Optimization and Evaluation of Heterologous Lysozyme Production in *Saccharomyces cerevisiae*

by

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Declaration

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Abstract

Optimization and Evaluation of Heterologous Lysozyme Production in *Saccharomyces cerevisiae*

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Hen egg white lysozyme (HEWL; muramidase; EC 3.2.1.17) is an enzyme present in high concentrations in chicken (*Gallus gallus*) egg whites. It hydrolyses the link between N-acetylmuramic acid and N-acetylglucosamine in Gram positive bacterial cell walls, resulting in cell death. It is thus active against lactic acid bacteria (LAB), which may be present in grape juices and musts. These bacteria are responsible for malolactic fermentation of wines although many species, particularly of the genera *Lactobacillus* and *Pediococcus*, are considered spoilage organisms. The growth of LAB is therefore closely monitored and controlled during winemaking. The most common means of control is growth inhibition by chemical treatment (usually with SO_2). Lysozyme is a commonly used wine processing aid, complementing the antimicrobial activity of SO_2 . It allows for lower doses of SO_2 to be used, thus improving the wholesomeness of wine. The OIV (*Organisation Internationale de la Vigne et du Vin*) approved its use in quantities up to 500 mg per liter of wine in 1997.

This study evaluated the effect of different secretion signals on the secretion of lysozyme by the haploid auxotroph *Saccharomyces cerevisiae* strain FY23. Secretion by an industrial strain (VIN13) transformed with a single copy of the *HEWL*

gene with the MF-α secretion signal under the control of the *PGK1* (phosphoglycerate kinase 1) prompter and terminator was also evaluated. In the case of FY23 four secretion signals were used, namely the native lysozyme signal and the *S. cerevisiae* mating factor-α signal as well as mutants of these signals. These mutants incorporated two additional arginines at the N-terminus of the signals immediately downstream of the terminal methionine. The effect of these mutations was to increase the positive charge of the secretion signal N-terminals. The secretion signal-lysozyme fusions were placed under the regulation of the *S. cerevisae PGK1* gene's promoter and terminator. The resulting expression cassettes were cloned into integrating vectors YIpLac211 and pDMPOF1b and episomal vector pHVX2. These were used to transform FY23 and VIN13.

FY23 as well as VIN13 transformants were evaluated in an artificial medium designed to reflect the nutrient content of grape juice, with particular attention being paid to assiminable nitrogen. Three hexose concentrations were tested in order to determine the effect thereof on lysozyme secretion titer.

Lysozyme secreted under all tested growth conditions was found to be too low for detection by either enzymatic assay or HPLC-FLD. For this reason secreted lysozyme was isolated and concentrated 10x by means of cation-exchange. Subsequently, lysozyme concentrations in the concentrates was determined by means of the aforementioned techniques. SDS-PAGE analysis of lysozyme concentrates was also performed.

No significant differences were found between native or MF-α secretion signals and their mutated counterparts in terms of secretion titer or proteolytic maturation. Lysozyme secreted with the MF-α signal was found to be misprocessed in all cases, with both an authentically processed and a larger form, in which the secretion signal was not completely removed, being present. Lysozyme secreted with the native signal appeared to be correctly processed in all cases. Secretion titer from high copy number episomal FY23 tranformants was similar to that of integrants containing a single copy of the gene. Sugar concentration affected lysozyme production, with higher quantities of the enzyme being secreted when higher initial sugar concentrations were used. Lysozyme titers were extremely low (< 0.25 mg/L) with all expression cassettes under all the tested conditions with both FY23 and VIN13. In the case of the VIN13's a lower final biomass was found for the secretor strain tested in comparrison to the VIN13 wild-type.

Uittreksel

Optimering en Evaluering van Heterloë lisosiem produksie deur *Saccharomyces cerevisiae*

("Optimization and Evaluation of Heterologous Lysozyme Production in Saccharomyces cerevisiae*")*

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Hoendereierwitlisosiem (HEWL; muramidase, EG 3.2.1.17) is ń ensiem teenwoordig in hoë konsentrasies in hoender (*Gallus gallus*) eierwitte. Dit hidroliseer die binding tussen N-asetielmuramiensuur en N-asetielglukosamien in Gram positiewe bakteriese selwande, wat tot seldood lei. Dit is dus aktief teen melksuurbakterieë (MSB), wat in druiwesap en mos teenwoordig kan wees. Hierdie bakterieë is verantwoordelik vir appelmelksuurgisting van wyne, hoewel baie spesies, veral van die genera *Lactobacillus* en *Pediococcus*, ook as bederforganismes beskou word. Die groei van MSB word dus noukeurig tydens wynbereiding gemoniteer en beheer. Die algemeenste wyse van beheer is groei-inhibisie deur chemiese behandeling (gewoonlik SO_2). Lisosiem is ń algemeen gebruikte wyntoevoegingsmiddel en vul die antimikrobiese aktiwiteit van SO_2 aan. Met lisosiem kan ń laer dosis van SO_2 gebruik word, wat lei tot ń verbetering van die heilsaamheid van die wyn. Die OIV (*Organisasie Internationale de la Vigne et du Vin*) het die gebruik daarvan goedgekeur tot en met 500 mg per liter wyn vanaf 1997. Hierdie studie het die effek van verskillende sekresieseine op die uitskeiding van lisosiem deur die

haploïede ouksotrofe *Saccharomyces cerevisiae* stam, FY23, geëvalueer. Uitskeiding deur ń industriële stam (VIN13), wat getransformeer is met ń enkelkopie van die HEWL-gene met die MF-α sekresiesein onder die beheer van die *PGK1* (Fosfogliseraat kinase 1) promotor en termineerder, is ook geëvalueer. In die geval van FY23 is vier sekresieseine gebruik, naamlik die inheemse lisosiemsein, *S. cerevisiae* MF-α sein, asook mutante van hierdie seine. Hierdie mutante het twee bykomende arginienresidu's by die N-terminus van die seine direk stroom-af van die terminale metionien. Die effek van hierdie mutasies was om die positiewe lading van die uitskeidingsein N-terminale te verhoog. Die gevolglike uitdrukkingskassette is in die integrasievektor YIpLac211 en pDMPOF1b, en die episomale vektor pHVX2, gekloneer. Dit is gebruik om VIN13 en FY23 te transformeer. FY23, sowel as VIN13-transformante, is geëvalueer in ń kunsmatige medium wat ontwerp is om die voedingsinhoud van druiwesap te weerspieël, met besondere aandag aan assimileerbare stikstof. Drie heksose konsentrasies is getoets om te bepaal wat die uitwerking daarvan op die lisosiemsekresietiter is. Onder alle groeitoestande was die isosiem wat uitgeskei is, te laag om deur ensimatiese toetse of HPLC-FLD bepaal te word. Om hierdie rede is uitgeskeide lisosiem geïsoleer en 10x gekonsentreer deur middel van katioon-uitruiling. Daarna is lisosiemkonsentrasies bepaal deur middel van bogenoemde tegnieke. SDS-PAGE-ontleding van lisosiemkonsentraat is ook uitgevoer. In terme van sekresietiter of proteolitiese maturasie, is geen beduidende verskille gevind tussen inheemse of MF-α sekresieseine en hul gemuteerde eweknieë nie. Lisosiem wat deur die MF-α sein uitgeskei is, is in alle gevalle foutief geprosesseer, met ń teenwoordigheid van beide die regte produk en ń groter produk, waarin die uitskeidingsein nie heeltemal verwyder word nie. Lisosiem wat met die inheemse sein uitgeskei is, blyk in alle gevalle korrek verwerk te wees. Sekresietiter van ń aantal hoë-kopie episomale FY23-transformante was soortgelyk aan dié van integrante met ń enkelkopie van die geen. Suikerkonsentrasie beïnvloed lisosiemproduksie, met ń hoër hoeveelheid van die ensiem wat uitgeskei word wanneer die aanvanklike suiker in hoër konsentrasies gebruik is. Lisosiemtiters was baie laag ($<$ 0.25 mg/L), met al die kassette onder al die getoetste toestande vir beide FY23 en VIN13. In die geval van die VIN13's, is ń laer finale biomassa vir die uitskeidingstam in vergelyking met die VIN13 wilde-tipe gevind.

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“So I have just one wish for you - the good luck to be somewhere where you are free to maintain the kind of integrity I have described, and where you do not feel forced by a need to maintain your position in the organization, or financial support, or so on, to lose your integrity. May you have that freedom.” – Richard P. Feynman

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Abbreviations

ATCC	American type culture collection
BiP	Binding protein
dNTP	Deoxynucleotide triphosphate
DAP	Diammonium phosphate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
ERAD	Endoplasmic reticulum associated degradation
FLD	Fluorescent detection
GM	Genetically modified
GMO	Genetically modified organism
GOX	glucose oxidase
HCl	Hydrochloric acid
HEWL	Hen egg white lysozyme
HPLC	High-performance liquid chromatography
IWBT	Institute for Wine Biotechnology
LB	Luria Bertani medium
LMG	Laboratorium voor microbiologie, Gent
LOD	Limit of detection
LOQ	Limit of quantification
MRS	De Man, Rogosa, Sharpe medium
NCDO	National culture of dairy organisms
OIV	Organisation Internationale de la Vigne et du Vin
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
SC	Synthetic complete medium
SRP	Signal recognition particle
TB	Terrific Broth
UPR	Unfolded protein response
YPD	Yeast peptone dextrose medium

Chapter 1

General Introduction, Project History and Specific Project Aims

1.1 Introduction

Anaerobic fermentation of grape juice, usually dominated by yeasts of the species *Saccharomyces cerevisiae*, is the principal biological process in the production of wine and primarily involves the conversion of grape sugars into ethanol through the central glycolytic pathway (Boulton *et al.*, 1996). Many microbes, however, play a role in the making of wine and are present in vineyards, wineries and on grapes (Fleet, 1993; Konig *et al.*, 2009). A second process involving the decarboxylation of malic acid to lactic acid, malolactic fermentation (MLF), may be performed by lactic acid bacteria either during or after alcoholic fermentation (Boulton *et al.*, 1996; Rankine, 1989; Lonvaud-Funel, 1999). Whether MLF occurs, to what degree it takes place and whether it is considered beneficial or deleterious to the quality of the finished wine is influenced by various factors. Perhaps foremost among these factors is the presence and quantity of indigenous must LAB which may include species from the genera *Lactobacillus*, *Oenococcus*, *Pediococcus* and *Leuconostoc* (Wibowo *et al.*, 1985; Fleet, 1993; Bae *et al.*, 2006; Rankine, 1989; Konig *et al.*, 2009; Lonvaud-Funel, 1999). Other factors include the style of the wine being made, fermentation temperature, pH of the must, contact with oxygen and the initial fermentable sugar concentration (Boulton *et al.*, 1996; Konig *et al.*, 2009; Rankine, 1989).

As a winemaking practice, MLF of most red wines is generally desirable, pri-

marily as a means of deacidification and microbial stabilization although it may also contribute to the organoleptic qualities of the wine. For example, LAB are responsible for the production of diacetyl (2,3-butanedione) which is perceived as a desirable buttery or butterscotch flavour (Ramos *et al.*, 1995; Francis & Newton, 2005; Boulton *et al.*, 1996). In regions with cool climates MLF is encouraged for the purpose of deacidification (Boulton *et al.*, 1996). In warm climates must pH is usually high (pH $3.5-4.0$) which renders deacidification unnecessary. In this case the purpose of MLF is primarily microbial stabilization. It may also be encouraged when specific adjustments to flavour or mouth feel are required. When MLF is deliberately conducted and a starter culture is used, *Oenococcus oeni* is the organism of choice in most cases (Boulton *et al.*, 1996). Members of the *Pediococcus*, *Leuconostoc* and *Lactobacillus* genera are, for the most part, considered spoilage organisms whose proliferation should be discouraged (Boulton *et al.*, 1996; Rankine, 1989).

In white wine production, complete prevention of MLF is usually desirable as volatile compounds perceived as fruity flavours are overpowered by diacetyl and their sensory effect is masked (Boulton *et al.*, 1996; Rankine, 1989; Konig *et al.*, 2009). With these wines, MLF's effect on flavour and mouth feel is more important than deacidification, especially in those produced in regions with warm climates. Whether to allow MLF is therefore of considerable importance where white wine production is concerned and it is generally not desired. Notable exceptions are Champagne production and some styles of new-world Chardonnay. With Champagne production MLF is usually carried out before bottle fermentation (the 2nd alcoholic fermentation) as a means of microbiological stabilization because the wine is left on the yeast lees. Failure to do this creates the risk of MLF occurring after bottling. If MLF after the initial alcoholic fermentation could be avoided without the risk of in-bottle MLF occurring the Champagne production process could possibly be shortened, which might be economically advantageous to the producer. Whether MLF of other white wines is encouraged may also depend on the traditions in the area of production (Boulton *et al.*, 1996).

Low temperatures and pH's are deleterious to the growth of LAB in must/wine. Fermentations with must at pH 3.3 and lower are generally regarded as safe (unlikely or very slow) against MLF (Boulton *et al.*, 1996; Konig *et al.*, 2009). In warm regions, such as South Africa, the pH of grape juices and wines is generally higher than in cool regions such as those found in most European wine produc-

ing regions. Additionally, in warmer regions the fermentable sugar concentrations of red grape juices (white grapes are harvested at 20-24 Brix, slightly earlier than reds) are generally higher, resulting in higher EtOH concentrations after alcoholic fermentation. High ethanol concentrations are inhibitory to LAB growth and therefore MLF, although this is possibly mitigated to some degree by the increased pH which is favourable to their growth. Accordingly, MLF is more likely to occur in musts produced in warmer regions.

In summary, depending on the style of wine, region in which it is to be produced and specifics of the particular fermentation (fruit quality, indigenous microbial population etc.), MLF may be encouraged, delayed or prevented completely (Boulton *et al.*, 1996). In order to manage these various factors many techniques have been developed over the years, providing winemakers with an array of tools with which to tailor their wines and manage the occurrence of MLF.

Possible interventions for preventing or controlling MLF include the following: removing wine from yeast lees (racking), use of antimicrobial agents such as dimethyl dicarbonate (the use of which requires that grape must be clarified), fumaric acid (which is difficult to dissolve in wine at effective concentrations) and SO_2, control of physical parameters such as fermentation temperature and must pH (Boulton *et al.*, 1996; Bartowsky, 2009). Amongst the chemical additives available is the enzyme lysozyme, isolated from chicken (*Gallus gallus*) egg whites (Gerbaux *et al.*, 1997).

There has been an increased demand for reduction in chemical preservatives used in winemaking, in particular SO_2 due to the sensitivity of some individuals to this substance (Weber *et al.*, 2009; Perez-Calderon *et al.*, 2007). Sulphur dioxide has antimicrobial properties against both yeast and bacteria and also functions as an antioxidant, hence its traditional use in winemaking (Romano & Suzzi, 1993). However, it has been shown that it can be added at lower concentrations when used in conjunction with lysozyme. In this way SO_2 is present in quantities sufficient to perform its antioxidant function while retaining a usable antimicrobial level by synergy with lysozyme. Lysozyme (enzyme 3.2.1.17) is an accepted processing aid, the OIV allowing additions of up to 500 mg per liter since 1997. It complements the antimicrobial action of SO_2, especially in musts with comparatively high pH's (>3.5) as SO_2 is more effective at low pH, whereas lysozyme has higher activity at higher pH's.

Addition of lysozyme to red musts at the end of alcoholic fermentation fol-

lowed by inoculation with commercial *O. oeni* starter cultures has been shown to decrease the duration of the malolactic fermentation (Lopez *et al.*, 2009), ostensibly because the inoculant is not forced to compete with a pre-existing native LAB population. From the winemakers' perspective this may be economically advantageous as wineries would benefit from decreased production times.

