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ORIGINAL ARTICLE

The heterologous expression of polysaccharidase-encoding genes with oenological relevance in *Saccharomyces cerevisiae*

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glucanase, pectinase, pectate lyase, polygalacturonase, wine, xylanase, yeast.

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Abstract

Aims: The main objective of this study was to develop polysaccharide-degrading wine strains of *Saccharomyces cerevisiae*, which are able to improve aspects of wine processing and clarification, as well as colour extraction and stabilization during winemaking.

Methods and Results: Two yeast expression/secretion gene cassettes were constructed, namely (i) a pectinase gene cassette (pPPK) consisting of the endo-polygalacturonase gene (*pelE*) from *Erwinia chrysanthemi* and the pectate lyase gene (*peh1*) from *Erwinia carotovora* and (ii) a glucanase/xylanase gene cassette (pEXS) containing the endo-β-1,4-glucanase gene (*end1*) from *Butyrivibrio fibrisolvens* and the endo-β-1,4-xylanase gene (*xynC*) from *Aspergillus niger*. The commercial wine yeast strain, VIN13, was transformed separately with these two gene cassettes and checked for the production of pectinase, glucanase and xylanase activities. Pinot Noir, Cinsaut and Muscat d'Alexandria grape juices were fermented using the VIN13[pPPK] pectinase- and the VIN13[pEXS] glucanase/xylanase-producing transformants. Chemical analyses of the resultant wines indicated that (i) the pectinase-producing strain caused a decrease in the concentration of phenolic compounds in Pinot Noir whereas the glucanase/xylanase-producing strain caused an increase in phenolic compounds presumably because of the degradation of the grape skins; (ii) the glucanase/xylanase-producing strain caused a decrease in wine turbidity, especially in Pinot Noir wine, as well as a clear increase in colour intensity and (iii) in the Muscat d'Alexandria and Cinsaut wines, the differences between the control wines (fermented with the untransformed VIN3 strain) and the wines produced by the two transformed strains were less prominent showing that the effect of these polysaccharide-degrading enzymes is cultivar-dependent.

Conclusions: The recombinant wine yeasts producing pectinase, glucanase and xylanase activities during the fermentation of Pinot Noir, Cinsaut and Muscat d'Alexandria grape juice altered the chemical composition of the resultant wines in a way that such yeasts could potentially be used to improve the clarity, colour intensity and stability and aroma of wine.

Significance and Impact of the Study: Aspects of commercial-scale wine processing and clarification, colour extraction and stabilization, and aroma enhancement could potentially be improved by the use of polysaccharide-degrading wine yeasts without the addition of expensive commercial enzyme preparations. This offers the potential to further improve the price : quality ratio of wine according to consumer expectations.

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Introduction

Originally, all wines were made by utilizing the natural microflora present on the grape berries for spontaneous fermentation. Various yeasts found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural wine fermentations. While the controlled growth of these indigenous species might in some circumstances enhance the chemical complexity and sensory quality of wines, there are other circumstances in which their contribution might result in spoilage (Fleet 1992). Today, most winemakers use pure *Saccharomyces cerevisiae* cultures to produce wine of reproducible quality. This microbiological simplification of the wine fermentation process has paved the way for the development and use of specialized wine yeasts tailored to achieve better outcomes for producers and consumers alike.

In wine production, maceration refers to the breakdown of grape solids following crushing of the grape berries and is always included in the initial phase of red wine production. The rupture of grape cells and consequent release of enzymes facilitates the liberation and solubilization of compounds such as phenolic derivatives and glycosidic precursors bound to the cells of the skin, flesh and seeds (Fernández-Zurbano *et al.* 1999). Properly conducted maceration can thus enhance wine quality as phenolic compounds (e.g. anthocyanins and tannins) are major components of red wine colour (Wightman *et al.* 1997), and the release of glycosidic precursors increase wine aroma upon their subsequent hydrolysis by glycosidase activities (Ganga *et al.* 1999).

Maceration plays a definitive role in the production of wine (for a review see Van Rensburg and Pretorius 2000). The enzymes responsible for maceration not only originate from the grape itself, but also from yeasts and other grape juice-related micro-organisms. Winemakers also reinforce and extend the action of these endogenous enzymes by the use of exogenous, industrial enzyme preparations (Fleet 1993).

Polysaccharides can influence the clarification and stabilization of must and wine. Polysaccharides, found in wines at levels between 300 and 1000 mg l^{-1}, can originate in the grape itself, fungi on the grape and/or micro-organisms present during the winemaking process (Whitaker 1990). The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, β-glucans and, to a lesser extent, hemicellulose (mainly xylans). Industrial enzyme preparations used to degrade these polysaccharides play an important role in improving the juice yields, the release of colour and flavour components, and improving the clarification and filtration of wine (Colagrande *et al.* 1994). Most of the commercial pectinase and glucanase preparations currently available are derived from *Aspergillus* and *Trichoderma* strains, respectively (Canal-Llauberes 1993).

