Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae*

by

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DECLARATION

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

Daniël Francois Malherbe **Date**

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SUMMARY

The winemaking process constitutes a unique ecological niche that involves the interaction of yeasts, lactic acid bacteria and acetic acid bacteria. *Saccharomyces cerevisiae* has established its importance as a wine yeast and also proven itself as a reliable starter culture organism. Its primary role is to convert the grape sugar into alcohol and, secondly, its metabolic activities result in the production of higher alcohols, fatty acids and esters, which are important flavour and aroma compounds that are essential for consistent and predictable wine quality. There is a growing consumer demand for wine containing lower levels of alcohol and chemical preservatives.

Glucose oxidase (GOX) has received considerable research interest regarding its potential application in the wine industry to reduce alcohol levels and as a biocontrol agent. Several physical processes are used for the removal or reduction of alcohol in wine and some of them are sometimes used in combination. These processes tend to involve expensive equipment and can be intensive from a processing point of view. An alternative approach was introduced with the concept of treating grape must with GOX to reduce the glucose content of the must, and therefore produce a wine with a reduced alcohol content after fermentation. Due to the demanding nature of modern winemaking practices and sophisticated wine markets, there is an ever-growing quest for specialised wine yeast strains possessing a wide range of optimised, improved or novel oenological properties.

The first and main objective of this study was to genetically engineer wine yeasts to produce wine with a reduced alcohol content. In order to do this, the structural glucose oxidase (*gox)* gene of *Aspergillus niger* 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 and terminator ($PGK1_{PT}$). This $PGK1_P$-$MF\alpha1_S$-*gox*-$PGK1_T$ gene cassette (designated *GOX1*) was introduced into a laboratory strain of *S. cerevisiae* (Σ1278). Results obtained indicated the production of biologically active glucose oxidase and showed that it is secreted into the culture medium. This would mean that the enzyme will convert the glucose to gluconic acid in the medium before the yeast cells are able to metabolise the glucose to ethanol. Microvinifications performed with Chardonnay grapes showed that the laboratory yeast starter cultures transformed with *GOX1* were indeed able to reduce the total amount of alcohol in the finished product.

The second objective of this study involved the potential application of GOX as a biocontrol agent. Screening was performed for wine spoilage microorganisms, such as acetic acid bacteria and lactic acid bacteria, using plate assays. The wine spoilage microorganisms tested formed different sized inhibition zones, indicating varying degrees of inhibition. The inhibition of some of the wine spoilage microorganisms was confirmed under a scanning electron microscope. The total collapse of the bacterial cell wall could be seen and might be explained by the fact that a final product of the GOX enzymatic reaction is hydrogen peroxide (H_2O_2). The produced 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 is the cause of the destruction of basic molecular structures, such as nucleic acids and cell proteins.

In this exciting age of molecular yeast genetics and modern biotechnology, this study could pave the way for the development of wine yeast starter culture strains for the production of wine with a lower alcohol content and reduced levels of chemical

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preservatives, such as sulphur dioxide. The use of genetically modified organisms (GMOs) within the wine industry is a limiting factor at present and credible means must be found to effectively address the concerns of traditionalists within the wine industry and the negative overreaction by some consumer groups. There is a vast potential benefit to the wine consumer and industry alike and the first recombinant wine products therefore should unmistakably demonstrate safe products free of potentially harmful compounds, and have organoleptic, hygienic and economic advantages for both the wine producer and consumer.

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OPSOMMING

Die wynmaakproses behels 'n ekologiese interaksie tussen gis, asynsuurbakterieë en melksuurbakterieë. *Saccharomyces cerevisiae* het homself alreeds bewys as 'n belangrike en betroubare inisiëringsgis in wyn. Die hoofdoel van die gis is om druifsuikers na etanol om te skakel. Tweedens lei die gis se metaboliese aktiwiteite tot die produksie van hoër alkohole, vetsure en esters, wat tot die konsekwente voorspelbare smaak en aromaverbindings in herhaalbare kwaliteit wyn bydra. Daar is 'n toenemende aanvraag na wyne met 'n laer alkoholinhoud en minder preserveermiddels.

Glukoseoksidase (GOX) het heelwat navorsing in die wynindustrie uitgelok omdat dit gebruik kan word om die alkoholinhoud in wyn te verlaag, asook as 'n biologiese beheermiddel kan funksioneer. Daar is reeds sekere fisiese prosesse wat gebruik kan word om die alkohol in wyn te verwyder of te verminder. Sommige van hierdie prosesse word soms in kombinasie gebruik. Die nadeel is egter dat hierdie prosesse baie duur en intensief is, veral ten opsigte van prosessering. 'n Alternatief om die alkoholinhoud van wyn te verlaag, het egter na vore gekom toe daar voorgestel is om die mos met GOX te behandel. As gevolg van die veeleisende aard van moderne wynmaakpraktyke en gesofistikeerde wynmarkte, is daar 'n nimmereindigende soektog na meer gespesialiseerde wyngisrasse wat 'n wye reeks van geoptimiseerde en verbeterde, en selfs unieke, wynkundige einskappe bevat.

Die hoofdoelwit van hierdie navorsingsprojek behels die genetiese manipulasie van 'n gisras sodat dit in staat is om wyn met 'n laer alkoholinhoud te produseer. Om hierdie doel te verwesentlik, is die strukturele glukoseoksidasegeen (*gox*) van *Aspergillus niger* in 'n integreringsvektor gekloneer. Transkripsie-inisiëring en -terminering is deur fosfogliseraatkinase-1-promotor en -termineerder ($PGK1_{PT}$) bewerkstellig. Die α-spesifieke gisferomoon-α-faktor ($MF\alpha 1_S$) is gebruik om die uitskeiding van GOX uit die gis te bewerkstellig. Saam vorm bogenoemde die $PGK1_P$-$MF\alpha 1_S$-*gox*-$PGK1_T$-geenkasset, wat as *GOX1* bekend is. *GOX1* is na 'n labaratoriumras van *S. cerevisiae* (Σ1278) getransformeer. Resultate dui aan dat biologies aktiewe GOX geproduseer en uitgeskei word. Dit beteken dat van die glukose in die medium reeds na glukoonsuur omgesit sal word voordat die gis dit kan begin benut en alkohol produseer. Kleinskaalse wynmaakprosesse wat met Chardonnay-druiwe en GOX-produserende labaratoriumgis uitgevoer is, het inderdaad tot laer alkoholpersentasies gelei.

Die tweede doelwit van die navorsingsprojek was om te bepaal of GOX die potensiaal as biologiese beheermiddel het. Daar is ondersoek ingestel na sekere wynbederfsorganismes soos asynsuur- en melksuurbakterieë en die inhibisie van die organismes is op agarplate gemonitor. Verskillende grade van inhibisie, soos die grootte van die inhibisiesone, was sigbaar vir die verskillende wynbederfsorganismes wat getoets is. Die inhibiese van sekere wynbederfsorganismes is ook met behulp van 'n skandeerelektronmikroskoop bevestig. Die totale ineenstorting van die bakteriële selwand was sigbaar en kan verklaar word deur die teenwoordigheid van waterstofperoksied (H_2O_2). Laasgenoemde is 'n byproduk van die laaste metaboliese reaksie en staan as 'n antimikrobiese middel bekend. Die byproduk (H_2O_2) gee aanleiding tot hiperbariese suurstoftoksisiteit, 'n gevolg van die peroksidasie van membraanlipiede en 'n sterk oksiderende effek t.o.v. die bakteriële selwand. Dit lei tot die vernietiging van die basiese molekulêre strukture, soos die nukleïensure en selproteïene.

Tydens hierdie opwindende era van molekulêre gisgenetika en biotegnologie kan hierdie navorsing die fondament lê vir die ontwikkeling van 'n wyngiskultuur wat in staat is

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om wyn met 'n laer alkoholinhoud te produseer. Die gebruik van geneties gemanupileerde organismes (GMO's) in die wynbedryf is egter nog 'n beperkende faktor. 'n Geloofwaardige manier moet dus gevind word om die bekommernisse van tradisionaliste, asook die negatiewe oorreaksies van sommige verbruikers, aan te spreek en hok te slaan. Daar is groot potensiaal en voordele vir beide die verbruiker en industrie. Dit is dus belangrik dat die eerste rekombinante wynprodukte wat die mark betree, veilig en vry van potensieel skadelike verbindings is, asook organoleptiese, higiëniese en ekonomiese voordele toon te opsigte van beide die wynprodusent en gebruiker.

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This thesis is dedicated to Prof. H.J.J. van Vuuren, who introduced me to the world of microbiology, and more specifically molecular biology.

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BIOGRAPHICAL SKETCH

Daniël Francois Malherbe was born in Bellville, South Africa on 1 February 1975. He attended Eikestad Primary School and matriculated at Paul Roos Gymnasium, Stellenbosch in 1993.

Danie entered the Stellenbosch University (Stellenbosch) and obtained a BSc degree in Genetics and Microbiology in 1998. In 1999 he completed a BScHons degree in Wine Biotechnology at the Institute for Wine Biotechnology. In 2000, Danie enrolled for an MSc degree in Wine Biotechnology.

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PREFACE

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

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Chapter 2 **LITERATURE REVIEW**
Glucose oxidase and its possible applications

Chapter 3 **RESEARCH RESULTS**
Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

Wine production has deep cultural, historical and economic roots in South Africa and has evolved over a period of 350 years from the traditions of the Dutch, German and French Huguenot colonists at the Cape. For the first 200 years after the establishment of the replenishment station at the southern tip of Africa in 1652, wine exports were the main source of "foreign exchange". Today, about 104 000 hectares are covered by vineyards and the annual wine production fluctuated between 800 and 900 million litres per year during the 1990s. Over the last 20 years, between 1980 and 1999, consumption of wine increased on average by a compound rate of only 2,5% per year. This places the South African wine industry as the 11th largest wine industry in the world in terms of volumes of wine produced (Spies, 2001).

As the drinking habits of consumers change and the implications of alcohol consumption for health and other related social issues (such as road safety) start to play a role in everyday life, one should look at other marketing strategies for international and domestic wine advertising. Over the past decade, the focus has shifted within the wine industry and there has been increased international interest in, and consumer demand for, low-alcohol, "reduced-alcohol" and de-alcoholised wines (Scudamore-Smith & Moran, 1997; Pickering *et al.*, 1998). In addition, in South Africa, which has a warm climate, grapes tend to have higher sugar concentrations and this leads to the production of wines with high alcohol levels. If South Africa wants to compete with wines from the Northern Hemisphere, which usually have an alcohol content of 11-12%, a possible solution is to harvest grapes earlier than usual, when the berries have lower sugar levels. This, however, could have an overall negative influence on wine quality, as not all the flavour and aroma components would have developed optimally. Commercial interest has also been stimulated by the potential for savings in tax and tariffs on the reduced alcohol content in these wines (Pickering *et al.*, 1998; Gladstones & Tomlinson, 1999; Gladstones, 2000).

Enzymes play a crucial role in the winemaking process, and many of these enzymes originate from the grape itself, from the indigenous microflora on the grapes and from the microorganisms present during winemaking. In addition to enzymes that occur in pre- and post-fermentation practices, there are at least ten different enzymes driving the fermentation kinetics that convert grape juice to wine. The endogenous enzymes of grapes, yeasts and other microorganisms present in must and wine often are neither efficient nor sufficient under winemaking conditions to effectively catalyse the various biotransformation reactions. Commercial enzyme preparations therefore are used widely to enhance wine fermentations. Consequently it is of key importance to understand the nature and behaviour of these enzymes and to create the optimal conditions to exploit those enzymes that are valuable, while inhibiting those that may be harmful to the quality of the wine. Research in this field is very active and continually expanding. Recently, it also was suggested to use aerobic yeasts for the production of low-alcohol wines (Erten & Campbell, 2001), but the prospect of developing wine yeast strains expressing heterologous enzymes is also available (Whittington *et al.*, 1990; Park *et al.*, 2000; Van Rensburg & Pretorius, 2000; Kapat *et al.*, 2001).

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Some of the important enzymes in winemaking can be classified as those that act in (i) the clarification and processing of wine (pectinases, glucanases, xylanases, proteases); (ii) the release of varietal aromas from precursor compounds (glycosidases); (iii) the reduction of ethyl carbamate formation (urease); and (iv) the reduction of alcohol levels (glucose oxidase) (Uhlig, 1998; Van Rensburg & Pretorius, 2000).

The concept of treating grape must with glucose oxidase (GOX), which has GRAS status (Generally Regarded As Safe), to reduce the glucose content of the must and thereby produce a wine with a lower alcohol content after fermentation (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998; Pickering *et al.*, 1999a, b, c) was recently introduced as an alternative approach to several physical processes that have been designed and used specifically for the removal or the reduction of alcohol in wine. Some of these processes may be used in combination and include: distillation, thermal evaporation, 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 can also be intensive from the point of view of processing (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998). Concerns have also been raised as to the sensory quality of the finished product (wine) and, in addition, the partial or incomplete fermentation of immature grapes with a low sugar content can have the inherent problem of excess residual sugar and a lack of flavour development in the resulting wine (Pickering *et al.*, 1998). When must is treated with GOX, the enzyme converts glucose into gluconic acid (which also has GRAS status), which is not metabolised by wine yeasts. Wines produced in this way should have reduced levels of ethanol and higher acidity. Furthermore, this technology could also be employed to produce a reserve of acidic musts or wines for blending purposes (Canal-Llaubères, 1993).

1.2 PROJECT AIMS

This study forms part of an important research project on reduced alcohol wines within the Institute for Wine Biotechnology and is funded by Winetech (Wine Industry Network of Expertise and Technology). As there is an increased international interest and consumer demand for low-alcohol, "reduced-alcohol" and de-alcoholised wines, it is important to create a fast, reliable and inexpensive method to reduce the alcohol in wine. In this study, we have attempted to genetically enhance a yeast strain to reach this goal. The possibility of controlling wine spoilage bacteria, such as lactic acid bacteria and acetic acid bacteria, was also investigated.

The specific aims of this study were the following:

(i) to sub-clone the glucose oxidase gene (*gox*) from *Aspergillus niger* into a multicopy yeast expression vector, as well as into an integration vector, and to introduce the *GOX1* gene cassette into *Saccharomyces cerevisiae*;

(ii) the analysis of yeast transformants for integration of the gene;

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(iii) screening of yeast transformants for the production of glucose oxidase with a plate assay;

(iv) to quantitatively determine the levels of glucose oxidase by glucose oxidase-producing yeast, both intracellularly and extracellularly;

(v) to evaluate the antimicrobial activity of *S. cerevisiae*-producing GOX against lactic acid bacteria and acetic acid bacteria;

(vi) to perform scanning electron microscopy to assess the morphological status of lactic acid bacteria and acetic acid bacteria treated with GOX; and

(vii) to perform microvinification trials to evaluate the glucose oxidase-producing laboratory *S. cerevisiae* strain for reduced alcohol levels.

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CHAPTER 2

LITERATURE REVIEW

Glucose oxidase and its possible applications

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2. LITERATURE REVIEW

Until the beginning of the previous century, wine was made by taking advantage of the natural yeasts present on the surface of grape skins. These yeasts were responsible for spontaneous natural wine fermentations. Today, yeasts are selected for specific characteristics, such as a high fermentation rate, low volatile acidity, low sulphur dioxide (SO_2) formation, etc. (Pretorius, 2000). Many of these characteristics are connected directly to specific enzymes and it is clear that they play a crucial role in the effective catalysation of various biotransformation reactions. This makes them of utmost importance to the winemaking process and is why we need to understand the nature and behaviour of these enzymes in order, to create optimal conditions and exploit those enzymes that are valuable, while inhibiting those that may be harmful to the quality of the wine. The use of glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) in the winemaking process will be the main focus of this literature study, with special emphasis on wines with reduced alcohol.

2.1. HISTORY OF MICROBIAL GLUCOSE OXIDASE

Maximov first discovered "enzyme" activity in 1904 in a dry powder from the mycelia of *Aspergillus niger*, but Müller was the first to relate this oxidative action to an enzyme (Müller, 1928; Uhlig, 1998a). Müller discovered the enzyme in *Aspergillus niger* and *Penicillium glaucum* and called it glucose oxidase. Franke and Lorenz partially purified the enzyme in 1937 and found that hydrogen peroxide was formed when this enzyme was allowed to act on glucose. They also suggested that this enzyme was a flavo-protein (Baldwin *et al.*, 1953). In 1943, another "substance" with antibacterial properties was reported. It was called penicillin B and was isolated from *Penicillium notatum*. Upon purification, the substance appeared to have the properties of a protein (but differs markedly from the penicillin discovered by Fleming) and was highly effective *in vitro* against both Gram-negative and Gram-positive bacteria (Roberts *et al.*, 1943). In the same year (1943), Van Bruggen and co-workers independently purified the same enzyme and also called it penicillin B (Van Bruggen *et al*, 1943). At this stage, this enzyme was known as penicillin B, penatin (Kocholaty, 1942) and glucose oxidase. In 1945, however, another enzyme, which was also able to oxidise glucose to gluconic acid and hydrogen peroxide in the presence of molecular oxygen and water, was purified, this time from *Penicillium notatum*, and named notatin (Coulthard *et al.*, 1942; Coulthard *et al.*, 1945; Keilin & Hartree, 1948; Keilin & Hartree, 1952). It was at this point that it was generally conceded that glucose oxidase, notatin and penicillin B were one and the same enzyme (Baldwin *et al.*, 1953).

Dwight Baker identified the potential commercial application of glucose oxidase in 1943, when he developed the first method of industrial production as he sought a means of coping with the oxidative changes in beer due to the high level of air in bottled beer (Uhlig, 1998b). The first commercial product, however, only entered the market in 1952. Large-scale enzymatic preparations are commonly produced from *A. niger* or *Penicillium amagasakiense* (Kusai *et al.*, 1960). Earlier, other *Penicillium* strains, such as *P. notatum* or *P. glaucum*, were also used (Keilin & Hartree, 1948).