Because of lysozyme's proven utility in the food industry in general and, more recently, in winemaking (Pitotti *et al.*, 1991; Cunningham *et al.*, 1991; Gerbaux *et al.*, 1997; Gould, 2000; Masschalck & Michiels, 2003) and *S. cerevisiae*'s ability to express heterologous proteins, the possibility of developing a lysozyme secreting *S. cerevisiae* for application in wine-making was considered worthy of investigation. This was largely because oenological lysozyme purified from eggs was, at the time of writing, prohibitively expensive. One kilogram (sufficient to treat 2000 – 10000 liters) retails for approximately ZAR 1000 increasing production costs significantly. This is an unacceptable expense for many wine producers. For this reason a yeast producing lysozyme in an oenologically useful manner might be welcomed by winemakers who use the enzyme routinely, provided consumer mistrust of foods produced with GMO's decreases.

1.2 Project History

In 1999 the possibility of expressing HEWL (Hen Egg White Lysozyme) by wine yeast strains was considered by the IWBT (Winetech project number: IWBT 1/9). The aim of the study entitled "Production of fusion lysozyme enzyme by wine yeasts active against Gram-positive and Gram-negative spoilage bacteria" was defined in 2002 as the development of wine yeast with "pasteurisation properties". This would allow for the use of lower SO_2 and chemical preservative levels and inhibit the "growth of spoilage bacteria in bottled wine" (Du Toit & Pretorius, 2002). This was to be accomplished by expressing the *HEWL* gene under the control of the constitutive *PGK1* promoter and terminator with either the native lysozyme or MF-α secretion signals. Also considered was a strategy for increasing lysozyme's spectrum of activity to include Gram-negative organisms, specifically AAB (Acetic Acid Bacteria), by addition of a pentapeptide tail to the enzyme as is described by (Ibrahim *et al.*, 1992).

Progress as outlined in the IWBT 2002 funder's report (Du Toit & Pretorius, 2002) submitted to Winetech may be summarized as follows. A cDNA copy of

the *HEWL* gene received from Infruitec was cloned into an episomal yeast expression plasmid and transformed into four laboratory strains (ISP52, Y294, Σ1278 and BJ3505). Lysozyme production by these strains in YPD (Yeast extract Peptone Dextrose) broth was then evaluated. While transcription of the genes took place, no enzyme activity could be detected in episomal transformant culture supernatants. When no activity was detected against *M. luteus*, supernatant activity was tested on a panel of wine-native LAB[1]. No activity was detected against the LAB and it was postulated that the pH of the medium was unsuitable for the assay used. Attempts were made to optimize the methods of detection, culminating in LOD's (limits of detection) of 7.9 mg/L. Even with improvement to the detection method, no activity was detected within either the culture supernatants or extracted intracellular fractions.

The expression cassettes were subsequently subcloned into a yeast integration vector and transformed into the same four laboratory strains. All of the yeast integrants showed lysozyme secretion when colonies grown on agar were overlaid with *M. luteus*. A lysozyme gene isolated from transgenic tobacco (*N. tabacum cv xanti*) was obtained from the University of Quebec. This HEWL differed somewhat from the Infruitec variant in terms of amino acid content. It too was cloned into episomal yeast expression vectors and transformed into the same yeast strains. The transgenic tobacco lysozyme, unlike the Infruitec variant, was successfully secreted by the laboratory strains containing the episomal plasmids. YIp's containing the new lysozyme variants were created and were to be investigated in the project titled "IWBT 5/04".

Progress made for the period of 2003–2005 is outlined in the Winetech progress report for 2005 but is difficult to summarize, the reader is referred to the document in question (Du Toit & Van Rensburg, 2005). In 2004 integration of the four expression cassettes into *S. cerevisiae* VIN13 was attempted but was unsuccessful. Progress made from 2003 to 2008 as described in 2008's Winetech progress report (Du Toit & Van Rensburg, 2008) is as follows: In project "IWBT 5/04", four lysozyme yeast integration vectors had been created and characterised. *S. cerevisiae* laboratory strains had been transformed and integrations confirmed. It was also claimed that the task of evaluating antimicrobial activity by lysozyme producing yeast was completed, presumably with regard to these laboratory strains although

[1]Note that *M. luteus* is considerably more sensitive to the lytic activity of lysozyme than any LAB

no mention of activity against LAB is mentioned. Two of the four lysozyme expression cassettes had been transformed into *S. cerevisiae* VIN 13 and confirmation of integration was at that time underway. Lysozyme secretion by putative VIN 13 transformants had been evaluated by plate assay against *M. luteus* but no secretion had been detected. A total of 16 South African LAB strains isolates were selected in order to determine their sensitivity to lysozyme and serve as a “sensitive panel” against which transgenic *S. cerevisiae* strains could be tested. Throughout this period no wines were made with transgenic yeast, nor were any attempts made to properly characterize lysozyme secreted under plate-culture conditions in terms of secretion titer or authenticity of proteolytic maturation. Secretion from the industrial strain VIN13 was not successful.

1.3 Specific Aims

In this study hen egg white lysozyme secretion by an auxotrophic haploid *S. cerevisiae* (FY23) was evaluated in terms of titer and signal-processing under oenological conditions in a medium simulating grape must. The effect of the mating factor-α and native secretion signals, as well as the effect of N-terminal charge alterations in the aforementioned secretion signals, on secreted lysozyme was determined quantitatively and qualitatively. Expression from yeast transformed with multicopy episomal expression vectors as well as single copy genomic integrations was compared. With modification of previously used techniques integration of single copies of the above-mentioned lysozyme expression cassettes in VIN13 was attempted and lysozyme secretion subsequently evaluated. Lysozyme expression cassettes containing hen egg white lysozyme under the control of the *PGK1* promoter and terminator were created. Four different secretion signals were evaluated. These secretion signals were the native HEWL and the yeast MF-α secretions signals and two mutant variants thereof. The mutants incorporated additional arginine residues immediately adjacent to the respective proprotein’s N-terminal methionines in order to alter the secretion signal charges. This was done in an effort to improve secretion titer.

These expression cassettes were cloned into yeast multicopy 2μ-episomal and single copy integrating vectors which were subsequently used to transform *S. cerevisiae* FY23. Single copy integrating vectors were also constructed for integration into *S. cerevisiae* VIN13. Fermentations were performed using a synthetic grape

must under oenological conditions. Lysozyme secreted by transformants during fermentation was isolated, concentrated and quantified in order to:

- Quantify and compare lysozyme titers secreted by episomal and integrative FY23 transformants
- Investigate relative secretion of lysozyme under control of MF-α and native secretion signals
- Determine whether N-terminal charge alteration of the secretion signals affected lysozyme secretion efficiency
- Investigate each secretion signal's effect on proteolytic maturation during secretory pathway transport
- Investigate the influence of fermentable sugar concentrations on lysozyme titer in finished wines
- Compare enzymatic and HPLC-FLD methods for lysozyme concentration determination
- Quantify and compare relative lysozyme titer in artificial grape juice fermented by FY23 and VIN13

Chapter 2

Literature Review

2.1 General Introduction

Hen egg white lysozyme is commonly used as a biological control agent in the making of wine. This review will therefore briefly discuss its applications in the process of winemaking. As the focus of the current project was development and evaluation of transgenic yeast secreting lysozyme in an oenological context, the bulk of the review will focus on related topics. These include a description of the yeast secretory pathway, a description of *Saccharomyces cerevisiae* as a host for heterologous protein secretion and a general outline of yeast engineering strategies. The latter topic is discussed both in general and in the context of yeasts designed for use in the winemaking industry. Side effects and pitfalls of heterologous expression are described. Finally, proposals for heterologous expression of lysozyme in oenological *S. cerevisiae* will be mentioned.

The yeast *S. cerevisiae* has a compact genome, the first eukaryotic genome to be fully sequenced, and a well-studied metabolism. It's tractable genetics, well-annotated genome and the availability of knockout-mutants for approximately 96% of its genes have made it useful in the dissection of metabolic pathways. It is also an industrially important organism for the production of proteins, and has several advantages over other eukaryotic and prokaryotic (e.g. *Escherichia coli*) expression systems.

Unlike prokaryotic expression systems, *S. cerevisiae* is capable of performing many of the post-translational modifications required for maturation of most proteins derived from higher eukaryotes, including folding, accurate disulphide bridge formation and N-linked glycosylation. In comparison to mammalian, insect and

plant cell lines, *S. cerevisiae*'s generation time is short and its cultivation is technically uncomplicated and inexpensive. Additionally, as *S. cerevisiae* does not naturally secrete significant amounts of protein (and few strains secrete proteases), recovery of heterologous secreted proteins from culture media is relatively straightforward. These characteristics make it a highly convenient host organism for the production of industrial and medically-important proteins.

2.2 Lysozyme's applications in winemaking

Lysozymes are classified according to their amino acid sequence alignment as well as their substrate specificity. There are various groups of lysozymes, based on their substrate specificity, examples being the chicken, goose, viral and bacteriophage-types. *Gallus gallus* or chicken lysozyme has widespread application in food preservation (Cunningham *et al.*, 1991) and its potential application in winemaking has been investigated since 1990 (Pitotti *et al.*, 1991). Following promising results from pilot studies (Gerbaux *et al.*, 1997; Pitotti *et al.*, 1991), extensive investigation into the enzyme's potential use was conducted at various research institutes and commercial wineries worldwide (Fordras). Consequently, it was accepted as a processing aid by the OIV in 1997 (*Organisation Internationale de la Vigne et du Vin*) and several winemaking applications for lysozyme (some of which are described below) have been subsequently been described.

A large part of the enzyme's appeal is that its use allows for significant reductions in the quantity of sulphur dioxide (SO_2) added during winemaking. While SO_2 is not always used, especially in cooler climates, it remains the most effective antioxidant currently available to winemakers (Boulton *et al.*, 1996). Despite its usefulness, SO_2 poses a potential health risk to the consumer (Jackson, 1994). Lysozyme is seen as supportive of SO_2's antimicrobial activity, allowing for reduced sulfite levels.

Recently, pre-fermentation addition of oenological tannins as a replacement for SO_2 has been investigated (Bellachioma *et al.*, 2008). Consequent to this Sonni *et al.* (Sonni *et al.*, 2009) produced sulphur-free white wines by using lysozyme in conjunction with added oenological tannins. The tasting panel employed in this study found the sulphur-free wines preferable to control wines produced with SO_2 in the usual manner.

2.2.1 Inhibition of MLF

Perhaps the most technical application of lysozyme with regards to winemaking is its use in completely inhibiting MLF. As such it is not always successful. This is, at least in part, due to the varying sensitivity of different LAB species to chemical control agents (Wassung, 2006). Prevention of MLF occurring spontaneously is difficult once a LAB population has become established in a winery (Boulton *et al.*, 1996). In white wines MLF may produce undesirable off-flavours, unwanted changes in acidity, loss of fruit aroma and loss of varietal characteristics (Rankine, 1989; Fordras; Boulton *et al.*, 1996), although exceptions do exist in which MLF is specifically encouraged (eg. Champagne production and the sur lies method with Chardonnay). Traditionally SO_2 has been applied in higher doses in white wines than in reds. In a trial run for three years with Champagne wines it was determined that addition of lysozyme allowed for reductions in SO_2 of up to four g/hL, a significant decrease for equivalent efficacy.

2.2.2 Delay of MLF

In red vinifications MLF will occasionally begin before the alcoholic fermentation has completed. This is especially so when carbonic maceration is used (Rankine, 1989) and two cases may be distinguished: Musts from warm climates with high (10^5-10^6 CFU/mL) LAB populations and those from cooler climates with low (10^3-10^4 CFU/mL). In the former lysozyme is added simultaneously with SO_2, while in the latter lysozyme may replace SO_2 altogether. If MLF occurs early there is a risk of lactic acid spoilage and maceration has to be stopped, negatively affecting the wine's quality. Lysozyme is used as a preventative in carbonic macerations considered to be at risk, with 100 mg of the enzyme per liter final juice volume being added immediately after tank filling. In ordinary vinifications 200 mg/L added at the end of AF has been found to be effective.

2.2.3 Treatment of sluggish fermentations

In cases where LAB multiply rapidly before the end of AF, the tempo of the AF may be adversely affected. This is especially the case in clarified white juices, which are already susceptible to stuck fermentations (Rankine, 1989). In cases where AF has become slow and LAB are proliferating, lysozyme may be added to control

LAB. Alternatively, lysozyme may be added as a preventative early in the alcoholic fermentation when it is suspected that the fermentation may become sluggish.

2.2.4 Microbiological stabilization after MLF

The possibility of LAB conducting in-bottle MLF exists. This is because LAB may exist in a viable but non-culturable state, thus evading detection by traditional culture techniques prior to bottling (Millet & Lonvaud-Funel, 2000). Fordras S.A. recommends adding 250-500 mg/L upon completion of the MLF to ensure microbiological stabilization in commercial fermentations (*Fordras S. A., Switzerland*).

2.3 Protein secretion in yeast - from peptide chain synthesis to exocytosis

Secretion of proteins is a fundamental, and complex, process in both prokaryotic and eukaryotic cells. It is vital to industrial protein production, particularly within the pharmaceutical industry. As a result exhaustive study into the processes involved has been undertaken.

The mechanisms of protein transportation across the cellular membranes and the targeting of proteins to their various destinations were longstanding questions in cell biology. The answers were finally provided in 1975 by Günter Blobel (Blobel & Dobberstein, 1975*a*,*b*) with his development of the "signal hypothesis". His discovery that proteins are directed to their various destinations by means of "intrinsic signals that govern their transport and localization within the cell" earned him the 1999 Nobel Prize in Physiology or Medicine[1]. In the years following his discovery much research has been conducted into intracellular protein trafficking and signals targeting a variety of destinations have been found (for an early compilation see (Watson, 1984)).

The process of protein secretion comprises several steps (Kikuchi & Ikehara, 1991). After initiation of translation in the cytosol a secretion signal targets the nascent polypeptide chain to the membrane of the ER (Endoplasmic Reticulum), from where it is translocated into the ER lumen. In the lumen it is folded, the secretion signal cleaved and, should it contain the correct signal, N-glycosylated. It is then exported from the ER in vesicles. Transportation to the Golgi apparatus

[1] http://nobelprize.org/nobelprizes/medicine/laureates/1999/press.html

follows, where the protein is further modified. Finally it is packaged in a secretory granule with other secretory proteins and released into the periplasmic space.

2.3.1 Secretion signals

In the case of proteins destined for secretion, targeting to the ER membrane is effected by secretion signal peptides. These secretion signals have three characteristic and distinct regions: a (usually) positively charged amino-terminal region (N-region), a hydrophobic core region (H-region), and a carboxy-terminal region (C-region) (Von Heijne, 1990; Kikuchi & Ikehara, 1991). The carboxy-terminal region includes a cleavage site for type I signal peptidases (SPase I) (Tuteja, 2005). Secretion signals are usually on the N-terminal of the secretory protein, although exceptions exist (Vergunst *et al.*, 2005). Functionally, the charges on the amino-terminal prevent complete translocation of the secretion signal. The charged residues prevent entry of the N-terminus into the membrane's hydrophobic core and are retained on the cytoplasmic side of the ER membrane (Sakaguchi *et al.*, 1992). The importance of positive charges at the N-terminus of secretion signalshas been demonstrated previously (Szczesna-Skorupa *et al.*, 1988). Mutations replacing neutral residues preceding cytochrome P-450's hydrophobic signal core with positively charged residues converted the membrane-halt signal to a secretory signal. This resulted in secretion of the ordinarily membrane-associated protein. Secretion titer may, in some cases, be affected by the magnitude of the N-terminal charge, as in the case of HEWL (Hashimoto *et al.*, 1998).

