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ORIGINAL PAPER

D. F. Malherbe · M. du Toit · R. R. Cordero Otero · P. van Rensburg · I. S. Pretorius

Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production

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Abstract There is a growing consumer demand for wines containing lower levels of alcohol and chemical preservatives. The objectives of this study were to express the *Aspergillus niger* gene encoding a glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) in *Saccharomyces cerevisiae* and to evaluate the transformants for lower alcohol production and inhibition of wine spoilage organisms, such as acetic acid bacteria and lactic acid bacteria, during fermentation. The *A. niger* structural glucose oxidase (*gox*) gene was cloned into an integration vector (YIp5) containing the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) and the phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). The $PGK1_P$-$MF\alpha1_S$-*gox*-$PGK1_T$ cassette (designated *GOX1*) was introduced into a laboratory strain (Σ1278) of *S. cerevisiae*. Yeast transformants were analysed for the production of biologically active glucose oxidase on selective agar plates and in liquid assays. The results indicated that the recombinant glucose oxidase was active and was produced beginning early in the exponential growth phase, leading to a stable level in the stationary phase. The yeast transformants also displayed antimicrobial activity in a plate assay against lactic acid bacteria and acetic acid bacteria. This might be explained by the fact that a final product of the GOX enzymatic reaction is hydrogen peroxide, a known antimicrobial agent. Microvinification with the laboratory yeast transformants resulted in wines containing 1.8–2.0% less alcohol. This was probably due to the production of D-glucono-δ-lactone and gluconic acid from glucose by GOX. These results pave the way for the development of wine yeast starter culture strains for the production of wine with reduced levels of chemical preservatives and alcohol.

Abbreviations *GOX* Glucose oxidase enzyme · *gox Aspergillus niger* glucose oxidase gene · *GOX1 Aspergillus niger gox* gene expressed in *Saccharomyces cerevisiae* as the $PGK1_P$-$MF\alpha1_S$-*gox*-$PGK1_T$ gene cassette · *MFα1S* Yeast mating pheromone α-factor secretion signal · *PGK1P* Yeast phosphoglycerate-kinase-1 gene promoter · *PGK1T* Yeast phosphoglycerate-kinase-1 gene terminator

D. F. Malherbe · M. Toit · R. R. Cordero Otero · P. Rensburg · I. S. Pretorius (✉)
Institute for Wine Biotechnology,
Department of Viticulture and Oenology, Stellenbosch University,
7600 Stellenbosch, South Africa
e-mail: isp@sun.ac.za
Tel.: +27-21-8084730
Fax: +27-21-8083771

Introduction

Selected strains of *Saccharomyces cerevisiae* are widely used as wine yeast starter cultures whose primary role are to convert the grape sugar into alcohol. In addition, the wine yeast's metabolic activities result in the production of higher alcohols, fatty acids and esters, which are important flavour and aroma compounds. In warmer climatic regions, the grape sugar levels are higher than in cooler climatic regions and wines therefore are prone to alcohol levels of 13.5–15% (v/v). In recent years, there has been increased international interest in and consumer demand for low-alcohol, "reduced-alcohol" and de-alcoholised wines, resulting in a search for new production methods (Scudamore-Smith and Moran 1997; Pickering et al. 1998). Commercial interest has also been stimulated by the potential for savings in tax and tariffs on the reduced alcohol content in these wines (Gladstones and Tomlinson 1999; Gladstones 2000).

Several physical processes have been used (sometimes in combination) for the removal or reduction of alcohol in wine, including thermal evaporation, distillation, membranes, extraction, adsorption, centrifugation, freeze concentration and partial fermentation (Bui et al. 1986; Pickering et al. 1999a; Mermelstein 2000). These processes tend to involve expensive equipment and require intensive processing. An alternative approach was intro-

Table 1 Microbial strains and plasmids used in this study. *ATCC* American Type Culture Collection (Rockville, Md., USA), *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), *LMG* LMG Culture Collection of the Laboratory of Microbiology (University of Gent, Belgium)

Strains/plasmids	Genotype or strain number	Source or reference or culture collection
Strains		
Bacteria		
E. coli DH5α	*supE*44Δ*lac*U169 (ø80*lacZ*ΔM15) *hsd*R17 recA1 *end*A1 gryA96 *thi*-1 relA1	Sambrook et al. (1989)
Lactobacillus fermentum	9328^T	ATCC
Lactobacillus brevis	8291	ATCC
Lactobacillus sakei subsp. *sakei*	20017	DSM
Pediococcus pentosaceus	13561	LMG
Oenococcus oeni	DSm 7008	Christian Hansen A/S (Denmark)
Acetobacter aceti	286	This laboratory (wine isolate)
Gluconobacter oxydans	7145^T	DSM
Yeast		
Saccharomyces cerevisiae Σ1278	JT4500 ura3	This laboratory
Saccharomyces cerevisiae ΣpGOXe	JT4500 ura3 $PGK1_P$-$MF\alpha1_S$-gox-$PGK1_T$	This study
Saccharomyces cerevisiae ΣpGOXi	JT4500 ura3 $PGK1_P$-$MF\alpha1_S$-gox-$PGK1_T$	This study
Plasmids		
pSK+3.Sma	*gox* Ap^R lacZ	Geisen (1995)
YEp352	$PGK1_P$- $MF\alpha1_S$-$PGK1_T$ Ap^R *URA3*	This laboratory
YIp5	Ap^R Tc^R	Struhl et al. (1979)
pGOXe	$PGK1_P$- $MF\alpha1_S$-gox-$PGK1_T$ Ap^R	This study
pGOXi	Ap^R Tc^R *URA3* $PGK1_P$- $MF\alpha1_S$-GOX-$PGK1_T$	This study

duced with the concept of treating grape must with glucose oxidase (GOX) to reduce the glucose content of the must, thereby producing a wine with a reduced alcohol content after fermentation (Villettaz 1987; Pickering and Heatherbell 1996; Pickering et al. 1998, 1999a, b,c).

Glucose oxidase from *Aspergillus niger*, which has GRAS (generally regarded as safe) status, is of considerable industrial importance and has previously been produced in heterologous host organisms (Frederick et al. 1990; De Baetselier et al. 1991; Hodgkins et al. 1993; Hong et al. 1998). Amongst others, GOX is used for the removal of residual glucose or oxygen from foods and beverages in order to improve their shelf life (Whittington et al. 1990; De Baetselier et al. 1991; Hammer 1998; Park et al. 2000; Kapat et al. 2001) and to maintain flavour and colour stability (Ohlmeyer 1957; Pickering 1998; Power 1998; Vemulapalli et al. 1998). The possible effect of GOX on wine parameters was previously investigated with satisfactory results (Pickering et al. 1999a, b, c). GOX metabolises glucose into gluconic acid, which also has GRAS status. Thus not all the glucose is metabolised into ethanol.