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Oxygen can be responsible for certain changes in food. Enzymes are the main catalysts of these oxidative processes or reactions and the specific enzyme group is known as the oxidoreductases. There are many examples of these oxidative reactions, such as the browning of fruit or vegetables caused by polyphenoloxidase, which is also responsible for the darkening of mushroom or potato tissue when it is injured (Uhlig, 1998a). Another oxidase, lipoxygenase, which occurs in soybeans, oxidises unsaturated fatty acids. Similar oxidation can occur with carotene and is used for bleaching flour in the manufacturing of baked goods. Furthermore, enzymatically catalysed oxidation plays a major role in the formation of aroma compounds, but some of these flavour compounds are unwanted off-flavours. In addition to the above-mentioned rancidity, oxidative rancidity can occur in milk fat due to lipolysis and oxidases can also destroy valuable vitamins, such as ascorbic acid (Uhlig, 1998a).

To eliminate the damaging effects of these oxidoreductases in food, it is necessary to inactivate these enzymes. This can be done by heat or by the total elimination of oxygen from the food (Uhlig, 1998a). The elimination of oxygen can be done with GOX, which catalyses the oxidation of β-D-glucose to β-D-gluconic acid and hydrogen peroxide in the presence of molecular oxygen.

Glucose oxidase is an intracellular enzyme that is not released in the medium during culturing, but remains within the cell. The exception is *Penicillium*, in which case the rapid release of the enzyme is observed during its growth. This phenomenon is explained by the autolysis of the mycelia.

The enzyme therefore has to be extracted from the cells during the manufacturing process. This is accomplished by mechanical destruction of the cell walls or by the introduction of autolytic processes, supported by exogenous enzymes, whereafter the stabilised enzymes are purified. This is done by filtration or ultrafiltration, and the enzymes are then concentrated and stabilised or dried (Uhlig, 1998a).

The idea that GOX acts as an antibiotic (notatin) was discarded when it was discovered that the antimicrobial activity resulted from the formation of hydrogen peroxide (Uhlig, 1998a). Subsequently, the enzyme has been purified from *Aspergillus niger* (Pazur & Kleppe, 1964; Swoboda & Massey, 1965), *Penicillium notatum* (Coulthard *et al.*, 1945; Keilin & Hartree, 1948), *Penicillium amagasakiense* (Kusai *et al.*, 1960) and a number of other sources. It generally has been assumed that GOX from different sources has the same properties. This proved not to be the case and it was shown that there indeed are differences regarding their physical and chemical properties (Swoboda & Massey, 1965). The enzymes of *Aspergillus* and *Penicillium* differ in amino acid composition, but otherwise have similar properties (**Table 2.1**) (Uhlig, 1998a).

Table 2.1 Comparison of glucose oxidase from different sources (Uhlig, 1998a)

Enzyme Source	Molecular Weight (Daltons)	K_m	iP
Penicillium amagasakiense	154 000	1.15×10^{-2}	4.35
Penicillium notatum	138 000 – 152 000	0.96×10^{-2}	-
Aspergillus niger	192 000	1.1×10^{-2}	4.5

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2.2 GLUCOSE OXIDASE FROM *ASPERGILLUS NIGER*

2.2.1 The gene

The *GOX* gene consists of 1815 base pairs (bp), encoding 605 amino acid residues. The mature protein contains 584 amino acids. The difference is due to 21 amino acids comprising the signal sequence that display an unusual feature. The amino acid in position –1 is a basic one, namely arginine, which causes an unexpected cleavage at the arginine-serine bond (Kriechbaum *et al.*, 1989). The mature enzyme consists of 583 amino acids and is preceded by a 22 amino acid pre-sequence (Frederick *et al.*, 1990). No introns or intervening sequences have been found or identified in the coding region of the GOX sequence (Kriechbaum *et al.*, 1989; Frederick *et al.*, 1990). The deduced amino acid sequence of the coding region is almost identical to the known parts of the protein sequence, although a small number of amino acid exchanges have been observed. The overall DNA sequence of the gene, as well as the corresponding amino acids, is shown in **Figure 2.1**.

2.2.2 Regulation

The 5'-noncoding region shows a CT-rich sequence 12 base pairs (bp) upstream of the ATG start codon. These regions are characteristic of highly expressed genes or genes lacking the TATA and CAAT boxes from *Saccharomyces cerevisiae* and several other filamentous fungi. Therefore it is suggested that these CT regions may act as promoter elements. In filamentous fungi, one transcription initiation site often appears in or immediately downstream from these pyrimidine-rich sequences. Two sites (CATC) showing homology to known *Aspergillus niger* transcription initiation sites were found within the region between the CT sequence and the start codon (Kriechbaum *et al.*, 1989).

2.2.3 The enzyme

2.2.3.1 Glucose oxidase (β-D-glucose:oxygen 1-oxido-reductase, EC 1.1.3.4)

Glucose oxidase from *Aspergillus niger*, a homodimer with a molecular mass of about 160 kDa, is a flavin-containing globular glycoprotein with a carbohydrate content of 16% (w/w) (Pazur *et al.*, 1965; Hayashi & Nakamura, 1981). The carbohydrate modules are of the high-mannose type, and the oligomeric polysaccharide is covalently attached to polypeptide chains via asparagine and serine or threonine residues (Takegawa *et al.*, 1989). The enzyme contains three cysteine residues and eight potential sites for N-linked glycosylation. The protein shows 26% identity with the alcohol oxidase of *Hansenula polymorpha* and the N terminus has a sequence homologous with the AMP-binding region of other flavo-enzymes, such as *p*-hydroxybenzoate hydroxylase and glutathione reductase (Frederick *et al.*, 1990). The enzyme also contains two tightly bound, but noncovalently linked, flavin adenine dinucleotide (FAD) molecules per dimer (Pazur & Kleppe, 1964) (**Figure 2.2**).

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M Q T L L V S S L V V S L A
5'- CAACCAGCCTTTCCTCTCTCATTCCCTCATCTGCCCATC **ATG** CAG ACT CTC CTT GTG AGC TCG CTT GTG GTC TCC CTC GCT

A A L P H Y I R S N G I E A S L L T D P K D V S G
GCG GCC CTG CCA CAC TAC ATC AGG AGC AAT GGC ATT GAA GCC AGC CTC CTG ACT GAT CCC AAG GAT GTC TCC GGC

R T V D Y I I A G G G L T G L T T A A R L T E N P
CGC ACG GTC GAC TAC ATC ATC GCT GGT GGA GGT CTG ACT GGA CTC ACC ACC GCT GCT CGT CTG ACG GAG AAC CCC

N I S V L V I E S G S Y E S D R G P I I E D L N A
AAC ATC AGT GTG CTC GTC ATC GAA AGT GGC TCC TAC GAG TCG GAC AGA GGT CCT ATC ATT GAG GAC CTG AAC GCC

Y G D I F G S S V D H A Y E T V E L A T N N Q T A
TAC GGC GAC ATC TTT GGC AGC AGT GTA GAC CAC GCC TAC GAG ACC GTG GAG CTC GCT ACC AAC AAT CAA ACC GCG

L I R S G N G L G G S T L V N G G T W T R P H K A
CTG ATC CGC TCC GGA AAT GGT CTC GGT GGC TCT ACT CTA GTG AAT GGT GGC ACC TGG ACT CGC CCC CAC AAG GCA

Q V D S W E T V F G N E G W N W D N V A A Y S L Q
CAG GTT GAC TCT TGG GAG ACT GTC TTT GGA AAT GAG GGC TGG AAC TGG GAC AAT GTG GCC GCC TAC TCC CTC CAG

A E R A R A P N A K Q I A A G H Y F N A S C H G V
GCT GAG CGT GCT CGC GCA CCA AAT GCC AAA CAG ATC GCT GCT GGC CAC TAC TTC AAC GCA TCC TGC CAT GGT GTT

N G T V H A G P R D T G D D Y S P I V K A L M S A
AAT GGT ACT GTC CAT GCC GGA CCC CGC GAC ACC GGC GAT GAC TAT TCT CCC ATC GTC AAG GCT CTC ATG AGC GCT

V E D R G V P T K K D F G C G D P H G V S M F P N
GTC GAA GAC CGG GGC GTT CCC ACC AAG AAA GAC TTC GGA TGC GGT GAC CCC CAT GGT GTG TCC ATG TTC CCC AAC

T L H E D O V R S D A A R E W L L P N Y O R P N L
ACC TTG CAC GAA GAC CAA GTG CGC TCC GAT GCC GCT CGC GAA TGG CTA CTT CCC AAC TAC CAA CGT CCC AAC CTG

O V L T G O Y V G K V L L S O N G T T P R A V G V
CAA GTC CTG ACC GGA CAG TAT GTT GGT AAG GTG CTC CTT AGC CAG AAC GGC ACC ACC CCT CGT GCC GTT GGC GTG

E F G T H K G N T H N V Y A K H E V L L A A G S A
GAA TTC GGC ACC CAC AAG GGC AAC ACC CAC AAC GTT TAC GCT AAG CAC GAG GTC CTC CTG GCC GCG GGC TCC GCT

V S P T I L E Y S G I G M K S I L E P L G I D T V
GTC TCT CCC ACA ATC CTC GAA TAT TCC GGT ATC GGA ATG AAG TCC ATC CTG GAG CCC CTT GGT ATC GAC ACC GTC

V D L P V G L N L O D O T T A T V R S R I T S A G
GTT GAC CTG CCC GTC GGC TTG AAC CTG CAG GAC CAG ACC ACC GCT ACC GTC CGC TCC CGC ATC ACC TCT GCT GGT

A G O G Q A A W F A T F N E T F G D Y S E K A H E
GCA GGA CAG GGA CAG GCC GCT TGG TTC GCC ACC TTC AAC GAG ACC TTT GGT GAC TAT TCC GAA AAG GCA CAC GAG

L L N T K L E O W A E E A V A R G G F H N T T A L
CTG CTC AAC ACC AAG CTG GAG CAG TGG GCC GAA GAG GCC GTC GCC CGT GGC GGA TTC CAC AAC ACC ACC GCC TTG

L I O Y E N Y R D W I V N H N V A Y S E L F L D T
CTC ATC CAG TAC GAG AAC TAC CGC GAC TGG ATT GTC AAC CAC AAC GTC GCG TAC TCG GAA CTC TTC CTC GAC ACT

A G V A S F D V W D L L P F T R G Y V H I L D K D
GCC GGA GTA GCC AGC TTC GAT GTG TGG GAC CTT CTG CCC TTC ACC CGA GGA TAC GTT CAC ATC CTC GAC AAG GAC

P Y L H H F A Y D P Q Y F L N E L D L L G Q A A A
CCC TAC CTT CAC CAC TTC GCC TAC GAC CCT CAG TAC TTC CTC AAC GAG CTG GAC CTG CTC GGT CAG GCT GCC GCT

T Q L A R N I S N S G A M O T Y F A G E T I P G D
ACT CAA CTG GCC CGC AAC ATC TCC AAC TCC GGT GCC ATG CAG ACC TAC TTC GCT GGG GAG ACT ATC CCC GGT GAT

N L A Y D A D L S A W T E Y I P Y H F R P N Y H G
AAC CTC GCG TAT GAT GCC GAT TTG AGC GCC TGG ACT GAG TAC ATC CCG TAC CAC TTC CGT CCT AAC TAC CAT GGC

V G T C S M M P K E M G G V V D N A A R V Y G V O
GTG GGT ACT TGC TCC ATG ATG CCG AAG GAG ATG GGC GGT GTT GTT GAT AAT GCT GCC CGT GTG TAT GGT GTG CAG

G L R V I D G S I P P T Q M S S H V M T V F Y A M
GGA CTG CGT GTC ATT GAT GGT TCT ATT CCT CCT ACG CAA ATG TCG TCC CAT GTC ATG ACG GTG TTC TAT GCC ATG

A L K I S D A I L E D Y A S M O
GCG CTA AAA ATT TCG GAT GCT ATC TTG GAA GAT TAT GCT TCC ATG CAG **TGA** GTGGTATGATGGGGATATGAGTGAGGATATT

AGGGGATGGTACTTAGATGCTGGGGAGGTATAATCATAGATTGGATAGAATTGGTAGGTTACATAGACAGGTTACATGAATAGACGTTCGTTATATGTG

AGCAGACATTACTACCAAACAAGGGCATTGTTCAGTT

Figure 2.1 Sequence of the GOX-encoding gene from *Aspergillus niger*. The DNA sequence and the derived amino acid sequence are given (Kriechbaum *et al.*, 1989).

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These flavin co-factors are responsible for the oxidation-reduction properties of the enzyme. GOX is an acidic protein, with a net negative charge at a neutral pH (Jones *et al.*, 1982), and shows resistance to sodium n-dodecyl sulphate (SDS) denaturation at pH 6.0, whereas most other globular proteins are denatured. However, it is susceptible to denaturation at a pH lower than 4.3 (Jones *et al.*, 1982). Dissociation of the subunits of GOX has been reported to be possible only under denaturing conditions and is accompanied by the loss of co-factor FAD (O'Malley & Weaver, 1972; Jones *et al.*, 1982). The apoenzyme produced on removal of the FAD has a molecular weight of 153 kDa and can exist in two conformational states, with sedimentation coefficients of 4.5 S and 8.0 S (Swoboda & Massey, 1965). However, it is suggested that the apoenzyme has the molecular weight of the subunit, 79 000 Da $\pm$ 4000 Da (Tsuge *et al.*, 1975). There is general agreement that the holoenzyme has two subunits, although O'Malley and Weaver (1972) argue that they are linked covalently by disulphide bonds. This has been found not to be the case (Jones *et al.*, 1982).

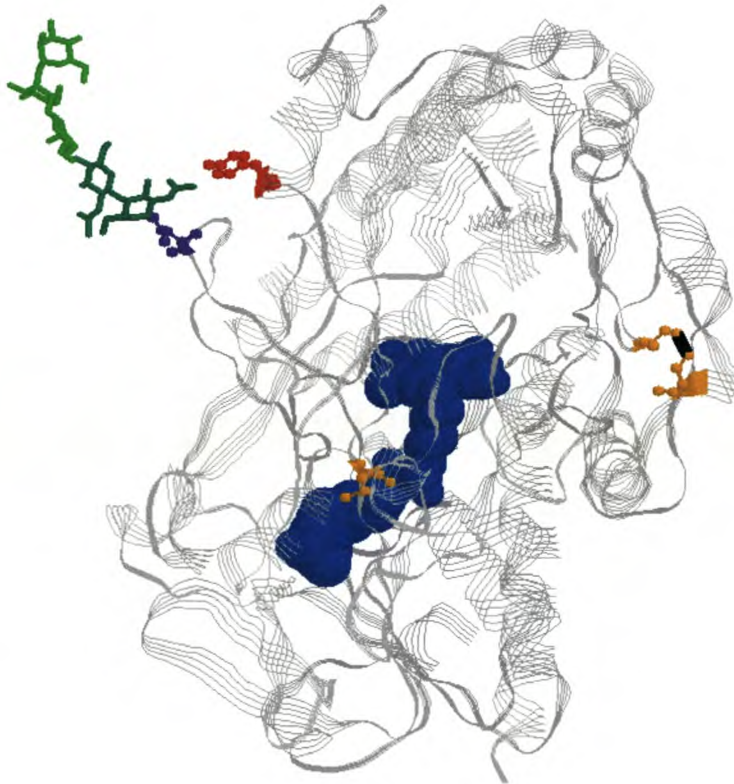

Figure 2.2. The GOX enzyme: a FAD molecule can be seen in the middle (blue), cysteine molecules are orange and the di-sulphide bridge is black (to the right). Green represents mannose (to the left) and green-blue is N-acetyl-D-glucosamine. Asparagine and tyrosine are red and purple respectively.

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2.2.3.2 Enzymatic reaction

Glucose oxidase catalyses the oxidation of β-D-glucose to δ-D-gluconolactone (Gibson *et al.*, 1964) (**Figure 2.3**). Previous examination of the substrate specificity has shown that the enzyme can also attack 2-deoxy-D-glucose, D-mannose, D-galactose and D-xylose (Keilin & Hartree, 1948). In addition, the rate of glucose oxidation catalysed by glucose oxidase is much faster than the oxidation of mannose, galactose and xylose, and the turnover numbers for these three sugars are low (Gibson *et al.*, 1964).