The hydrophobic core is inserted into, and is long enough to span, the membrane. This brings the C-region within reach of the signal-peptidase complex, located on the lumen side of the ER membrane. During peptide translocation the signal peptide is cleaved from the translocating peptide by this complex. The cleaved signal peptide is then released back into the cytoplasm where it is degraded (Lyko *et al.*, 1995).

Some secretion signals are "stronger" than others, by which is meant that polypeptides using them are prioritized for translocation when competing with other signals (Nicchitta, 2002). The *Gallus gallus* hen egg white lysozyme signal[2] is considered to be one of the strongest eukaryotic secretion signals.

[2] Used in this study

2.3.2 Peptide transport from the cytoplasm into the ER lumen

Polypeptide translocation into the ER occurs through gated pores, first identified in yeast in 1987 (Deshaies & Schekman, 1987). These pores are tightly sealed[3] on the ER lumen side, in the absence of associated ribosomes, by BiP (Hamman *et al.*, 1998) (Binding Protein, Kar2p in *S. cerevisiae*), a member of the Hsp70 family (Vogel *et al.*, 1990) of proteins. Two distinct mechanisms exist, with translocation being either co- or post-translational. Translocation from the cytosol to the ER is either SRP (Signal Recognition Particle) dependant or SRP independent and preproteins may be translocated by either of these pathways, or both (Ng *et al.*, 1996). In the former pathway import occurs through the Sec61 complex (which is conserved in prokaryotes and eukaryotes) and, in the latter, through a Sec61 complex associated with a tetrameric accessory Sec62 complex. Which pathway a polypeptide will use is determined by its H-region hydrophobicity, with more hydrophobic sequences being targeted to the post-translocational pathway (Ng *et al.*, 1996). Adaptation to the loss of this pathway has been shown to involve induction of heat shock genes and down regulation of protein gene translation on a global level (Mukta & Walter, 2001). This results in a decrease in growth rate which the authors propose is the yeast's means of compensating for a decreased ability to correctly sort protein. Post-translational membrane translocation is the only pathway whereby polypeptides with C-terminal sorting signals may be translocated. The mechanisms involved have been recently reviewed (Wickner & Schekman, 2005; Rapoport, 2007).

2.3.3 Co-translational membrane transport

This is the major mechanism for ER polypeptide import in eukaryotes. In co-translational transport the N-terminal secretion signal of the nascent polypeptide chain is recognised shortly after translation initiation by a cytosolic SRP, described in Keenan *et al.* (Keenan *et al.*, 2001). The SRP binds to the ribosome/nascent polypeptide chain, slowing chain growth until the resulting complex recognizes and binds to the cytosolic side of a trans-ER-membrane SRP receptor (Walter & Blobel, 1982). Were chain growth not slowed, folding of the nascent-peptide chain in the cytosol before association with the SRP receptor (SR) would prevent translocation. The entire complex, consisting of the nascent polypeptide chain, the ri-

[3] In order to preserve the oxidising environment of the ER, which is important for protein folding

bosome and an SRP subsequently binds to Sec61, a single copy of which forms the trans-membrane pore (Van den Berg *et al.*, 2004). Despite its functionality in monomeric form, it is possible that Sec61p forms oligomers (see arguments in (Rapoport, 2008)). The signal sequence then causes the translocon to open in what is known as the start-transfer sequence. GTP binding to SRP and the SR causes release of the signal sequence. Subsequent hydrolysis of bound GTP allows disassociation of SRP from the SR (Connoly & Gilmore, 1989). At this point translation resumes and the nascent polypeptide chain passes through the translocon. Translation provides the energy for translocation, with the protein being forced through the Sec61 translocon as it is synthesized. Following completion of protein synthesis, the ribosome remains attached to the translocon (Potter & Nicchita, 2002). This may be a device to increase translational efficiency of ER targeted protein as a single mRNA could then pass through several membrane bound ribosomes simultaneously. Association of ribosomes with the outer ER membrane in high density during co-translational transport is responsible for the appearance of the rough ER. Interestingly, cells in which SRP translocation is non-functional gradually develop the ability to translocate SRP-dependant proteins (Ogg *et al.*, 1992).

2.3.3.1 Post-translational membrane transport

Post translational ER import requires the Sec61 and Sec62/63 complexes as well as BiP (Panzner *et al.*, 1995). The loss of BiP completely blocks translocation (Vogel *et al.*, 1990). Two models have been proposed, the mechanisms of which are not mutually exclusive, and have been comprehensively discussed (Elston, 2002). In both models translocation occurs through the Sec62/63 translocon, where BiP binds polypeptides with low sequence specificity as they exit the translocon complex (Misselwitz *et al.*, 1998). BiP hydrolyses ATP when it binds protein (Matlack *et al.*, 1997) in the presence of the Sec63p J domain, which is located in the ER-lumen (Corsi & Schekman, 1997). The first mechanism (or motor model) sees BiP acting to generate force for the import of the polypeptide. BiP binds both the polypeptide and the J-domain of the Sec61 complex. It then undergoes a conformational change, actively pulling the polypeptide into the ER lumen (Glick, 1995; McClellan *et al.*, 1998). Successive cycles of BiP binding and releasing the polypeptide chain result in translocation. The second (or Brownian ratchet) model proposes that BiP works as a molecular ratchet (Simon *et al.*, 1992; Matlack *et al.*, 1999). BiP binds to the translocating polypeptide as it emerges from the translocon and

prevents it from moving backwards. The peptide may however move forward by passive diffusion, whereupon more BiP binds the newly emerged polypeptide segment. Because only forward motion is allowed complete translocation eventually occurs. This mechanism can be used in prepro-α factor[4] ER import (Rothblatt & Meyer, 1986; Matlack *et al.*, 1999). Polypeptide chains must be maintained in an unfolded conformation in the cytosol if they are to pass through membrane pores. Post-translational transport therefore requires the presence of cytosolic chaperones to prevent folding and maintain polypeptide solubility.

2.3.4 Processing within the endoplasmic reticulum

The ER is the site for protein glycosylation, folding and disulphide bridge formation. Polypeptides entering the ER through the Sec61 channel have little secondary structure, allowing easy recognition of C-domain cleavage signals by the signal peptidase complex and glycosylation motifs by the OST (Oligosaccharyltransferase) complex (Chen & Liang, 2001). Both of these complexes are associated with the lumenal side of the ER membrane. Upon recognition of a glycosylation signal (Asn-X-Thr/Ser where X may be any amino acid except proline), the OST complex attaches N-linked $Glc_3Man_9GlcNac_2$ glycan to asparagine. Glycosylation of secretory proteins is beyond the scope of this review as HEWL does not naturally contain any glycosylation signals. Lack of secondary structure also facilitates folding chaperone binding, as these chaperones recognize and bind to hydrophobic residues (Bukau & Horwich, 1998). Disulphide bond formation requires an oxidative environment and is catalysed by PDI (Protein Disulphide Isomerase), a soluble lumen chaperone (Tsai *et al.*, 2001).

2.3.5 Quality control within the endoplasmic reticulum

The correct folding of protein in the ER is closely monitored, with incorrectly or incompletely folded proteins being translocated back into the cytoplasm for degradation in a process called ERAD (Endoplasmic Reticulum Associated Degradation) (Tsai *et al.*, 2002; McCracken & Brodsky, 1996). In cases where BiP becomes depleted as a result of accumulating misfolded protein, the UPR (Unfolded Protein Response) is triggered. BiP is associated with the transmembrane Ire1p but binds preferentially to unfolded protein. Disassociation of BiP from Ire1p results in Ire1p

[4]The secretion signal of which is used in this study

dimerization and autophosphorylation, triggering a cascade ultimately resulting in upregulated expression of UPR genes (folding chaperones, protein trafficking and ERAD associated genes) (Okamura *et al.*, 2000; Casagrande *et al.*, 2000; Travers *et al.*, 2000; Patil & Walter, 2001).

2.3.6 Export from the ER to the Golgi apparatus

Properly folded proteins are exported from the ER in vesicles which form in COPI and COPII-protein coated sections of the smooth ER (Antonny & Schekman, 2001). These vesicles are targeted to and fuse to the Golgi apparatus.

2.3.7 Processing in and secretion from the Golgi apparatus

The Golgi apparatus is the organelle in which secretory proteins are sorted and targeted to their various destinations. Additionally, the Golgi apparatus is involved in MF-α proteolytic processing and addition of mannose residues to the outer-chain of the α-factor secretion signal. The organelle contains Kex2p, which cleaves the Lys-Arg residues at the MF-α secretion signal's carboxy terminal. This separates the MF-α signal from the α-factor peptide and the α-factor peptides from each other. Subsequently, as the peptides are being transported out of the Golgi in secretory vesicles, Ste13p trims X-Ala (where X may be Ala, Glu or Val) from their amino-terminals. Kex1p removes Lys-Arg from their carboxy-terminals.

2.3.8 Exocytosis and passage through cell wall

Ultimately, secretory vesicles are transported to the cell's plasma membrane where they fuse and release their contents into the periplasmic space between the membrane and the cell wall. Pore sizes in the yeast cell wall increase with increased growth rate, probably as a result of insufficient rates of synthesis with rapid cell replication. Protein cross section is implicated, so when it is desirous to secrete large molecular weight proteins, or protein with large minimum cross sections in conditions characterized by slow cell growth (Smith *et al.*, 1985).

2.4 Heterologous gene expression in *Saccharomyces cerevisiae*

Since the yeast *S. cerevisiae* was first successfully transformed with foreign DNA (Hinnen *et al.*, 1978) it has seen extensive use as a host for protein expression, both in research and industrial applications. A wide variety of heterologous proteins have been successfully expressed in the yeast *S. cerevisiae*, and a number of reviews outlining strategies for its use in protein production exist (Romanos *et al.*, 1992; Ostergaard *et al.*, 2000; Nevoigt, 2008). The same may be said for other yeast species (Sreekrishna *et al.*, 1997). *S. cerevisiae*'s usefulness as a research tool is derived from its eukaryotic nature, rapid growth, simple culture requirements and the wide variety of molecular tools available for its manipulation. Within industry, as a vector for protein production, it has several advantages over prokaryotic and plant or animal tissue culture systems (Rai & Padh, 2001). Firstly, as a food organism it has GRAS (Generally Regarded As Safe) status, which distinguishes it from many of the other yeast expression systems. It correctly catalyses formation of disulphide bridges in proteins containing multiple bridges. Active peptide production is generally successful without the formation of inclusion bodies. Secretion is generally higher than in plant or animal systems, and the use of special transformation, culture and recovery processes are not required. Finally, unlike prokaryotic systems, it is capable of removing N-terminal methinonine from synthesized peptides (Mine *et al.*, 1999).

2.5 Engineering strategies for improved protein secretion

Many aspects of yeast protein expression machinery have been closely investigated. This has ultimately resulted in both a better understanding of the biochemistry of gene expression and the establishment and growth of a global multi-billion dollar bio-pharma industry. The need to further improve industrial protein production capacity as well as improve the economy of production continues to drive research in this field. Many factors influencing protein expression have been described (Fleer, 1992). With intelligent manipulation of expression systems, 50-fold increases in expression of heterologous proteins have been reported (Shusta *et al.*, 1998). The

benefits of careful strategic planning are plain to see. As a result a number of general strategies and guidelines for successful expression have emerged (Rai & Padh, 2001; Sevastsyanovich *et al.*, 2009). In general, factors to be considered at the beginning of any project involving heterologous protein production include such basic considerations as selection of an appropriate host organism or strain, optimization of growth media and culture conditions, selection of an appropriate promoter and (if necessary) secretion signal, expression level tuning and codon optimization. Physical culture parameters such as pH and temperature should be controlled. More advanced strategies involve, for example, modification of redox equivalent levels and/or co-expression with transcriptional activators or chaperone proteins catering to the specific processing requirements of the protein in question. Another approach is to increase the folding stability of the protein to be secreted, thereby expediting its migration through the yeast secretory pathway (Kjeldsen *et al.*, 2002; Kowalski *et al.*, 1998). As our understanding of yeast biology improves, new approaches are developed. For example, a recent investigation revealed that identical expression cassettes are transcribed at different levels depending on the site of chromosomal integration (Flagfeldt *et al.*, 2009), allowing for new possibilities in expression level tuning.

Metabolic engineering is an iterative process, and much reliable primary research must be undertaken before an engineering approach can even be attempted (Wittrup, 1999; Raab *et al.*, 2005). The results of metabolic engineering strategies are often unpredictable and in some cases counter-intuitive. Limitations imposed either by the intrinsic characteristics of the modified organism or the environment in which it is to perform must be taken into account. Often several rounds of experimentation and modeling are required (Nielson, 2001). Modeling complex metabolic networks, especially under constantly evolving conditions such as those in ongoing batch fermentations, is especially problematic (Kacser & Burns, 1997) and may even be impossible in complex systems. In the words of Nevoight "Only with a complete understanding of the complex global metabolic network and its responses to changing environmental conditions would one be able to predict all the secondary responses of a metabolic engineering approach" (Nevoigt, 2008).

2.5.1 Choice of host system

Organisms vary greatly in their capacity to secrete protein, this may be due to intrinsic properties of the organism or the nature of the secreted protein itself. An idea of an organism's capacity for processing and secretion of secretory proteins can be formed by examining naturally secreted protein levels in unmodified strains. For example, many reports of gram per liter production in *Pichia pastoris* (examples are given in (Cereghino *et al.*, 2002; Cos *et al.*, 2006)) exist, whereas 20 mg/L of secreted protein is often considered an excellent yield in *S. cerevisiae* in the case of immunoglobin production (Shusta *et al.*, 1998). With wine production, selection of the host organism is a limiting factor and so the viability of projects will depend on the maximum secretory capacity of the comparatively few yeasts and bacteria routinely used for fermentation. As secretory capacity is changeable, depending on many interrelated factors such as media composition, energy metabolism, redox capacity, growth temperature and the properties of the protein in question it is impossible to predict secretion titers in highly changeable environments. For this reason, it should be determined experimentally in each case.

An example of a high yield of secreted, unglycosylated protein from *S. cereviseae* is 180 mg/L for BPTI (Bovine Pancreatic Trypsin Inhibitor) (Parekh & Wittrup, 1997) after careful genetic optimization and under tightly controlled growth conditions. To emphasize the difficulty with which this result was obtained a brief description of the conditions of the study follows. A gene-dose optimized strain producing BPTI and overexpressing folding chaperones was grown at optimal pH and temperature controlled conditions. Oxygen was maintained at 20% saturation for the duration of the experiment. Glucose was maintained at 5 g/L once the initial growth phase had completed. Growth was for 120 h, at the end of which the protein concentration was determined.

2.5.2 Selection of an appropriate secretion signal

Expression level may be affected to a great extent by the choice of secretion signal. It is impossible to predict which secretion signal will result in the most efficient secretion of a particular protein and it is therefore normal to experiment with many different signals until a satisfactory one is found (Hashimoto *et al.*, 1998). Initial attempts to produce foreign proteins in bacteria and yeast made use of those proteins' native secretion signals. These signals were not always recognised by the

host organism's secretory apparatus and so experimentation with fusions of genes to yeast-endogenous signals began (Hitzeman *et al.*, 1981). One of the first yeast signals used was the secretion signal for *S. cerevisiae* mating-factor-α (Brake *et al.*, 1984; Zsebo *et al.*, 1986). A study conducted by Koganesawa *et al.* provides a good example of the variable effects secretion signals can have on secretion titers. When silkworm and human lysozyme were secreted with the MF-α signal the titers were 0.043 and 6.4 mg/L respectively. When secreted with their own signals the opposite was seen, with human lysozyme at 0.039 mg/L and silkworm lysozyme at 1.1 mg/L (Koganesawa *et al.*, 2001).