The addition of commercial enzyme preparations during winemaking can be quite expensive and could contain impurities, which might adversely affect the quality of wine. Therefore, researchers from several laboratories around the world have searched for natural strains of *S. cerevisiae*-producing enzymes with oenological relevance or for genetic mechanisms by which the enzymatic activities of wine yeasts could be broadened (for reviews see Pretorius 1997, 2000; Van Rensburg and Pretorius 2000; Pretorius and Bauer 2002; Pretorius and Høj 2005; Pretorius 2006).

In this paper, we describe the construction of two recombinant wine yeast strains. In the first instance, endo-β-1,4-glucanase gene (*end1*) from *Butyrivibrio fibrisolvens* together with the endo-β-1,4-xylanase gene (*xynC*) from *Aspergillus niger* was integrated into the genome of a commercial wine yeast, *S. cerevisiae* VIN13. In the second instance, the pectate lyase-encoding gene (*pelE*) from *Erwinia chrysanthemi* and the polygalacturonase-encoding gene (*peh1*) from *Erwinia carotovora* subsp. *carotovora* was integrated into the genome of VIN13. The glucanase and pectinase genes were fused in-frame downstream of the *S. cerevisiae ADH1* promoter and *Mfα1* secretion sequence and upstream of the *TRP5* terminator. The xylanase gene, including its native signal sequence, was cloned between the *ADH1* promoter and terminator sequences. The aim of this study was to investigate whether these polysaccharide-degrading VIN13 wine yeast strains were able to improve aspects of wine processing and clarification, as well as colour extraction and stabilization during winemaking.

Materials and methods

Microbial strains and growth conditions

The plasmids and microbial strains used in this study are listed with their sources and relevant genotypes in Table 1.

Ampicillin-resistance (Ap^R) transformants of *Escherichia coli* were grown in Luria Bertani broth (Sambrook *et al.* 1989) supplemented with ampicillin at a concentration of 100 μg ml^{-1}. *Saccharomyces cerevisiae*, transformed with the plasmid pEXS, containing the *SMR1* (sulfometuron methyl resistance; Sm^R) marker gene and the glucanase (*end1*) and xylanase (*xyn*C) genes, were grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) supplemented with concentrations of sulfometuron methyl ranging between 50 and 100 μg ml^{-1}. *Saccharomyces cerevisiae* transformed with the plasmid pPPK containing the

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Table 1 Microbial strains and plasmids used in this study

Strain and plasmid	Genotype/description	Source/reference
Escherichia coli		
DH5α	*supE44 placU169* (φ80*lacZ*ρ*M15*) *hsdR17 recA1 gyrA96 thi-1 relA1*	GIBCO/Bethesda†
Saccharomyces cerevisiae		
VIN13	commercial diploid strain	Anchor Yeast Technologies, South Africa
VIN13-EXS	*MATα, ura3 :: ADH1$_P$ MFα1$_S$ TRP5$_T$ end1 ADH2$_{PT}$ xynC*	This study
VIN13-PPK	*MATα, ura3 :: ADH1$_P$ MFα1$_S$ TRP5$_T$ pelE ADH1$_P$ MFα1$_S$ TRP5$_T$ peh1*	This study
Plasmids		
YIp5	*bla URA3*	Struhl *et al.* (1979)
pUG6	*bla KanMX*	Güldener *et al.* (1996)
pDLG31	*bla PGK1$_P$-LKA1-PGK1$_T$-SMR1*	Gundllapalli Moses *et al.* (2002)
pAR5	*bla KanMX ADH1$_P$-MFα1$_S$-end1-TRP5$_T$ ADH1$_P$-MFα1$_S$-pelE-TRP5$_T$ ADH1$_P$-MFα1$_S$-peh1-TRP5$_T$ LEU2*	Van Rensburg *et al.* (1994)
pEX	*bla ADH1$_P$-MFα1$_S$-end1-TRP5$_T$ ADH2$_P$-xynC-ADH2$_T$-URA3*	Petersen *et al.* (1998)
YIp5S	*bla SMR1 URA3*	This study
pK	*bla KanMX URA3*	This study
pEXS	*bla ADH1$_P$-MFα1$_S$-end1-TRP5$_T$ ADH2$_P$-xynC-ADH2$_T$-SMR1 URA3*	This study
pPPK	*bla ADH1$_P$-MFα1$_S$-pelE-TRP5$_T$ ADH1$_P$-MFα1$_S$-peh1-TRP5$_T$ KanMX URA3*	This study

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KanMX (geneticin resistance; Gt^R) marker gene and the pectinase genes (*peh1* and the *pelE*) were grown in YPD with concentrations of geneticin ranging between 100 and 400 mg ml^{-1}. Solid media contained 2% agar. Bacteria and yeast were grown at 37°C and 30°C, respectively.