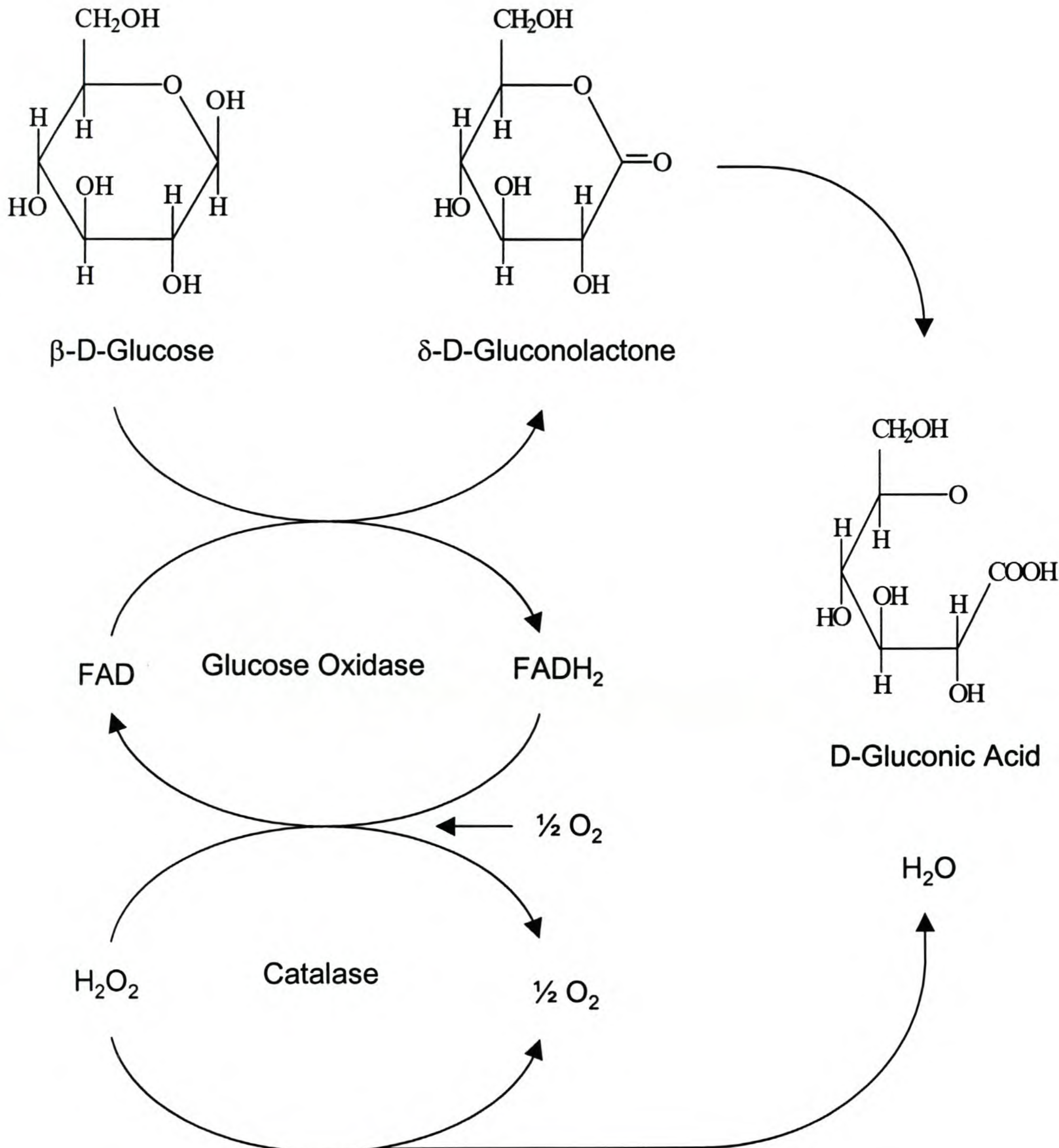

Figure 2.3 The reaction mechanism of a glucose oxidase-catalase system (adapted from Pickering *et al.*, 1998).

The slower rates of oxidation of mannose, galactose and xylose can be attributed directly to a glucose oxidase-catalysed reaction. It has previously been shown that the final products in the enzymatic oxidation of galactose and mannose, when isolated, show these to be the corresponding aldonic acids (Müller, 1928). Thus, galactose and mannose are oxidised directly to the aldonic acids without a prior conversion to glucose. A 1 or 2 mol excess of galactose slowly but completely reduces the glucose oxidase flavin, and a similar argument can be put forward for the other two sugars (Gibson *et al.*, 1964).

Similarly to other flavoenzymes, the enzymatic reaction can be divided into two steps. Firstly, the substrate β-D-glucose is oxidised by the transfer of two hydrogen atoms to molecular oxygen, thereby producing hydrogen peroxide and δ-D-gluconolactone (shown in **Figure 2.3**). Secondly, δ-D-gluconolactone is hydrolysed non-enzymatically to gluconic acid. If the catalase is present, the hydrogen peroxide can be reduced to water and molecular oxygen (Ough, 1975; Uhlig, 1998a).

2.3 HETEROLOGOUS EXPRESSION OF THE *ASPERGILLUS NIGER* GLUCOSE OXIDASE GENE IN YEASTS

Glucose oxidase from *Aspergillus niger* has been expressed in yeast. In *Hansenula polymorpha* it was used as a reporter gene to isolate regulatory mutations (Hodgkins *et al.*, 1993) and in *Saccharomyces cerevisiae* it was used to enhance glucose oxidase production in batch cultures (Kapat *et al.*, 2001), for high-level production of extracellular glucose oxidase (Whittington *et al.*, 1990; De Baetselier *et al.*, 1991), for kinetic analysis of a yeast-derived enzyme (Frederick *et al.*, 1990) and for commercial mass production (Park *et al.*, 2000).

2.3.1 Expression of the glucose oxidase gene in *Hansenula polymorpha* - a reporter gene

H. polymorpha strain A16 was transformed with an episomal plasmid and, after several generations, a stable clone was recovered from which GOX was expressed using a methanol oxidase promoter (*MOX*) that showed dual control, being repressed by either glucose or ethanol and induced by methanol. Furthermore, the *MOX* terminator region was used to terminate transcription and the yeast mating pheromone α-factor secretion signal ($MF\alpha 1_S$) sequence from *S. cerevisiae* was used to direct secretion. The goal of this study, using glucose oxidase as a reporter gene along with a simple plate assay, was to identify strains showing disturbances of *MOX* gene regulation, to provide a means of analysing the regulatory mechanisms acting upon the *MOX* promoter and to identify overproducing mutants. It was also clear that glycosylation in *H. polymorpha* occurs to a higher extent than in *S. cerevisiae* (Hodgkins *et al.*, 1993).

2.3.2 Expression of the glucose oxidase gene in *Saccharomyces cerevisiae*

2.3.2.1 Kinetic analysis – first example of an active flavoenzyme secreted from *S. cerevisiae*

Plasmids were used to transform the *GOX* gene into *S. cerevisiae* strain GRF181, in which the mature glucose oxidase gene was under the control of a yeast hybrid alcohol dehydrogenase II-glyceraldehyde-3-phosphate dehydrogenase promoter, either the yeast α-factor pheromone leader or the glucose oxidase pre-sequence and a glyceraldehyde-3-phosphate dehydrogenase terminator (Frederick *et al.*, 1990).

The synthesis and secretion of between 75 and 400 μg/ml of active glucose oxidase was observed. Further analysis of this *S. cerevisiae*-derived enzyme showed that the enzyme has comparably specific activity and more extensive N-linked glycosylation than the *A. niger* protein. Furthermore, a difference was observed in the glycosylation of glucose oxidase between the yeast and *A. niger,* with the yeast using two different leader sequences, namely the leader sequence of the *GOX* gene and the $MF\alpha 1_S$ leader sequence. The reason for this observation is unknown. However, yeast is known to hyperglycosylate some foreign proteins that are secreted, especially those using the α-factor leader peptide (Frederick *et al.*, 1990).

2.3.2.2 Production in batch cultures

Glucose oxidase was cloned into *S. cerevisiae* and expressed under two strong promoters, the *ADH1* promoter and the *GAL10* promoter. Secretion was controlled by the $MF\alpha 1_S$ secretion signal of *S. cerevisiae* and the α-amylase secretion signal sequence of *Aspergillus oryzea*. The *GAL7* and *GOX* terminators were responsible for termination. Four vectors in total were constructed. The recombinant yeast produced the enzyme and it was found that 85% of the total enzyme was extracellular. *GOX* with the α-amylase signal sequence under the *GAL10* promoter and *GAL7* terminator produced the highest amount of extracellular GOX, namely 8.7 U/ml. Another interesting observation was the much larger molecular size of the *S. cerevisiae*-produced GOX. The size was about 250 kDa, which is much bigger than the 170 kDa of the GOX produced by *A. niger*. This phenomenon can be explained by the overglycosylation of foreign proteins by *S. cerevisiae* (Hong *et al.*, 1998).

2.3.2.3 High-level production of extracellular glucose oxidase

While the secretion of many heterologous proteins from *S. cerevisiae* has been reported, the production levels obtained are quite low in some cases. In particular, proteins with a molecular mass higher than 50 000 Dalton have secretion problems and a large amount of the protein sometimes remains inside the cells. *S. cerevisiae* was engineered to secrete into the medium levels of >200 U/ml of *A. niger* glucose oxidase, which corresponds to more than 3 g/L of recombinant GOX. The expression levels are highly dependent on the secretion signal used. Using the MFα-factor preprosequence instead of the GOX signal, a 5- to 10-fold decrease in expression was observed eventhough GOX was efficiently secreted into the medium (> 80%). At this point in time, it is not clear if the difference occurs at the transcriptional or the translational level. The molecular weight of

the recombinant GOX was estimated by Sephadex chromatography to be 350 000 – 400 000 Dalton, which is primarily due to extensive N-linked glycosylation. The recombinant GOX enzyme that was produced was more stable at higher temperatures and a wider pH range than the native *A. niger* enzyme, and it is also free of contaminating amylase, cellulase and catalase (De Baetselier *et al.*, 1991).

2.4 COMMERCIAL APPLICATIONS OF GLUCOSE OXIDASE

Glucose oxidase from *Aspergillus niger*, which has GRAS status (Generally Regarded As Safe), is of considerable industrial importance. It has also been incorporated into various biosensors and is widely applied for the detection and quantification of glucose in industrial solutions, as well as in bodily fluids. In the food industry, it is used to prevent the Maillard reaction and oxidative processes, mediated by atmospheric oxygen. It is also used for the stabilisation of various foods and beverages in order to improve their shelf life (Whittington *et al.*, 1990; De Baetselier *et al.*, 1991, Hammer, 1998; Park, 2000; Kapat *et al.*, 2001), and to prevent colour alterations and taste changes (Ohlmeyer, 1957; Power, 1998; Pickering, 1998; Vemulapalli *et al.*, 1998).

The Maillard reaction occurs between the aldehyde and amino groups in food. The aldehyde groups are associated mainly with glucose, while the amino groups are derived from amino acids or indirectly from proteins (Uhlig, 1998b). The final product is pigmented with melanoidins that are responsible for browning and decolouration (Low *et al.*, 1989). The Maillard reaction is not always redundant and may even be desired if the browning of baked goods, roasted meat or potatoes is required. On the other hand, this reaction is undesirable for egg products, for canned fruits and vegetables, or in fruit juices and wine. One Maillard reaction partner, the aldehyde group, can be decreased in number or eliminated from the reaction by the glucose oxidase catalase system (Uhlig, 1998b).

Glucose oxidase-catalase has been used commercially for a long time, and although there are many applications of this enzyme system, only a few have been successful commercially. One of the major obstacles was the unforeseen problem with the hydrogen peroxide formed by the reaction; others were mainly due to economics or the equipment available at the time (Power, 1998).

2.4.1 Oxygen removal

Glucose oxidase-catalase has been used for many years to deoxygenate beverages; however, its commercial use currently is limited to certain specific applications. The successful use of glucose oxidase has also been reported for the deoxygenation of cheese, bread, mayonnaise, citrus concentrates, beverages, beer and wine (Power, 1998; Uhlig, 1998b).

2.4.1.1 Cheese

Glucose oxidase is used as a sealant against oxygen. A thin film of glucose oxidase is sprayed onto the packaging material to provide an oxygen barrier. Vacuum packaging, however, has lately shown to be a superior method to prevent oxygenation. A new

application of this enzyme has been reported for cheese powder stabilisation. A mixture of glucose and powdered glucose oxidase replaces about 50% of the customarily used anticlumping agent. After the package is sealed, the enzyme is activated by residual moisture in the cheese powder and the remaining oxygen is removed (Uhlig, 1998b).

2.4.1.2 Bread

Oxidants are frequently used to strengthen gluten in flour. A similar, but weaker, effect has been achieved with glucose oxidase. A positive influence on the baking properties of flour was also noticed when glucose oxidase was used in the presence of catalase and ascorbic acid (Uhlig, 1998b; Vemulapalli *et al.*, 1998).

2.4.1.3 Mayonnaise

Mayonnaise, the product, is an emulsion of oil and water that contains oxygen. When it is exposed to light, oxidation may occur at room temperature and considerable taste changes can take place. Mayonnaise can be stabilised when 15 to 50 glucose oxidase units per kilogram of product are added (Uhlig, 1998b).

2.4.1.4 Citrus concentrates and beverages

Both citrus concentrates and beverages contain solubilised oxygen that produces peroxide when it is exposed to sunlight. These peroxides are responsible for considerable flavour changes in citrus beverages. Both orange and grapefruit juice concentrates can experience oxidative changes and it thus is desirable to remove the oxygen. A decrease in the potential growth of yeast was shown when glucose oxidase was added to freshly pressed orange juice (Baldwin *et al.*, 1953; Uhlig, 1998b).

2.4.1.5 Beer

The deteriorating effects of oxygen on the flavour and shelf life of beer have been a headache for brewers and scientists serving the brewing industry for some time. The possibility of beer stabilisation using glucose oxidase therefore was studied intensively. Glucose oxidase-catalase inhibits the oxidation reactions in pasteurised beer and decreases iron pick-up from the can. Its effectiveness in retarding the spoilage of unpasteurised beer, by inhibiting the growth of yeast, was demonstrated clearly. Pasteurised beer that is deoxidised with glucose oxidase can maintain brewery freshness for many months and even makes it possible to keep unpasteurised beer for several weeks without refrigerating (Baldwin *et al.*, 1953; Ohlmeyer, 1957; Uhlig, 1998b).

2.4.1.6 Wine

Glucose oxidase-catalase can also be used for the deoxygenation of wine and stabilisation against both browning and organoleptic changes. However, large-scale use has never occurred. One of the primary causes of these unutilised successes is the inability of fungal catalase to function catalytically in the presence of ethanol. Without catalase activity, glucose oxidase still deoxidises the product, but it is in reality an effective

hydrogen peroxide producer until either glucose or oxygen is depleted or the enzyme is inactivated. This system still has some organoleptic benefits and can be used in ethanol-containing beverages if other compounds, such as sulphur dioxide (SO_2) or ascorbic acid, are present. These compounds scavenge the peroxide formed by the glucose oxidase and the rate of oxygen permeation into the finished product over time is very low (McLeod & Ough, 1970; Temple & Ough, 1975; Pickering *et al.*, 1998; Power, 1998; Uhlig, 1998b).

2.4.2 Glucose removal

Scott was primarily responsible for developing an important application for the glucose oxidase-catalase system in 1953, namely desugaring or desaccharification to stabilise commercial liquid egg white (Baldwin *et al.*, 1953). The removal of glucose for the minimisation of Maillard browning is also performed commercially for other food ingredients, using procedures similar to those used for egg desugaring. In these applications, the enzymatic process is preferred over fermentation because of the minimal change in the flavour of the treated product. Two more glucose oxidase/catalase desugaring procedures will be discussed. The reduction of glucose in potatoes for the minimisation of browning will be discussed briefly (Low *et al.*, 1989), while the conversion of glucose to gluconic acid in grape juice for the production of low/reduced alcohol wine will be discussed more extensively, as it is the main focus of this study (Villettaz, 1987).

2.4.2.1 Desugaring of egg white

Dried egg white containing natural sugar deteriorates rapidly upon storage. The conventional egg white processes used, use bacteria and yeast fermentation to get rid of the excess sugar. Except when used to remove the desired sugar concentrations, these fermentation processes are rather difficult to control, since proteolytic and other undesired reactions take place. The application of glucose oxidase represents a marked improvement in the desugaring of egg white and show promise for markedly good storage characteristics. To create a stable product with extended shelf life, the concentration of free glucose must be reduced to less than 0.1%. Egg white is normally concentrated about eight-fold, whole egg is concentrated about four-fold and egg yolk only about two-fold. The glucose concentration in fresh egg white therefore will be reduced the most (Baldwin *et al.*, 1953; Uhlig, 1998b).

2.4.2.2 Reduction of glucose in potatoes

Non-enzymatic browning poses a serious problem for many potato products, such as granules, flakes, chips, French fries and hash browns, and the problem has become acute particularly in the case of products destined for foreign markets, since the use of sulphites as anti-browning agents has been curtailed or prohibited altogether, because of the potential health risk. Considering the universality of sulphites as preservatives, the urgency in the search for alternatives has become very important. Glucose oxidase was used to treat potato samples and the results were very compelling. The enzyme could be used successfully as an efficient aid for potato products to decrease the glucose content and,

consequently, the colour problems caused by the non-enzymatic browning reaction (Low *et al.*, 1989).

2.4.2.3 Reduction of glucose in grape juice

The glucose oxidase-catalase desugaring process is used in wine for the conversion of glucose to gluconic acid and thereby to produce low/reduced alcohol wines (Power, 1998). Because there is a growing consumer demand for wine containing lower levels of alcohol and chemical preservatives, and GOX has proved to be a possible solution for both problems, more intensive research has been done on this enzyme and its possible application in wine (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998; Pickering *et al.*, 1999a, b, c).

2.4.3 A growing market for wines with reduced alcohol

Over the past decade, it has become apparent that there is a drop in the consumption of high alcohol beverages (10-13%) and, at the same time, the consumption of beverages with a lower alcohol content has shown an increase. This might be partly because of drink-driving legislation, the awareness of health risks arising from excessive alcohol intake (Erten & Campbell, 2001), or only a new fashion trend. It is a clear indication that there is a growing market for low alcohol products. Therefore it comes as no surprise that the focus within the wine industry has also shifted towards low-alcohol, "reduced-alcohol" and de-alcoholised products as a result of the increased international interest and consumer demand (Pickering & Heatherbell, 1996; Scudamore-Smith & 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 (Pickering *et al.*, 1998; Gladstones & Tomlinson, 1999; Gladstones, 2000).

Several processes therefore have been designed and used for the removal or the reduction of alcohol in wine, sometimes in combination. These include distillation, thermal evaporation, membranes, extraction, adsorption, centrifugation, freeze concentration and partial fermentation (Bui *et al.*, 1986; Mermelstein, 2000). Not only has concern been raised as to the sensory quality of the finished product (wine), but these processes unfortunately also tend to involve expensive equipment and can also be intensive from a processing point of view (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998). In addition, partial or incomplete fermentation can have the inherent problem of excess residual sugar, whereas the fermentation of immature grapes with a lower sugar content can be responsible for wine with less flavour and aroma (Pickering *et al.*, 1998). Recently, it was also suggested that aerobic yeasts could be used for the production of low-alcohol wines (Erten & Campbell, 2001).