Engineering of secretion signals has been attempted in several studies. A successful strategy involving the hen egg white lysozyme secretion signal increased the positive charge on the N-terminal of the signal by incorporating additional arginines (Tsuchiya *et al.*, 2003). This strategy resulted in double the amount of human lysozyme secreted by the mutant hybrid than by the wild-type fusion.

Incomplete cleavage of secretion signals sometimes occurs, resulting in more than one processing variant being secreted. This is especially the case with c-type lysozymes secreted with the yeast MF-α secretion signal (Archer *et al.*, 1990; Hashimoto *et al.*, 1998; Koganesawa *et al.*, 2001; Kawamura *et al.*, 2003).

2.5.3 Selection of an appropriate promoter

Promoter selection for heterologous protein production is of critical importance. Promoters vary widely in strength, this being defined as the ability to facilitate initiation of transcription at a high rate. Those from the central glycolytic pathway are generally used as they have been shown to result in high level expression (Fleer, 1992). Recent studies have, however, shown that utilization of *ADH2* and *PGK1* promoters in episomal expression vectors for xylanase production result in decelerated growth, decreased flux through glycolysis and lower biomass yield (Gorgens *et al.*, 2001). This has been attributed to both the negative effects of protein burden (Snoep *et al.*, 1995) and, in the case of *PGK*1, competition for rare transcription factors. Previous studies in which each of the central glycolytic pathway enzyme were overexpressed under the control of their own promoter individually reported no negative effects on glycolytic flux (Hauf *et al.*, 2000), lending credence to the theory that competition for transcription factors was the cause of the growth effects seen by Gorgens and Snoep (Snoep *et al.*, 1995; Gorgens *et al.*, 2001). This has to

be contrasted with Gonzalez's findings that heterologous expression results in a decrease in flux through native protein synthesis, including enzymes, with consequent decreases in specific growth rate (Gonzalez-Candelas *et al.*, 2000). These findings are of particular importance when GMO yeast are being developed for wine fermentations, as any effect on yeast growth may have deleterious effects on the quality on the final product. It has been demonstrated that in nitrogen limiting conditions the rate of an alcoholic fermentation is directly related to yeast biomass (Valera *et al.*, 2004).

As a result of this the use of strong promoters not coupled to central metabolic pathways has been suggested. An example of such a promoter is *HXT7*, which codes for a glucose transporter expressed during sugar starvation and repressed during growth on glucose(Karpel, 2008). A truncated version of *HXT7* lacking glucose repression has been shown to be about 3 times as active as *PGK1* in LacZ fusion protein expression (Hauf *et al.*, 2000). Another potential candidate is the yeast metallothioein (*CUP1*) promoter (Mascorro-Gallardo *et al.*, 1996). Some genes are down regulated in the presence of glucose so would require removal of the repressor regulatory sequence for constitutive expression. A potential problem with engineered promoters is the possibility of unforeseen/unpredictable regulatory artifacts (Mijakovik *et al.*, 2005).

2.5.4 Modification of secretory capacity by genetic engineering

Peptide chain folding within the ER is known to be a major bottleneck in protein secretion (Parekh & Wittrup, 1995). As the details of this process are now better understood attempts have been made to increase folding capacity, with most of these attempts involving with overexpression of foldases and chaperones such as BiP, PDI and calnexin (Klabunde *et al.*, 2007). Modulation of PDI levels has been found to increase in secretion of single-chain antibody fragments (Shusta *et al.*, 1998) and other proteins (Bao & Fukuhara, 2001; Moralejo *et al.*, 2001; Robinson *et al.*, 1994) in a variety of organisms, with varying degrees of success. Bao *et al.* found that increasing expression of either polyubiquitin or PDI increased secretion titer of protein with large numbers of disulphide bridges (Bao & Fukuhara, 2001). Conversely, reduction of BiP levels has been shown to decrease the amount of BPTI secreted by *S. cerevisiae* (Robinson *et al.*, 1996). At time of writing not enough is known about the specific properties to allow for reliable in-silico prediction of

protein secretion potential, although such factors as ease of folding are known to play a role (Kowalski & Wittrup, 1998).

It has been found that organisms, including yeast and bacteria, respond to various stresses by overexpression of ribosomal RNA genes (increasing a cell's number of ribosomes is a well-documented stress response, allowing for rapid adjustment to changing environmental conditions (Klappenbach *et al.*, 2000)) and increasing the surface area of the ER (Becker *et al.*, 1999). This increased ER surface area results in an increase in the number of secretory and folding "units", with a concomitant increase in secretory capacity. This is sometimes coupled to a marked decrease in growth rate. For this reason it is usual to uncouple production of the target protein from the host's growth phase.

Shusta *et al.* found that overexpression of Gal4p, a transcriptional activator for the *GAL*1-10 promoter in a optimally tuned scFv secreting strain resulted in a marked decrease in secretion (Shusta *et al.*, 1998). The cause of this effect was revealed to be incomplete cleavage of the pro-sequence normally cleaved by the Kex2 protease. The effect of increased activator concentrations on native pathways was not investigated.

2.5.5 Copy number or gene dosage

Wittrup *et al.* showed that the effect of copy number on secretion is highly dependant on the qualities of the protein itself (Wittrup *et al.*, 1995). They were able to demonstrate the existence of a "synthesis optimum" for heterologous secretory proteins, beyond which the titer of secreted protein may actually decrease. Increasing a target gene's copy number generally results in an increase in transcription, although not necessarily in an increase in protein secretion (Wittrup *et al.*, 1995). Therefore, the relationship between gene copy number and level of expression is not linear, or rather, is only linear within a defined range for a specific protein expression system. Wittrup found that accumulation of translation product exceeding the capacity of the host organism's secretory pathways could result in complete disruption of secretion, so much so that in the case of GPDI, a strain possessing a single copy of the gene secreted more protein than a strain transformed with a high copy episomal plasmid. Subsequent gene dosage optimization in *S. cerevisiae*led to secreted BPTI concentrations of 180 mg/L, double the quantity of BPTI secreted by *Pichia pastoris* (Parekh & Wittrup, 1997).

Copy numbers of ribosomal RNA genes in bacteria may range from one to dozens (Schmidt, 1997). The more copies of the gene an organism has, the more rapidly it can adapt to changing environmental conditions. This confers obvious competitive as well as survival advantages in changeable environments (Klappenbach *et al.*, 2000). A side effect of this is potential slower growth due to the increased burden placed on the cell's biosynthetic machinery. This same effect is seen in studies where foreign genes are expressed in *S. cerevisiae* and *E. coli*, although the magnitude of the effect is dependant on several additional factors.

2.5.6 Codon optimization

Codon usage varies widely between different, sometimes even closely related, organisms (Lloyd & Sharp, 1992) and it was demonstrated comparatively early on that non-optimal codon utilisation could result in drastic reductions in secreted heterologous protein yield (Kotula & Curtis, 1991). It follows that the first step taken when engineering high level heterologous protein expression (once an appropriate host expression system has been selected) should be optimization of the target gene's codon content. For a comprehensive explanation of codon bias see Sharp *et al.* (Sharp & Cowe, 1991). The earliest study in which HEWL was expressed and secreted in *S. cerevisiae* (Oberto & Davidson, 1985) attributed the comparatively low titer (1.5% of total cellular protein) at least in part to the large codon bias (0.049 compared to 0.99 for glyceraldehyde-3-phosphate) for this protein in yeast (Bennetzen & Hall, 1981).

There are various examples of codon optimization culminating in improved expression and even host organism performance. Gustafsson *et al.* provides a summary of studies where codon optimization has resulted in improved protein production and outlines a sensible procedure for gene design and codon optimization when considering heterologous expression (Gustafsson *et al.*, 2004). For example, Wiedeman *et al.* demonstrated the first successful metabolic pathway engineering by the use of codon optimization (Wiedeman & Boles, 2008). *S. cerevisiae* containing codon-optimized genes engineered to ferment L-arabinose to ethanol showed a marked increase in both rate of production (2.5-fold) and ethanol yield per gram dry weight (0.24–0.36 g ethanol/g L-arabinose consumed).

2.5.7 Medium composition and physical parameters of cultivation

Choi and co-workers (Choi *et al.*, 2004) demonstrated increased productivity of human lysozyme by controlling the pH of batch cultures of *S. cerevisiae*. This effect was more marked for the high copy number plasmid than the low copy number plasmid, indicating that the secretory apparatus was saturated, although shifts in localization from the intracellular space to the periplasm and extracellular environment occurred as time went on. This result may be interpreted in two ways, either the cells were able to clear the backlog of intracellularly retained lysozyme once they entered stationary phase and production had slowed or the stress of high level production induced enlargement of the ER surface area, thus increasing secretory capacity. A pH shift from 5.5 to 3.0 once stationary phase was attained resulted in increased secretion in a significantly shorter time but resulted in lower cellular biomass. Gorgens *et al.* has investigated the effects of amino acid and nitrogen supplementation on production of xylanases (Gorgens *et al.*, 2005). Temperature of cultivation has also been shown to directly influence the titer of heterologously produced proteins. For example, when *Trametes versicolor* laccase A was expressed in *S. cerevisiae*, secreted activity was found to be 16-times higher when cultivated at 19°C culture than in an otherwise-identical 28°C culture (Cassland & Jonsson, 1999).

2.6 Challenges and pitfalls of heterologous protein production

There are various consequences to altering an organism's metabolism, particularly when recombinant proteins are being produced (Mattanovich *et al.*, 2004; Snoep *et al.*, 1995; Gorgens *et al.*, 2001; Martinez *et al.*, 1999). For example, in *S. cerevisiae* heterologous protein expression has been found to result in up-regulation of amino acid and purine synthesis (Steffensen & Pedersen, 2006). Changes to an organism's metabolism outside of whatever it has specifically been engineered to do are of particular importance in organisms intended for food production. Alterations, while intended to impart beneficial qualities, may have negative effects on the organism's fermentation performance and contribution to the character of the food in question. Some of the factors capable of contributing to undesirable performance

are described below.

2.6.1 The metabolic burden of expression

The effect of the "metabolic burden of expression" by heterologous production (particularly secretion) of proteins on host organism's growth and metabolism was first identified in 1957 (Novick & Weiner, 1957). It has since been thoroughly investigated. The effect was termed "protein burden" in 1995 (Snoep *et al.*, 1995) and defined as "the negative effect on any part of cell function caused by an overexpression of a protein independent of its catalytic activity". In the context of that study the effect was due to overexpression of glycolytic enzymes but the term has come to encompass heterologously expressed genes as well. Most often these effects are seen as decreased cell growth rate, a decrease in flux through glycolysis and low final biomass yield in batch fermentation. Further effects include alterations in end-level concentration of metabolites such as higher alcohols, a factor that should greatly concern winemakers (Nevoigt, 2008). The metabolic and protein burden of expression has, to the knowledge of this author, never been specifically investigated in genetically engineered wine yeast. This is particularly worrying in especially cases where high titer secretion of a protein is the goal.

2.6.2 Saturation of yeast secretory pathways

As described above, Wittrup *et al.* demonstrated the existence of an "optimum expression level" for heterologous protein secretion (Wittrup *et al.*, 1995). The existence of a secretory maximum implies that said maximum can be exceeded. The question then becomes what the implications of over-secretion are, both in terms of production of the protein in question and with regard to cell function in general. One consequence is saturation of the cell's protein folding capacity (Parekh & Wittrup, 1995). This leads to both decreased secretion and induction of cellular stress responses such as the UPR (unfolded protein response) and ERAD (endoplasmic reticulum associated degradation). It has been demonstrated that once the cellular secretion machinery has been disrupted, it is difficult to restore its function (Huang *et al.*, 2008; Miller *et al.*, 2005).

A fallacy that persisted for some time in the protein engineering community was the apparent superiority of *P. pastoris* over *S. cerevisiae* as a host for expression. While *P. pastoris* AOX integration is generally the strategy pursued, in *S. cerevisiae*

2μ-multicopy plasmids have traditionally been used (Parekh & Wittrup, 1997). Direct comparison of the two systems is not possible in most cases, as was demonstrated by Parekh and Wittrup with gene dosage optimized BPTI secretion from *S. cerevisiae* (Parekh & Wittrup, 1997). They postulated that in many *S. cerevisiae* secretion studies where 2μ-based plasmids were used, protein synthesis may have been occurring at a rate greater than that optimal for the organism. This could potentially have led to blockage of the secretory pathways and subsequent low protein secretion. This hypothesis is supported by reported differences in protein expression levels from different transformants when 2μ-based plasmids were used, indicating stable, heritable copy number variation of these plasmids (Purvis *et al.*, 1987).

2.6.3 Potential for errors in heterologous protein production

It was noted comparatively early (Eckart & Bussineau, 1996; Olins, 1996) that the authenticity of a heterologously expressed protein is at least as important as its yield. At that time focus was on a host organism's ability to correctly process foreign proteins in terms of folding and post-translational covalent modification. For example, *E. coli* do not possess the ability to perform N-linked glycosylation and are a poor choice for expression of most mammalian lumen proteins. The last two decades have seen expansion of our understanding of both translation and post-translational processing, which has subsequently allowed for better selection of strain and culture conditions in expression studies. However, aside from what are by now obvious considerations (eg. matching a protein's processing requirements to an appropriately equipped expression strain) the possibility of various types of translational errors, and the factors causing them, should always be taken into account.

Kurland *et al.* highlighted the importance of investigating translational errors associated with heterologous gene expression (Kurland & Gallant, 1996). Among the issues they raised was the possibility that medically important proteins containing microheterogeneity could induce immunogenic effects. The errors to which they referred affect the quality rather than the quantity of protein produced. Any negative effects resulting from their presence may only become apparent once the product for which they were intended is in use. These errors may be classified according to their effects on a) the function of the protein produced, or b) the producer organism's biology. Missense amino acid substitutions occur naturally in native proteins at very

low frequencies, but under certain conditions may occur as frequently as 1 substitution per 100 residues. Such circumstances include high level expression of a protein containing unusual proportions of amino acids compared to those used by the producer organism, or in cases where an important amino acid is limiting. Translation errors are classified as either missense errors or processivity errors (Sundararajan *et al.*, 1999), the former producing sequence changes and the latter premature termination of translation. Missense errors can be further sub-divided into errors causing ribosomal in-frame hopping, amino acid substitutions, frameshift mutations and termination codon read-through (Kurland & Gallant, 1996). These errors are distinct from ribosome stalling, in which peptide chain elongation is slowed resulting in lower yields of accurately translated protein.

Detection of processivity errors may be difficult, especially if errors occur at low frequency or mutants co-purify with correctly translated protein. Any negative effects resulting from their presence may only become apparent once the product for which they were intended is in use. However, as factors contributing to their appearance become better understood it should be possible to predict and therefore avoid them through appropriate planning of the expression study.

2.6.4 Codon bias

Organisms vary greatly in their use of various codons. For this reason the use of optimized codons has been extensively investigated. Results vary widely, in many cases optimised codon use has resulted in greatly increased yields but this result is not consistent for all proteins. For this reason, the usefulness of optimised codons has to be determined experimentally. Important observations include the existence of "hungry codons". If two or more codons requiring low-abundance tRNAs are immediately next to each other, premature termination of translation or a frameshift mutation may occur. This is especially the case when the protein production machinery is operating close to maximum capacity, as is likely to be the case during heterologous production. Misprocessed product then accumulates. It is possible that the increase in misprocessed products seen by Hashimoto (Hashimoto *et al.*, 1998) was due to this effect.