Another consumer demand concerns the reduction of chemical preservatives used in the food and beverage industries. Various possibilities have been investigated by the wine industry, including bacteriocins (Radler 1990a, b; Schoeman et al. 1999; Du Toit and Pretorius 2000), lysozyme (Gerbaux et al. 1997) and glucose oxidase (Pickering 1998).

The antimicrobial compound that results from the GOX reaction is hydrogen peroxide (H_2O_2) (De Vuyst and Vandamme 1994; Geisen 1999). H_2O_2 has been shown to be active against gram-positive and gram-negative bacteria. Therefore, GOX could have a dual purpose when used in the winemaking process.

The objective of this study was to express the *A. niger gox* gene in *S. cerevisiae* and to evaluate the GOX-producing yeast transformant under winemaking conditions for its ability to reduce the total amount of ethanol produced, as well as to assess the antimicrobial activity against acetic acid bacteria and lactic acid bacteria.

Materials and methods

Microbial strains and plasmids

The microbial strains and plasmids used in this study are listed in Table 1.

Culture conditions and media

Escherichia coli DH5α was grown as previously described (Sambrook et al. 1989). Luria-Bertani (Biolab, Merck, South Africa) medium supplemented with ampicillin (100 mg/ml) was used to select *E. coli* transformants containing plasmids. *E. coli* was routinely cultured at 37 °C. Lactic acid bacteria were cultured in De Man Rogosa Sharpe (MRS) broth or on agar plates (Biolab, Merck, South Africa), while acetic acid bacteria were grown in glucose yeast extract (GY) medium [containing 5% glucose (w/v) and 1% yeast extract (w/v)] or on GYC agar plates [containing 3% $CaCO_3$ (w/v), 5% glucose (w/v), 1% yeast extract (w/v) and 2% agar (w/v)] (Drysdale and Fleet 1988). Acetic acid bacteria and lactic acid bacteria were cultured routinely at 30 °C. Yeast cells were cultured in yeast peptone dextrose (YPD) medium [containing 1% yeast extract (w/v), 2% peptone (w/v) and 2% glucose (w/v)] at 30 °C. Yeast transformants were isolated on uracil selective (SC^{-Ura}) agar plates [containing 0.67% yeast nitrogen base (w/v) without amino acids (Difco), 2% glucose (w/v) and all the required growth factors except uracil].

PCR amplification of the glucose oxidase gene

The *gox* gene of *A. niger* was amplified by PCR from the plasmid pSK+3.Sma (Geisen 1995). Primer prGOXmfa (5'-GATCAAGCTTCTCAGACTCTCCTTGTGAGC-3'), containing part of the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) and a *Hin*dIII restriction site (underlined), was used as the forward primer. The reverse primer, prGOX (5'-GATCCTCGAGACCACTCACTGCATGGAAGC-3'), contained a *Xho*I restriction site (underlined). Both primers were obtained from Integrated DNA Technologies (Coraville, Iowa). PCR was carried out in a final volume of 50 μl of reaction mixture using the TRIO-Thermoblock (Biometra, Goettingen, Germany). The reaction mixture consisted of 1 μl pSK+3.Sma as template DNA (6 ng/μl), 2.5 μl of primer prGOXmfa (1.5 pmol/μl), 2.5 μl of primer prGOX (1.5 pmol/μl), 1 μl of dNTP mixture (1.25 mM, final concentration for added nucleotides), 2 μl of $MgCl_2$ (25 mM), 1 μl (3.5 units) of Expand DNA polymerase (Roche Diagnostics, Mannheim, Germany), 4 μl of PCR reaction buffer without $MgCl_2$ (10 mM Tris-HCl pH 8.3, 50 mM KCl and 0.01% gelatin [w/v]) and 36 μl of deionised water.

The reaction was run for 30 cycles: denaturation was at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 3.5 min. An initial denaturation at 94 °C for 2 min and a final extension at 72 °C for 5 min were used. The resulting 1.8-kb PCR product, containing the *gox* gene fused in-frame to $MF\alpha1_S$, was analysed by standard agarose gel electrophoresis.

DNA manipulations and plasmid construction

DNA manipulations were carried out in the yeast-*E. coli* shuttle vector YEp352 containing the constitutive yeast phoshopglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences with the $MF\alpha1_S$ secretion signal. The episomal plasmid pGOXe (Table 1) was obtained by subcloning a 1.8-kb PCR product, containing the *gox* gene fused in-frame to $MF\alpha1_S$, into the *Hin*dIII and *Xho*I restriction sites of YEp352. A GOX-encoding gene cassette (designated *GOX1*) was produced consisting of $PGK1_P$-$MF\alpha1_S$-*gox*-$PGK1_T$. The G*OX1* gene cassette was subcloned as a *Pvu*II restriction fragment from pGOXe into the *Pvu*II site of YIp5 containing the *URA3* marker gene, rendering the integration plasmid pGOXi (Fig. 1). Standard methods for plasmid DNA isolation, restriction enzyme digestion, ligation reactions and *E. coli* DH5α transformations were used (Sambrook et al. 1989). A lithium acetate transformation method (Gietz and Schiestl 1991) was used for the transformation of *S. cerevisiae* Σ1278.

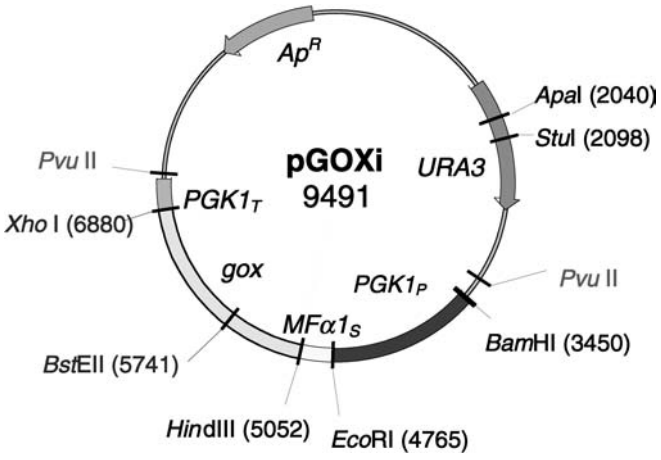

Fig. 1 The yeast imtegration plasmid (pGOXi) containing the *GOX1* gene cassette. *GOX1* consists of the $PGK1_P$-$MF\alpha1_S$-*gox*-$PGK1_T$ construct and was inserted into the YIp5 plasmid

