumaric acid, and is therefore not efficient enough for the improvement of the aroma of wine.

Several bacteria and fungi contain phenolic acid decarboxylases as part of their metabolism involved in the biodegradation of plant wastes. Several bacteria are able to grow on these compounds as the sole carbon source as was shown for *Pseudomonas putida* WCS358 (*8*), for *Acinetobacter* (*9*), and for *Pseudomonas fluorescens* (*10*). In all of these bacteria, *p*-coumaric acid and ferulic acid are converted to *p*-hydrobenzoic acid and vanillic acid, respectively, which in turn are transformed into protocatechuic acid and integrated into the tricarboxylic acid cycle via the $\beta$-ketoadipate pathway.

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10.1021/jf026224d CCC: $25.00 © 2003 American Chemical Society
Published on Web 07/12/2003

**Table 1.** Microbial Strains and Plasmids Used in This Study

| strains and plasmids | genotypes | source/ref |
|---|---|---|
| *S. cerevisiae* strains | | |
| Σ1278b | *MAT*α, *ura3* | Liu et al., 1993 (*18*) |
| WE 372 | commercial diploid strain | Anchor Yeast Technologies (SA) |
| VIN13 | commercial diploid strain | Anchor Yeast Technologies (SA) |
| Σ1278b[YPADC] | *MAT*α, *ura3* :: $PGK1_{PT}$ *padc* | this study |
| Σ1278b[YPDC] | *MAT*α, *ura3* :: $PGK1_{PT}$ *pdc* | this study |
| Σ1278b[YPAD1] | *MAT*α, *ura3* :: $PGK1_{PT}$ *PAD1* | this study |
| VIN13[YSPADC] | *MAT*α, *ura3*:: *padc* | this study |
| VIN13[YSPDC] | *MAT*α, *ura3*:: *pdc* | this study |
| VIN13[POF] | *MAT*α, *URA3, PAD1* | this study |
| *Escherichia coli* strain | | |
| DHα | *sup*E44 ρ*lac*U169 (φ*80lacZ*ρM15) *hsd*R17 *rec*A1 *gyr*A96 *thi*–1 *rel*A1 | GIBCO/Bethesda Research Laboratories[a] |
| plasmids | | |
| pHPAD | $Em^R$ Δ*lacZ PADC* | Cavin et al., 1998 (*2*) |
| pJPDC1 | $Em^R$ Δ*lacZ PDC* | Cavin et al., 1997b (*13*) |
| PPGK1 | $Ap^R$ $Tc^R$ *URA3* $PGK1_P$ $PGK1_T$ | Eksteen et al., 2002 (*19*) |
| YIp5 | $Ap^R$ $Tc^R$ *URA3* | Struhl et al., 1979 (*20*) |
| pSH47 | $Ap^R$ *URA3* $GAL1_P$ $CYC1_T$ *CRE* | Güldner et al., 1996 (*15*) |
| 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 (*21*) |
| 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 Dr., Ichinnan Business Park, Paisley PA.

Strains of the yeast *Brettanomyces* are also well-known for their ability to form volatile phenols in wine (*11*). *Brettanomyces*, however, are associated with the unpleasant odor formation of the ethylphenols and are considered in wineries to be spoilage organisms. Phenolic acid is decarboxylated to the corresponding vinylphenol via a carboxylase enzyme. Then, with the action of an oxidoreductase enzyme, the vinylphenols are converted to the corresponding ethylphenols (*4*). These activities are not inhibited by other grape phenolics, resulting in a high conversion of the vinylphenols to ethylphenols by *Brettanomyces*.

Certain microbial phenolic acid decarboxylase enzymes decarboxylate phenolic acids to substituted phenylpropionic acids, and then the microorganism forms 4-vinyl derivatives, which can be reduced to 4-ethyl derivatives. In this regard, the *fdc* gene from *Bacillus pumilus*, *pdc* from *Lactobacillus plantarum*, and *padc* from *Bacillus subtilis* were cloned, characterized, and expressed in *Escherichia coli* (*2*, *12*, *13*). These bacterial enzymes use the same metabolic pathway for the decarboxylation of phenolic acid as that of the phenylacrylic acid decarboxylase (Pad1p) of *S. cerevisiae*.