Some of the processes that attempt to selectively remove alcohol while minimising the loss of wine quality parameters are discussed below. A new door was opened when it was suggested that reduced alcohol wine could be produced when the enzyme GOX is used. Such wines were prepared (Villettaz, 1987) and are also discussed below.

2.4.4 Physical removal of alcohol

2.4.4.1 Distillation

Distillation was the first method used by Carl Jung in Germany in 1920 to produce dealcoholised wines. The product was not very good, because this process not only removes the alcohol, but also removes the flavour volatiles (Mermelstein, 2000).

2.4.4.2 Thin-film evaporation

In this method, wine is fed to a centrifugal evaporator consisting of several hollow cones. A thin film of product travels across the inner surface of the cone, which is in turn heated by steam flowing through the hollow chamber within the cone. Exposure of the film to elevated temperatures for a relatively short period of time under vacuum allows the alcohol to be removed as vapour. This method is used to produce non-alcoholic wine (Mermelstein, 2000).

2.4.4.3 Reverse osmosis

When a solution is placed on one side of a semi-permeable membrane, and water is placed on the other side, water will diffuse through the membrane to the solute side until the concentration is the same on both sides of the membrane. This phenomenon is called osmosis.

In reverse osmosis, a cold filtration method (7.2-12.8°C), wine is pumped against a membrane at a pressure (450 psi) greater than the osmotic pressure, causing compounds with a smaller molecular weight, such as ethanol and water, to diffuse selectively through the membrane, thereby removing the alcohol from the wine and retaining the natural flavour of the wine (Mermelstein, 2000). The membrane rejects or passes compounds based on their molecular weight and the membrane pore size. Since ethanol and water are small relative to the wine matrix (grape juice or must), the larger compounds, such as organic acids and phenolics, are retained in the wine at a higher concentration. In many countries, the addition of extraneous water is strictly prohibited (Bui *et al.*, 1986). The water originally removed by reverse osmosis is added back to the concentrated wine to restore the initial balance of these materials and to produce a non-alcoholic or reduced-alcohol wine (**Figure 2.4**) (Mermelstein, 2000). Specific membranes are currently available for the processing of wine and other beverages by reverse osmosis (Bui *et al.*, 1986). The commercial objective is to produce wine of unchanged organoleptic quality, but with reduced alcohol. There is a market for this type of product in traditional wine-producing countries (Bui *et al.*, 1986).

2.4.4.4 Thermal gradient processing

In this method, wine is cooled to form ice crystals, which float to the top of the tank, increasing the alcohol concentration in the liquid. About half of the contents are then drained from the bottom of the tank. The tank is then heated to melt the ice crystals, essentially diluting the liquid in the tank and thereby producing wine with reduced alcohol

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concentrations. This is a very energy-intensive method and is not currently being used commercially (Mermelstein, 2000).

2.4.4.5 Osmotic distillation

This is another method of dealcoholising wine. Wine is passed through a hydrophobic hollow-fibre membrane and degassed water is passed along the other side. The difference in vapour pressure results in some of the alcohol in the wine evaporating into the water. This is done at room temperature without elevated pressures, except to gently pump the wine, whereas reverse osmosis uses high pressure and vacuum distillation uses elevated temperatures, both being conditions that may be detrimental to the wine. This method is not yet approved for commercial use but is being tested in some wineries. A disadvantage of the process is that it generates a lot of stripping water containing alcohol, which must be taken into consideration by any company that wants to use this process. However, the alcohol can be recovered by traditional distillation (Mermelstein, 2000).

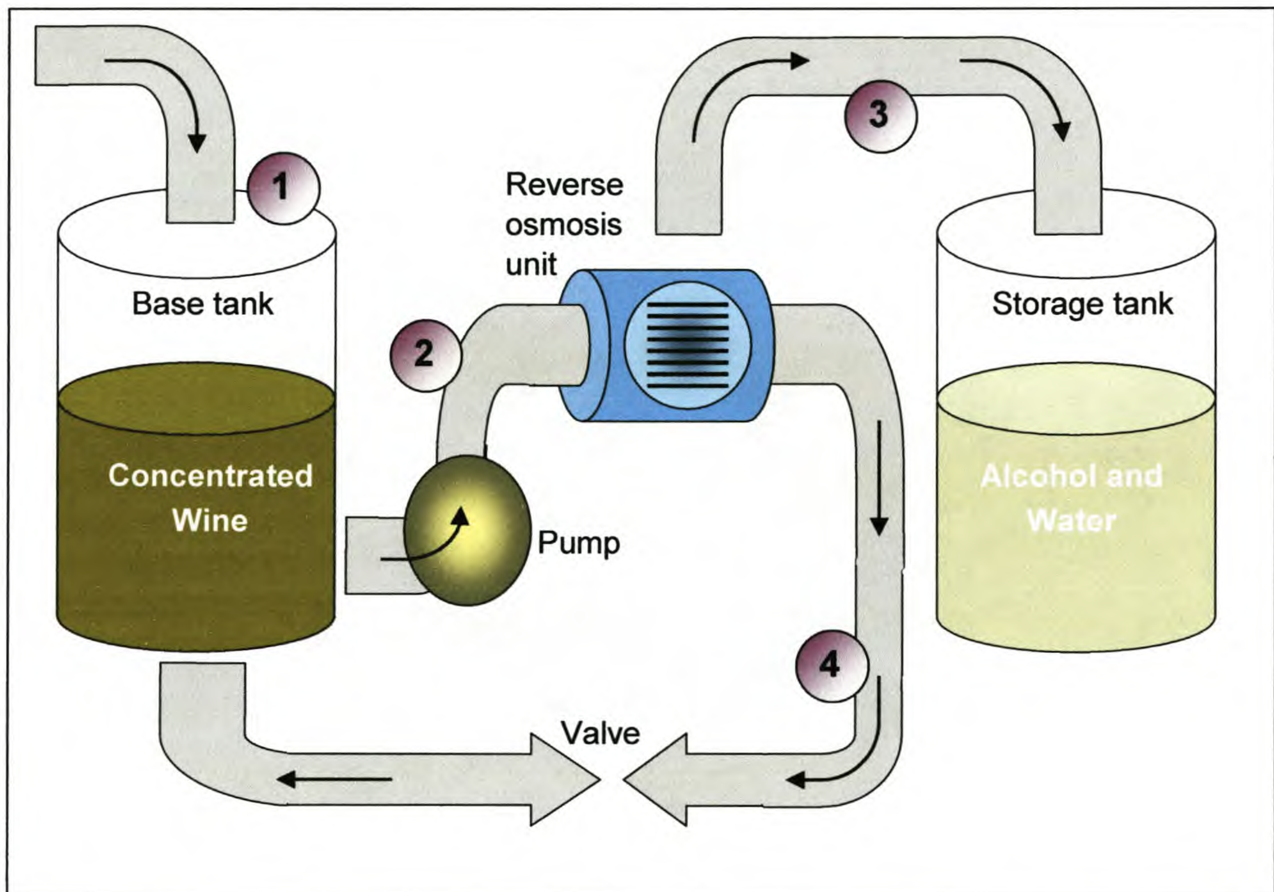

Figure 2.4. Reverse osmosis in wine. ❶ The base tank which is initially filled with wine. ❷ A pump that pushes the wine into the reverse osmosis unit. ❸ Cylinders containing membranes are used to separate a syrupy concentrate from the alcohol and wine. ❹ Water and alcohol are pumped/flow into a storage tank and the concentrate is returned to the base tank. This process lasts 10 to 20 cycles (adapted from Mermelstein, 2000).

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2.4.4.6 Spinning cone column

The spinning cone column (SCC) is a thin-film, multistage stripping column that uses centrifugal force to enhance distillation. The column, 101.6 centimeter in diameter and 4 m high, contains a series of alternating stationary and rotary truncated cones. Wine is fed into the top of the column, flows down the upper surface of the first stationary cone and onto the surface of the first spinning cone, where centrifugal force spreads it into a turbulent thin film that flows off the cone and drops onto the next stationary cone. This process is repeated further down the column.

The stripping gas, generally low-pressure steam, enters the column at the bottom and flows upward, stripping out the flavour volatiles from the thin film. The volatiles are then condensated and collected. The wine without the volatiles is then run through the column again at a slightly higher temperature to reduce the alcohol content to the desired level. The volatiles are then added back to the alcohol-reduced wine to produce a low-alcohol wine, which retains its original flavour (Mermelstein, 2000). A schematic representation of the process can be seen in **Figure 2.5**. The alcohol can be recovered as a by-product.

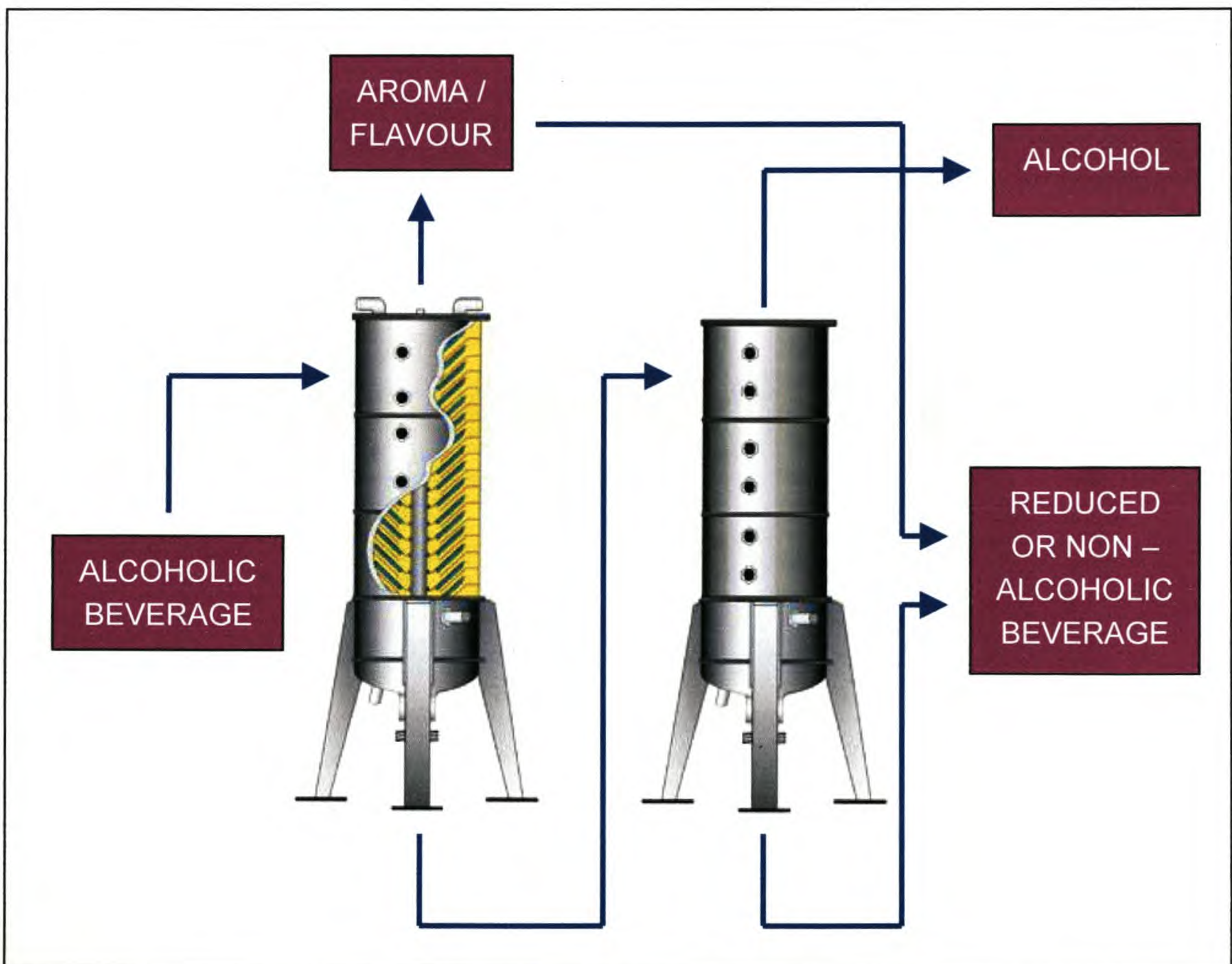
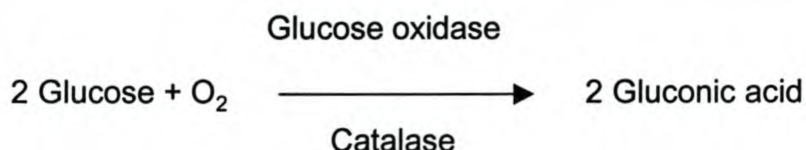


Figure 2.5. Reduced or non-alcohol beverage production by the Spinning Cone Column (SSC) (image used with permission of Flavourtech Pty., Ltd).

2.4.5 Enzymatic removal of alcohol

2.4.5.1 Enzymatic treatment of grape juice

An alternative approach to using expensive equipment is the concept of treating grape juice from the mature fruit with GOX to reduce the glucose content of the juice, which produces wine with a reduced alcohol content after fermentation (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering, 1998; Pickering *et al.*, 1998; Pickering *et al.*, 1999a, b, c). The basic net enzymatic reaction involved is shown below.



Alcoholic fermentation is started after the grape juice has been treated with GOX for a short period of time. Since gluconic acid is not converted to alcohol by wine yeast, the wine obtained after the fermentation of the treated juice contains less alcohol than a conventional wine. The excess acid (gluconic acid) in the low-alcohol wine can be removed, e.g. by chemical deacidification, until satisfactory organoleptic properties are obtained (Villettaz, 1987).

The compositional, stability and sensory characteristics of white wine made from glucose-oxidase treated juice have been reported, with satisfactory results. High levels of grape juice glucose conversion efficiencies were reached and final ethanol concentrations of 6.3% to 6.5% v/v were obtained, corresponding to a 36% to 40% reduction in alcohol (Pickering *et al.*, 1999a). The lower levels of alcohol could be a direct effect of the juice aeration that was carried out, as GOX needs oxygen for optimum glucose conversion. **Figure 2.6** shows a simplified form of the enzymatic treatment of grape juice. **Figure 2.7** and **Figure 2.8** show the basic winemaking process on an industrial scale. For the enzymatic treatment of grape juice with GOX, it would have to be added after juice clarification (**Figure 2.7**), but before the primary alcoholic fermentation (**Figure 2.8**). Large amounts of gluconic acid were formed and were present in the finished wine, although a reduction occurred as a result of precipitation reactions during the alcoholic fermentation. Processing trials showed that the low pH of grape juice could be the dominant limiting factor in the rate and extent of glucose conversion by GOX.

Raising the pH of the juice with calcium carbonate, prior to treatment with GOX, appeared to have been effective and helped to minimise the processing time and increase the degree of glucose conversion (Pickering *et al.*, 1999a).

Reduced alcohol wine produced by glucose oxidase-catalase treatment has significantly modified taste and appearance attributes. However, the aroma, aroma-by-mouth and mouthfeel characteristics are relatively unaffected, except for the fruity aromas, which are generally less intense in GOX wines due to the juice aeration that is required. The length of the flavour is also reduced and the high acidity is a detracting characteristic, but this could be corrected by adding sweet reserve, a common practice in the production

of low-alcohol wines. Further research could determine the optimum ratio and composition of sweet reserve required to maximise the sensory quality (Pickering *et al.*, 1999c).

One of the technological limits of the glucose oxidase-catalase system is that these GOX wines have an increased SO_2-binding power compared to wines made by conventional methods. This high SO_2-binding capacity is cause for concern, given the statutory regulations governing the maximum permitted SO_2 levels in wine, as well as the general trends towards lower SO_2 use in the wine industry. The higher concentration of carbonyl compounds may account for this increased demand for SO_2. More sulphates are also formed in GOX wines. GOX wines, however, are more stable against browning and have a more golden colour, which is possibly a result of increased quinone production and the regeneration of oxidisable phenolic substrates (Pickering *et al.*, 1999b).

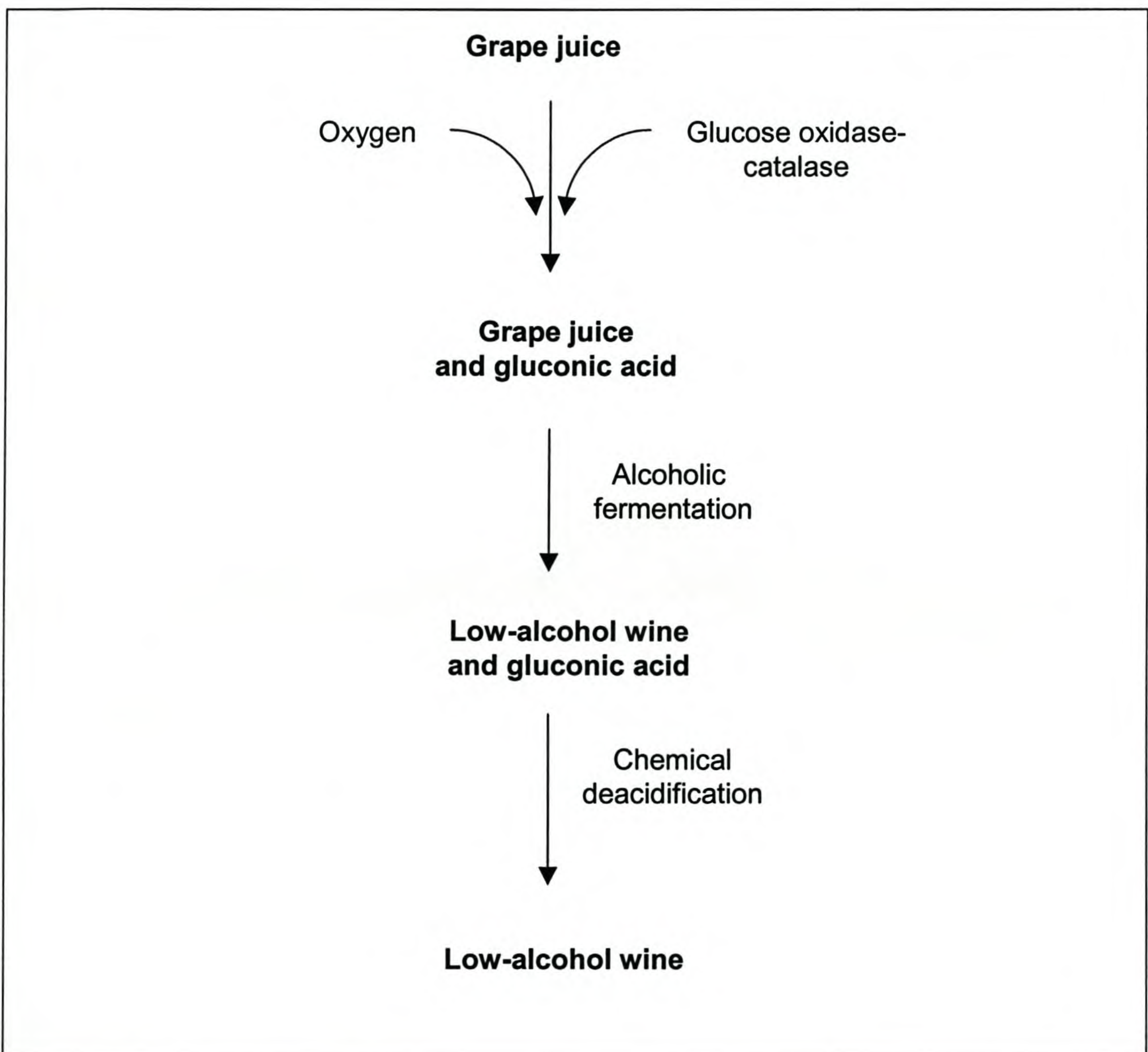


Figure 2.6. Low-alcohol wine production by enzymatic treatment of grape juice (adapted from Villettaz, 1987).