Southern blotting

Standard methods were used for yeast genomic DNA isolations (Sambrook et al. 1989). The genomic DNA was digested with *Hin*dIII and *Xho*I (for gene integration confirmation) and with *Bst*EII and *Nsi*I (for single-copy integration confirmation). The different DNA fragments were separated on a 1% (w/v) agarose gel. The DNA was depurinated, denatured and the gel neutralised before the DNA was transferred to a Hybond-N nylon membrane (AEC-Amersham, South Africa). The *gox* gene was used as probe and the digoxigenin nonradioactive nucleic acid labelling and detection system was used for Southern hybridisation to detect the integration and to verify single-copy integration into *S. cerevisiae* strain Σ1278 (Fig. 2). The DIG Labelling Kit (Roche Biochemical Products, South Africa) was used.

Glucose oxidase plate assay

A modified method of Hodgkins et al. (1993) was used for the screening and selection of GOX-producing yeast colonies. Yeast colonies that were previously selected on SC^{-Ura} plates and identified as positive transformants were spotted onto YPD plates and incubated for 2–3 days at 30 °C. The plates were then overlaid with 10 ml of 0.1 M McIlvaine buffer, pH 7.0 [containing 1% (w/v) agarose, 10 g glucose/l , 100 mg *o*-dianisidine dihydrochloride/l (Sigma, South Africa) and 15 U horseradish peroxidase type II/ml (Sigma, South Africa)]. The overlay was allowed to set and the plates were incubated at 37 °C for 1 h. The untransformed *S. cerevisiae* strain (Σ1278) was used as negative control. As GOX metabolises glucose into gluconic acid, H_2O_2 is formed as a byproduct. The H_2O_2 was used by horseradish peroxidase to oxidise *o*-dianisidine dihydrochloride and a colour change was visible on the agar plates. Transformed yeast colonies secreting active recombinant GOX were surrounded by a brown halo.

Glucose oxidase spectrophotometric assay

The *S. cerevisiae* strain carrying the YIp5 vector (control strain) and the transformant containing the *GOX1* construct were inoculated to 10^6 cells per ml in SC^{-Ura} liquid media. Samples were taken every 3 h over a 24-h period using a modified method of Park et al. (2000). Cells were harvested at 5,000 rpm for 5 min. The supernatant was used for the extracellular enzyme assay. The remaining cells were resuspended in 5 ml of 50 mM Tris (pH 7.5, containing 10 mM $NaCl_2$) buffer. Approximately 0.1 g of 0.2-mm

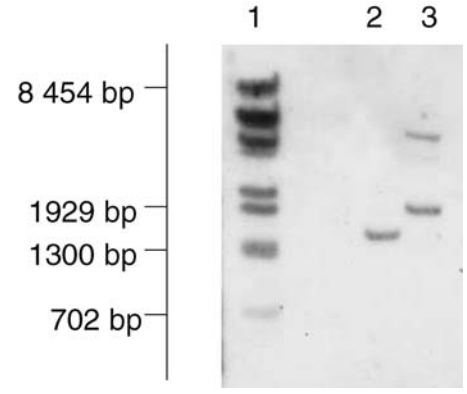

Fig. 2 A Southern blot autoradiogram demonstrating integration of the *GOX1* gene cassette in the genomic copy of the *ura3* marker locus of the yeast host strain: *Lane 1* λ DNA marker digested with *Bst*EII, *lane 2* yeast genomic DNA digested with *Hin*dIII and *Xho*I verifying gene integration, *lane 3* yeast genomic DNA digested with *Bst*EII and *Nsi*I verifying single copy integration. *Open lane* Untransformed control host strain, Σ1278. The PCR-amplified *gox* gene from *Aspergillus niger* was used as a probe for the Southern blot hybridisation

glass beads was added and the cells were vortexed vigorously for 3 min. After centrifugation at 6,000 rpm for 2 min, the supernatant, containing the intracellular protein extract, was carefully removed and the samples were stored. Each sample (30 μl) that was tested was mixed with 1.5 ml of 1.0 M citrate-phosphate buffer at pH 4.5, 0.3 ml of 1.0 M D-glucose, 0.1 ml of 60 U horseradish peroxidase/ml and 1.0 ml of 0.31 mM *o*-dianisidine dihydrochloride. These reaction mixtures were incubated for 30 min at 37 °C and thereafter stopped on ice by adding 0.3 ml of 4 M H_2SO_4. The absorbance was measured at 500 nm (A_{500}) and the units of glucose oxidase per ml produced were determined relative to a standard curve of absorbance vs enzyme amount. All values given are the means of triplicate samples; all experiments reported were carried out at least three times with similar results.

Antibacterial activity plate assays

The untransformed control yeast strain (Σ1278) and the transformant containing the *GOX1* gene cassette (ΣpGOXi) were "line-spotted" vertically on GYC agar plates and incubated for 24 h. Acetic acid bacteria (Table 1) were grown on GYC agar plates. A single colony was selected and resuspended in 10 μl of distilled water. The acetic acid bacteria were streaked horizontally over the already spotted yeasts on the same GYC agar plates and incubated at 30 °C until inhibition zones were visible.

The untransformed control yeast strain (Σ1278) and the transformant (ΣpGOXi) were also spotted on YPD plates and incubated for 24 h. Lactic acid bacteria (Table 1) were cultured in MRS broth overnight. A 20-μl culture of the lactic acid bacteria was added to MRS soft agar [0.7% (w/v)] and lawned onto the YPD plates over the yeast colonies. Plates were allowed to set and then incubated at 30 °C until inhibition zones were visible (Table 2).

d-Gluconic acid/d-glucono-δ-lactone assay and HPCE

A D-gluconic acid/D-glucono-δ-lactone assay kit (Roche Diagnostics) was used to determine whether there was any D-glucono-δ-lactone present in the Chardonnay juice samples used for the microvinifications.