DNA manipulations and plasmid construction

Standard methods for manipulating and subcloning of DNA fragments, plasmid DNA isolations, transformation of *E. coli* DH5α and yeast cell electroporation were used (Sambrook *et al.* 1989; Ausubel *et al.* 1996). DNA amplification by the polymerase chain reaction (PCR) technique and Southern blot hybridization were performed as described by Laing and Pretorius (1992) and Sambrook *et al.* (1989).

The sulfometuron methyl gene (*SMR1*) was cloned as a *Bam*HI DNA fragment from plasmid pDLG31 and ligated into the *Bam*HI site of the yeast integrating plasmid YIp5. The *SMR1* gene was cloned as a 3·2-kb *Eco*RV DNA fragment from plasmid YIp5(SMR1) and ligated into the *Sma*I site of plasmid pEX (Petersen *et al.* 1998), resulting in plasmid pEXS (Fig. 1a).

For the construction of plasmid pPPK, the yeast integrating plasmid YIp5 was used as base plasmid. Plasmid, pUG6, was digested with *Pvu*II and *Eco*RV to obtain the 1·5-kb *KanMX* gene. This *Pvu*II–*Eco*RV DNA fragment

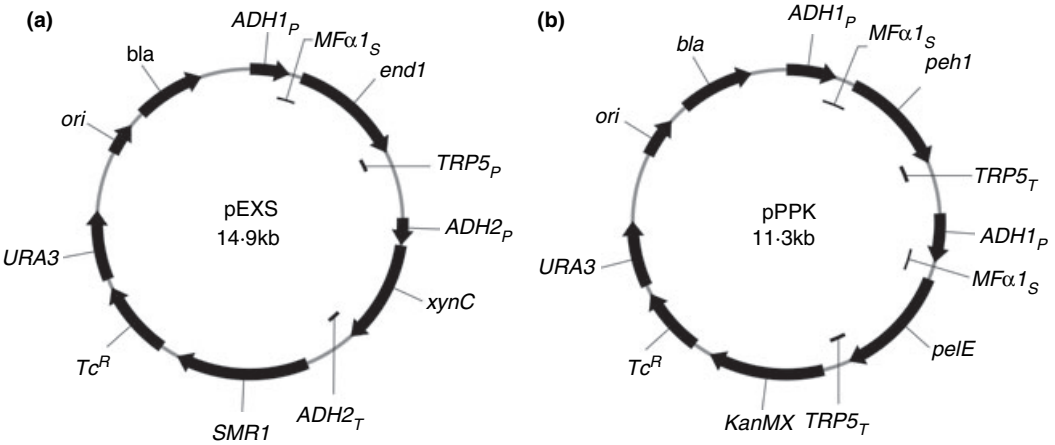

Figure 1 Schematic representation of recombinant plasmids pEXS (a) and pPPK (b).

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was then integrated into the *Pvu*II site of YIp5, generating plasmid pK. The 4·3-kb DNA fragment containing the pectate lyase gene (*pelE*) and polygalacturonase gene (*peh1*) was cut from plasmid pAR5 with *Not*I, filled up to produce blunt ends and ligated into the *Sma*I site of pK, resulting in plasmid pPPK (Fig. 1b).

Plate assays

Pectinase activity was determined as follows: *Saccharomyces cerevisiae* transformants were grown in 10 ml YPD for 24 h. Ten microlitre aliquot of the preculture was plated onto polygalacturonic acid agarose diffusion plates (0·1 mol l^{-1} citrate, 0·2 mol l^{-1} Na_2HPO_4, 0·5% ammonium oxalate, 1% type II agarose and 0·01% polygalacturonic acid; pH adjusted to 3·5). After overnight incubation at 25°C, the plates were stained with 0·02% (w/v) ruthenium red for 60 min at 37°C and the cells gently washed off with water. Transformants with pectinase activity showed clear zones around the colony.

Glucanase activity was tested by spotting the transformants onto YPD plates containing 0·1% (w/v) barley β-glucan (Sigma-Aldrich, St Louis, MO, USA) or 0·4% (w/v) lichenan (Sigma-Aldrich) plates. The plates were incubated for 3–5 days at 37°C before staining with 0·03% (w/v) Congo red (Teather and Wood 1982). After destaining with water, positive transformants showed clear zones around the colonies.

Transformants were screened for xylanase activity by spotting the transformants onto YPD plates containing 0·2% 4-*O*-methyl-D-glucurono-D-xylan-remazol brilliant blue (RBB)-xylan (Sigma) (Biely 1985). Xylanase cleaves RBB-xylan into a colourless product.