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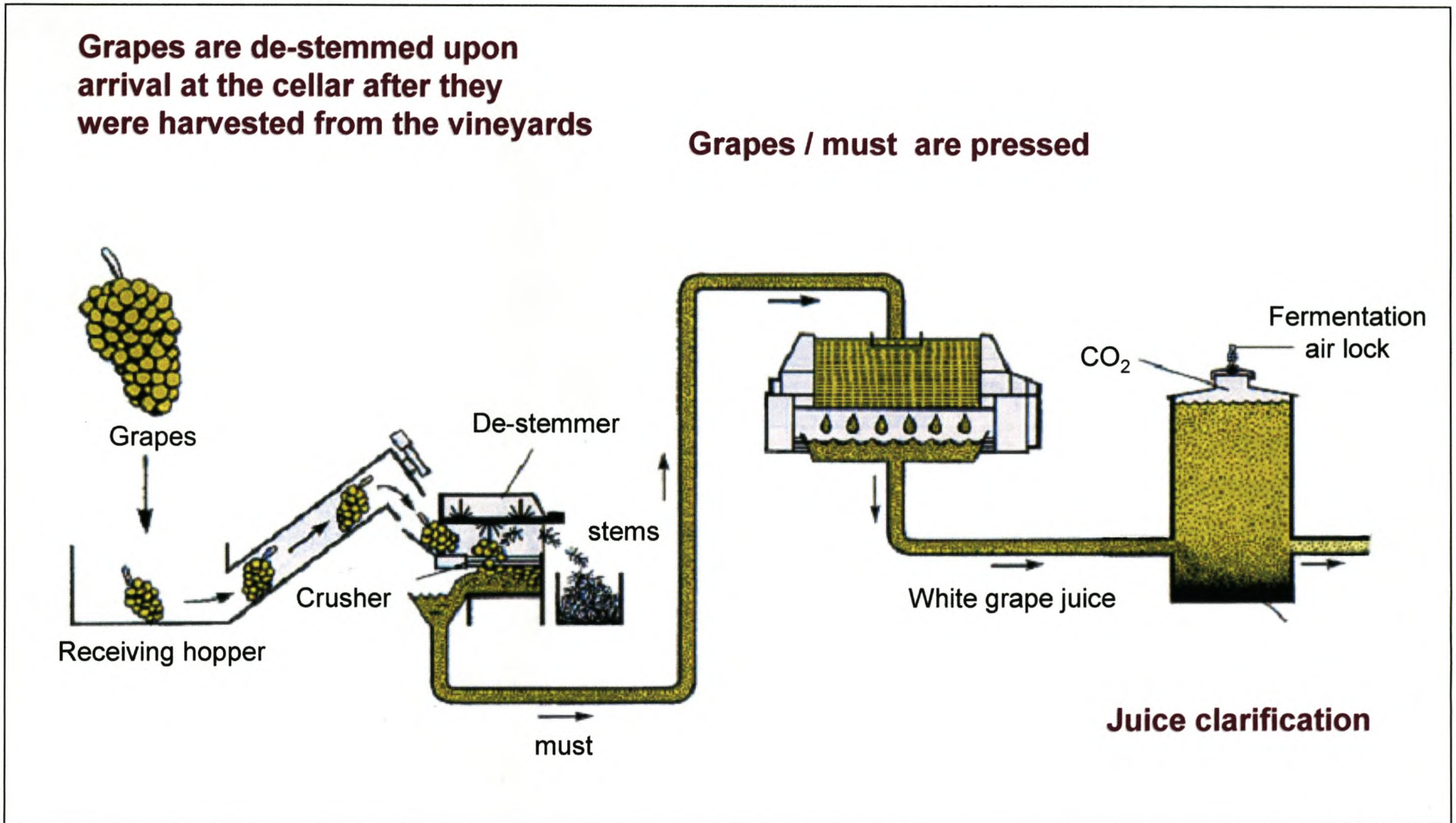

Figure 2.7. The basic winemaking process (adapted from Ziraldo, 1995).

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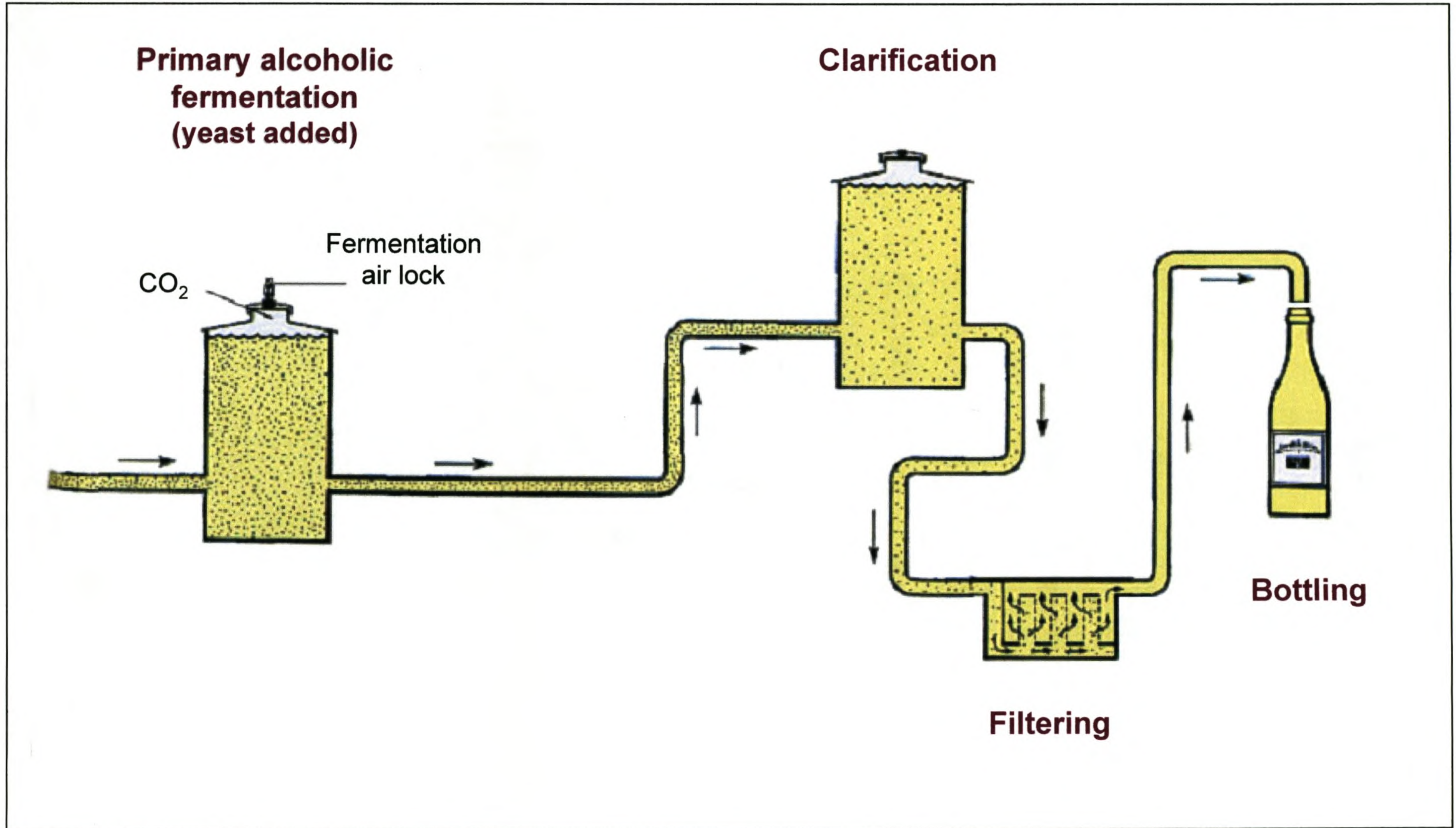

Figure 2.8. The basic winemaking process (adapted from Ziraldo, 1995).

Another limitation of the glucose oxidase-catalase system is that, since the amount of glucose contained in grape juice accounts for 50% of the total sugar fraction, the highest alcohol reduction obtainable (dry wine production) corresponds to 50% of the potential alcohol formed, using conventional technology (Villettaz, 1987). The uses of oxygen rather than air is recommended to reduce the time required for the GOX treatment and thus to minimise undesirable aerobic metabolism/growth (Pickering *et al.*, 1999a).

Further research could focus on the development or evaluation of GOX preparations with higher activity at wine pH. The use of immobilised GOX may have some processing advantages in grape juice and could be evaluated. There were no obvious indications of significant inhibition of the system by SO_2; it may be sensible to examine this further, because GOX can be inhibited by SO_2 in wine (Pickering, 1998).

2.4.6 Glucose oxidase as biological control agent

Various inhibitory techniques that have GRAS status can be used for the biological preservation of foods and these may be more appealing to some consumers than the use of chemical additives. Some of these are bacteriocins, lysozyme and glucose oxidase (Geisen, 1998). As GOX oxidises the glucose to gluconolactone, which is then spontaneously converted to gluconic acid, the pH decreases and the other secondary product, hydrogen peroxide, which inhibits the growth of spoilage organisms, is formed. Both the decrease in pH and the hydrogen peroxide act as biological control agents, but the hydrogen peroxide is the major factor in the inhibitory effect (Yoo & Rand, 1995).

The increase in hydrogen peroxide concentration could lead to hyperbaric oxygen toxicity. Hyperbaric oxygen toxicity is a direct result of the peroxidation of membrane lipids, which would lead to increased membrane permeability. This would not only cause the strong oxidation effect on the bacterial cell wall, but could also contribute to the destruction of the basic molecular structures of nucleic acids and cell proteins. The hydrogen peroxide may also react with other components to form additional inhibitory compounds (De Vuyst & Vandamme, 1994).

The glucose oxidase system was used to enzymatically preserve fish, leading to improved refrigerated shelf-life. The GOX was either applied as a surface dip, immobilised in the ice used to chill the fish, or entrapped in algin blankets used to separate the fish layers. All three approaches resulted in preservative benefits and delayed the onset of spoilage (Field *et al.*, 1986). It also showed antimicrobial effects when used on several other seafood products. This system also inhibited the microbial growth of a wide variety of meat spoilage microorganisms and some pathogens (Yoo & Rand, 1995).

The production of microbiologically safe mould-fermented foods is very important, as the growth of pathogenic or toxinogenic bacteria, such as *Listeria monocytogenes* and *Staphylococcus aureus*, can have serious consequences and result in health risks. The GOX gene from *Aspergillus niger* was expressed in tests in *Penicillium nalgiovense*, a fungal starter culture for the production of fermented foods. The transformant showed higher inhibition activity and it was concluded that hydrogen peroxide is responsible for the antibacterial activity in the GOX-producing *Penicillium*. GOX production by genetic engineering therefore is an effective means to introduce broad-spectrum antibacterial activity into fungal starter cultures or any other industrially important starter culture (Geisen, 1998; Schoeman *et al.*, 1999).

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GOX from *Talaromyces flavus* was shown to control several soil-borne plant pathogens, such as *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. It also showed great potential as a biocontrol agent against *Verticillium dahliae*, another plant pathogen, as it was able to inhibit the germination of its microsclerotia (Stosz *et al*., 1996).

As hydrogen peroxide has been implicated in the induction of plant defence genes and programmed cell death, the expression of a chimeric fungal glucose oxidase gene was evaluated in transgenic tobacco and canola plants as a possible tool for engineering plant cell death and defence gene induction. The results showed that the effectiveness of glucose oxidase expression for the induction of cell death unfortunately is restricted by the glucose supply in the plants (Kazan *et al*., 1998).

The use of glucose oxidase as a biological control agent for the preservation of foods against a broad spectrum of spoilage microorganisms without the use of chemical additives therefore may satisfy some consumers. The over-expression of the GOX gene in certain microorganisms can enhance the inhibitory effect of glucose oxidase. The laws against the use of genetically modified organisms (GMOs) are limiting factors at the moment.

2.5 FUTURE PROSPECTS IN THE WINE INDUSTRY

Glucose oxidase has received considerable research interest regarding its potential application in the wine industry to reduce alcohol levels and as a biocontrol agent. Due to the demanding nature of modern winemaking practices and sophisticated wine markets, there is an ever-growing quest for specialised wine yeast strains possessing a wide range of optimised, improved or novel oenological properties (Pretorius, 2000).

S. cerevisiae has established its importance as a wine yeast and also has proved itself as a reliable starter culture organism when rapid and reliable fermentations are essential for consistent flavour and predictable wine qualities. As it was shown throughout this chapter how important it is for the wine industry to adapt to the market-pull and changing consumer demands, one should not be trapped by conventional yeast breeding methods, but should explore those that are new and available in this exiting age of molecular yeast genetics and modern biotechnology. Expressing GOX in *S. cerevisiae* to produce reduced-alcohol wines is one of these opportunities and, by doing so, it could be possible to produce a wine yeast that could save time and money for the winemaker, as well as satisfy the consumer.

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CHAPTER 3

RESEARCH RESULTS

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

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3. RESEARCH RESULTS

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

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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/reduced alcohol production and inhibition of wine spoilage organisms, such as acetic acid bacteria and lactic acid bacteria, during fermentation. The structural glucose oxidase (*gox*) gene was cloned into an integration vector (YIp5) containing the yeast mating pheromone α-factor secretion signal ($MF\alpha 1_S$) and the phospho-glycerol kinase 1 gene promoter and terminator ($PGK1_{PT}$). The $PGK1_P$-$MF\alpha 1_S$-GOX-$PGK1_T$ cassette is designated *GOX1*. *GOX1* was introduced into a laboratory strain of *S. cerevisiae* (Σ1278). Yeast transformants were analysed for the production of biologically active glucose oxidase on plates and in liquid assays. The results indicated that the recombinant glucose oxidase is active and is produced from early in the exponential growth phase, leading to a stable level in the stationary phase. The 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 (H_2O_2), which is a known antimicrobial agent. Microvinification with the laboratory yeast transformants resulted in wines containing 1.8 to 2.0% less alcohol. There was also an increase in titratable acidity, with a concomitant decrease in pH. This is probably due to the production of gluconic acid from glucose by the GOX. This study therefore paves the way for the development of wine yeast starter culture strains for the production of wine with lower alcohol content, higher acidity and reduced levels of chemical preservatives, such as sulphur dioxide.

Keywords: Recombinant yeast, glucose oxidase, reduced-alcohol wine, microbial spoilage, hydrogen peroxide

Abbreviations: *gox* gene, *Aspergillus niger* glucose oxidase gene; *GOX* gene, *Aspergillus niger gox* gene expressed in *Saccharomyces cerevisiae*; GOX, glucose oxidase enzyme; *GOX1*, GOX cassette consisting of $PGK1_P$-$MF\alpha 1_S$-GOX-$PGK1_T$ in frame

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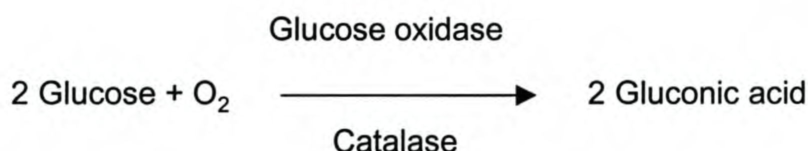
3.1 INTRODUCTION

The winemaking process is a complete ecological niche that involves the interaction of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). *Saccharomyces cerevisiae* is widely used as a wine starter culture and its primary role is to convert the grape sugar into alcohol. Secondly, its 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 have been increased international interest in and consumer demand for low-alcohol, "reduced-alcohol" and de-alcoholised wines, resulting in a search for new methods that will address this question (Scudamore-Smith & 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 & Tomlinson, 1999; Gladstones, 2000).

Several physical processes have been used for the removal or reduction of alcohol in wine, sometimes in combination, 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 can be intensive from the view of processing and an alternative approach was introduced with the concept of treating grape must with GOX to reduce the glucose content of the must, thereby producing a wine with a reduced alcohol content after fermentation (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998; Pickering *et al.*, 1999a, b, c).

Glucose oxidase from *Aspergillus niger*, which has GRAS status (Generally Regarded As Safe), is of considerable industrial importance. It has also been incorporated into various biosensors and is widely applied for the detection and quantification of glucose in industrial solutions as well as in bodily fluids. It is also 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 the 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 alcohol. The basic net enzymatic reaction involved is shown below.