The strategy used in this study was to clone the *B. subtilis padc* gene, the *L. plantarum pdc* gene, and the *S. cerevisiae PAD1* gene between the constitutive yeast phosphoglyceratekinase I gene promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences and to integrate these gene constructs into the genomes of a laboratory strain of *S. cerevisiae*, Σ1278b. The two bacterial gene constructs were also integrated into the genome of an industrial wine yeast strain, *S. cerevisiae* VIN13. As a control the *PAD1* gene was disrupted in VIN13.

The aim of this study was to determine the effect of the overexpression of heterologous and homologous 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 was to determine the ability of these recombinant yeast strains to influence the aroma of wine.

## MATERIALS AND METHODS

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

**Media.** The *E. coli* DH5α cells were cultured in Luria Bertani (LB) medium (5 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ tryptone, and 10 g $L^{-1}$ sodium chloride). For the selection of *E. coli* transformants, 100 $\mu$g $mL^{-1}$ ampicillin was included in the LB medium. Yeast cells were cultured in yeast peptone dextrose (YPD) medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ peptone, and 20 g $L^{-1}$ glucose). For the selection of yeast transformants, $SC^{-Ura}$ medium (6.7 g $L^{-1}$ yeast nitrogen base without amino acids, 20 g $L^{-1}$ glucose, and 8.3 g $L^{-1}$ of each of the growth factors Leu, His, and Trp) was used to provide auxotrophic pressure. Solid media contained 20 g $L^{-1}$ agar. Bacteria and yeasts were cultured at 37 and 30 °C, respectively.

**Cloning and Sequencing Procedures.** The Polymerase Chain Reaction (PCR) method was used for the isolation of the *padc*, *pdc*, and *PAD1* genes. Primers (**Table 2**) were synthesized by Integrated DNA Technologies (Coralville, IA). The *padc* and *pdc* genes were amplified from pHPAD and pPDC1, respectively (*2*, *13*). The genomic DNA from a commecial red wine yeast, *S. cerevisiae* WE372, was used as template for *PAD1* amplification. Using a TRIO-Thermoblock (Biometra) cycler, 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 s (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 (Roche Diagnostics, Mannheim, Germany). The reaction mixture that was used contained 1.25 mM nucleotides (dNTPs), 2 ng $\mu L^{-1}$ template, 0.3 $\mu$M of each primer, and 0, 2.5, or 5 $\mu$L of 25 mM $Mg^{2+}$. The PCR products were purified and underwent restriction digests for preparation for the cloning process.

The plasmid pEG6 (a modified version of pUG6 where the *ENO1* promoter was inserted instead of the *Ptef*) was used as a template for

**Table 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′-ATCAACTCTATTAGTAGTTGAGTAACGTAATAAAATGCTCAGAAAAAGTCCAACTAGTGGATCTGATATCA-3′ | |
| $pPOF_L$ | 5′-TGATTTCAATCTACGGAGTCCAACGCATTGAGCAGCTTCAATTGAGTAGATCTTCGTACGCTGCAGGTCGAC-3′ | |

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 (geneticin resistance) was obtained with two flanking regions with homology outside the *PAD1* gene. 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.

Standard methods were used for the restriction, isolation, purification, and ligation of DNA, plasmid transformation into *E. coli*, and agarose−gel electrophoresis (*14*). T4 DNA ligase and all the restriction endonucleases were purchased from Roche Diagnostics.

After PCR amplification, *PAD1* and *pdc* were digested with *Eco*RI and *Xho*I and inserted into the $PGK1_{PT}$ cassette of pPGK1. The *padc* gene was digested with *Bgl*II and *Xho*I for insertion into the $PGK1_{PT}$ cassette of pPGK1. Plasmid YEp352 was also digested with *Eco*RI and *Xho*I and with *Bgl*II and *Xho*I for cloning of the *PAD1*, *padc*, and *pdc* genes to yield plasmids YEpPADC, YEpPDC, and YEpPAD1, respectively (**Figure 1**).

All three $PGK1_{PT}$ cassettes containing the three different decarboxylase genes were excised from YEpPADC, YEpPDC, and YEpPAD1 with *Hin*dIII and run through an agarose gel for DNA isolations and cloning into YIp5 integrational plasmid after *Hin*dIII linearization. The following integration plasmids were obtained: YPADC, YPDC, and YPAD1 (**Figure 1**).