Microvinification

All fermentations were carried out in triplicate. The varieties used were Pinot Noir, Cinsaut and Muscat d'Alexandria. Grapes of each variety (20 kg) were destemmed and crushed, 40 p.p.m. of SO_2 was added, and inoculated with an actively growing yeast culture to a final concentration of $1–2 \times 10^6$ cells ml^{-1} for each sample. As the grape must was not sterilized before inoculation with the control strain (VIN13) and two transformants (VIN13-EXS and VIN13-PPK), standard procedures were used to verify the dominance of these strains in the population of yeast cells at the end of fermentation. These strain verification procedures included (i) replica plating of yeast colonies (obtained from postfermentation samples) from a nonselective agar medium (YPD) onto a selective medium containing either sulfometuron methyl (for VIN13-EXS identification) or geneticin (for VIN13-PPK) and (ii) the amplification of the glucanase, xylanase and pectinase gene constructs (Fig. 1) from the Sm^R (VIN13-EXS) and Gt^R (VIN13-PPK) colonies.

The must of the red varieties (Pinot Noir and Cinsaut) was fermented at 25°C, and white must (Muscat d'Alexandria) at 15°C. Samples (20 ml) were collected at four stages of fermentation: (i) at the beginning of fermentation; (ii) at the end of fermentation; (iii) after press and (iv) after filtration from Cinsaut and Pinot Noir for colour and phenolic testing. This was done by measuring the absorption of the samples at different wavelengths (280, 420 and 520 nm) (Zoecklein *et al.* 1995). The wines made from Muscat d'Alexandria were tested after filtration for total hydroxycinnamates and total phenolics. The total volumes of free-flow wine and pressed wine were determined for both Cinsaut and Pinot Noir. Free-flow wine was collected from the press before any pressure was applied to the skins; this was followed by the collection of the press wine when the skins were pressed. The volume of free-flow and press wine was measured in a measuring flask and together were measured as total wine yield. The turbidity of the wine was measured with a turbidimeter (Nephelometer) in NTU.

Determination of the chemical composition of wines

The concentrations of ethanol (%v/v), reducing sugar, pH, titratable acidity, volatile acidity, malic acid and lactic acid in the finished wines were determined by using standard methods described by Iland *et al.* (2000).

Gas–liquid chromatography

The following apparatus and chromatographic conditions were used for the detections: an HP 6890 series gas chromatograph fitted with a flame ionization detector (FID), a split-splitless injector, a 7683 automatic sampler and a Supelco SPB5 column (60 m × 0·32 mm internal diameter, 0·25-μm film thickness). Chromatographic conditions entailed the following: helium as carrier gas, head pressure of 140 kPa; total flow of 12·5 ml min^{-1}; purge flow of 7·0 ml min^{-1}; 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^{-1}, then to 160°C at 5°C min^{-1} and then to 220°C at 10°C min^{-1} and held for 10 min; make-up gas N_2 at 30 ml min^{-1}; detector FID, H_2 at 40 ml min^{-1}; air, 450 ml min^{-1}; injected volume, 2 μl.

The following method was used: 10 ml of wine was introduced into the extraction tube, and 200 μl of Freon 113 (1,1,2-trichloro-1,2,2-trifluoroethane, obtained from Sigma-Aldrich) was added as an extracting agent, as well as 2 μl of a solution of 2,6-dimethylheptenol (400 mg l^{-1} in ethanol as internal standard). NaCl (1·2 g)

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was also added. The tubes were capped and shaken for 30 min in an automatic shaker at maximum speed. The tubes were centrifuged (5 min at 3950 ***g***) and the organic phase was recovered with a Pasteur pipette, then transferred over 50 mg Na_2SO_4 into an HP 2-ml vial with a 200-μl glass insert, and analysed under the chromatographic conditions described above. After the chromatographic analysis, the relative areas or heights of the calibrated peaks were interpolated from calibration graphs created with synthetic wine solutions (ethanol 12% for white wine, 16% for red wine; tartaric acid 6 g l^{-1}; pH 3·2) having an alcohol content similar to that of the analysed wine.

ANOVA was performed to compare the measured chemical composition of the Pinot Noir, Cinsaut and Muscat d'Alexandria wines. For significance tests, a critical level of 5% was used and 95% CI was calculated. This was done using the STATISTICA processing package.

Results

Genetic transformation of the industrial wine yeast strain VIN13

Plasmid pEXS containing the pectinase (*ADH1$_P$-MFα1$_S$-end1-TRP5$_T$*) and xylanase (*ADH1$_P$-xynC-ADH2$_T$*) gene cassettes and *SMR1* marker gene, and plasmid pPPK, containing the pectinase (*ADH1$_P$-MFα1$_S$-peh1-TRP5$_T$* and *ADH1$_P$-MFα1$_S$-pelE-TRP5$_T$*) gene cassettes and *KanMX* marker gene were transformed separately into *S. cerevisiae* VIN13. Plasmid pEXS was integrated into the *Nco*I site of the genomic copy of *URA3* and plasmid pPPK was integrated into the *Stu*I site of the same gene. Integration of these plasmids in these VIN13 transformants was confirmed with Southern blotting (data not shown). The expression of the glucanase/xylanase and pectinase gene cassettes, and production of biologically active glucanase, xylanase and pectinases were confirmed by screening on selective agar plates. Positive transformants were also confirmed by PCR (data not shown).