2.6.5 Differences between host and heterologous protein amino acid utilization ratios

One of the problems heterologous expression faces is a potential imbalance between the needs of a foreign gene and the ability of the translational apparatus to supply them. The concept of the “hungry codon” was introduced to explain this situation (Kane, 1995). A hungry codon is a codon at which translation stalls, either because the tRNA recognising it is rare in the host species or because the population of acylated tRNAs is depleted due to amino acid starvation (Barak *et al.*, 1996). If both of these conditions are met simultaneously for the same codon in a large population of mRNAs, the situation may become dire. In special cases of ribosome stalling, frameshifting can occur (Barak *et al.*, 1996). Frameshift mutations may be spontaneous (Sundararajan *et al.*, 1999) (and references therein) with tRNA slippage during pausing resulting from amino acid starvation.

2.7 Lysozyme expression studies

Molecular biology has provided powerful tools for investigating the mechanisms of living cells. Lysozyme being a well characterized, highly stable secretory protein was naturally recruited as a reporter for investigation of protein secretory pathways in yeast. Hen egg white lysozyme (HEWL) was first expressed in *S. cerevisiae* in 1985 (Oberto & Davidson, 1985), demonstrating that its 18 amino acid secretion signal is recognised and correctly processed by the yeast secretory apparatus. This study provided supporting evidence for the then-emergent theory that yeast secretory pathways are capable of both recognizing and correctly processing secretion signals from higher eukaryotes. It also helped establish *S. cerevisiae* as a convenient model organism for eukaryotic secretion due to the ease with which it can be manipulated. The following year human lysozyme was successfully secreted from *S. cerevisiae* using the HEWL signal (Jigami *et al.*, 1986). HEWL has since been adopted as a model protein in the study of secretion and its secretion signal recognised as one of the strongest eukaryotic signals. It, and other lysozymes, have been expressed in a variety of organisms for various purposes. Hashimoto *et al.* examined the effect of secretion signal on the amount of HEWL secreted (Hashimoto *et al.*, 1998). They found that, while the MFα secretion signal produced the highest protein yields (in comparison to the signals for invertase (*SUC*2), killer toxin

type 1 (*KILM*1) and acid phosphatase (*PHO*1)) it also produced misprocessed subproducts. These products increased in proportion to correctly processed lysozyme as the duration of the cultivation increased. The gene was expressed in AH22 transformed with a pHA394 yeast expression vectors differing in secretion signal. Of the four signals (*KILM*1, *SUC*2, *PHO*1 and MFα) tested, MFα produced up to ten times the amount of active lysozyme compared to the other signals. Importantly, in the case of both MFα and the killer toxin signal, misprocessed products were detected which increased with length of cultivation.

Chicken lysozyme is not naturally glycosylated. It is thus useful in deconvoluting the mechanisms of glycosylation in yeast, as well as the effect of glycosylation on enzyme properties such as solubility and specific activity. Glycosylation of lysozyme by *S. cerevisiae* has been studied extensively. In one of the earliest investigations HEWL was subjected to site directed mutation introducing N-linked (Asn-*X*-Ser/Thr) glycosylation signals (Nakamura *et al.*, 1993). This study was conducted to investigate hyperglycosylation (polymannosyl glycosylation) in yeast, the purpose of which was at the time unknown, and found that lysozyme glycosylated at position 49 had comparable enzymatic properties but greater heat stability than the unglycosylated wild-type. Notably, it was found that polymannose side chains attached to HEWL were larger ($200-350$ residues/lysozyme molecule) than those of proteins naturally secreted by *S. cerevisiae*. Further investigation of another mutant (R21T) indicated that attachment of abnormally large polymannosyl side chains occurred regardless of the position of the glycosylation signal (Kato *et al.*, 1994). At this point it was postulated that this hyperglycosylation was a result of a long residence time in the Golgi apparatus due to HEWL's rigid nature as well as its high isoelectric point (pH 10.2). Glycosylation of HEWL being demonstrated at positions 19 and 49, Ueda *et al.* further demonstrated that lysozyme could be glycosylated at position 87 and that glycosylation increased thermal stability in comparison to the wild type (Ueda *et al.*, 1996). Lysozyme, engineered to contain N-glycosylation signals, was put forward as a useful reporter protein for studying processing and secretion of glycosylated proteins.

Kato *et al.* incorporated multiple asparagine-linked glycosylation signals, investigating the positional effect of introduced glycosylation signals on glycosylation patterns (Kato *et al.*, 1994). Their findings revealed that the proximity of both the (Asn-X-Thr/Ser) signal to the N-terminal and the molecular surface of a folded protein increase the likelihood of glycosylation. Additionally, the effect of glycosyla-

tion on enzyme activity was determined. Equimolar amounts of polymannosylated lysozyme were found to have significantly lower activity than the wild-type, while oligomannosylated lysozyme's activity was undiminished. Their findings seemed to indicate that lysozyme is retained in the Golgi apparatus far longer than is typical of yeast mannoproteins in general. Expression chase experiments indicated that lysozyme is retained by the yeast's secretory apparatus far longer than native secreted proteins (Kato *et al.*, 1994). This effect may be due to the protein's unusually rigid structure and high isoelectric point (Kato *et al.*, 1994; Blanco *et al.*, 2007). The effect site-specific glycosylation of lysozyme immunogenicity was investigated by Usui *et al.* (Usui *et al.*, 2004) with an eye on development of non-allergenic food proteins.

In structure-function studies, the roles of individual amino acid residues within the enzyme's active site were determined, demonstrating for example that Trp-62 both interacts with substrate carbohydrates and helps maintain the protein's active site's structure (Maenaka *et al.*, 1994). Following this work Hashimoto *et al.* used HEWL mutants expressed in *S. cerevisiae* to finally confirm lysozyme's catalytic mechanism (Hashimoto *et al.*, 1998). Further studies using lysozyme as a model secretory protein include investigation of calnexin's role in secretion of glycosylated proteins (Song *et al.*, 2001*a*), the effect of glycosylation on lysozyme emulsification properties (Shu *et al.*, 1998) and amyloid formation modelling human disease (Song *et al.*, 2001*b*) and the enzyme's robustness against mutation (Kunichika *et al.*, 2002). Increasing protein thermostability by intramolecular cross-linking has also been investigated (Ueda *et al.*, 2000).

Lysozyme's range of antimicrobial activity has been expanded through the inclusion of a C-terminal pentapeptide tail (Ibrahim *et al.*, 1992). A lysozyme effective against both Gram-positive (lactic acid bacteria) and Gram-negative (acetic acid bacteria) would no doubt be useful in winemaking. While the use of lysozyme is approved by the OIV, it is not clear that a heterologously expressed form modified in such a fashion would be accepted, especially without extensive trials. Additionally, the titer of the modified lysozyme was significantly lower than that of the wild-type (Ibrahim *et al.*, 1992). A later study demonstrated enhanced secretion of this fusion-lysozyme by inclusion of a N-terminal glycosylation signal and removal of *S. cerevisiae*'s calnexin gene (Arima *et al.*, 1998). As calnexin plays a role in quality control of glycosylated proteins (Song *et al.*, 2001*a*) the consequences of its deletion in a wine strain might not present a solution to the problem.

Table 2.1: Titers of lysozyme expressed in various organisms

Host organism	Lysozyme titer	Reference
S. cerevisiae	1.5% TYP[a]	(Oberto & Davidson, 1985)
S. cerevisiae	0.2 mg/L	(Kumagai *et al.*, 1987)
S. cerevisiae AH22	0.01 – 0.07 mg/L	(Ueda *et al.*, 1996)
S. cerevisiae AH22	1.0 – 13.9 mg/L	(Hashimoto *et al.*, 1998)
S. cerevisiae	0.005 mg/L[b]	(Arima *et al.*, 1998)
P. pastoris GS115	20 mg/L	(Mine *et al.*, 1999)
P. pastoris	30 mg/L	(Saito *et al.*, 2003)
P. pastoris X33	400 mg/L[c]	(Masuda *et al.*, 2005)
A. niger	12 mg/L	(Archer *et al.*, 1990)
K. lactis	0.5 mg/L	(Tanaka *et al.*, 2000)
Lactococcus lactis subsp. *lactis*	low[d]	(Van de Guchte *et al.*, 1989)

[a] Total Yeast Protein
[b] Unstable H5 lysozyme with carboxy hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro)
[c] Cell culture had a wet mass of 306 g/L in this study
[d] Lysozyme was visualized on SDS-PAGE but concentration was too low for quantification by enzymatic activity

While many studies in which hen egg white lysozyme is heterologously expressed do not report secreted titers (for examples see (Ibrahim *et al.*, 2001, 1994; Arima *et al.*, 1997; Kato *et al.*, 1994; Kunichika *et al.*, 2002)), those that do generally report low levels of secretion, examples being listed in Table 2.1. The expression of hen egg-white lysozyme (HEWL) by oenological *S. cerevisiae* strains was proposed in 2000 (Pretorius, 2000). The central argument for lysozyme expression during winemaking is the high cost of the commercial enzyme. This could ostensibly be addressed by expressing it during fermentation, at no extra cost to the winemaker.

2.8 Genetically modified yeast in the wine industry

As motivation for development of GM *S. cerevisiae* strains, it has been claimed that there is a demand amongst wine makers for "specialized yeast with special traits" resulting from genetic modification (Pretorius, 2000; Romano *et al.*, 2003; Mannuzza *et al.*, 2002). This should be balanced against claims of consumer resistance to GM wines (Nevoigt, 2008). As the development of useful strains may take a long time (Pretorius, 2000), it is possible that consumer fears will have subsided globally by the time GMO yeasts are ready for regular use in large-scale wine production. Thus far at least two GMO wine yeast have been approved for industrial

use (Akada, 2002), although to date only one has been successfully commercialized (Husnik *et al.*, 2006) (as has a yeast used in Sake production).

Targets for genetic improvement of wine yeasts include grape juice processing, control of wine spoilage organisms, improved fermentation performance, improved sensory characteristics and wine wholesomeness (Nevoigt, 2008). Specific targets for improvement, proposed in 1998 (Blondin & Dequin, 1998), have since been discussed in a number of reviews (Pretorius, 2000; Dequin, 2001; Pretorius & Bauer, 2002; Pretorius *et al.*, 2003; Schuller & Casal, 2005) and are presented in Table 2.2.

Special engineering challenges in the modification of commercial yeast strains exist (Nevoigt, 2008). These fall into four categories. Firstly, industrial strains of *S. cerevisiae* differ significantly from easily manipulated laboratory strains. Secondly, alteration of metabolic pathways or incorporation of new characteristics should not alter the basic qualities of the wine produced in ways other than intended. (Requirements of a good wine yeast strain include predictable and reproducible fermentation characteristics, vigorous fermentation and reliable flavour characteristic development (Boulton *et al.*, 1996)). Fermented beverages' (such as wine) sensory characteristics are determined by both quantity and balance of various compounds. These are a function of juice characteristics, cellar practices as well as the fermenting strain (Boulton *et al.*, 1996) and care must be taken not to perturb the yeast's contribution to balance when modifying strains. As an example of possible side-effects of metabolic engineering, when glycerol 3-phosphate dehydrogenase (*GPD*1) was overexpressed in an effort to divert carbon flux towards glycerol (Michnick *et al.*, 1997), large changes in by-product formation were observed. Acetaldehyde, acetate, acetoin $2,3$ butane-diol and succinic acid all increased in the fermented medium. Notably, a substantial decrease in biomass dry weight (4.2 ± 0.8-2.55 ± 0.07 g/L) by the overexpressing strain was also observed. Thirdly, said changes should not affect the fermentation process itself in undesirable ways. They should especially not require expensive process adaptation at the winery. The final category is consumer resistance to the use of genetically modified organisms (GMO) in alcoholic beverage production. This final detail has been discussed extensively with regard to wine yeast (Pretorius & Bauer, 2002).

Difficulties with some GM wine yeast projects appear to exist. Projects requiring the presence of large amounts of molecular oxygen in oxygen-limited systems (such as wine and beer fermentations) are one example (Heux *et al.*, 2006; Henricsson *et al.*, 2005; Malherbe *et al.*, 2003; Malherbe, 2010). Glucose oxidase (GOX)

expression in wine yeast as a means to decrease ethanol concentrations in wine was found to have no little effect on ethanol concentrations (Malherbe, 2010). Significant differences in terms of metabolite production between modified and wild-type strains were, however, reported. Some studies have been performed on auxotrophic laboratory strains of *S. cerevisiae*. Information garnered from such studies at best represents a "first step" in an ongoing oenological strain development project. Haploid auxotrophic strains are never used in commercial winemaking and extrapolation from their performance to that of industrial strains cannot reliably be made. Evidence against the use of auxotrophs in metabolic engineering and protein expression studies has been presented (Gorgens *et al.*, 2004). Furthermore, Hensing *et al.* highlighted important differences between lab-scale and industrial fermentations (Hensing *et al.*, 1995). Strain performance in a laboratory may differ vastly from performance under industrial conditions.

The multidisciplinary nature of the field of wine biotechnology should be taken into account when considering past and future research. Problem identification (and realistic solution development, where possible) requires critical input from experts from various fields. A molecular biologist, biochemist or bioinformaticist is unlikely to have extensive practical knowledge of industrial winemaking processes. Nor is a winemaker or process engineer likely to understand the very real complexities, limitations and potential side effects of metabolic engineering. Open consultation between knowledgeable authorities from appropriate fields is required for realistic project design.

Table 2.2: *S. cerevisiae* improvement studies, adapted from Schuller, 2005

Improvement	Metabolism		gene	Source	Construction				
					P	T	Pla	M	Chr
Sensorial	Aroma liberating enzymes	Endogluconase	*AGL*1	*Trichoderma longibrachiatum*	*ACT*	-	2μ	*CYH*2	-
		Arabinofuranidase	*abfB*	*Aspergillus niger*	*ACT*	-	2μ	*CYH*2	-
		Endoxylanase	*xlnA*	*Aspergillus nidulans*	*ACT*	-	2μ	*CYH*2	-
	Acidity adjustment	Malate permease	*MAE*1	*Schizosaccharomyces pombe*	*PGK*1	*PGK*1	2μ	*SMR*1 – 140	+
		Malic enzyme	*MAE*2	*Schizosaccharomyces pombe*	*PGK*1	*PGK*1	2μ	*SMR*1 – 140	+
		Malolactic enzyme	*mleS*	*Lactococcus lactis*	*PGK*1	*PGK*1	2μ	*URA*3	
		Glycerol-3-phosphate	*GPD*1	*Saccharomyces cerevisiae*	*ADH*1	ADH1	2μ		-
	Glycerol production	Glycerol-3-phosphate	*GPD*1	*Saccharomyces cerevisiae*	*ADH*1	ADH1	2μ	*ble(TN5)*	-
	Acetate ester production	Alcohol	*AFT*1	*Saccharomyces cerevisiae*	*PGK*1	PGK1	2μ	*LEU*2	+
Health and safety	Resveratrol production	β-glucosidase	*bglN*	*Candida molischiana*	*ACT*	*ACT*	2μ	*CYH*2	-
	Ethyl carbamate elimination	Block urea secretion	*CAR*1	*Saccharomyces cerevisiae*					
	Ethanol reduction	Glucose oxidase	*GOX*	*Aspergillus niger*	*PGK*1	*PGK*1		*IME*1	+