A high-performance capillary electrophoresis (HPCE) method with indirect absorbance detection that was developed by Lee and Lin (1996) was used for the determination of the carbohydrates, glucose, fructose and gluconic acid in the Chardonnay juice samples. The HPCE analyses were carried out on a HP3D CE system (Hewlett-Packard) equipped with a diode array detector. The detector wavelength was fixed at 550 nm with 222 nm as the reference wavelength. A bare silica capillary column with an internal diameter of 50 μm (total length of 112.5 cm and effective length of 104 cm) was used. Samples and standards were injected hydrodynamically (50 mbar for 4 s). A constant voltage of +30 kV was applied during the separation run and the temperature of the column was set at 25 °C. A running buffer (NAA) was prepared by adjusting a 2 mM 1-naphthylacetic acid solution to a pH of 12.2 (using 1 M NaOH). This solution was filtered through a 0.22-μm filter. The collected data were analysed on HP Chemstation Software. Before each run, the column was flushed with the NAA-buffer for 2 min. After each run, the column was flushed with 1 M NaOH (3 min), water (2 min) and NAA-buffer (3 min). Glucose, fructose and gluconic acid solutions (0.1 M) were prepared. These were used to generate suitable standards for the simultaneous analysis of glucose, fructose and gluconic acid (e.g. 70 mM glucose, 30 mM fructose and 2 mM gluconic acid). Samples were centrifuged (8 minx12 000 rpm) before diluting them five- or 10-fold. Standards were run between the samples in order to create valid calibration curves for each component, which could then be used by the HP Chemstation Software to calculate the concentrations of glucose, fructose and gluconic acid in the samples.

Table 2 Rate of inhibition of the lactic acid bacteria seen as clear zones on lawned agar plates

Strains	Inhibition zone[a]
Lactobacillus fermentum	++
Lactobacillus brevis	++++
Lactobacillus sakei subsp. *sakei*	+++
Pediococcus pentosaceus	+++
Oenococcus oeni	–

Scanning electron microscopy

Acetic acid bacteria (*Acetobacter aceti*), lactic acid bacteria (*Lactobacillus brevis*), the untransformed control yeast strain (Σ1278) and the yeast transformant carrying the *GOX1* gene cassette were incubated for 18 h at 30 °C in their particular growth media as well as in combination. Nucleopore Track-Etch Membranes (1 μm) were installed into Millipore (non-sterile) Swinnex filters. The microbial cells were filtered through the membrane using a 5 ml syringe, whereafter the filters were transferred to small glass bottles in which the remainder of the experiment was done. The protocol described by Chung and Hancock (2000) was followed from this point onwards. The filters were fixed for 30 min in 2.5% (w/v) glutaraldehyde in a 0.1 M sodium phosphate buffer at 4 °C and afterwards washed three times for 5 min in 0.1 M of sodium phosphate buffer, pH 7.2. The cells were then fixed with 1% (w/v) osmium tetroxide in a 0.1 M sodium phosphate buffer for 30 min, rinsed in distilled water for 5 min, fixed with 1% (w/v) tannic acid for 20 min and rinsed again in distilled water for 5 min. The samples were finally fixed with 1% (w/v) osmium tetroxide and rinsed with distilled water for 5 min. The samples were progressively treated with different alcohol concentrations (all v/v): 50%, 70%, 80%, 95% (twice) and 100% (three times) each for 5 min to dehydrate, after which they were dried in a Balzers Critical Point Dryer, sputtered with gold and viewed under a Leica Stereoscan Scanning Electron Microscope.

Microvinifications

Chardonnay grape juice with a sugar concentration of 230 g/l and a pH of 3.85 was used for this study. The untransformed control yeast strain (Σ1278) and the yeast transformant containing the *GOX1* gene cassette were inoculated to 10^6 cells per ml into 150 ml of juice for the microvinification. The accumulated weight loss was measured over a period of 14 days, after which the alcohol concentration was determined by ebulliometry using the "Church-ward Technique" (Iland et al. 2000).

Results

Cloning and expression of the *A. niger gox* gene in *S. cerevisiae*

The *A. niger* structural glucose oxidase (*gox*) gene was fused to the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$) and then placed under the control of the constitutive phosphoglycerate-kinase-1 gene promoter ($PGK1_P$) and terminator ($PGK1_T$). This gene cassette (designated *GOX1*) was inserted into a yeast integration vector (YIp5) (Fig. 1) and transformed into a laboratory strain (Σ1278) of *S. cerevisiae*. Integration of a single copy of the *GOX1* construct into the *ura3* marker locus of strain Σ1278 was verified by Southern blot analysis (Fig. 2). The Ura^+ integrants were screened for the secretion of biologically active GOX by selecting the colonies surrounded by a brown halo in the GOX agar plate assays (Fig. 3).

The time course of production (intra- and extracellular) of recombinant GOX during the growth of the *S. cerevisiae* transformant (ΣpGOXi) containing an integrated copy of the *GOX1* gene cassette was monitored spectrophotometrically. High amounts of GOX were produced and secreted into the culture medium during the first 8 h, which corresponds to the exponential growth phase of the transformed yeast (Fig. 4). In addition, most of the GOX activity was measured in the extracellular fraction and only a small fraction remained intracellularly. The secreted GOX activity is required for the conversion of the glucose to gluconic acid in the media before the yeast cells are able to metabolise the glucose to ethanol.

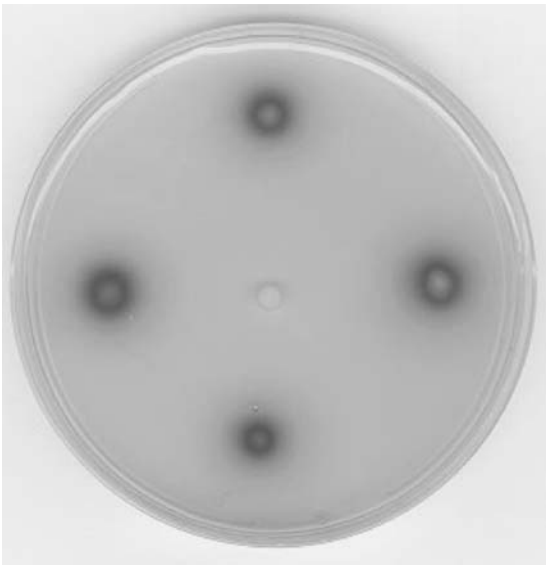

Fig. 3 Glucose oxidase plate assay. The untransformed *Saccharomyces cerevisiae* host strain, Σ1278, was used as negative control. Transformed yeast colonies secreting active recombinant GOX were surrounded by a brown halo and were identified as positives. In this figure, four different positive transformed yeast colonies are visible around the negative control in the middle of the plate