The *SMR1-410* gene of *S. cerevisiae*, which codes for sulfometuron-methyl (SMM) resistance, was isolated from plasmid pDLG31 as a *Bam*HI fragment. Plasmids YPADC and YPDC were also linearized with *Bam*HI, and the *SMR1-410* gene was inserted into both of these plasmids, thereby generating plasmids YSPADC and YSPDC (**Figure 1**).

The final constructs were verified by sequence analysis using ABI PRISM Big Dye Terminator cycle sequencing ready reaction kits with an ABI PRISM 377 DNA sequencer (PE/Applied Biosycstems). Both the coding and noncoding strands were sequenced to ensure the reliable identification of all constructs.

**Transformation into *S. cerevisiae* Σ1278b and VIN13.** Standard methods for yeast transformation were used (*14*). Plasmids YPADC, YPDC, and YPAD1 were linearized with *Nco*I (a unique restriction site within the *URA3* locus) to yield flanking *URA3* regions. Linearized plasmids were transformed into *S. cerevisiae* Σ1278b and selected on $SC^{-Ura}$ plates. Similarly, YSPADC and YSPDC were also linearized with *Nco*I in the *URA3* gene and transformed into VIN13. Transformants were selected on SC plates containing 80 $\mu$g mL$^{-1}$ SMM.

The POF PCR product (*PAD1-loxP-kan$^R$-loxP-PAD1*) was used to disrupt one of the *PAD1* alleles in the diploid wine yeast strain, VIN13, and transformants were selected on YPD agar plates containing geneticin (400 mg L$^{-1}$). The plasmid pSH47 (*15*) was then transformed into this VIN13 strain containing the single Δ*pad1* deletion 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 $kan^R$ marker gene. Selection was done on SC plates containing 80 $\mu$g L$^{-1}$ SMM and confirmed by testing on YPD media containing 400 mg L$^{-1}$ geneticin. The POF PCR product was then retransformed to disrupt the second allele of *PAD1* in VIN13[Δ*pad1*], thereby generating a VIN13 strain in which both *PAD1* alleles were deleted. This VIN13 strain carrying the double Δ*pad1* deletion was used as one of the control strains during microvinification trials and enzyme assays.

**Southern Blot Hybridization.** Standard methods were used for yeast genomic DNA isolations (*14*). The digoxigenin nonradioactive nucleic acid labeling and detection system was used to perform the Southern hybridization for the detection of integration in the different Σ1278b and VIN13 strains. The DIG labeling kit from Roche Biochemical Products (South Africa) was used.

For confirming the integration of the various plasmids in strains (1278b[YPADC], (1278b[YPDC], (1278b[YPAD1] VIN13[YSPADC], and VIN13[YSPDC], the genomes of these yeast transformants were cut once inside the respective decarboxylase gene cassettes and once in the genomes just outside the URA3 locus. For these experiments, the following enzymes were used, respectively: *Nsi*I; *Nsi*I and *Spe*I; *Nsi*I, *Nsi*I, *Nsi*I, and *Spe*I. For VIN13[POF], the enzymes *Sna*BI and *Cla*I were used to cut once in the geneticin marker and once in the genome. The probes used for the various Southern blots were *padc*, *pdc*, *PAD1* gene DNA, and the geneticin gene, respectively. On all of these Southern blots two labeled DNA fragments were detected.

**Enzymatic Assays.** Protein extractions were performed on cells grown to an optical density at 600 nm ($OD_{600}$) 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 resuspended in 50 mM Tris (pH 7.5)/10 mM NaCl. After 0.1 g of 0.2 mm glass beads had been added, the mixture was vortexed for 3 min and spun down at 6000 rpm for 2 min. 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 *p*-coumaric acid was added with the extracted proteins. After 24 h, the reactions were stopped by diluting them 16-fold with 20 mM Tris-HCl/3 g L$^{-1}$ sodium dodecyl sulfate (pH 6). The method for the detection of decarboxylase activity has previously been described (*16*, *17*). Briefly, *p*-coumaric acid decarboxylation can be detected spectrophotometrically due to the loss of the double bonds. This leads to a hypochrome UV spectrum displacement (*16*). *p*-Coumaric acid shows a peak at 285 nm, and the vinyl derivatives show a peak at 255 nm (*17*). Detection of the disappearance of the 285-nm peak and formation of a 255-nm peak indicate the degradation of *p*-coumaric acid and the formation of the corresponding vinyl derivatives.