Table 2.2 – *Continued*

Improvement	Metabolism		gene	Source	Construction				
					P	T	Pla	M	Chr
Control of spoilage	Antimicrobial enzyme production	Pediocin	*pedA*	*Pediococcus acidilactici*	*ADH*1	*ADH*1	2μ	*URA*3	-
		Chitinase	$CTS1-2$	*Saccharomyces cerevisiae*	*PGK*1	*PGK*1	2μ		-
		Leucocin	*lcaB*	*Leuconostoc carnosum*	*ADH*1	*ADH*1	2μ	*URA*3	
		Chicken lysozyme	*lcaB*	*Gallus gallus*					
Processing efficiency	Prevention of filter clogging and improved extraction	Endopolygalactoronase	*PGU*1	*Saccharomyces cerevisiae*	*PGK*1	*PGK*1	LEU2		-
		Endopolygalactoronase	*PGU*1	*Saccharomyces*	*PGK*1	-	*CYH*		+
		Pectate lyase	*PELA*	*Fusarium solani*	*ACT*	-	*CYH*	2μ	-
	Flocculation timing	Flocculin	*FLO*1, *FLO*11	*Saccharomyces cerevisiae*	*HSP*30				

Table abbreviations: *P* Promoter, *T* Terminator, *Pla* Plasmid, *M* Marker, *Chr* Chromosomal integration

Studies are listed in descending order (Perez-Gonzalez *et al.*, 1993; Sanchez-Torrez *et al.*, 1996; Ganga *et al.*, 1999; Volschenk *et al.*, 2001, 1997; Michnick *et al.*, 1997; Remize *et al.*, 1999; Lilly *et al.*, 2000; Gonzalez-Candelas *et al.*, 2000; Pretorius *et al.*, 2003; Malherbe, 2010; Schoeman *et al.*, 1999; Carstens *et al.*, 2003; Pretorius *et al.*, 2003; Pretorius, 2000; Vilanova *et al.*, 2000; Fernandez-Gonzalez *et al.*, 2005; Gonzalez-Candelas *et al.*, 1995; Pretorius *et al.*, 2003)

2.9 Rational yeast engineering strategies for protein secretion in the wine-making industry

As has been described, in developing novel secretory yeast strains it is first necessary to understand the basic distinction between "science" and "engineering" (Wittrup, 1999). Science seeks to understand a system, whereas engineering uses this understanding to modify the system for a defined purpose. The process of engineering a commercial yeast may be divided into four parts. It begins with identification of a practical, significant application and clear definition of the objective. Secondly, a comprehensive evaluation of the state of existing knowledge relevant to the subject should be undertaken. Focused scientific enquiry should then be undertaken in areas of primary research where necessary knowledge is lacking. Finally a workable strategy for obtaining the objective, based on existing scientific knowledge, should be created. For a lysozyme producing *S. cerevisiae* to be of oenological relevance the following points should be addressed:

- The real-world application of such a strain should be properly defined.
- Effects of high-level secretion on fermentation performance and organoleptic qualities should be determined[5].
- Lysozyme should be reliably produced to oenologically useful levels under a realistically wide range of conditions.

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[5] Efforts to minimize any anticipated effects should be incorporated into expression system design during the planning phase

Chapter 3

Materials and Methods

All standard biological and molecular protocols used in this study are described in Current Protocols in Molecular Biology - Ausubel *et al.* (2003).

3.1 Microorganisms, media and culture conditions

Saccharomyces cerevisiae strains as well as *Escherichia coli*, along with their respective genotypes, are listed in Table 3.1. Lactic acid bacteria and *Micrococcus luteus*strains were used in lysozyme sensitivity assays and are listed in Table 3.2.

S. cerevisiae strains were routinely cultured at 30°C in yeast extract, peptone, dextrose (YPD) medium [1% yeast extract, 2% glucose and 2% peptone, (Biolab, Merck, R.S.A.) or synthetic complete (SC) medium [2% glucose, 0.67% yeast nitrogen base without amino acids (Difco, Detroit, USA)]. SC medium was supplemented with essential amino acids but lacked leucine (SC^{-LEU}) or uracil (SC^{-URA}) where appropriate in order to maintain selective pressure. Selection of G418 susceptible (*kan*MX) yeast transformants was performed on YPD agar containing 80 μg/mL Geneticin® (Gibco BRL, Germany)[1].

E. coli strain DH5α was used for propagation of plasmid DNA and was routinely cultured at 37°C. Ampicillin resistant transformants were selected for on Luria Bertani (LB) agar (Biolab, Merck, R.S.A.) containing ampicillin (100 mg/mL). *E. coli* transformants were propagated in Terrific Broth (TB) (Biolab, Merck, South Africa) with 100 mg/mL ampicillin as higher yields of plasmid DNA are produced in this medium.

[1]The activity of this additive is batch dependant

Lactic acid bacteria were grown microaerophilically in De Man, Rogosa, Sharpe medium (MRS) (Biolab, Merck, R.S.A.).

3.2 DNA operations for creation and analysis of constructs

Four expression cassettes directing constitutive expression and secretion of hen egg white lysozyme (HEWL) in *S. cerevisiae* were constructed. Secretion of lysozyme under the control of four different secretion signals was investigated. The parent template for all molecular manipulations was pGEM®-T Easy H1X, constructed at the IWBT, which contained HEWL with its native secretion signal. Table 3.3 lists plasmids used and constructed during this study. Plasmid maps are provided in Figures 3.1, 3.2 and 3.3[2].

All constructed plasmids were analysed by restriction digest and, subsequently, DNA sequencing (Central Analytical Facility, Stellenbosch University). Restriction digests were performed in order to demonstrate correct size of inserts. Prior to sequencing pDMPOF1b and YIpLac211 constructs underwent restriction digestion in order to select plasmids with identical insert orientation as ligations for these plasmids were non-directional. pDMPOF1b derivatives were sequenced in forward and reverse direction with primers pDMPOF-F and pDMPOF-R. Plasmids with pHVX2 or pDrive backbone were sequenced with commercial (Qiagen, Southern Cross Biotechnology, Cape Town) primers SP6 and M13-reverse. YIpLac211 constructs' sequences were determined with SP6 and M13-reverse primers. Restriction enzyme digestions were performed with DNA restriction endonucleases obtained from Fermentas (Penzberg, Germany) and Roche (Roche Diagnostics, Mannheim, Germany). DNA fragments resulting from PCR or restriction digestion were separated by means of agarose gel electrophoresis. Isolation of DNA from gels was performed with a Zymoclean DNA recovery kit (Zymo Research Corporation, U.S.A.). Ligations were performed in 20 μL volumes with 1 unit of T4 DNA ligase (Fermentas, Vilnius, Lithuania).

[2]Note that RM and RN secretion signals are represented by (+) MF-alpha1 SS and (+) Native SS in Figures 3.1, 3.2 and 3.3

3.2.1 PCR procedures for fragment amplification

PCR (polymerase chain reaction) primers used in this study are listed in Table 3.4. Reaction conditions are described in Table 3.5. Descriptions of all PCR products are contained in Table 3.6. TaKaRa ExTaq™ DNA polymerase (TAKARA Bio Inc, Shiga, Japan) was used in all PCR for manipulation of DNA. *Taq* polymerase (Bioline U.S.A. Inc, U.S.A.) was used for PCR screening reactions according to the manufacturer's guidelines. Reactions were carried out using a Hybaid thermocycler (Hybaid PCR express, UK). PCR reaction mixtures consisted of 1 μL template DNA (10 ng/μL), 5 μL of each primer (1.5 pmol/μL), 8 μL dNTP mixture (final concentration 1.25 mM), 5 μL of 10x PCR reaction buffer and 0.5 μL of DNA polymerase. $MgCl_2$ was included in the reaction buffer. Analytical grade sterile dH_2O was used to adjust reaction mixtures to 50 μL.

PCR products were separated by gel electrophoresis in 0.8% agarose gels. Gels were stained with ethidium bromide and banding patterns subsequently visualized using and AlphaImage™2200 camera with AlphaEase™software (Analytical and Diagnostic Products, Weltevrede Park, South Africa).

3.2.2 Construction of Plasmids Containing the Native and Mutated-Native Secretion Signals

Lysozyme and its native secretion signal were amplified from pGEM™-T Easy H1X with the primer pairs LysBglII-F/LysXhoI-R and RLysBglII-F/LysXhoI-R yielding products designated BH1X and BRH1X, respectively. These reaction products were cloned into pDrive™ (Qiagen, Cape Town, R.S.A), resulting in plasmids pDrive-BH1X and pDrive-BRH1X. These plasmids were subsequently digested with *Bgl*II and *Xho*I and the resultant fragments ligated into pHVX2 (previously linearized with the same enzymes). These operations resulted in episomal expression plasmids designated "pHVX2-N" and "pHVX2-RN". Plasmids pHVX2-N and pHVX2-RN were used as templates for PCR with the primer set PGK_p-5'F-BamHI/PGK_t-3'R-BamHI. The products of these reactions, designated B-PGK-N and B-PGK-RN, were ligated into pDrive™. This resulted in plasmids "pDrive B-PGK-N" and "pDrive B-PGK-RN". These plasmids were in turn digested with *Bam*HI and the resultant fragments ligated into *Bam*HI-linearized YIpLac211 and pDMPOF1b. This resulted in single-copy integrating plasmids "YIpLac211-N", "YIpLac211-RN", "pDMPOF1b-N" and "pDMPOF1b-RN".

3.2.3 Construction of MFα-Lysozyme Hybrid

Plasmid pGEM®-H1X was used as template for PCR with primer pair MFαHEWL-F/LysXhoI-R, the product of which was mega-primer *linker*HEWL. Plasmid pHVX2-MFα was used as template for PCR with primer pair BglII-MFα-F/MFαHEWL-R, resulting in mega-primer MFα*linker*. These mega-primers have a 32 bp region of overlap on their respective 5' terminals, in combination they represent a fusion of authentic MFα to lysozyme. After gel-purification both megaprimers were used as template in a single reaction with the primer pair BglII-MFα-F/LysXhoI-R. The product of this reaction was ligated into pDrive™, producing pDrive BMFαH1X.

3.2.4 Construction of plasmids containing the MFα and mutated-MFα secretion signals

PCR with primer set BglII-RR-MFα-F/LysXhoI-R was performed on template pDrive BMFαH1X, resulting in fragment BRMFαH1X. This was ligated into pDrive™ resulting in pDrive BRMFαH1X. These plasmids were subsequently digested with *Bgl*II and *Xho*I and the resultant fragments ligated into *Bgl*II/*Xho*I linearized pHVX2. This resulted in episomal expression plasmids "pHVX2-M" and "pHVX2-RM". As with the native secretion signals constructs, pHVX2-M and pHVX2-RM were used as templates for PCR with the primer set PGK$_p$-5'F-BamHI/PGK$_t$-3'R-BamHI. The reaction products, designated B-PGK-M and B-PGK-RM, were ligated into pDrive™, producing plasmids "pDrive B-PGK-M" and "pDrive B-PGK-RM". These plasmids were digested with *Bam*HI and the resultant fragments ligated into *Bam*HI-linearized YIpLac211 and pDMPOF1b. Single-copy integrating plasmids "YIpLac211-M", "YIpLac211-RM", "pDMPOF1b-M" and "pDMPOF1b-RM" resulted.

3.3 Yeast transformation

Plasmids YIpLac211-M, YIpLac211-RM, YIpLac211-N and YIpLac211-RN were linearized with *Nco*I and transformed into *S. cerevisiae* FY23 by lithium acetate transformation Ausubel *et al.* (2003). Successful integration disrupted the non-functional *URA*3 gene, repairing it and complementing the strain's uracil auxotrophy. Putative transformants were selected for on SC^{-URA}. Untransformed FY23 was used as negative control. Stability of integration in positive transformants was demonstrated as previously described Ausubel *et al.* (2003).