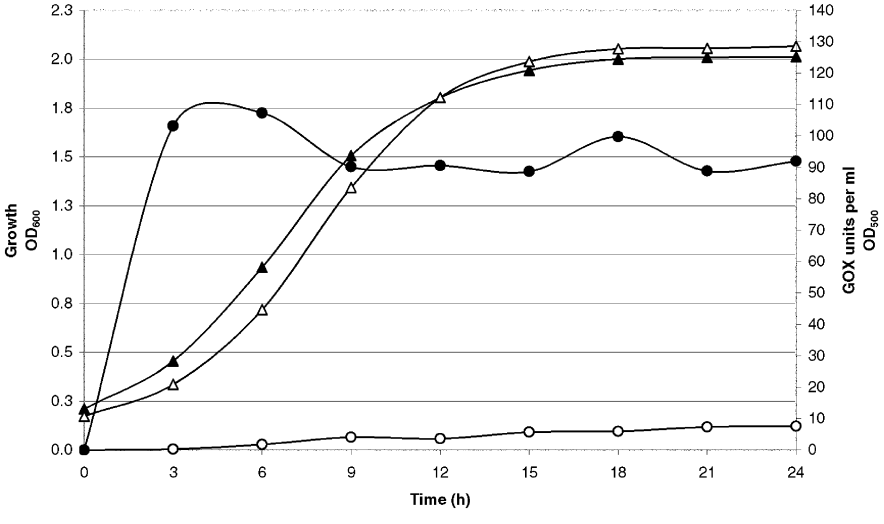

Fig. 4 The growth of *S. cerevisiae* strains and the time course production of glucose oxidase (units/ml) (extracellular and intracellular) over a 24-h period are presented as *lines* on the graph. △ Growth of the untransformed control strain (Σ1278), ▲ growth of the transformed strain (ΣpGOXi) containing *GOX1*, ◯ GOX activity associated with the intracellular fraction, ● extracellular GOX activity. The graph is a representative example of fermentation experiments performed in triplicate. The standard deviation was less than 5%

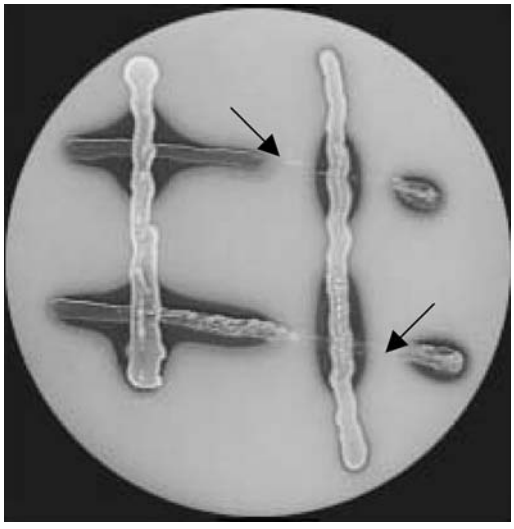

Fig. 5 Antibacterial activity plate assays depicting the inhibition of acetic acid bacteria, *Acetobacter aceti* (*top horizontal line*) and *Gluconobacter oxydans* (*bottom horizontal line*), by the GOX-producing yeast strain. The yeast strains were streaked out vertically. The untransformed control strain of *S. cerevisiae* (Σ1278) is on the *left* and the *S. cerevisiae* strain (ΣpGOXi) transformed with *GOX1* is on the *right* of the agar plate. Inhibition zones, where no growth is visible, can clearly be seen around the yeast transformant (*arrows*)

The inhibition of wine spoilage bacteria by the GOX-producing yeast

Using antibacterial activity plate assays, it was noted that the yeast expressing *GOX1* seemed to inhibit the growth of both acetic acid bacteria and lactic acid bacteria, which are the major bacterial spoilage microorganisms in wine. A clear zone was visible around the yeast expressing *GOX1* where the growth of the acetic acid bacteria and lactic acid bacteria were inhibited, with no zone visible around the control yeast strain (Fig. 5). The varying degrees of inhibition for lactic acid bacteria are recorded in Table 2. The antibacterial activity was ascribed to the secreted end-product of the GOX enzymatic reaction, hydrogen peroxide (H_2O_2 is a known antimicrobial agent) rather than to the GOX enzyme itself.

Samples of the bacterial cells that were exposed to the GOX-producing yeast were prepared and viewed under a scanning electron microscope. Total collapse of the bacterial cell wall was observed (Fig. 6).

The production of low-alcohol wine by the GOX-producing yeast

The accumulated weight loss of Chardonnay juice inoculated with an untransformed Σ1278 strain of *S. cerevisiae* and with the ΣpGOXi strain transformed with the *GOX1* gene cassette was measured over a period of 14 days, after which the ethanol and sugar concentrations were determined (data not shown). The sugar concentration was reduced from 230 to 64 g/l. The alcohol concentration produced by the ΣpGOXi transformant was 10.1% (v/v), while the Σ1278 control strain had an ethanol concentration of 11.9% (vol/vol). This 1.8% reduction in ethanol content was presumably due to the shift of the carbon flux way from ethanol formation in favour of gluconic acid. However, there was no significant decrease in pH in the wine. HPCE analysis and a D-gluconic acid/D-glucono-*δ*-lactone assay showed that D-glucono-1,5-lactone was accumulated within the first 3 days (Fig. 7). It is known that some yeasts use D-glucono-1,5-lactone (the intermediate between glucose and gluconic acid) as a carbon source. If this is the case, it could explain why the pH of the wine remained constant in contrast to the decline expected because of gluconic acid accumulation.

Discussion

Prudent wine drinkers are increasingly fastidious about the presence of asthmatic chemical preservatives (e.g., sulphites) and high levels of alcohol in wine (Armstrong et al. 2001; Schoeman et al. 1999; Du Toit and Pretorius 2000; Pretorius 2000, 2003). Therefore, the objective of this study was to investigate the feasibility of controlling both the growth of spoilage bacteria and the formation of high levels of ethanol during wine fermentation by developing tailored strains of *S. cerevisiae* for biopreserved wines with a lower alcohol content. To test this novel concept, an antimicrobial yeast starter culture system, which produces D-glucono-1,5-lactone and gluconic acid at the expense of ethanol, was established as a prototype model for further development and improvement as a one-step, biological alternative to chemical preservation and the physical removal of ethanol from wine.

In this study, a single copy of the *A. niger* GOX-encoding gene, integrated into the genome of a laboratory strain of *S. cerevisiae,* was constitutively expressed under the control of the yeast *PGK1* transcriptional sequences (Figs. 1 and 2). Secretion of the *GOX1*-encoded enzyme was directed by the yeast *MFα1*$_S$ leader sequence (Fig. 3). The production of extracellular GOX activity was growth associated (Fig. 4) and the secreted GOX converted some of the glucose in the culture medium to H_2O_2, D-glucono-1,5-lactone and gluconic acid.