**Microvinification.** Weisser Riesling juice was used to perform microvinifications with recombinant laboratory strains. The juice had a sugar content of 230 g L$^{-1}$ (23 °Balling) and a pH of 3.4. Sulfur dioxide (30 mg L$^{-1}$) was added to avoid the risk of indigenous yeast and bacteria growth. Half the juice was complemented with 0.04 mM *p*-coumaric acid, ferulic acid, and caffeic acid, and the other half was used without complementation. A total volume of 150 mL of juice was used for each microvinification. The strains (1278b[YPADC], (1278b[YPDC], and (1278b[YPAD1] were inoculated to 3 × 106 cells mL$^{-1}$ after the overnight culture in YPD was washed with distilled water. All microvinifications were repeated three times, along with a (1278b strain as control. Fermentations were done over a period of 2 weeks, after which cells were collected and samples were taken for gas chromatographic (GC) analysis.

For the vinifications with the industrial strains, the same Weisser Riesling juice was used, along with Chardonnay juice with a sugar concentration of 220 g L$^{-1}$ (22 °Balling) and a pH of 3.3. The VIN13-[YSPADC], VIN13[YSPDC], and VIN13[POF] strains were inoculated into 1 L of Chardonnay juice and Riesling juice. Untransformed VIN13 was used as a control strain. All samples were fermented in duplicate

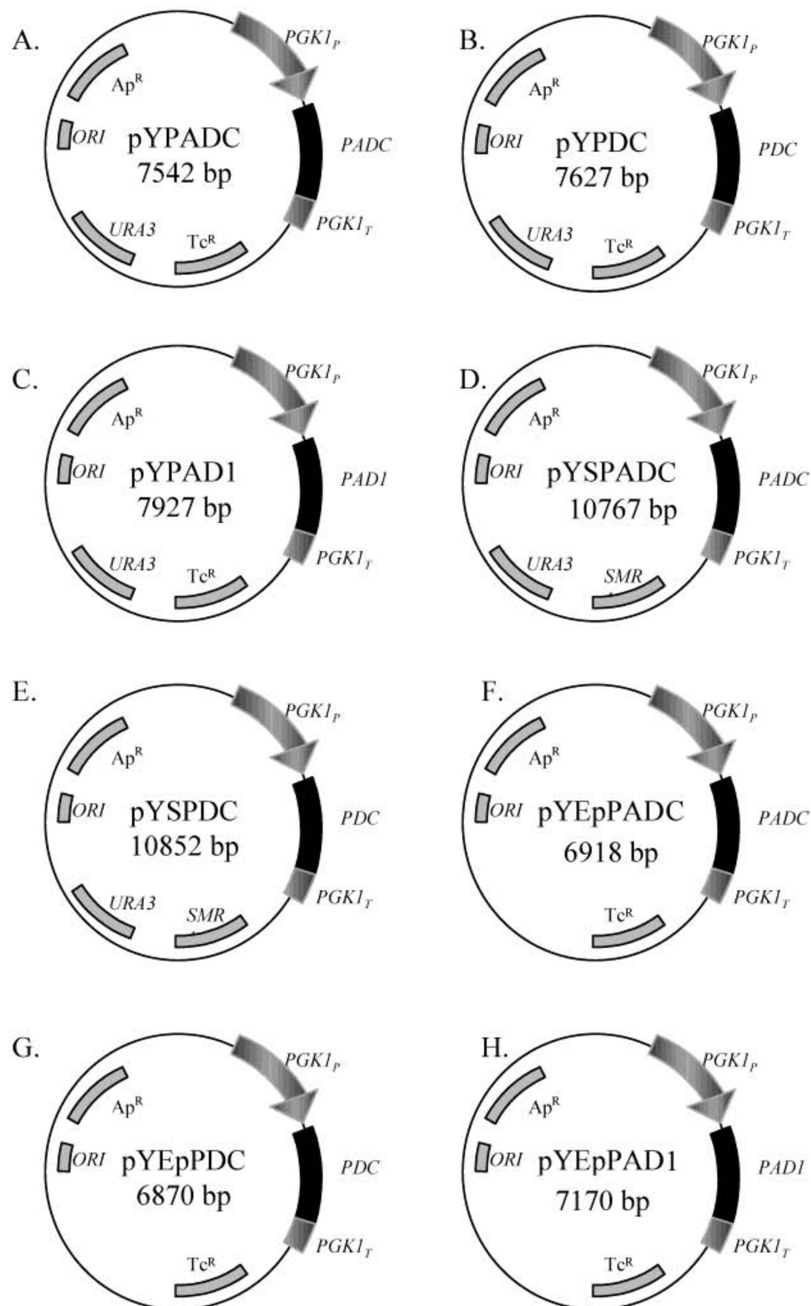


**Figure 1.** Plasmids constructed during this study.

at 15 °C. The juice was fermented until the sugar concentration was below 4 g $L^{-1}$ and sampled for GC analysis.