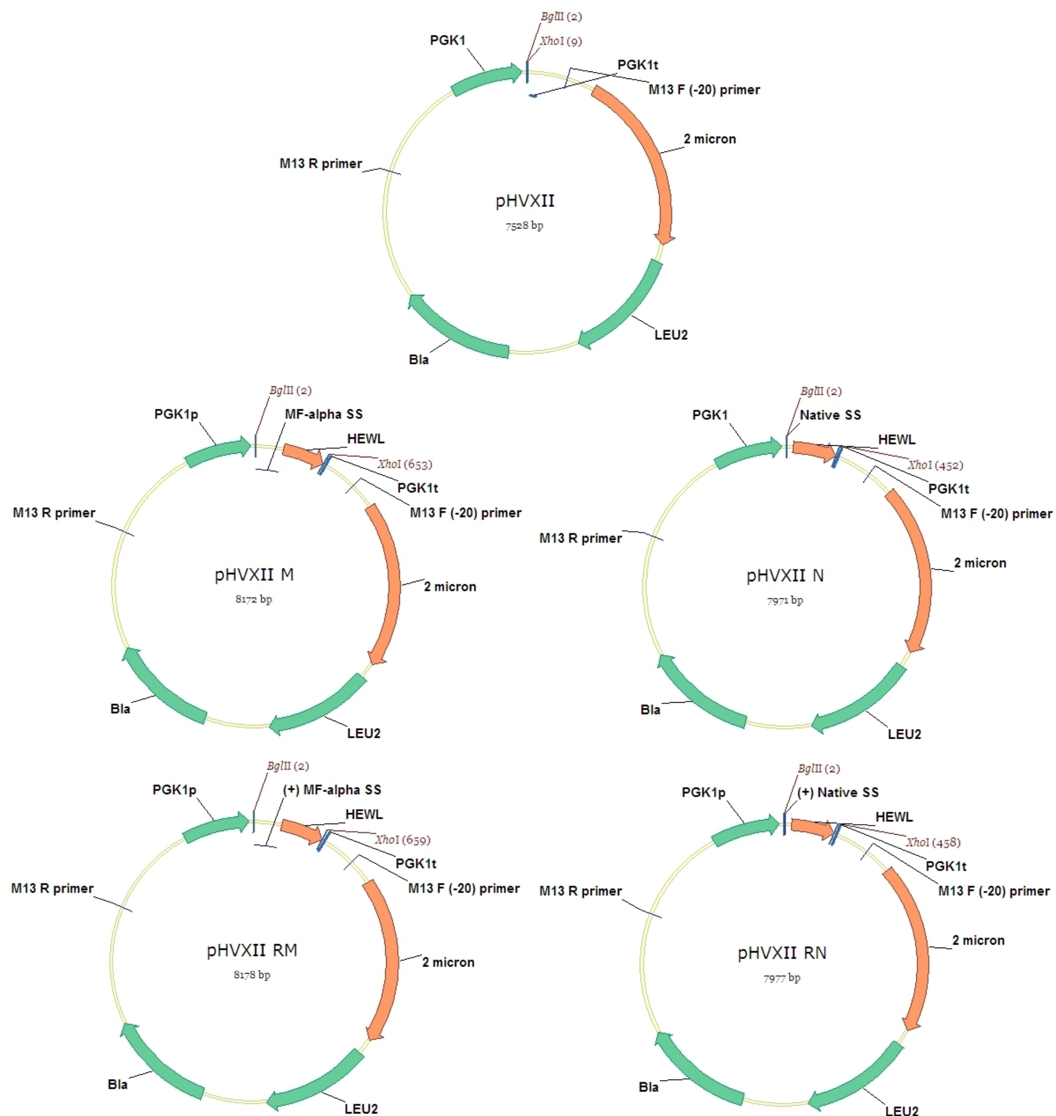


Figure 3.1: Plasmid maps of pHVX2 plasmids created or used in this study

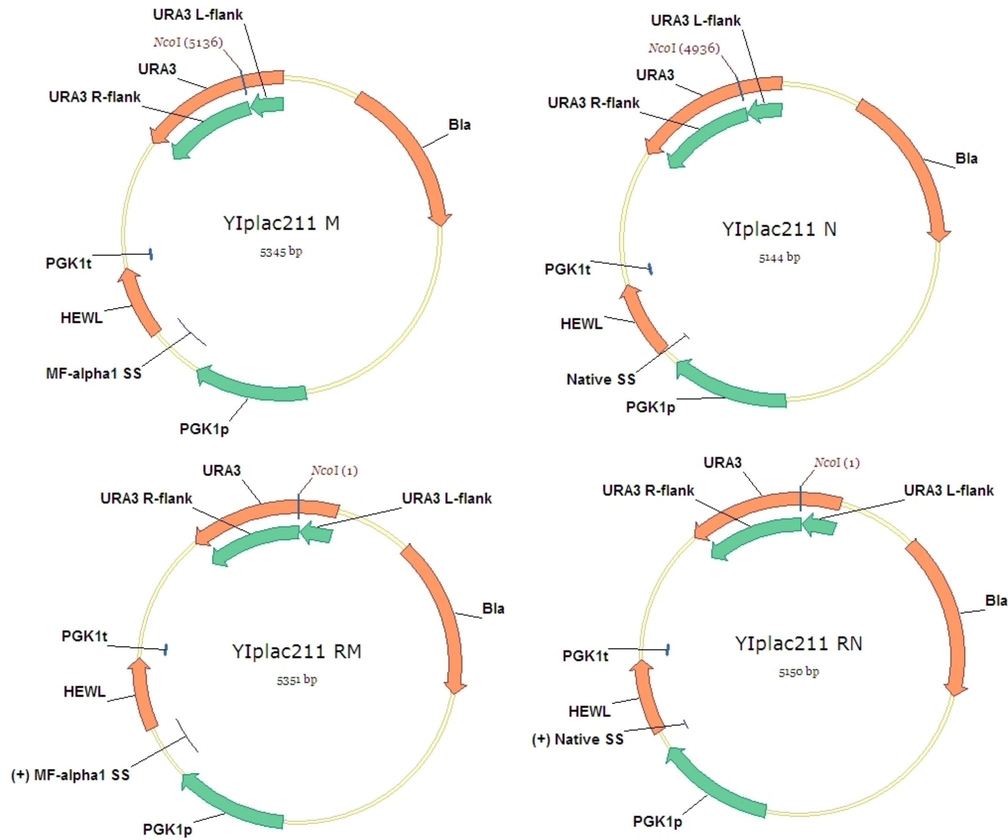


Figure 3.2: Plasmid maps of YIpLac211 plasmids created in this study

Plasmids pDMPOF1b-M, pDMPOF1b-RM, pDMPOF1b-N and pDMPOF1b-RN were digested with *Nco*I and transformed into *S. cerevisiae* VIN13 PAD::Kmx[3] by electroporation at 1.5kV and 200 Ω. The *PAD1* gene on chromosome *XIII* has been replaced with Kmx4 conferring resistance to geneticin in VIN13 PAD::Kmx. In transformations resulting in successful chromosomal integration at this site, the Kmx resistance gene is replaced with a single copy lysozyme expression cassette. Transformants are therefore rendered sensitive to geneticin. Confirmed integrants were cured of plasmid DNA by culturing them in non-selective YPD broth for approximately 60 generations.

[3]Note that *PAD1* and *POF1* describe the same gene

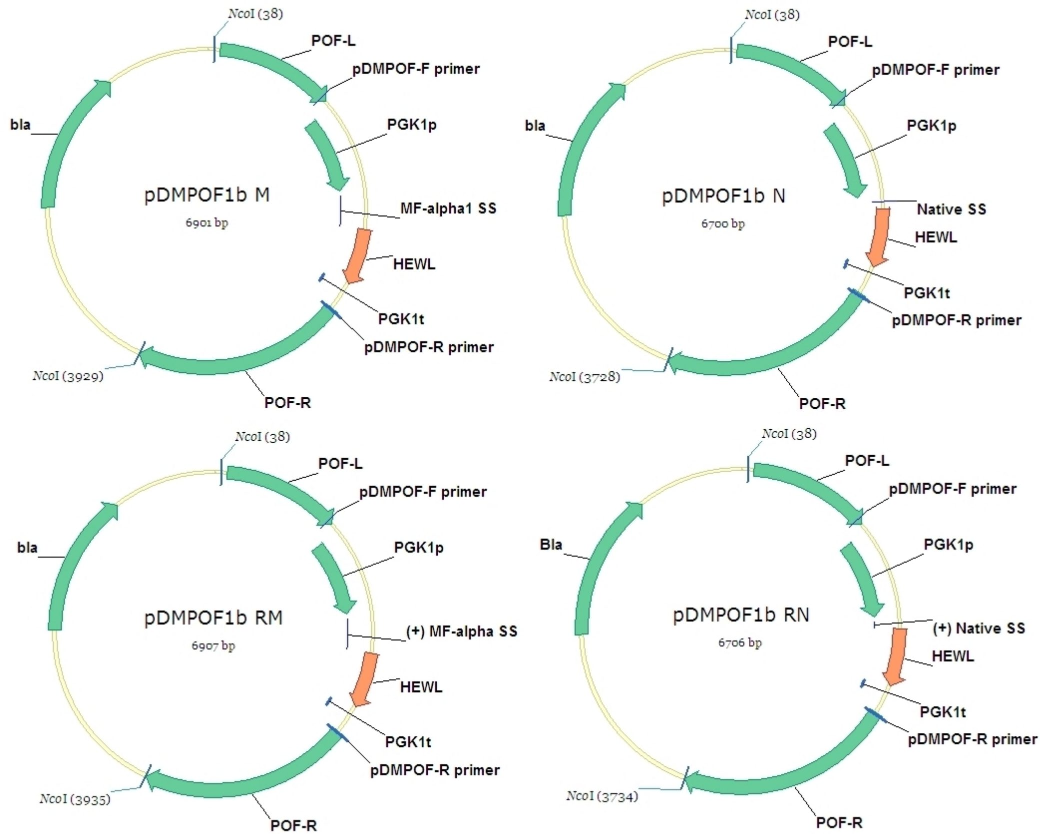


Figure 3.3: Plasmid maps of pDMPOF1b plasmids created in this study

3.4 Analysis of yeast transformants

Genomic DNA from *S. cerevisiae* FY23 and VIN13 was isolated using the yeast alkaline lysis method. FY23 putative transformants were selected on SC^{-URA}. Clones displaying lytic activity against *M. luteus* were further analysed by PCR and Southern blotting. VIN13 putative transformants were replica-plated on YPD agar and YPD agar containing 80 mg/L G418. Colonies incapable of growing on the G418 supplemented agar were tentatively considered to be successful transformants. Integration was subsequently confirmed by PCR.

3.4.1 PCR confirmation of expression cassette integration

Cassette integration into VIN13 was confirmed by PCR with primer pairs ExPOF-L/HEWL-F and ExPOF-L/pDMPOF-F. In the case of a successful integration a single product was expected for the first primer pair as the HEWL-F primer shared homology with the *HEWL* gene but not with the yeast genome. Two products were

expected for the second primer pair as VIN13 is diploid.

3.4.2 Southern blots

Integration of heterologous genes into the *URA*3 locus of the FY23 genome was confirmed by Southern hybridization analysis. Yeast genomic DNA was prepared according to standard procedures(Ausubel *et al.*, 2003). Separate digestions of the genomic DNA were performed with *Sma*I and *Bst*EII. Subsequently, the digested DNA was separated on 0.8% [w/v] agarose gels and depurinated, denatured and neutralized according to standard procedures - Sambrook & Russel (2001). DNA fragments were transferred onto a positively-charged nylon membrane (AEC-Amersham, South Africa).

The digoxigenin (DIG) non-radioactive nucleic acid labeling assay system (Roche Diagnostics, Mannheim, Germany) was used for Southern hybridization to confirm integrations. DIG labelled probe was generated by PCR with primer pair HEWLprobe-F and HEWLprobe-R, resulting in a 300 bp probe with no homology to yeast genomic DNA. Lambda phage DNA digested with *Bst*EII was used as a molecular weight marker. Chemiluminescent detection was carried out according to the manufacturer's application manual for filter hybridization.

Table 3.1: Microorganisms used and constructed

Micro organism[a,b,e]	Genotype	Source
Escherichia coli		
DH5α	$supE44\Delta lacU169$ $(\phi80lacZ\Delta M15)hsdR17recA1$ *end*A1 *gry*A96 *thi*-1 *rel*A1	GIBCO-BRL/Life[c]
Saccharomyces cerevisiae		
FY23	MAT*a* *leu*2 *trp*1 *ura*3	Winston *et al.* (1995)
VIN13	MAT*a*/MATα	Anchor yeast[d]
VIN13 PAD::Kmx	MATere*a*/MAT$\alpha PAD :: Kmx$	IWBT
FY23-Mi	MAT*a* *leu*2 *trp*1 URA3 PGK1$_P$-$MF\alpha1$-$HEWL$-PGK_T	This study
FY23-Ni	MAT*a* *leu*2 *trp*1 URA3 PGK1$_P$-$NativeSS$-$HEWL$-PGK_T	This study
FY23-RMi	MAT*a* *leu*2 *trp*1 URA3 PGK1$_P$-$RMF\alpha1$-$HEWL$-PGK_T	This study
FY23-RNi	MAT*a* *leu*2 *trp*1 URA3 PGK1$_P$-$RNativeSS$-$HEWL$-PGK_T	This study
VIN13-Mi	MAT*a*/MATα Kmx::PGK1$_P$-$MF\alpha1$-$HEWL$-PGK_T	This study
VIN13-Ni	MAT*a*/MATα Kmx::PGK1$_P$-$NativeSS$-$HEWL$-PGK_T	This study
VIN13-RMi	MAT*a*/MATα Kmx::PGK1$_P$-$RMF\alpha1$-$HEWL$-PGK_T	This study
VIN13-RNi	MAT*a*/MATα Kmx::PGK1$_P$-$RNativeSS$-$HEWL$-PGK_T	This study

[a] Double arginine insertions immediately after a secretion signal's N-terminal methionine are indicated with an "R"
[b] An "i" suffix indicates strain created by chromosomal integration of exogenous DNA
[c] GIBCO/Bethesda Research Laboratories Paisley, PA (USA)
[d] Anchor Yeast (Cape Town, South Africa)
[e] FY23 episomal transformants are not listed

Table 3.2: Organisms tested for lysozyme sensitivity

Micro organism	Culture number[c]
Lactic acid bacteria	
Lactobacillus nagelli	ATCC 700692
Lactobacillus plantarum	K57[a]
Lactobacillus pentosus	DSM 20314
Lactobacillus brevis	J23[a]
Lactobacillus buchneri	DSM 20057
Lactobacillus casei	LMG 13552
Lactobacillus curvatus	LMG 13553
Lactobacillus fermentum	ATCC 9328
Lactobacillus fermentum	LMG 13554
Lactobacillus hilgardii	ATCC 8290
Lactobacillus hilgardii	N52[a]
Lactobacillus kunkeei	DSM 12361
Lactobacillus plantarum	LMG 13556
Pediococcus pentosaceus	NCDO 514
Pediococcus pentosaceus	LMG 13561
Pediococcus pentosaceus	LMG 13560
Pediococcus pentosaceus	NCDO 813
Other	
Micrococcus luteus[b]	ATCC 4698

[a] These are wine isolates from the IWBT culture collection

[b] Lyophilized cell walls supplied by Sigma-Aldrich

[c] Expanded culture collection names are listed in the abbreviations section

Table 3.3: Plasmids used and constructed

Name	Genotype	Source or reference [a b]
pHVXII	2μm *bla LEU2 PGK1$_P$-PGK1$_T$*	(Volschenk *et al.*, 1997)
pHVXII MFα	2μm *bla LEU2 PGK1$_P$-MFα-PGK1$_T$*	IWBT
YIplac211	2μm *bla lacZ URA3*	(Gietz & Sugino, 1988)
pDMPOF1b	2μm *loxP-kanMX-loxP* POF1b-*L*-*Nco*I-POF1b-*R*	AWRI
pDrive BH1X	*bla Bgl*II-NatSS-HEWL-*Xho*I	this study
pDrive BRRH1X	*bla Bgl*II-RRNatSS-HEWL-*Xho*I	this study
pDrive BMFαH1X	*bla Bgl*II-MFα-HEWL-*Xho*I	this study
pDrive BRRMFαH1X	*bla Bgl*II-RRMFα-HEWL-*Xho*I	this study
pDrive B PGK N	*Bam*HI-*PGK1$_P$*-NatSS-HEWL-*PGK1$_T$*-*Bam*HI	this study
pDrive B PGK RN	*Bam*HI-*PGK1$_P$*-RRNatSS-HEWL-*PGK1$_T$*-*Bam*HI	this study
pDrive B PGK M	*Bam*HI-*PGK1$_P$*-MFα-HEWL-*PGK1$_T$*-*Bam*HI	this study
pDrive B PGK RM	*Bam*HI-*PGK1$_P$*-RRMFα-HEWL-*PGK1$_T$*-*Bam*HI	this study
pHVXII M	2μm *bla LEU2 PGK1$_P$-MFα-HEWL-PGK1$_T$*	this study
pHVXII RM	2μm *bla LEU2 PGK1$_P$-RRMFα-HEWL-PGK1$_T$*	this study
pHVXII N	2μm *bla LEU2 PGK1$_P$-NatSS-HEWL-PGK1$_T$*	this study
pHVXII RN	2μm *bla LEU2 PGK1$_P$-RRNatSS-HEWL-PGK1$_T$*	this study
YIpLac211 N	2μm *bla lacZ URA3 PGK1$_P$*-NatSS-HEWL-*PGK1$_T$*	this study
YIpLac211 RN	2μm *bla lacZ URA3 PGK1$_P$*-RRNatSS-HEWL-*PGK1$_T$*	this study
YIpLac211 M	2μm *bla lacZ URA3 PGK1$_P$*-MFα-HEWL-*PGK1$_T$*	this study
YIpLac211 RM	2μm *bla lacZ URA3 PGK1$_P$*-RRMFα-HEWL-*PGK1$_T$*	this study
pDMPOF1b M	2μm *bla* POF1b-*L*-selected forxtitPGK1$_P$-MFα-HEWL-*PGK1$_T$*-POF1b-*R*	this study
pDMPOF1b RM	2μm *bla* POF1b-*L*-*PGK1$_P$*-RRMFα-HEWL-*PGK1$_T$*-POF1b-*R*	this study
pDMPOF1b N	2μm *bla* POF1b-*L*-*PGK1$_P$*-NatSS-HEWL-*PGK1$_T$*-POF1b-*R*	this study
pDMPOF1b RN	2μm *bla* POF1b-*L*-*PGK1$_P$*-RRNatSS-HEWL-*PGK1$_T$*-POF1b-*R*	this study

[a] AWRI : The Australian Wine Research Institute (Adelaide, SA, Australia)
[b] IWBT : The Institute for Wine Biotechnology (Stellenbosch, R.S.A.)