Microvinification

Microvinification experiments were carried out with the transformed VIN13 yeast strains, VIN13[pEXS] and VIN13[pPPK], and the VIN13 host strain as control. The standard chemical analyses of the wines are shown in Table 2. Spectrophotometric tests were performed at different stages during the fermentation for the determination of the effects of the recombinant yeast on wine colour and phenolic composition of the wine (Table 3).

In the case of Pinot Noir, it is known that the extraction of sufficient colour pigments from the skins tends to be a problem. The VIN13[pEXS] transformant caused an increase in colour density in Pinot Noir wine. Colour density values below six are generally considered to be light-coloured wines; colour densities from six to ten are considered to be medium-coloured wines. Using these general benchmarks, in the first three stages of measurements, the wines made with the VIN13[pEXS] transformant were considered to be medium-coloured wines while the wines made with the VIN13 control strain and the VIN13[pPPK] transformant were considered to be light-coloured wines. Interestingly, more than half of the colour density (50–55%) and total red pigments (50 to 60%) was lost due to filtration. The same effect of filtration was observed with Cinsaut (data not shown). The increase in colour density was nearly double with transformant VIN13[pEXS] compared to the control and VIN13[pPPK] transformant in all four stages tested. This is presumably because of better degradation of the grape skins leading to a higher concentration of colour

Table 2 Chemical analysis of wines

Wine/strain	Ethanol (%, v/v)	Reducing sugar (g l^{-1})	pH	Volatile acid (g l^{-1})	Total acid (g l^{-1})	Malic acid (g l^{-1})	Lactic acid (g l^{-1})
Cinsaut							
VIN13	11·62 ± 0·06	0·57 ± 0·04	3·08 ± 0·02	0·23 ± 0·02	6·43 ± 0·09	2·85 ± 0·21	0·14 ± 0·03
VIN13[PPK]	11·87 ± 0·36	0·48 ± 0·01	3·16 ± 0·02	0·17 ± 0·03	5·62 ± 0·01	2·01 ± 0·07	0·24 ± 0·01
VIN13[EXS]	12·36 ± 0·06	0·59 ± 0·01	3·16 ± 0·01	0·18 ± 0·02	6·15 ± 0·06	2·79 ± 0·01	0·26 ± 0·03
Pinot Noir							
VIN13	10·98 ± 0·13	0·46 ± 0·01	2·95 ± 0·02	0·21 ± 0·02	7·07 ± 0·03	4·16 ± 0·19	0·1 ± 0·01
VIN13[PPK]	10·22 ± 0·06	0·37 ± 0·05	2·96 ± 0·04	0·2 ± 0·01	7·11 ± 0·21	4·51 ± 0·3	0·07 ± 0·01
VIN13[EXS]	11·66 ± 0·11	0·62 ± 0·1	2·95 ± 0·01	0·19 ± 0·02	6·96 ± 0·05	3·35 ± 0·05	0·14 ± 0·01
Muscat d'Alexandria							
VIN13	12·17 ± 0·02	1·83 ± 1·21	3·19 ± 0·02	0·06 ± 0·02	4·9 ± 0·08	2·34 ± 0·02	0·2 ± 0·07
VIN13[PPK]	12·07 ± 0·03	0·36 ± 0·01	3·12 ± 0·01	0·05 ± 0·01	5·11 ± 0·01	2·15 ± 0·01	0·26 ± 0·01
VIN13[EXS]	12·16 ± 0·01	0·52 ± 0·01	3·15 ± 0·01	0·04 ± 0·02	5·04 ± 0·02	2·32 ± 0·01	0·25 ± 0·01

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Table 3 Red wine colour and phenolic measurements of Pinot Noir wines tested at different stages in the winemaking process