The formation of H_2O_2 by the *GOX1*-containing yeast inhibited the growth of indicator strains of wine-related acetic acid bacteria and lactic acid bacteria (Figs. 5 and 6) with varying degrees of efficiency (Table 2). The mechanism by which the indicator bacterial strains were inhibited was not investigated in this study, but according to De Vuyst and Vandamme (1994), H_2O_2 leads to hyperbaric oxygen toxicity, a result of the peroxidation of the membrane lipid, and a strong oxidising effect on the bacterial cell, which causes the destruction of basic molecular structures, such as nucleic acids and cell proteins.

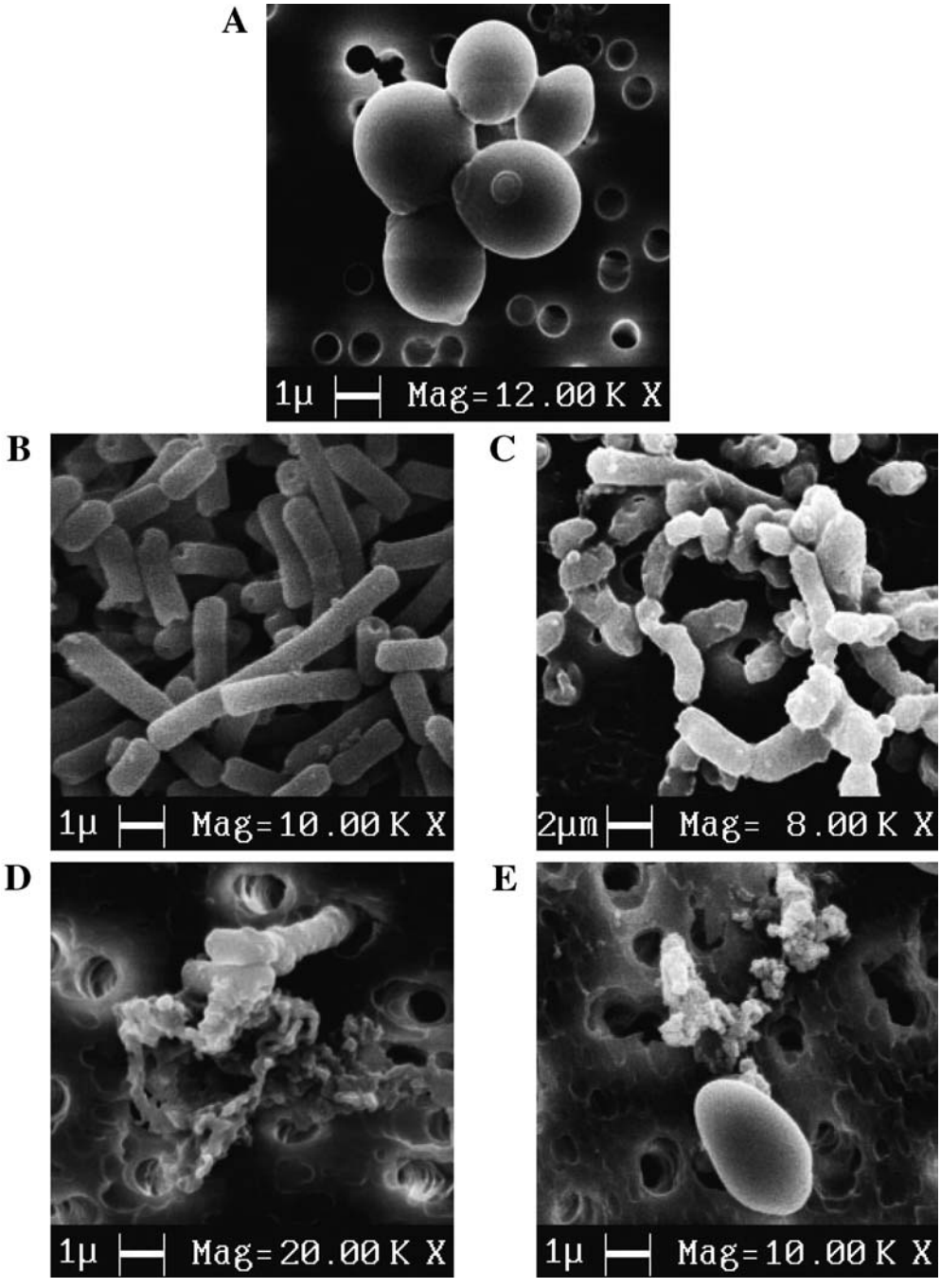

Fig. 6A–E Scanning electron micrograph to assess the morphological state of different yeast and bacteria cell samples. **A** Healthy *Saccharomyces cerevisiae* Σ1278 yeast cells, **B** healthy *Lactobacillus brevis* cells, **C** healthy *Acetobacter aceti* cells, **D** *L. brevis* with a disintegrated cell wall, **E** *A. aceti* with a disintegrated cell wall next to a healthy *S. cerevisiae* ΣpGOXi yeast cell. The presence of hydrogen peroxide in the media is the cause of inhibition

The observation by Geisen (1999), that the measure of the inhibitory activity is dependent on the concentration of glucose in the medium and the amount of GOX enzyme, suggests that the effectiveness of this antimicrobial prototype yeast could be improved by an increase in the expression of *GOX1* and the secretion of the yeast-derived GOX. Furthermore, we are also investigating the possibility of co-expressing *GOX1* together with gene cassettes encoding bacteriocins (pediocin and leucocin) and antimicrobial enzymes (chitinase, glucanase and lysozyme) (Schoeman et al. 1999; Du Toit and Pretorius 2000; Pretorius 2000, 2003; Pretorius and Bauer 2002). The purpose of such a strategy is to increase both the level and spectrum of antimicrobial activity expressed by a tailored wine yeast starter culture strain. Therefore, the ultimate goal would be to incorporate all of these antimicrobial activities into a single wine yeast, thereby counteracting all contaminating spoilage bacteria (e.g. *Acetobacter, Gluconobacter, Lactobacillus* and *Pediococcus*), yeasts (e.g. *Brettanomyces, Pichia* and *Zygosaccharomyces*) and moulds (*Aspergillus, Botrytis, Penicillium* and *Trichoderma,* etc.) in winemaking (Du Toit and Pretorius 2000; Pretorius 2000, 2003; Pretorius and Bauer 2002).