Another consumer demand is for 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 & Pretorius, 2000), lysozyme (Gerbaux *et al.*, 1997) and glucose oxidase (Pickering, 1998).

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The compound that possesses the antimicrobial activity in the GOX reaction is hydrogen peroxide (H_2O_2) (De Vuyst & 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 *gox*-encoding gene from *A. niger* has previously been expressed in *S. cerevisiae* and *Hansenula polymorpha* (Frederick *et al.*, 1990; De Baetselier *et al.*, 1991; Hodgkins *et al.*, 1993; Hong *et al.*, 1998).

The objective of this paper is to evaluate a new GOX-producing *S. cerevisiae* transformant under winemaking conditions for its ability to reduce the total amount of ethanol produced, as well as to assess the antimicrobial activity against LAB and AAB.

3.2 METHODS

3.2.1 Microbial strains and plasmids

The different microbial strains and plasmids used in this study are listed in **Tables 3.1** and **3.2**.

3.2.2 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^{-1}) was used to select *E. coli* transformants containing plasmids. *E. coli* was routinely cultured at 37°C. Lactic acid bacteria (LAB) were cultured in De Man Rogosa Sharpe (MRS) broth or agar plates (Biolab, Merck, South Africa), while acetic acid bacteria (AAB) were grown in Glucose Yeast Extract (GY) medium [containing 5% (w/v) glucose and 1% (w/v) yeast extract] or on GYC agar plates [containing 3% (w/v) $CaCO_3$, 5% (w/v) glucose, 1% (w/v) yeast extract and 2% (w/v) agar] (Drysdale & Fleet, 1988). LAB and AAB were cultured routinely at 30°C. Yeast cells were cultured in Yeast Peptone Dextrose (YPD) medium [containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] at 30°C. Yeast transformants were isolated on uracil selective (SC^{-Ura}) agar plates [containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose and all the required growth factors except uracil].

3.2.3 PCR amplification of the glucose oxidase gene

The *gox* gene of *A. niger* was amplified by polymerase chain reaction (PCR) from the plasmid pSK+3.Sma (Geisen, 1995). Primer prGOXmfa (5'-GAT CAA GCT TCT CAG ACT CTC CTT GTG AGC -3') that contains part of the $MF\alpha 1_S$ secretion signal and has a *Hin*dIII restriction site (underlined) was used as the forward primer. The reverse primer, prGOX (5'-GAT CCT CGA GAC CAC TCA CTG CAT GGA AGC -3'), has a *Xho*I restriction site (underlined). Both primers were obtained from Integrated DNA Technologies (Integrated DNA Technologies, Inc., 1710 Commercial Park, Coraville, IA 52241). PCR was carried out in a final volume of 50 μl reactions using the TRIO-Thermoblock (Biometra). The reaction mixture consisted of 1 μl pSK+3.Sma as template (6 ng μl^{-1}), 2.5 μl of primer

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prGOXmfa (1.5 pmol μl^{-1}), 2.5 μl of primer prGOX (1.5 pmol μl^{-1}), 1 μl of dNTP-mixture (1.25 mM, final concentration), 2 μl of $MgCl_2$ (25 mM), 1 μl of Expand DNA polymerase (Boehringer Mannheim), 4 μl of PCR reaction buffer without $MgCl_2$ [10 mM Tris-HCl pH 8.3, 50 mM KCl and 0.01% (w/v)gelatin] 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 final extension at 72°C for 5 min were used. The resulting PCR product (GOX$MF\alpha1_S$) was analysed by standard agarose gel electrophoresis (agarose gels, 0.8% w/v), stained with 10 mg ml^{-1} of ethidium bromide, visualised under UV illumination (Spectroline model TL-312A), and photographed with a Polaroid camera (Polaroid MP4+ Instant Camera System).

Table 3.1. Different microbial strains and plasmids used in the genetic construction of recombinant *Saccharomyces cerevisiae* strain.

Strains/Plasmids	**Genotype**	**Source or reference**
Strains		
E. coli DH5α	*supE*44 Δ*lac*U169 (ø80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gry*A96 *thi*-1 *rel*A1	Sambrook *et al.* (1989)
S. cerevisiae Σ1278	JT4500 ura3	This laboratory
S. cerevisiae ΣpGOXe	JT4500 ura3 $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$	This study
S. 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	This laboratory
YIp5	Ap^R Tc^R *URA3*	Struhl *et al.* (1979)
pGOXe	$PGK1_P$- $MF\alpha1_S$-GOX-$PGK1_T$ Ap^R	This study
pGOXi	$PGK1_P$- $MF\alpha1_S$-GOX-$PGK1_T$ Ap^R Tc^R *URA3*	This study

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Table 3.2. Different lactic acid bacteria and acetic acid bacteria used in this study.

Strains	Strain number	Culture collection
Lactic acid bacteria:		
Lactobacillus fermentum	9328^T	ATCC*
Lactobacillus brevis	8291	ATCC
Lactobacillus sakei subsp. *sakei*	20017^T	DSM**
Pediococcus pentosaceus	13561	LMG***
Oenococcus oeni	Viniflora oenos	Chr. Hansen A/S
Acetic acid bacteria:		
Acetobacter aceti	286	This laboratory (Wine isolate)
Gluconobacter oxydans	7145^T	DSM**

* ATCC : American Type Culture Collection, Rockville, Maryland, USA

** DSM : Deutshe Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

*** LMG : LMG Culture Collection of the Laboratory of Microbiology, University of Gent, Belgium

3.2.4 DNA manipulations and plasmid construction

DNA manipulations were performed in the yeast-*E. coli* shuttle vector YEp352 containing the *PGK1* promoter and terminator sequences with the yeast mating pheromone α-factor secretion signal ($MF\alpha1_S$). The expression vector pGOXe (**Table 3.1**) was obtained by subcloning the PCR product GOX$MF\alpha1_S$ of 1.8 kb into YEp352 between the *Hin*dIII and *Xho*I restriction sites (**Figure 3.1**). A GOX-encoding gene cassette (designated *GOX1)* was produced, consisting of $PGK1_P$-$MF\alpha1_S$-GOX-$PGK1_T$ in frame. *GOX1* was removed from pGOXe using *Pvu*II and cloned into YIp5 containing the *URA3* marker gene, rendering the integration plasmid pGOXi (**Figure 3.2**). Standard methods for plasmid DNA isolation, restriction enzyme digestion, ligation reactions and *E. coli* DH5α

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transformations were used (Sambrook *et al.*, 1989). A lithium acetate transformation method (Gietz & Schiestl, 1991) was used for the transformation of *S. cerevisiae* Σ1278.

3.2.5 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). An 1% (w/v) agarose gel was used to separate the different bands. 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 to perform the Southern hybridisation for the detection of the integration and verification of single copy integration into *S. cerevisiae* strain Σ1278 (**Figure 3.2**). The DIG Labelling Kit from Roche Biochemical Products (South Africa) was used.

3.2.6 Assays

3.2.6.1 Glucose oxidase plate assay

A modified method of Hodgkins *et al.* (1993) was used for the screening and selection of GOX-producing yeast colonies. Different yeast colonies that were previously selected on SC^{-Ura} plates, and identified as 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 L^{-1} glucose, 100 mg L^{-1} o-dianisidine dihydrochloride (Sigma, South Afrrica) and 15 IU ml^{-1} horseradish peroxidase Type II (Sigma, South Africa)]. The overlay was allowed to set and the plates were incubated at 37°C for 1 h. Untransformed *S. cerevisiae* 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.

3.2.6.2 Glucose oxidase spectrophotometric assay

Transformed *S. cerevisiae* ΣpGOXe and ΣpGOXi were inoculated to $1x10^6$ cells ml^{-1} in SC^{-Ura} liquid media. Untransformed *S. cerevisiae* Σ1278 was inoculated to $1x10^6$ cells ml^{-1} as control also 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 5000 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. 0.1 g of 0.2 mm glass beads was added and the cells were vortexed vigorously for 3 min. After centrifugation at 6000 rpm for 2 min, the supernatant, containing the intracellular protein extract was carefully removed and the samples were stored. 30 μl of each sample to be 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 ml^{-1} horseradish peroxidase and 1.0 ml of 0.31 mM o-dianisidine dihydrochloride. These reaction mixtures were incubated for 30 minutes at 37°C and thereafter stopped on ice by adding 0.3 ml of 4.0 M H_2SO_4. The absorbance was measured at A_{500nm} and the units of glucose oxidase per ml produced were determined

relative to a standard curve of absorbance versus enzyme amount. All values given are the means of triplicate samples; all experiments reported were carried out at least three times with similar results. (The units of glucose oxidase per ml produced were determined relative to a standard curve of absorbance versus enzyme amount).

3.2.6.3 Acetic acid bacteria and lactic acid bacteria plate assays

20 μl of untransformed *S. cerevisiae* Σ1278 (control) and ΣpGOXi (transformant) were "line-spotted" vertically on GYC plates and incubated for 24 h. Acetic acid bacteria (**Table 3.2**) were grown on GYC plates. A single colony was selected and resuspended in 10 μl of distilled water (dH_2O). 20 μl of acetic acid bacteria were "line-spotted" horizontally over the already spotted yeasts on the same GYC plates and incubated at 30°C until inhibition zones were visible.

20 μl of untransformed *S. cerevisiae* Σ1278 (control) and ΣpGOXi (transformant) were spotted on YPD plates and incubated for 24 h. Lactic acid bacteria (**Table 3.2**) were cultured in MRS broth overnight. 20 μl of the lactic acid bacteria were added to MRS soft agar [0.7% (w/v) agar] and lawned onto the YPD plates containing the yeast colonies. Plates were allowed to set and then incubated at 30°C until inhibition zones were visible (**Table 3.3**).

3.2.7 D-gluconic acid / D-glucono-δ-lactone assay and HPCE

A D-gluconic acid/D-glucono-δ-lactone assay kit (Boehringer Manheim, South Africa) was used to determine if there was any D-glucono-δ-lactone present in Chardonnay juice samples used for microvinifications.

A High-Performance Capillary Electrophoresis (HPCE) method with indirect absorbance detection that was developed by Lee & Lin (1996) was used for the determination of the carbohydrates, glucose, lactose and gluconic acid in the Chardonnay juice samples. The CE 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 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 sec). A constant voltage of +30 kV was applied during the separation run and the temperature of the column was set at 25ºC. A running buffer (NAA) was prepared by adjusting a 2 mM 1-naphthylacetic acid solution to pH of 12.2 (using 1 M NaOH). This solution was filtered through a 0.22 μm filter. The collected data were analyzed 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 prepare 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 min x 12 000 rpm) before diluting them 5- 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.

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3.2.8 Scanning electron microscopy

Lactic acid bacteria (*L. brevis*), acetic acid bacteria (*A. aceti*) and yeast *S. cerevisiae* Σ1278 (control) and ΣpGOXi (transformant) 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 performed. The protocol described by Chung & 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), *viz.* 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.

3.2.9 Microvinifications

Chardonnay grapes with a sugar concentration of 230 g/L and a pH of 3.85 were used for this study. The yeasts, *Saccharomyces cerevisiae* Σ1278 (non-transformed control) and *S. cerevisiae* ΣpGOXi (transformant), were inoculated to 1×10^6 cells/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 "Churchward Technique" (Iland *et al.*, 2000).

3.3 RESULTS AND DISCUSSION

3.3.1 Cloning and transformation of the *A. niger gox* gene, and Southern Hybridisation to confirm gene integration into *S. cerevisiae*

Using molecular biology techniques, the *A. niger* glucose oxidase gene was sub-cloned into two different plasmids that were both successfully transformed into the yeast *S. cerevisiae* Σ1278. The first of the plasmids that was constructed by sub-cloning the PCR product, GOX$MF\alpha 1_S$, into an episomal vector, YEp352 (**Figure 3.1**) resulting in the plasmid pGOXe. Transformants containing the episomal vector were grown under selective conditions (SC^{-Ura}) to maintain the plasmid (Parent *et al.*, 1985; Romanos *et al.*, 1992). The second plasmid that was constructed (**Figure 3.2**) is a yeast integration plasmid. *GOX1* was cloned into YIp5, resulting in plasmid pGOXi which contained yeast chromosomal DNA (the *URA3* gene) for targeting integration into the chromosomal *URA3* locus by homologous recombination. The *gox* gene was used as probe and the digoxigenin non-radioactive nucleic acid labelling and detection system was used to

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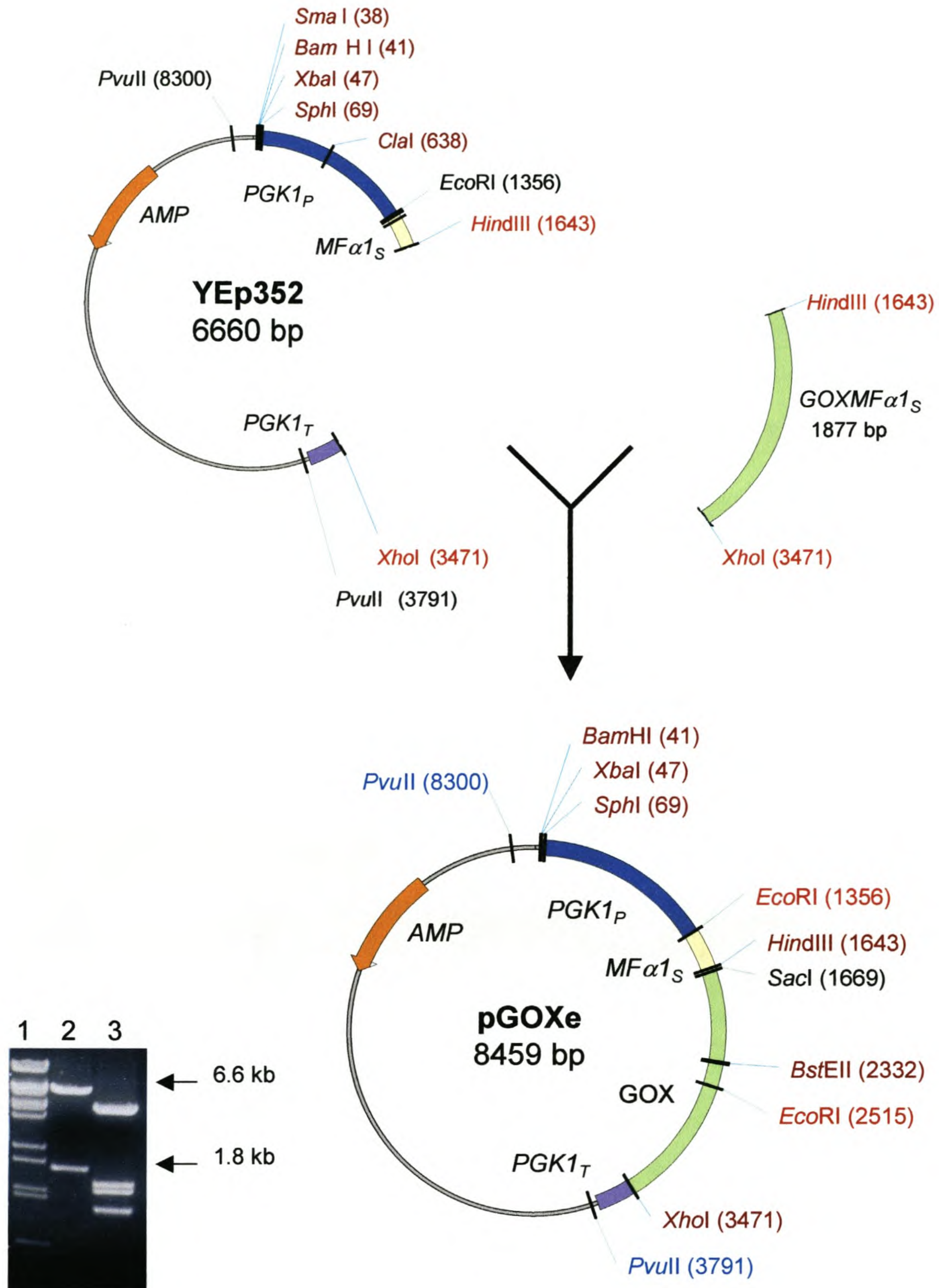

Figure 3.1. Strategy used to construct the expression plasmid, pGOXe. The PCR product, GOX*MFα1*$_S$, was cloned into the YEp352 plasmid. A 0.8% (w/v) agarose gel was used to verify the correct band sizes. The *Bst*EII marker is used in lane 1. Lane 2, pGOXe digested with *Hin*dIII-*Xho*I and lane 3, pGOXe digested with *Eco*RI-*Pvu*II.