Stage	Yeast strain	Colour density	Colour hue	Total red pigments	Degree of red pigment colour	Total phenolics	Modified wine colour density	Modified wine colour hue
Beginning of fermentation	VIN13	5·32 ± 0·59	0·5 ± 0·03	24·34 ± 1·14	14·57 ± 1·99	41·85 ± 4·86	3·72 ± 0·03	0·72 ± 0·02
	pEXS	8·23 ± 0·27	0·5 ± 0·02	34·90 ± 2·93	15·79 ± 2·10	53·87 ± 0·43	5·24 ± 0·52	0·83 ± 0·08
	pPPK	4·46 ± 0·76	0·89 ± 0·03	24·09 ± 4·21	10·06 ± 3·26	38·52 ± 4·00	4·04 ± 0·17	1·20 ± 0·17
Before press	VIN13	6·64 ± 0·21	0·4 ± 0·02	32·47 ± 0·32	14·52 ± 0·79	26·1 ± 2·71	4·65 ± 0·08	0·51 ± 0
	pEXS	9·23 ± 0·54	0·38 ± 0·01	33·14 ± 0·73	20·15 ± 0·64	31·65 ± 1·29	6·19 ± 0·15	0·5 ± 0
	pPPK	5·14 ± 0·14	0·47 ± 0·01	32·35 ± 0·30	10·81 ± 0·43	21·81 ± 0·93	3·71 ± 0·01	0·56 ± 0·04
After press	VIN13	5·48 ± 0·15	0·41 ± 0	37·67 ± 1·58	10·30 ± 0·71	20·19 ± 0·50	3·69 ± 0·07	0·53 ± 0·03
	pEXS	8·11 ± 0·01	0·37 ± 0	38·57 ± 2·04	15·35 ± 0·86	26·40 ± 1·00	5·26 ± 0·07	0·49 ± 0·02
	pPPK	4·10 ± 0·12	0·41 ± 0·01	29·30 ± 4·76	10·10 ± 1·86	15·34 ± 1·21	2·84 ± 0·12	0·54 ± 0·01
After filtration	VIN13	2·42 ± 0·26	0·46 ± 0	10·15 ± 0·64	16·31 ± 0·74	16·65 ± 0·07	3·20 ± 0·05	0·47 ± 0·01
	pEXS	4·26 ± 0·57	0·42 ± 0·01	14·70 ± 0·07	20·39 ± 2·94	25·24 ± 0·07	4·68 ± 0·05	0·44 ± 0·01
	pPPK	1·82 ± 0·07	0·5 ± 0·01	8·13 ± 0·64	14·88 ± 0·70	14·74 ± 0·50	2·57 ± 0·20	0·47 ± 0

pigments in the wine. Surprisingly, the VIN13[pPPK] transformant gave less colour than was expected. Some pectinases are capable of reducing red wine colour through pigment modification and subsequent degradation (Wightman *et al.* 1997); this might be the case for VIN13[pPPK] as well. The effect on colour was much lower in Cinsaut (data not shown), from which enough colour pigments are usually extracted under normal winemaking conditions.

Because of the increased degradation of the grape skins by the endo-glucanase and endo-xylanase produced by the VIN13[pEXS] transformant, a higher concentration of phenolics was released into the Pinot Noir wine. About 30% more phenolics than the reference wine were obtained after filtration. In Cinsaut, the same transformant seemed to cause no significant difference in the amount of phenolics (data not shown). It might be that the phenolics are bound differently in Cinsaut than in Pinot Noir or that there are some differences in the grape skin of these cultivars. In both varieties, a decrease in phenolics was observed towards the end of fermentation. This can be because of the binding and precipitation of the phenols. The greater amount of extracted phenolics could help stabilize the colour and prove interesting to observe when measuring the colour levels after 1–2 years of maturation.

The amounts of free-flow and pressed Pinot Noir and Cinsaut wine are compared in Fig. 2a. In both varieties, the two transformants (VIN13[pEXS] and VIN13[pPPK]) gave more free-flow wine than the control (VIN13) presumably because of the enzymatic degradation of the polysaccharides in the grape skins, although only pectinases produced by VIN13[pPPK] seem to have had a significant effect. Here, the VIN13[pPPK] transformant gave about 5% more free-flow wine by degrading the pectins in the cell walls. In this experiment, the polysaccharases

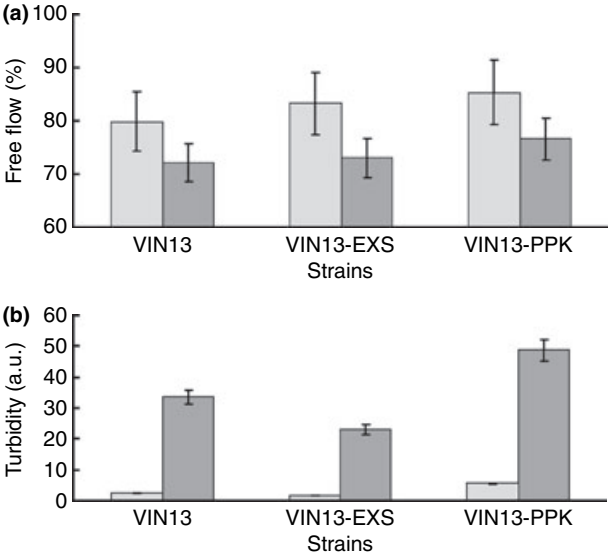

Figure 2 (a) Wine yield trial. (b) Wine turbidity of Pinot Noir (empty bars) and Cinsaut (filled bars). The values represented are from three independent experiments plus/minus the deviation of the samples from the average.

produced by the yeast transformants again showed a stronger effect in Pinot Noir than in Cinsaut wine. This indicates that enzymes secreted by the wine yeasts do not always have the same effects on juice from all different grape cultivars.