**Gas Chromatography.** The following apparatus and reagents were used for the detection of volatile phenols: The pure chromatographic reagent standards of analytical quality were obtained from Fluka and Aldrich. Synthetic wine solutions [a semisweet Chenin Blanc (ethanol 10% v/v)] were used as background matrix in which calibrations were done. The synthetic wine solutions were spiked with each of the four volatile phenols, so that their concentrations were 1 mg $L^{-1}$ each. 1,1,2-Trichloro-1,2,2-trifluoroethane (Freon 113) of HPLC quality was obtained from Aldrich.

The volumetric material included calibrated Hamilton syringes with volumes of 10 and 100 $\mu$L. Centrifuge glass tubes with conical bottoms (12 cm × 17 o.d.) and glass stoppers were used as extraction tubes. A labinco rotary mixer was used as a mechanical shaker. An HP 6890 series gas chromatograph, fitted with an MSD, fitted with a splitless injector, and an automatic sampler 7673 were used to analyze the samples. The GC was fitted with a Supelco HP-5MS (HP 19091S-433) column of 30 m × 0.25 mm i.d., 0.25 $\mu$m film thickness. The initial flow was 1.7 mL $min^{-1}$ and the average velocity 48 cm $s^{-1}$. A constant flow was maintained during analyses. Chromatographic conditions entailed the following: He as carrier gas; head pressure of 99.5 kPa; total flow of 11.8 mL $min^{-1}$; purge flow of 7.4 mL $min^{-1}$; purge time of 0.5 min; injector (splitless) 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 $min^{-1}$, then to 160 at 5 °C $min^{-1}$, and then to 220 °C at 10 °C $min^{-1}$ and held for 10 min; injected volume of 2 $\mu$L. The data were recorded and processed by Hewlett-Packard Chemstation software (tune file and acquisition mode: stune.u in scan). The thermal auxiliary entailed an MSD transfer temperature for 280 °C. A solvent delay of 3.9 min was applied.

The following method was used: 10 mL of wine was introduced into the extraction tube, and 200 $\mu$L of Freon 113 was added as extracting agent, as well as 2 $\mu$L of a solution of 2,6-dimethylheptenol (400 mg $L^{-1}$ in ethanol as internal standard). NaCl (1.2 g) was also added. The tubes were capped and shaken for 30 min in an automatic shaker at maximium speed. The tubes were centrifuged (3000 rpm for 5 min), and the organic phase was recovered with a Pasteur pipet,

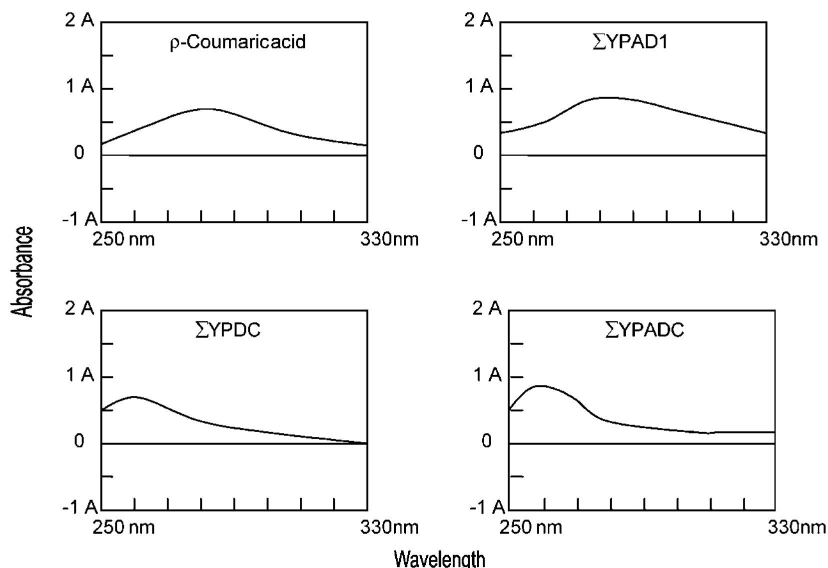


**Figure 2.** Absorption peaks for *p*-coumaric acid degradation by the cell crude extracts of Σ1278b[YPAD1], Σ1278b[YPDC], and Σ1278b[YPADC] strains buffered at pH 6: 255-nm peak, volatile phenol derivatives; 285-nm peak, *p*-coumaric acid.