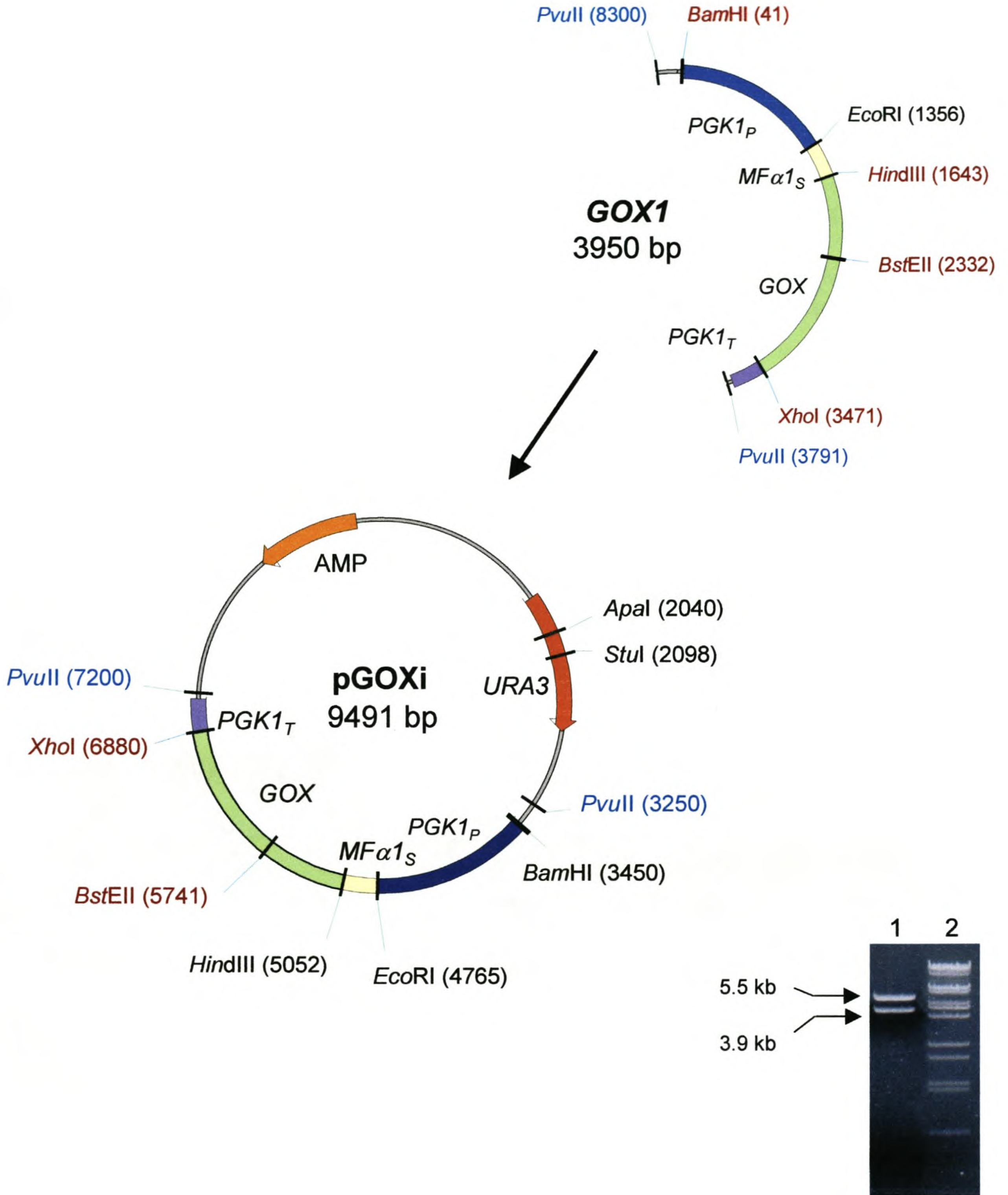

Figure 3.2. Strategy used to construct the integration plasmid, pGOXi. The GOX cassette was cloned into the YIp5 plasmid. A 0.8% (w/v) agarose gel was used to verify the correct band sizes. The *Bst*EII marker is used in lane 2. Lane 1, pGOXi digested with *Pvu*II.

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perform the Southern hybridisation for the detection of the integration and verification of single copy integration into *S. cerevisiae* strain ∑1278 (**Figure 3.3**).

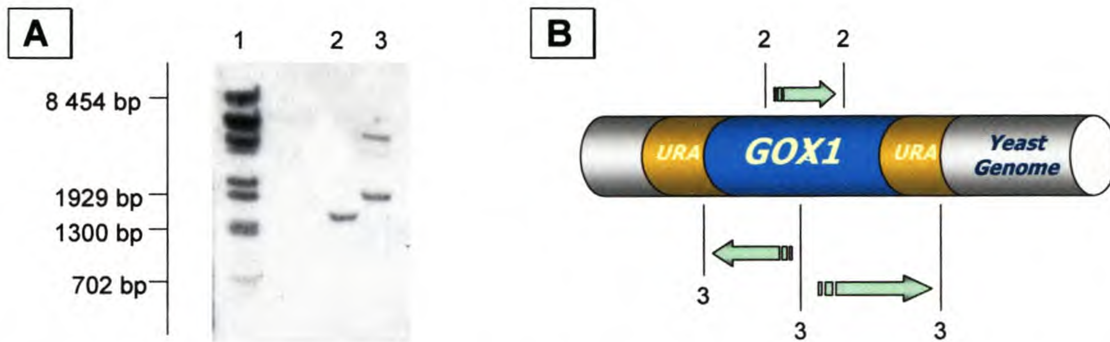

Figure 3.3. Strategy used to verify gene integration and single copy integration. **A:** The autoradiogram. Lane 1: The marker (λ digested with *Bst*EII). Lane 2, genomic DNA digested with *Hin*dIII and *Xho*I verifying gene integration. Lane 3, genomic DNA digested with *Bst*EII and *Nsi*I verifying single copy integration. **B:** A schematic representation of the yeast genome with the integrated *GOX1* and expected amount of annealed probes. The numbers in **B** corresponds with the numbers of the lanes in **A**, so does the amount of annealed probes and amount of bands.

3.3.2 Confirmation of GOX secretion

Confirmation of GOX secretion is verified by a simple colour reaction that is visible on a plate. Different yeast colonies, that were previously selected on SC^{-Ura} plates and identified as 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 (containing all the reagents necessary for the colour reaction). The overlay was allowed to set and the plates were incubated at 37°C for 1 h. Untransformed *S. cerevisiae* strain Σ1278 was used as negative control. As GOX metabolises glucose into gluconic acid and H_2O_2 which is formed as a by-product. The H_2O_2 is used by horseradish peroxidase to oxidise o-dianisidine dihydrochloride and a colour change is visible on the plates. Transformed yeast colonies secreting active recombinant GOX were surrounded by a brown halo and were identified as positives (**Figure 3.4**).

3.3.3 Determining the levels of GOX produced by transformed yeasts in liquid media

Liquid assays were done with *S. cerevisiae* ΣpGOXe and ΣpGOXi to compare the levels of enzyme production between the two transformants. Intra- and extracellular protein samples were assayed spectrophotometricaly. (The units of glucose oxidase per ml produced were determined relative to a standard curve of absorbance versus enzyme amount). A growth curve is included on the same graph to show the rate of growth of the different strains. The results are shown in **Figure 3.5**.

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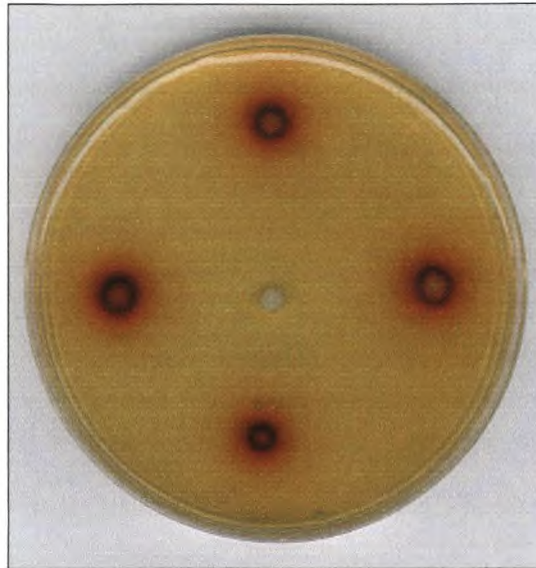

Figure 3.4. Glucose oxidase plate assay. Untransformed *S. cerevisiae* strain Σ1278 was used as negative control. Transformed yeast colonies secreting active recombinant GOX were surrounded by a brown halo and were identified as positives. In this figure four different positive transformed yeast colonies are visible around the negative control in the middle of the plate.

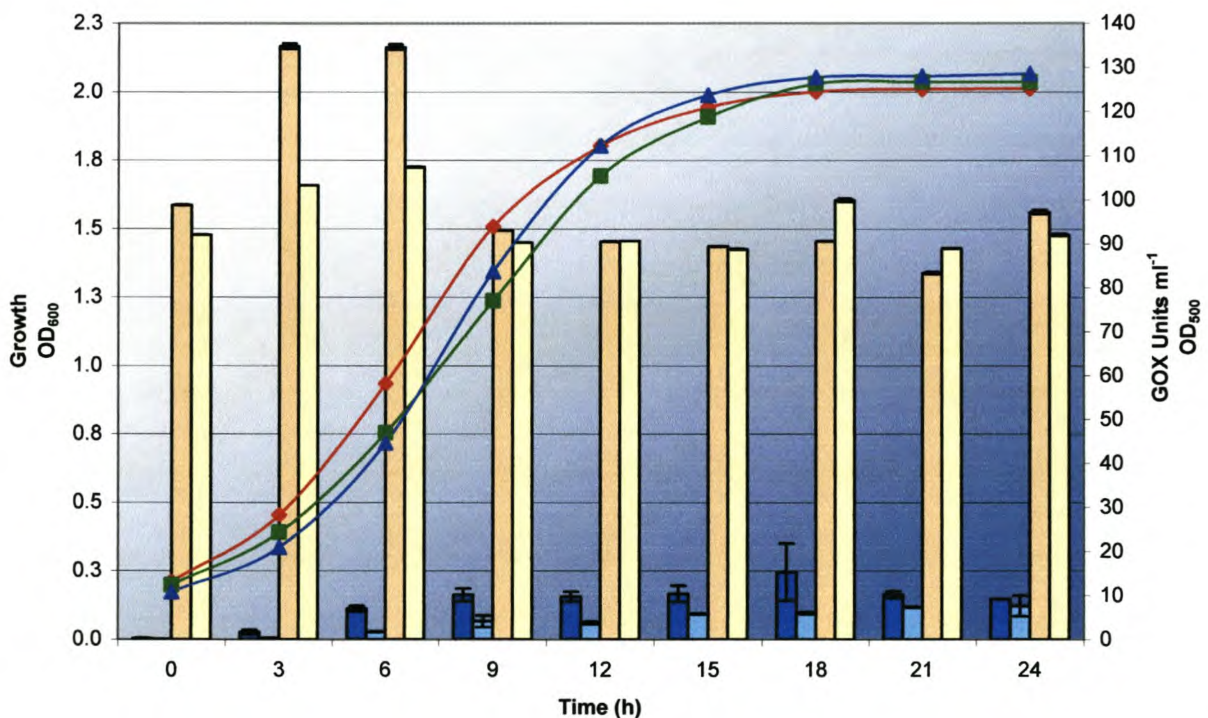

Figure 3.5. The growth of *S. cerevisiae* strains are presented as lines on the graph. They are: ◆, untransformed *S. cerevisiae* Σ1278 (control); ■, transformed ΣpGOXe; and ▲, transformed ΣpGOXe. The bars on the graph represent the levels of glucose oxidase (units ml^{-1}) measured extracellular and intracellular over a 24 h period. The glucose oxidase fractions measured are: ■, intracellular fraction produced by ΣpGOXe; ■, intracellular fraction produced by ΣpGOXi; ■, extracellular fraction produced by ΣpGOXe; and □, extracellular fraction produced by ΣpGOXi. The graph is a representative example of fermentation experiments performed in duplicate.

The assay showed that high amounts of GOX are produced and secreted into the media in the first 8 h, which corresponds to the exponential growth fase of the transformed yeast. It is also clear that the yeast containing the episomal plasmid produces more GOX. However, this is only the case for the first 8 h. Thereafter the two yeasts, ΣpGOXe and ΣpGOXi, produce the same amount of GOX. It was also observed that both yeasts secrete most of the enzymes produced and that only a small fraction is intracellular. This is an excellent result, because it means that the enzyme will convert the glucose to gluconic acid in the media before the yeasts are able to metabolise the glucose to ethanol.

3.3.4 Inhibition of acetic acid bacteria and lactic acid bacteria

Using plate assays, it was also noted that the yeasts expressing the *GOX* gene (ΣpGOXi) seem to inhibit the growth of acetic acid bacteria and lactic acid bacteria, which are the major bacterial spoilage organisms in wine. The varying degrees of inhibition for LAB can be seen in **Table 3.3**. It is the final product of the GOX enzymatic reaction, hydrogen peroxide (H_2O_2) a known antimicrobial agent, which is responsible for the inhibitory activity and not the GOX itself. The inhibitory activity is dependent on the concentrations of the enzyme and the glucose in the medium (Geisen, 1999). The hydrogen peroxide that is formed is secreted into the media. A clear zone around the yeast expressing the *GOX* gene can be seen where the LAB and AAB are inhibited, with no zone visible around the control (**Figures 3.6** and **3.7**).

To confirm the inhibition of the LAB and AAB, samples were prepared and viewed under a scanning electron microscope. The total collapse of the bacterial cell wall could be seen (**Figure 3.8**). The hydrogen peroxide that is produced leads to hyperbaric oxygen toxicity, a result of the peroxidation of the membrane lipid, and a strong oxidising effect on the bacterial cell, which is the cause of the destruction of basic molecular structures, such as nucleic acids and cell proteins (De Vuyst & Vandamme, 1994). Further studies will commence to confirm that effects observed were due to H_2O_2.

Table 3.3. Inhibition spectrum of *S. cerevisiae* ΣpGOXi against lactic acid bacteria.

Strains	Strain number	Inhibition zone*
Lactic acid bacteria:		
Lactobacillus fermentum	9328^T	++
Lactobacillus brevis	8291	++++
Lactobacillus sakei subsp. *sakei*	20017^T	+++
Pediococcus pentosaceus	13561	+++
Oenococcus oeni	Viniflora oenos	-

* A diameter of 1 mm of growth inhibition zones was taken as +

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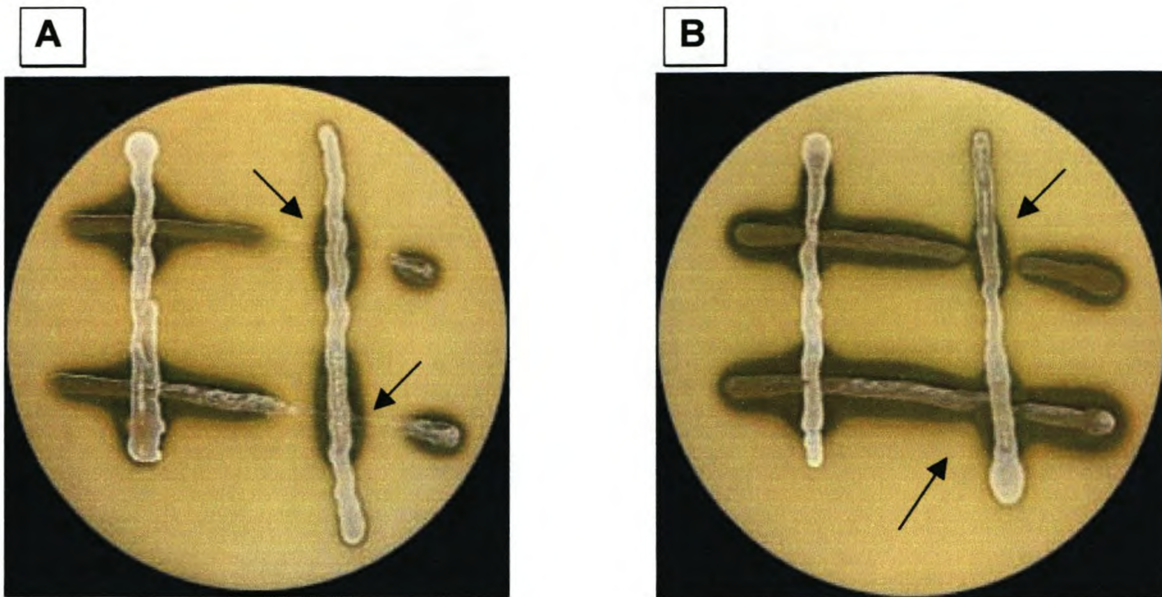

Figure 3.6. Inhibition of acetic acid bacteria **A**: *Acetobacter aceti* and **B**: *Gluconobacter oxydans*. The yeast was streaked out vertically. Untransformed *S. cerevisiae* Σ1278, the control, is on the left and *S. cerevisiae* ΣpGOXi is on the right of each plate. The acetic acid bacteria were streaked out horizontally and inhibition zones, where no growth is visible, can clearly be seen around the yeast transformant.

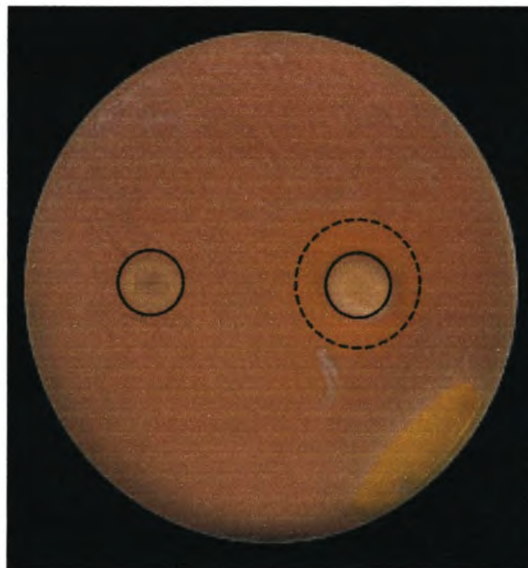

Figure 3.7. Inhibition of lactic acid bacteria, *Pediococcus pentosaceus*. On the left, untransformed *S. cerevisiae* Σ1278, the control. On the right is *S. cerevisiae* ΣpGOXi with an inhibition zone around the colony.