In terms of turbidity, the wines fermented with the VIN13[pEXS] transformant generally showed less turbidity whereas the wines fermented with the VIN13[pPPK] transformant showed higher turbidity than the control wine fermented with the VIN13 host strain (Fig. 2b). The turbidity of the wine control was 2·55 (Pinot Noir) and 33·5 (Cinsaut) compared with turbidity readings 1·61 (Pinot Noir) and 22·91 (Cinsaut) of wines fermented with

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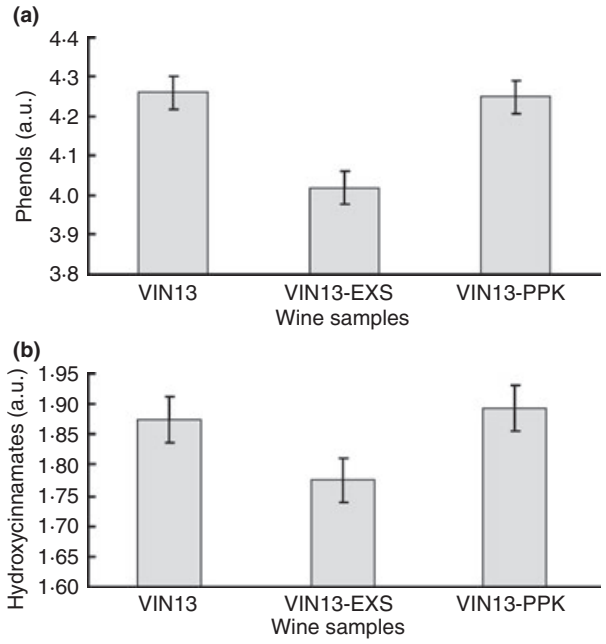

Figure 3 (a) The total phenolics in the Muscat d'Alexandria wines made with the three different wine yeasts. (b) The total hydroxycinnamates in the Muscat d'Alexandria wines made with the control and the two recombinant wine yeasts.

VIN13[pEXS]. It seems that VIN13[pPPK] either released more pectic particles leading to an increase in turbidity or it degraded the pectin only partially causing one molecule to be degraded into a number of smaller particles and thereby increasing the degree of turbidity.

Experiments were also done on a white variety, Muscat d'Alexandria, with both transformants. This variety is very fleecy, making it very difficult to press the juice from the skins. The continuous pressing of the grapes resulted in the extraction of a large amount of phenolics and other components, typically resulting in a bitter taste in the juice. From the results presented in Fig. 3, it is clear that fermentation with VIN13[pEXS] resulted in a wine with less phenolics and hydroxycinnamates than the control wine fermented with the untransformed VIN13 strain. Wines made with the VIN13[pEXS] transformant will probably be less astringent compared with the control wine. Fermentation with VIN13[pPPK] showed no significant differences relative to the control.

Finally, the gas–liquid chromatography results showed only minor differences in the wine composition profiles of the two transformants and the control. By comparing the concentrations of ethanol and residual sugar in the various wines, it appears that the VIN13[pEXS] transformant, in both the Cinsaut and Pinot Noir, consistently produced slightly higher ethanol concentrations, as well as marginally higher residual sugar concentrations than the control (Table 2). It might be that the secreted enzymes degraded the grape-derived polysaccharides, and therefore there was more sugar in the fermenting must. In both Pinot Noir and Muscat d'Alexandria wines, the VIN13[pPPK] transformant showed lower alcohol concentration than both the VIN13[pEXS] transformant and the VIN13 control strain.

VIN13[pPPK] seemed to produce greater concentrations of higher alcohols than the VIN13 control in Pinot Noir and Cinsaut, with mainly higher amounts of butanol-3-methyl and butanol-2-methyl (Table 4). In Muscat d'Alexandria, the use of VIN13[pPPK] also resulted in greater quantities of higher alcohols and short- to medium-chain ethyl esters, aldehydes and terpenes. Both transformants produced less long-chain ethylesters than the control. When ANOVA testing was carried out on the chemical composition (as measured by GC analysis) of the wines, it was found that the only significant differences observed were with butanoic acid-3-methylethylester in Pinot Noir and decanoic acid ethylester in Muscat d'Alexandria (results not shown).

Discussion

Until the early 1990s, studies directed towards the improvement of wine quality were mainly concentrated on the selection of new grape varieties and viticultural practices, or on fermentation and winemaking practices. However, over the last decade or so, an ever-increasing number of studies have been undertaken with regard to the genetic improvement of the organisms that play a vital role in the whole winemaking process, namely, the wine yeasts (Pretorius *et al.* 2003, 2006).

It is generally believed that the use of genes from fungal origin in recombinant yeast has advantages because their encoded enzymes are normally not inhibited by the pH and temperature ranges of must and wine or by the SO_2 added to the wine to protect it against oxidation and other micro-organisms. Genes of bacterial origin are often inhibited by the aforementioned factors; however, in this study, the *B. fibrisolvens* endo-β-1,4-xylanase, the *E. chrysanthemi* pectate lyase and the *E. carotovora* endo-polygalacturonase appeared to be tolerant of these parameters encountered in winemaking.