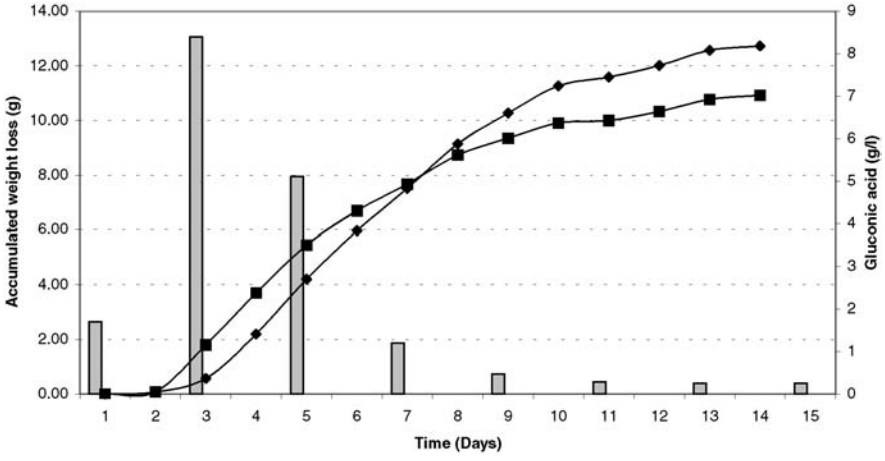

Fig. 7 Glucono-δ-lactone production by the *S. cerevisiae* transformant (ΣpGOXi) in grape must fermentation over a period of 14 days. *Lines* accumulated weight loss of the recombinant strain ΣpGOXi (■) and untransformed *S. cerevisiae* control strain, Σ1278 (◆). *Bars* Levels of glucono-δ-lactone measured for ΣpGOXi (Î). No glucono-δ-lactone was detected for the control strain (Σ1278). The standard deviation for this experiment was less than 1%

In addition to the antimicrobial activity of the yeast expressing the *GOX1* gene cassette, this study has also demonstrated that this prototype yeast was able to reduce the amount of ethanol in wine by 1.8% (v/v). This opens the possibility to bio-adjust the levels of ethanol in wine by optimising the production of GOX in a wine yeast strain tailored for the fermentation of grape juice containing excessively high levels of sugar. It is also envisaged that this strategy could be used in conjunction with another biological approach to the production of low-alcohol wine, namely the overproduction of glycerol at the expense of ethanol. It was reported that the overexpression of the glycerol-3-phosphate dehydrogenase genes (*GPD1* and *GPD2*), together with the constitutive expression of the glycerol transport facilitator gene (*FPS1*) and the deletion of the *ALD6* acetaldehyde dehydrogenase gene, successfully redirected the carbon flux towards glycerol and the extracellular accumulation of glycerol without the formation of acetate. Depending on the genetic background of these engineered strains, 1.5- to four-fold increases in glycerol levels were obtained, with concomitant decreases in ethanol concentrations (Michnick et al. 1997; Remize et al. 1999; De Barros Lopes et al. 2000; Eglinton et al. 2002; Nevoigt et al. 2002). However, it remains to be proved whether the heterologous expression of the *A. niger GOX1* gene, together with the overexpression of the *S. cerevisiae GPD1, GPD2* and *FPS1* genes in *ald6* deletion wine yeast strains, would provide an effective means of bio-adjusting the alcohol content to appropriate levels in commercial wines.

In conclusion, this study has resulted in progress towards the development of antimicrobial wine yeast starter culture strains for the production of wine with reduced levels of chemical preservatives and alcohol. Together with the other concurrent strategies of producing antimicrobial peptides and enzymes, and overproducing glycerol at the expense of ethanol formation, the GOX-producing yeast might offer a viable way to meet consumers' demands for affordable low-alcohol wine with no or decreased levels of sulphites. However, further research is essential to ensure that such a yeast would not compromise the safety and sensory quality of the wine. Furthermore, it is important to realise that no commercial wine is currently being produced by a genetically modified yeast and that the wine industry will not use such strains as starter cultures unless both the industry and the consumers are satisfied that they are safe and beneficial. Thus, several obstacles relating to scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues (Vivier and Pretorius 2003) remain to be overcome in the short to medium term before a GOX-producing yeast could be harnessed for the commercial-scale production of low-alcohol wine free from chemical preservatives.

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References

Armstrong GO, Lambrechts MG, Mansvelt EPG, Van Velden DP, Pretorius IS (2001) Wine and health. S Afr J Sci 97:279–282

Bui K, Dick R, Moulin G, Galzy P (1986) A reverse osmosis for the production of low ethanol content wine. Am J Enol Vitic 37:297–300

Chung W, Hancock REW (2000) Action of lysozyme and nisin mixtures against lactic acid bacteria. Int J Food Microbiol 60:25–32

De Baetselier A, Vasavada A, Dohet P, Ha-Thi V, De Beukelaer M, Erpicum T, De Clerck L, Hanotier J, Rosenberg S (1991) Fermentation of a yeast producing *A. niger* glucose oxidase: scale-up, purification and characterization of the recombinant enzyme. Biotechnology 9:559–561

De Barros Lopes M, Rehman A-U, Gockowiak H, Heinrich AJ, Langridge P, Henschke PA (2000) Fermentation properties of a wine yeast overexpressing the *Saccharomyces cerevisiae* glycerol 3-phosphate dehydrogenase gene (*GPD2*). Aust J Grape Wine Res 6:208–215

De Vuyst L, Vandamme EJ (1994) Antimicrobial potential of lactic acid bacteria. In: De Vuyst L, Vandamme EJ (eds) Bacteriocins of lactic acid bacteria. Blackie, New York, pp 99–103

Drysdale GS, Fleet GH (1988) Acetic acid bacteria in winemaking: a review. Am J Enol Vitic 39:143–154

Du Toit M, Pretorius IS (2000) Microbial spoilage and preservation of wine: using weapons from nature's own arsenal a review. S Afr J Enol Vitic 21:74–96

Eglinton JM, Heinrich AJ, Pollnitz AP, Langridge P, Henschke PA, De Barros Lopes M (2002) Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the *ALD6* aldehyde dehydrogenase gene. Yeast 19:295–301

Frederick KR, Tung J, Emerick RS, Masiarz FR, Chamberlain SH, Vasavada A, Rosenberg S, Chakraborty S, Schopter LM, Massey V (1990) Glucose oxidase from *Aspergillus niger*: Cloning, gene sequence, secretion from *Saccharomyces cerevisiae* and kinetic analysis of a yeast-derived enzyme. J Biol Chem 265:3793–3802