**Table 3.** GC Analysis of the Microvinifications by Laboratory Strains

| | 4-vinylphenol ($\mu$g L$^{-1}$) | | 4-vinylguaiacol ($\mu$g L$^{-1}$) | |
|---|---|---|---|---|
| | uncomplemented Riesling juice | complemented juice (0.04 mM) | uncomplemented Riesling juice | complemented juice (0.04 mM) |
| Σ1278b (control) | 81 | 1795 | 52 | 1683 |
| Σ1278b[YPADC] | 142 | 2869 | 98 | 2526 |
| Σ1278b[YPDC] | 149 | 2951 | 154 | 2643 |
| Σ1278b[YPAD1] | 93 | 2355 | 68 | 1636 |

transferred over 50 mg of $Na_2SO_4$ into an HP 2 mL vial with a 200 $\mu$L glass insert, and analyzed under the chromatographic 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.

For the calibration graph, five calibration points were prepared by diluting the above-mentioned synthetic wine solution 1:100, 1:50, 1:20, 1:10, and 1:2. These diluted wine solutions were extracted, and the calibration graphs were drawn with the GC results. The target ions used to calibrate each compound were as follows: 4-ethylphenol, 107, 122, and 77; 4-vinylphenol, 120, 91, and 65; 4-ethylguaiacol, 137 and 152; 4-vinylguaiacol, 150, 135, and 107. A plot representing chromatographic height versus concentration in wine was made for each compound, using the five calibration points.

## RESULTS

**Expression of Homologous and Heterologous Phenolic Decarboxylation Genes in Laboratory and Industrial Yeast Strains.** The aim of the study was to determine the effect of overexpression of the *padc* (*B. subtilis*), *pdc* (*L. plantarum*), and *PAD1* (*S. cerevisiae*) genes on the levels of 4-ethyl- and 4-vinylphenols in wines. As a control, a disruption cassette for the deletion of the *PAD1* gene was also constructed. The *padc*, *pdc*, and *PAD1* were successfully cloned by PCR amplification into pPGK1 to yield plasmids YEpPADC, YEpPDC, and YEpPAD1 (**Figure 1**), which were sent for sequencing to confirm correct sequences. The $PGK1_P$-*padc*-$PGK1_T$, $PGK1_P$-*pdc*-$PGK1_T$, and $PGK1_P$-*PAD1*-$PGK1_T$ gene cassettes were cloned into YIp5 and transformed into *S. cerevisiae* Σ1278b (Σ1278b[YPADC], Σ1278b[YPDC], and Σ1278b[YPAD1]) (**Figure 1**). Positive transformants were isolated from the $SC^{-Ura}$ medium, and integration was confirmed by Southern blotting (data not shown).

The PCR product obtained for the disruption of VIN13, the *PAD1* gene, 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. VIN13[YSPADC] and VIN13[YSPDC] were also confirmed to contain the *padc* and *pdc* genes, respectively. Integration of the VIN13[POF] disruption was confirmed through Southern blotting.

**Enzymatic Assays.** The crude cell extracted proteins of the Σ1278b[YPADC] and Σ1278b[YPDC] strains showed enzymatic activity responsible for a steady decrease in *p*-coumaric acid. Although the Σ1278b control and the Σ1278b[YPAD1] strains also showed a decrease in *p*-coumaric acid, the decrease was much smaller than that shown for the overexpressed bacterial enzymes. The readings from the spectrophotometer are shown in **Figure 2**.

**Microvinifications and Industrial Fermentations.** GC analysis of the microvinifications detects a 1.7-fold increase in the production of 4-vinylphenol by the Σ1278b[YPADC] and Σ1278b[YPDC] strains, in comparison to the control and the Σ1278b[YPAD1] strain (**Table 3**). For the production of 4-vinylguaiacol a 1.6-fold increase was observed with the Σ1278b[YPADC] strain and a 2.6-fold increase with the Σ1278b[YPDC] strain.