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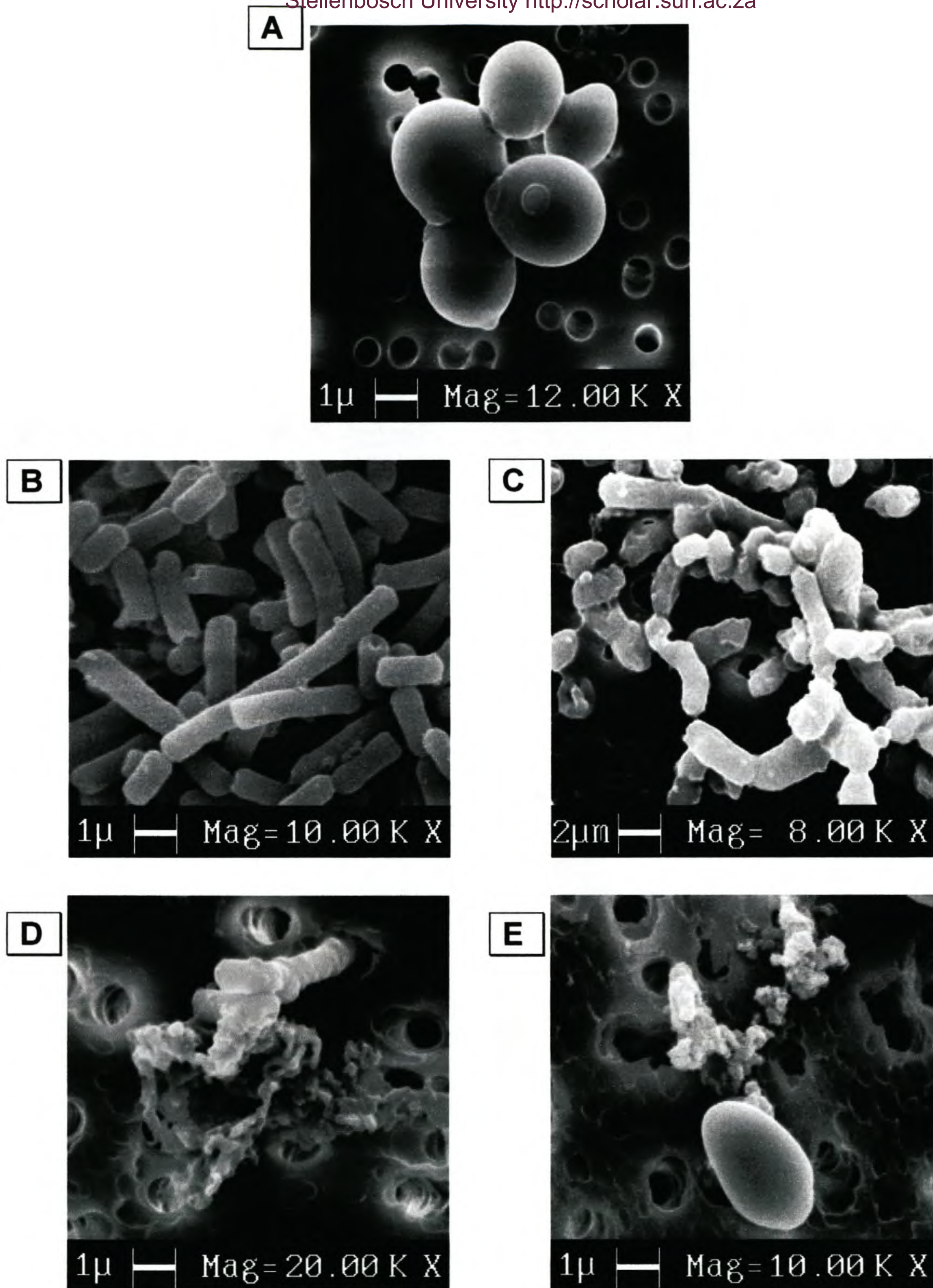

Figure 3.8. Scanning electron microscopy photos to asses the morphological state of different yeast and bacteria cell-samples. **A**: healthy *S. cerevisiae* Σ1278 yeast cells; **B**: healthy *L. brevis* (LAB) cells; **C**: healthy *A. aceti* (AAB) cells; **D**: *L. brevis* with a disintegrated cell wall and **E**: *A. aceti* with 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. High levels of hydrogen peroxide lead to hyperbaric toxicity and the collapse of the cell walls.

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3.3.5 Microvinifications

The accumulated weight loss of Chardonnay juice inoculated with an untransformed *S. cerevisiae* Σ1278, and transformed strain (ΣpGOXi), was measured over a period of 14 days, after which the alcohol and sugar concentrations were determined (**Figure 3.9**). The sugar concentration for both microvinifications was reduced from 230 g L^{-1} to 64 g L^{-1}. The alcohol concentration of ΣpGOXi was 10.1% (vol/vol), while the control had an alcohol concentration of 11.9% (vol/vol). Thus the yeast producing the GOX enzyme produced ±1.8 % vol/vol less ethanol than the control yeast.

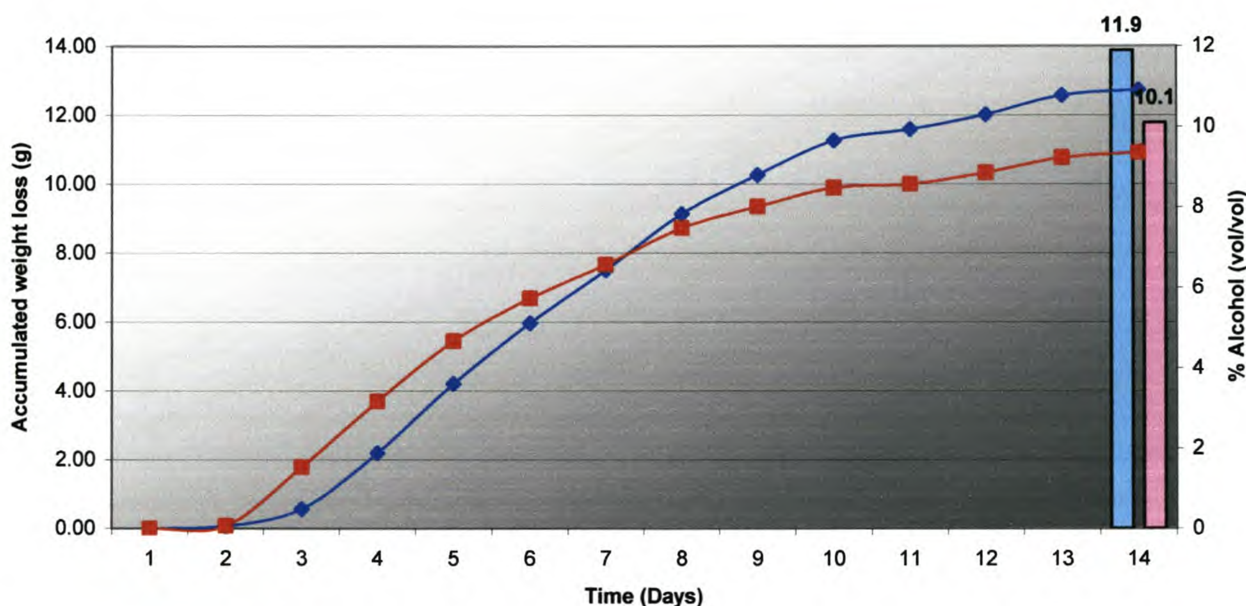

Figure 3.9. Accumulated weight loss of Chardonnay juice over a period of 14 days, fermented with; ◆, untransformed *S. cerevisiae* Σ1278 (control) and ■, transformed strain ΣpGOXi. The bars on the graph represent the levels of alcohol (vol/vol) measured at day 14. ▮, % alcohol produced by Σ1278 (control); ▮, % alcohol produced by ΣpGOXi. The standard deviation for this experiment was less than 1%.

However, there was a difference in the pH of both microvinifications, with both showing a slight increase from pH 3.15 to pH 3.18. This came as a surprise, because it was expected that gluconic acid would be formed, which would lower the pH. CE analysis and a D-gluconic acid/D-glucono-δ-lactone assay showed that there was a fast accumulation of D-glucono-1,5-lactone within the first 3 days (**Figure 3.10**). It is known that some yeasts could 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 the slight increase in the pH in contrast to the decline expected because of gluconic acid accumulation.

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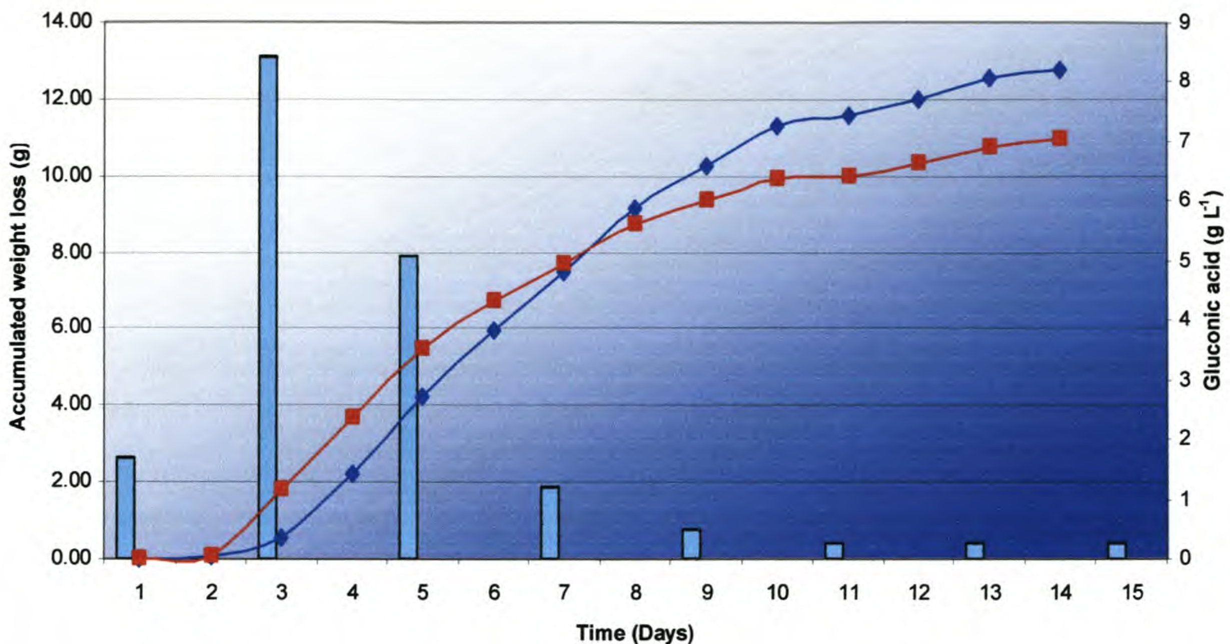

Figure 3.10. Glucono-δ-lactone production by the recombinant strain ΣpGOXi in must fermentation over a period of 14 days. The lines represent the accumulated weight loss of; ■, the recombinant strain ΣpGOXi and ◆; untransformed *S. cerevisiae* Σ1278, control. The bars represent the levels of glucono-δ-lactone measured for ΣpGOXi (▯). No glucono-δ-lactone was detected for the control strain (Σ1278). The standard deviation for this experiment was less than 1%.

3.4 CONCLUSIONS

This is the first time that *S. cerevisiae* has been genetically engineered to specifically reduce the alcohol content of wine during fermentation. The yeast was able to reduce the amount of alcohol of the finished product by 1.5–2% (v/v). This would make wines produced in the warmer parts of the world, which sometimes have a higher alcohol concentration, more competitive when exported to countries producing wines with alcohol concentrations of 12-12.5% (v/v). These wines could also compete with other reduced-alcohol wines and beverages. Furthermore, savings in tax and tariffs could have positive financial implications for the producer and exporter of these wines.

In addition, the by-product, H_2O_2, showed potential to be used as a control agent against unwelcome wine spoilage microorganisms, such as acetic acid bacteria and lactic acid bacteria, in the wine. It is likely, however, that the hydrogen peroxide could affect the wine composition by oxidising some phenolic compounds and causing off-flavours.

Further work is required to investigate the composition of these wines and to determine if these off-flavours exist. The specific kinetics of the glucose oxidase enzyme and its activity after it is produced in recombinant wine yeasts and used in conventional winemaking methods, which is an anaerobic reaction, should also be investigated.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

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4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

Like most other consumer products, yeast fermented beverages such as wine and beer are continuously subjected to the forces of market-pull and technology-push. The focus within the wine industry has shifted as a result of the interest of international and domestic consumes in low-alcohol, "reduced-alcohol" and de-alcoholised wines (Scudamore-Smith & Moran, 1997; Pickering *et al.*, 1998; Mermelstein, 2000), commercial interest in the potential for savings in tax and tariffs (Pickering *et al.*, 1998; Gladstones & Tomlinson, 1999; Gladstones, 2000), changed drinking habits and the implications of alcohol consumption for health and other related social issues.

As new trends in the upper end of these highly competitive and sophisticated wine markets continue to call for a diversity of innovations and due to the demanding nature of modern winemaking practices, there is an ever-growing quest for specialised wine yeast strains possessing a wide range of optimised, improved or novel oenological properties (Pretorius, 2000). Research in this field is very active and continually expanding and the prospect of developing wine yeast starter culture strains expressing heterologous enzymes is also available (Whittington *et al.*, 1990; Park *et al.*, 2000; Van Rensburg & Pretorius, 2000; Kapat *et al.*, 2001).

The yeast *Saccharomyces cerevisiae* is the most widely used industrial microorganism with GRAS status and has long been considered one of the most popular organisms for molecular genetic studies. With the announcement of the complete nucleotide sequence of the yeast genome on 24 April 1996, there was a renewed interest in the genetic improvement of commercial yeast strains (Goffeau *et al.*, 1996; Mewes *et al.*, 1997). Although gene technology has already contributed enormously to our basic understanding of the yeast's biochemical and physiological processes during fermentations and to the genetic fingerprinting of various yeast strains, it will be unwise to entertain unrealistic expectations of short-term benefits (Pretorius & Van der Westhuizen, 1991). Two decades after the first successful transformation of *S. cerevisiae* with foreign genetic material, there is still no recombinant yeast that is used on a commercial scale in the brewing or wine industries. Credible means must be found to effectively address the concerns of traditionalists within the wine industry and the negative overreaction of some consumer groups. There are enormous benefits to both the wine consumer and the industry in the application of this exciting new technology and the first recombinant wine products therefore should unmistakably demonstrate organoleptic, hygienic and economic advantages for the wine producer and consumer.

Furthermore, it is very important to realise that it is very dangerous to have unrealistic expectations about rapid commercialisation and short-term benefits for recombinant DNA technology in the wine industry. The successful application, as well as the commercialisation of transgenic wine yeasts, should not affect the wine's most enchanting and fascinating aspects, namely its diversity of style, flavour and aroma (Pretorius, 2000).

Several processes exist that are used specifically for the removal or the reduction of alcohol in wine. These processes tend to involve expensive equipment and can also be intensive from the view of processing (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998; Mermelstein, 2000). Concerns have also been raised about the

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sensory quality of the finished product (wine) and, in addition, partial or incomplete fermentation and the fermentation of immature grapes with a low sugar content can have the inherent problem of excess residual sugar and a lack of flavour development in the resulting wine (Pickering *et al.*, 1998).

The concept of treating grape must with the enzyme glucose oxidase (GOX), which has GRAS status (Generally Regarded As Safe), to reduce the alcohol level in wine after fermentation (Villettaz, 1987; Pickering & Heatherbell, 1996; Pickering *et al.*, 1998; Pickering *et al.*, 1999a, b, c), was recently introduced as an alternative approach to the expensive and controversial physical methods. Wines produced in this way should have reduced levels of ethanol and higher acidity. This technology could also be employed to produce a reserve of acidic musts or wines for blending purposes (Canal-Llaubères, 1993).

This thesis outlines the investigation of yeast starter cultures to produce reduced alcohol wines. The yeast *S. cerevisiae* was genetically modified to encode the *Aspergillus niger* glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) for the production of reduced-alcohol wines. The enzyme GOX also has GRAS status and converts glucose into gluconic acid, which also has GRAS status and cannot be metabolised by wine yeasts. This is the first time that the yeast S. *cerevisiae* was genetically engineered to produce reduced-alcohol wines.

In this study we have successfully integrated a stable copy of the *gox* gene encoding the *Aspergillus niger* glucose oxidase into the genome of *S. cerevisiae*. By means of assays, it was determined that GOX is indeed transcribed and secreted into the media. The transformed yeast expressed the highest amount of GOX during its exponential growth fase. It was also observed that the transformed yeasts secrete most of the total enzymes produced and that only a small fraction is intracellular. This is an excellent result, because it means the enzyme will convert the glucose to gluconic acid in the media before the yeast is able to metabolise the glucose to ethanol.

Microvinifications were used to show a decline in alcohol production between wines produced by transformed yeasts (ΣpGOXi) and those produced by the control strain. ΣpGOXi had an alcohol concentration of 10.1% (v/v) and the control had an alcohol concentration of 11.9% (v/v). Thus the yeast producing the GOX enzyme produced 1.8% (v/v) less ethanol than the control yeast.

In addition, it also was shown that hydrogen peroxide (H_2O_2), one of the end products of the metabolic reaction, which also has GRAS status, can be used as a biological control agent against lactic acid bacteria (LAB) and acetic acid bacteria (AAB). This aspect may appeal to some consumers as being preferable to the use of chemical additives (Pickering, 1998). To confirm the inhibition of LAB and AAB, samples were prepared and viewed under a scanning electron microscope. The total collapse of the bacteria cell wall could be observed. The inhibitory activity is dependent on the concentration of the enzyme and the glucose in the medium (Geisen, 1999). However, it is likely that the H_2O_2 could affect the wine composition by oxidising some phenolic compounds, resulting in the formation of off-flavours.

Further work is required to investigate the composition of these wines and to determine if these off-flavours exist. The specific kinetics of the glucose oxidase enzyme and its activity after it is produced in recombinant wine yeasts and used in conventional winemaking methods, which is an anaerobic reaction, should also be investigated.

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In conclusion, it is very clear that there is a growing consumer demand for wine containing lower levels of alcohol and chemical preservatives. The objectives of this study were achieved, namely to express the *Aspergillus niger* gene encoding a glucose oxidase (GOX; β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) in *Saccharomyces cerevisiae*, to indicate that glucose oxidase is active and produced from early in the exponential growth phase to a constant level in the stationary phase, and to show by means of microvinifications that the laboratory yeast transformant resulted in wines containing 1.8 to 2.0% (v/v) less alcohol. This study therefore paves the way for the development of wine yeast starter culture strains for the production of wine with a reduced alcohol content and with lower levels of chemical preservatives such as sulphur dioxide.

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