Geisen R (1995) Expression of the *Aspergillus niger* glucose oxidase gene in *Penicillium nalgiovense*. World J Microbiol Biotechnol 11:322–325

Geisen R (1999) Inhibition of food-related pathogenic bacteria by god-transformed *Penicillium nalgiovense* strains. J Food Protect 62:940–943

Gerbaux V, Villa A, Monamy C, Bertrand A (1997) Use of lysozyme to inhibit malolactic fermentation and to stabilize wine after malolactic fermentation. Am J Enol Vitic 48:49–54

Gietz RD, Schiestl RH (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast 3:253–263

Gladstones J (2000) Implications of lowering wine alcohol content. Wine Industry J 15:45–46

Gladstones J, Tomlinson B (1999) A proposal by the Independent Winemakers Association for volumetric taxation based on alcohol content. Wine Industry J 14:92–99

Hammer FE (1998) Oxidoreductases. In: Nagodawithana T, Reed G (eds) Enzymes in food processing. Academic, New York, pp 251–254

Hodgkins M, Mead D, Ballance DJ, Goodey A, Sudbery P (1993) Expression of the glucose oxidase gene from *Aspergillus niger* in *Hansenula polymorpha* and its use as a reporter gene to isolate regulatory mutation. Yeast 9:625–635

Hong SY, Choi SK, Lee YH, Pek UH, Jung JK (1998) Overproduction and high level secretion of glucose oxidase in *Saccharomyces cerevisiae*. Korean J Appl Microbiol Biotechnol 26:68–75

Iland P, Eward A, Sitters J, Markides A, Bruer N (2000) Determination of alcohol by ebulliometry. In: Techniques for chemical analysis and quality monitoring during winemaking. Patrick Iland Wine Promotions, Australia, pp 34–35

Kapat A, Jung J-K, Park Y-H (2001) Enhancement of glucose oxidase production in batch cultivation of recombinant *Saccharomyces cerevisiae*: optimization of oxygen transfer condition. J Appl Microbiol 90:216–222

Lee Y-H, Lin T-I (1996) Determination of carbohydrates by high-performance capillary electrophoresis with indirect absorbance detection. J Chrom B 681:87–97

Mermelstein NH (2000) Removing alcohol from wine. Food Technol 54:89–92

Michnick S, Roustan J-L, Remize F, Barre P, Dequin S (1997) Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for *GPD1* encoding glycerol 3-phosphate dehydrogenase. Yeast 13:783–793

Nevoigt E, Pilger R, Mast-Gerlach E, Schmidt U, Freihammer S, Eschenbrenner M, Garbe L, Stahl U (2002) Genetic engineering of brewing yeast to reduce the content of ethanol in beer. FEMS Yeast Res 2:225–232

Ohlmeyer DW (1957) Use of glucose oxidase to stabilize beer. Food Technol October, pp 503–507

Park EH, Shin Y-M, Lim Y-Y, Kwon T-H, Kim D-H, Tang M-S (2000) Expression of glucose oxidase by using recombinant yeast. J Biotechnol 81:35–44

Pickering G (1998) The use of enzymes to stabilise colour and flavour in wine—an alternative to SO_2. Aust Grapegrower and Winemaker September, pp 101–103

Pickering G, Heatherbell DA (1996) Characterisation of reduced alcohol wine made from glucose oxidase treated must. Food Technol 26:101–107

Pickering GJ, Heatherbell DA, Barnes MF (1998) Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. Food Res Int 31:685–692

Pickering GJ, Heatherbell DA, Barnes MF (1999a) The production of reduced-alcohol wine using glucose oxidase treated juice. Part I. Composition. Am J Enol Vitic 50:291–298

Pickering GJ, Heatherbell DA, Barnes MF (1999b) The production of reduced-alcohol wine using glucose oxidase treated juice. Part II. Stability and SO_2-binding. Am J Enol Vitic 50:299–306

Pickering GJ, Heatherbell DA, Barnes MF (1999c) The production of reduced-alcohol wine using glucose oxidase treated juice. Part III. Sensory. Am J Enol Vitic 50:307–316

Power J (1998) Enzymes in brewing. In: Nagodawithana T, Reed G (eds) Enzymes in food processing. Academic , New York, pp 455–456

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16:675–729

Pretorius IS (2003) The genetic improvement of wine yeasts. In: Arora D (ed) Fungal biotechnology. Decker, New York (in press)

Pretorius IS, Bauer FF (2002) Meeting the consumer challenge through genetically customised wine yeast strains. Trends Biotechnol 20:426–432

Radler F (1990a) Possible use of nisin in winemaking. I. Action of nisin against lactic acid bacteria and wine yeasts in solid and liquid media. Am J Enol Vitic 41:1–6

Radler F (1990b) Possible use of nisin in winemaking. II. Experiments to control lactic acid bacteria in the production of wine. Am J Enol Vitic 41:7–11

Remize F, Roustan JL, Sablayrolles JM, Barre P, Dequin S (1999) Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. Appl Environ Microbiol 65:143–149

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory , Cold Spring Harbor, New York

Schoeman H, Vivier MA, Du Toit M, Dicks LMT, Pretorius IS (1999) The development of bacterial yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (*pedA*) in *Saccharomyces cerevisiae*. Yeast 15:647–656

Scudamore-Smith P, Moran J (1997) A growing market for reduced alcohol wines. Wine Ind J 12:165–167

Struhl K, Stincomb DT, Scherer S, Davis RW (1979) High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc Natl Acad Sci USA 76:1035–1039

Vemulapalli V, Miller KA, Hoseney RC (1998) Glucose oxidase in breadmaking systems. Cereal Chem 75:439–442

Villettaz JC (1987) A new method for the production of low alcohol wines and better balanced wines. Proceedings of Sixth

Australian Wine Industry Technical Conference, Australian Industrial Publishers, Adelaide

Vivier MA, Pretorius IS (2002) Genetically tailored grapevines for a wine industry that is sustainable and focussed on quality. Trends Biotechnol 20:472–478

Whittington H, Kerry-Williams S, Bidgood K, Dodsworth N, Peberdy J, Dobson M, Hinchliffe E, Ballance DJ (1990) Expression of the *Aspergillus niger* glucose oxidase gene in *A. niger*, *A. nidulans* and *Saccharomyces cerevisiae*. Curr Genet 18:531–536