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

**Table 4.** GC Analysis Results of Volatile Phenols Formed during Vinifications by Industrial Yeast Transformants

| wine | strain | 4-vinylphenol ($\mu$g L$^{-1}$) | 4-ethylphenol ($\mu$g L$^{-1}$) | 4-vinylguaiacol ($\mu$g L$^{-1}$) |
|---|---|---|---|---|
| Riesling | VIN13 | 3400 | 1089 | 51.11 |
| | VIN13[YSPADC] | 3879 | 1139 | 80.28 |
| | VIN13[YSPDC] | 3462 | 1107 | 68.02 |
| | VIN13[POF] | 2912 | 1131 | ND[a] |
| Chardonnay | VIN13 | 5712 | 1194 | 61.22 |
| | VIN13[YSPADC] | 6795 | 1235 | 60.81 |
| | VIN13[YSPDC] | 5970 | 1167 | 60.63 |
| | VIN13[POF] | 5500 | 1117 | 53.02 |

[a] Not detected.

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

## DISCUSSION

From these results it is clear that the use of the recombinant strains of *S. cerevisiae* containing the *padc* and *pdc* genes show a definite increase in the utilization of phenolic acids when compared to the parent strains without the *padc* and *pdc* genes. However, the overexpressed yeast *PAD1* gene does not significantly improve the functioning of the Pad1p enzyme. In accordance with previous findings (*7*), this might indicate that post-transcriptional regulation might be critical for the functioning of Pad1p. The enzymatic assay results illustrate the decarboxylation of *p*-coumaric acid (a peak at 285 nm for *p*-coumaric acid) and the formation of volatile phenols. The second graph in **Figure 2** shows the same peak for the assay done on *p*-coumaric acid decarboxylation in the presence of the Pad1p enzyme expressed by Σ1278b. No peak was detected at 255 nm. This indicates that *p*-coumaric acid was not degraded in the presence of Pad1p. The third and fourth graphs in **Figure 2** indicate the decarboxylation of *p*-coumaric acid in the presence of Pdcp and Padcp, the two bacterial decarboxylase enzymes expressed in yeast. These graphs show 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 *p*-coumaric acid to its corresponding vinyl derivatives.

When the wines made by the recombinant strains were taken into consideration, 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 with the control and the Σ1278b[YPAD1] strain, there was a 1.7-fold increase for 4-vinylphenol by the Σ1278b-[YPADC] and Σ1278b[YPDC] strains and for 4-vinylguaiacol a 1.6-fold increase by the Σ1278b[YPADC] strain and a 2.6-fold increase by the Σ1278b[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 of 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$^{-1}$ and that for the Chardonnay wines between 5 and 7 mg L$^{-1}$. As the aroma can be perceived negatively at 4 mg L$^{-1}$ and the ideal concentration is $\sim$2.2 mg L$^{-1}$, the overproduction of 4-vinylphenol might be too extensive for aroma improvement (*5*, *6*). 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.

From all of the results, it is clear that the recombinant 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 in decarboxylating phenolic acids to their corresponding 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* (VIN13[POF]) indicate that there is still some decarboxylase activity in this recombinant strain. It is tempting to speculate that the industrial strain was aneuploid and that not all of the copies of the gene were disrupted or the yeast may have other enzymatic activities involved in phenolic acid metabolism (*22*). To obtain a clearer picture of the decarboxylation enzymes active in the industrial wine yeast strains, it is recommended that the concentration of phenolic acids (the precursors) present in the grape juice be measured. 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 due to the decarboxylase enzyme activities.

In conclusion, this study offers prospects for the development of new wine yeast strains for the production of optimal levels of volatile phenols during alcoholic fermentation, thereby improving the sensorial quality of wine.

## ACKNOWLEDGMENT

Our sincere gratitude is given to Méshelle Gey van Pittius for the GC analysis and to J.-F. Cavin for providing us with pHPAD and pPDC1.

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**Received for review December 17, 2002. Revised manuscript received March 27, 2003. Accepted April 23, 2003. Our sincere gratitude is given to the National Research Foundation (NRF) and the South African wine industry (Winetech) for financial support.**

JF026224D