Collectively, the results obtained in this study showed that enzymes directly secreted by the wine yeast can play a significant role in the end product of fermentation. They also showed that the same enzyme could have quite different effects on different grape cultivars. This can be attributed to inherent differences in the composition of different cultivars. These differences include skins that are naturally more difficult to press, skins that are thicker, or cultivars having berries with higher juice content than

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Table 4 Gas–liquid chromatography analysis of volatile components formed by the engineered strains in different wines

	Cinsaut			Muscat d'Alexandria			Pinot Noir		
Volatile compound (mg l^{-1})	VIN13	VIN13-EXS	VIN13-PPK	VIN13	VIN13-EXS	VIN13-PPK	VIN13	VIN13-EXS	VIN13-PPK
Butanol-3-methyl	92·25	102·39	112·40	81·2	73·57	122·82	82·13	ND	ND
Butanol-2-methyl	29·03	29·73	36·77	28·93	25·61	34·74	36·54	ND	ND
Acetic acid-2-phenyl ester	0·48	0·42	0·63	1·37	1·58	1·83	0·03	0·07	0·09
Butanoic acid-3-methylethyl ester	ND	ND	ND	ND	ND	ND	0·04	0·05	ND
Hexanol	ND	ND	ND	1·20	1·31	1·28	1·55	1·65	1·75
Decanoic acid ethyl ester	ND	ND	ND	0·3	0·29	0·26	0·04	0·09	0·08
Furfural	ND	ND	ND	0·02	0·25	0·03	ND	ND	ND
Linalool	ND	ND	ND	0·42	0·44	0·47	ND	ND	ND
Nonaldehyde	ND	ND	ND	0·04	0·04	0·05	ND	ND	ND
Octanoic acid ethyl ester	ND	ND	ND	1·04	0·96	1·00	ND	ND	ND
Terpineol	ND	ND	ND	0·14	0·14	0·16	ND	ND	ND
Citronellol	ND	ND	ND	0·02	0·02	0·02	ND	ND	ND

The numbers represent mean values from the mean of three independent fermentations. Measurements varied <10%.
ND, not detected.

others. There are numerous reasons why the effect of the same enzyme differs between cultivars. Glucanases and xylanases clearly decreased turbidity; this is especially true in Pinot Noir. Pectinases seemed to increase turbidity in both red cultivars. However, in the case of Muscat d'Alexandria, the wines produced by the glucanase-xylanase-producing yeast transformants were similar to the wine produced by the control yeast strain. The pectinase-degrading yeast transformants, on the other hand, resulted in the extraction of more juice from all three cultivars tested. Although more phenolic compounds were extracted by the yeast-producing glucanase and xylanase enzymes, the phenolic compounds had no effect on the yeast fermentation performance. Fermentation with the glucanase- and xylanase-secreting strains resulted in an increase in free-flow wine. These results are consistent with the information in other related publications, which have reported increased free-run juice yield when treated with commercial macerating enzymes (Haight and Gump 1994). Louw *et al.* (2006) also found a significant increase in free-run wine when Ruby Cabernet was fermented with different polysaccharide-degrading yeast strains.

The results of the gas-chromatographic analysis of the wines indicated that the secretion of the pectinase, glucanase and xylanase enzymes into the must during fermentation caused alterations in the chemical composition of the musts and wines. Some of these chemical changes in the wines are believed to have effects on their fruity aromas; however, no formal sensory analyses of these wines were undertaken during the cause of this study. Such analyses will most certainly be done in follow-up studies.

In conclusion, the wine yeasts producing pectinases, glucanases and xylanases had positive effects on various aspects of the three varietal wines tested. It was also clear that the results these yeast-derived enzymes gave differed depending on the grape variety, and it is therefore important to conduct similar experiments on a wider selection of grape varieties.

Based on the promising preliminary findings of this study, it appears that several aspects of commercial-scale wine processing and clarification, colour extraction and stabilization and perhaps even aroma enhancement could potentially be improved by the use of polysaccharide-degrading wine yeasts without the addition of expensive commercial enzyme preparations. However, it is important to note that the widespread use of genetically modified (GM) wine yeasts in commercial wine production has not yet occurred; so far, only a few wineries in North America have applied two recently commercialized GM yeasts, i.e. (i) a yeast that has the capacity to conduct the malolactic fermentation during the alcoholic fermentation and thereby reducing the risk of biogenic amine formation by bacteria (Husnik *et al.* 2006) and (ii) a yeast that secretes much less urea, which, in turn, limits the production of ethyl carbamate (Coulon *et al.* 2006). Both these GM wine yeasts have been granted GRAS (*G*enerally *R*egarded *A*s *S*afe) status by the Food and Drug Administration in the USA. However, while the strong anti-GM sentiment prevails in key European and other export markets, the positions of the South African and Australian wine industries remain that no GMOs will be used in the production of commercial wines. When this situation changes, the yeasts developed in this study could offer the potential to further improve the price : quality ratio of wine according to consumer expectations.

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