Table 3.4: Primers used in this study

Primer	Sequence (5' ⟶3')	Feature	Size (bp)	Tm (°C)	Design[a]
BglII-MFα-F	AGA TCT ATG AGA TTT CCT TCT ATT TTT ACT GCT GTT TTA TTC GCT	*Bgl*II	45	67.3	IWBT
BglII-RRMFα-F	AGA TCT ATG AGA **AGA AGA** TTT CCT TCT ATT TTT	*Bgl*II, AGA AGA[c]	33	56.6	IWBT
LysBglII-F	AGA TCT ATG AGG TCT TTG CTA ATC T	*Bgl*II	25	49.2	IWBT
LysXhoI-R	CTC GAG TCA CAG CCG GCA GCC TCT G	*Xho*I	25	68.6	IWBT
MFαHEWL-F	GTT TCT TTG GAT AAA AGA AAA GTC TTT GGA CGA TGT GAG CTG GCA GCA		48	77.2	IWBT
MFαHEWL-R	ACA TCG TCC AAA GAC TTT TCT TTT ATC CAA AGA AAC ACC TTC TTC TTT AGC AG		53	74.3	IWBT
PGK_p-5'F-BamHI	GGA TCC GTG AGG AAC TAT CGC ATA CCT GCA TT	*Bam*HI	32	69	IWBT
PGK_t-3'R-BamHI	GGA TCC AAA GGC ATT AAA AGA GGA GCG	*Bam*HI	27	63.7	IWBT
RR-Lys-BglII-F	AGA TCT ATG AGG AGG AGG TCT TTG CTA ATC TTG GT	*Bgl*II	35	65	IWBT
HEWLprobe-F	TGA AGC GTC ACG GAC TTG AT		20	53.0	IWBT
HEWLprobe-R	GCC AGG CGA CCC ACG CGT TC		34	66.3	IWBT
pDMPOF-F	TTA CTT GCT TTT TAT TCC TTC CCA ACG AGT		30	61.5	IWBT
pDMPOF-R	ACC CAA GAG CCT TCA TGA CCT ATT AGA ACA		30	62.3	IWBT
SP6 promoter primer	CAT TTA GGT GAC ACT ATA G		19		Qiagen
T7 promoter primer	GTA ATA CGA CTC ACT ATA G		19		Qiagen
M13 Forward	GTA AAA CGA CGG CCA GT		17		Qiagen
M13 Reverse	AAC AGC TAT GAC CAT G		16		Qiagen

[a] IWBT : Primers ordered at The Institute for Wine Biotechnology (IWBT) from Inqaba biotech (R.S.A.)
[b] underlined = introduced restriction sites
[c] primer introduces 2 arginines into MF-α secretion signal

Table 3.5: Primer pairs and PCR programs

Program number	Primer pair [a]	Template	Initial denaturation		Number of cycles	Amplification cycles		Final elongation		Fragment name [b]
			Temp (°C)	Time (sec)		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	
1	LysBglII-F LysXhoI-R	pGEM-HI1X	94	120	30	94 50 72	60 30 30	72	180	H1X
2	RR-Lys-BglII-F LysXhoI-R	pDrive BH1X	94	120	30	94 50 72	60 30 30	72	180	BRRH1X
3	MFαHEWL-F LysXhoI-R	pGEM HI1X	94	120	30	94 50 72	60 30 30	72	180	*linker*HEWL
4	BglII-MFα-F MFαHEWL-R	pHVX MFα	94	120	30	94 50 72	60 30 30	72	180	MFα*linker*
5	BglII-MFα-F LysXhoI-R	MFα*linker* *linker*HEWL[c]	94	120	30	94 50 72	60 60 60	72	180	BMFαH1X
6	BglII-RR-MFα-F LysXhoI-R	pDrive BMFαH1X	94	120	30	94 50 72	60 60 60	72	180	BRRMFαH1X

Table 3.5 *continued on following page…*

Table 3.5 – *Continued*

Program number	Primer pair [a]	Template	Initial denaturation		Number of cycles	Amplification cycles		Final elongation		Fragment name [b]
			Temp (°C)	Time (sec)		Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	
7	PGK_p-5'F-BamHI	pHVXII N	94	120	30	94	60	72	180	B PGK N
	PGK_p-3'R-BamHI	pHVXII RN				55	30			B PGK RN
		pHVXII M				72	100			B PGK M
		pHVXII RM								B PGK RM

[a] See table 3.4 for additional information on individual primers
[b] See Table 3.6 for detailed information regarding each PCR fragment
[c] Linker PCR products used as mega-primers in conjunction with listed pair as per Higuchi (1989)

Table 3.6: Fragments generated by PCR

Fragment name	Anticipated size (bp)	PCR program [a]	Plasmids [b]
BH1X	456	1	pDrive BH1X
BRRH1X	462	2	pDrive BRRH1X
*linker*HEWL	414	3	N/A
MFα*linker*	459	4	N/A
BMFαH1X	657	5	pDrive BMFαH1X
BRRMFαH1X	663	6	pDrive BRRMFαH1X
B-PGK-N	1353	7	pDrive B-PGK-N
B-PGK-RN	1359	7	pDrive B-PGK-RN
B-PGK-M	1554	7	pDrive B-PGK-M
B-PGK-RM	1560	7	pDrive B-PGK-RM

[a] PCR program details are described in Table.3.5
[b] PCR products were cloned into Qiagen pDrive^{TM} or Fermentas CloneJet^{TM}. Individual plasmids are described in Table 3.3.

3.5 Microvinifications

All fermentation experiments were conducted in a grape juice-like chemically defined medium containing macro and micro nutrient concentrations reflective of those found in white grape juice (Bell & Henschke, 2003; De Klerk, 2010). This medium's composition is described in Table 3.7. Yeast strains selected for fermentation were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of preculture medium and cultured overnight at 30°C. These overnight cultures were used to inoculate artificial grape juice medium to an OD_{600} of 0.100 ±0.010. Fermentations were carried out anaerobically in 250 mL Erlenmeyer flasks stoppered with fermentation locks. Loss of CO_2 was monitored daily by measuring culture vessel weight loss. Fermentations were regarded as completed when the mass of the Erlenmeyers stopped decreasing and subsequently remained stable for three days.

3.5.1 Preparation of grape juice-like artificial fermentation medium

Special attention was paid to the concentrations of amino acids and available ammonium ions. Stock solutions of trace elements, vitamins and amino acids were prepared and carefully divided into smaller volumes in such a way that each stock aliquot was sufficient for the preparation of one liter of the final medium. Two stocks of amino acids were prepared, one complete and one lacking leucine. These were stored at −20°C until needed. Trace elements were prepared as individual stocks in order to avoid formation of insoluble metal salts. These were autoclaved

at 121°C for 15 minutes before being stored at room temperature (24°C).
The base medium was prepared by dissolving sugars, organic acids and macro-elements in warm, deionised H_2O. After cooling to room temperature the appropriate amino acid stock was added and the medium's pH adjusted to 3.3 in the case of the synthetic juice and 5.5 in the case of the preculture medium. Adjustments to pH were made with KOH. The resultant solution(s) were sterilized by vacuum filtration through a (0.22μm) cellulose-acetate membrane[4] into a sterile 1 L Schott® bottle. The medium was then transferred into a sterile 1 L volumetric flask to which trace element, vitamin stocks and other components were added. The volume was then adjusted to 1 L with sterile dH_2O. This operation was repeated up to 5 times and the resultant media transferred into a sterile 5 L Schott® bottle and thoroughly mixed. This master stock was then decanted (by mass 150 g ± 0.9 g) into 250 mL Erlenmeyer flasks immediately prior to inoculation and stoppering with fermentation caps. A reserve of each media master batch was held back as a control for contamination. The stock solution of essential lipids was added prior to inoculation and was prepared by dissolving lipids in absolute ethanol at 80°C. Oleic acid was supplied in the form of Tween 80.

3.6 Isolation and concentration of lysozyme by cation-exchange chromatography

Lysozyme was isolated from culture supernatants and concentrated by means of cation-exchange. SPE 5 mL cartridges were packed with 2 mL settled bed volume SP Sepharose Fast Flow separation media (GE Healthcare, USA). This media was equilibrated according to the manufacturer's instructions. 100 mL of fermentation supernatants (determined by weight) were applied to this media at a rate of 1 mL per minute.

It was determined in preliminary experiments [results not shown] that lysozyme eluted within the first 6 mL of elution buffer. Lysozyme was eluted with 8 mL of 0.1 M, pH 6.5 potassium phosphate buffer containing 0.5 M NaCl. This eluents were acidified by addition of 900 μL of 1 N HCl and volumes subsequently adjusted to 10 ml in A-grade volumetric flasks. This resulted in 10x concentrates.

[4] It had previously been shown that, unlike protein (see results section), amino acid binding to this membrane was negligible(De Klerk, 2010)

Table 3.7: Artificial Grape Juice

Component	Amount per Liter	Component	Amount per liter
Sugars [a,b]		Essential lipids	
Glucose	25/50/80 g	Oleic acid	120 mg
Fructose	25/50/80 g	Ergosterol	15 mg
Organic acids [a]		Amino acids	
Malic acid	2.5 g	Tyrosine	5 mg
Tartaric acid	2.5 g	Tryptophan [c]	5 mg
Macro elements		Isoleucine	25 mg
K_2HPO_4	1.14 g	Aspartic acid	100 mg
$MgSO_4.7H_2O$	1.23 g	Glutamic acid	250 mg
$CaCl_2.2H_2O$	0.44 g	Arginine	450 mg
Trace elements		Leucine [c,d]	300 mg
$MnCl_2.4H_2O$	200 μg	Threonine	250 mg
$ZnCl_2$	135 μg	Glutamine	350 mg
$FeCl_2$	30 μg	Alanine	150 mg
$CuCl_2$	15 μg	Valine	50 mg
$CoCl_2.6h_2O$	30 μg	Methionine	5 mg
$NaMoO_2$	25 μg	Phenylalanine	25 mg
H_3BO_3	5 μg	Serine	100 mg
KIO_3	10 μg	Histidine	20 mg
Vitamins		Lysine	80 mg
Pyridoxine·HCl	2 mg	Asparagine	150 mg
Ca pantothenate	1.5 mg	Proline	100 mg
Thiamine·HCl	0.5 mg	Other	
Nicotinic acid	0.2 mg	$(NH_4)_2SO_4$	94 mg
Riboflavin	0.2 mg	Uracil[c]	120 mg
p-Aminobenzoic acid	0.2 mg		
Folic acid	0.2 mg		
Biotin	0.125 mg		
myo-Inositol	100 mg		

[a] Preculture contained 25/25 g/L glucose/fructose and 2.5 g/L citric acid
[b] Total sugar concentrations were varied: 50, 100 or 160 g per Liter
[c] Haploid strain FY23 is auxotrophic for the compounds indicated
[d] Leucine concentration was tripled from 100 mg/l

To evaluate method recovery, three fermentation flasks were spiked with lysozyme standard solutions to 10 mg/L immediately after inoculation. In this manner, the cumulative effect of potential protein loss through cell wall association, protease degradation, inefficiencies in the method of recovery etc. could be estimated. Method recovery for this concentration was 90%.

3.7 Lysozyme detection and concentration estimation

Lysozyme secretion by *S. cerevisiae* transformants was assayed for by means of radial diffusion assay. Lysozyme concentrations in culture supernatants resulting

from fermentation of artificial grape juice, as well as in cation exchange concentrates thereof, were determined by microtiter plate assay against *M. luteus* and by HPLC-FLD (High Performance Liquid Chromatography - Fluorescent Detection).

3.7.1 Radial diffusion assays

Overnight yeast cultures were diluted to an OD_{600} of 1.0 with physiological salt solution (0.9% w/v NaCl). 10 μl of the resulting cell suspensions which were spotted onto SC^{-Leu} and SC^{-Ura} agar. After cultivation at 30°C for 24 h plates were overlaid with 10 mL of agar containing *M. luteus* cell walls. Overlays were further cultivated at 30°C for three days to allow for zone formation. In the case of VIN13 transformants zones formation did not occur within 3 days and so cultivation was continued for a further seven days.

3.7.2 Microtiter plate assays

Lysozyme activity in finished fermentation supernatants and ion-exchange concentrates was estimated by a modified version of a published method (Shugar, 1952). Powerwave X microtiter plate reader (BIO-TEK Instruments, Inc. Japan). KC4 Kineticalc V 3.0 for Windows. The plate reader tray was pre-heated to 30°C. Plates were shaken for 5 seconds at intensity 4 before each reading. Readings were taken every 30 seconds for 10 minutes at 450 nm. 190 μL of a *M. luteus* suspension (*Micrococcus luteus* ATCC 4286 lyophilized cell walls (Sigma-Aldrich) at an OD_{450} of 0.80 in 0.1 M sodium phosphate buffer, pH 7.2) was added to each microtiter plate well. 10 μL of lysozyme sample or standard was added to each well and the plate was immediately placed into the reader. Six repeats were performed for each sample. Decrease in optical density at OD_{450} was measured as a function of time. Lysozyme concentrations in samples were determined by comparison with a reference curve established with lysozyme standards prepared in cation-exchange elution buffer.

3.7.3 HPLC- quantification of lysozyme

Lysozyme concentrations in raw fermentation supernatants as well as ion-exchange concentrates of said supernatants were determined by an established method (Riponi *et al.*, 2007). The effect of sample acidification on fluorescence was investigated.

3.7.3.1 Reagent and sample preparation

Acetonitrile of HPLC grade was purchased from Merck (Darmstadt, Germany). HPLC quality water was purified with a Millipore Milli-Q system (Millipore, Bedford, MA, U.S.A.). Lysozyme stock solutions were prepared in both a model wine solution (consisting of 12% ethanol, 2 g/L tartaric acid, adjusted to pH 3.2 with potassium tartrate) and the cation-exchange elution buffer (0.5 M NaCl in 0.1 M potassium phosphate buffer pH 6.5).

3.7.3.2 HPLC

High-performance liquid chromatography was conducted according to the method of Riponi *et al.* (Riponi *et al.*, 2007). An Agilent 1100 series system with a binary pump (G1312), 100 μL loop, photodiode detector (G1315B), fluorometric detector (G1321) and column oven was used. The column used was a Tosoh Bioscience (Stuttgart, Germany) TSK-GEL Phenyl-5PW RP (4.6 mm ID x 7.5 cm L) with a guard column containing the same resin. UV detection was performed at 225 nm. Fluorescent detection was performed with λex 276 nm and λem 345 nm (gain 10, spectrum bandwidth 18). Runs were acquired and analysed with Chemstation for LC 3D (Agilent technologies) software. Limits of detection (LOD) and quantification (LOQ) were set as 3 and 10 times the signal to noise (S/N) ratio, respectively. Lysozyme was identified by comparing its peak elution time to those of the standard solutions and absence of similar peaks in the samples prepared from fermentations not containing lysozyme. Quantification was done by comparing peak areas to those of lysozyme standard solutions (external standards) at 6 different concentrations, injected in pentuplicate. Two set of standards were run in order to investigate the effect of acidification on lysozyme fluorescent emission. In the first set, a dilution series was acidified with 9% v/v 10 N HCl prior to volumetric adjustment. In the second set, adjustment was made without acidification. Method linearity was established by square correlation coefficients (R^2) of the calibration curve.

The solvents were 1% CH_3CN, 0.2% TFA, 98.8% H_2O (solvent A) and 70% CH_3CN 0.2% TFA 29.8% H_2O (solvent B). Gradient elution was performed as follows: 100% Solvent A for 3 min, to 65.0% A in 7 min maintained for 5 min, then to 40.5% A in 12 min, then to 100% B in 2 min maintained for 5 min and back to 100% A in 2 min followed by a 10 minute re-equilibration with 100% A. Run conditions were as follows: the column was maintained at 30°C with a flow rate of

1 ml/min.

3.7.4 SDS-PAGE of lysozyme concentrates

SDS-PAGE analysis of cation-exchange concentrates was carried out according to the protocol outlined in the BioRad Mini-Protean 3 Cell instruction manual (Bio-Rad, Hercules, CA, USA). Coomassie brilliant blue and silver staining of acrylamide gels were carried out according to the protocol outlined in Current Protocols in Molecular Biology Ausubel *et al.* (2003). A Pro-Q Emerald 300 glycoprotein gel stain kit was used according to the manufacturer's instructions (Invirogen Detection Technologies, Paisley, UK) to test for glycosylation.

3.8 Statistics and data analysis

Statistical differences between groups of biological repeats were tested for with the use of t-tests. The level of significance was set at $P < 0.05$. Sigmaplot 11.0 from Systat software, Inc. was used to perform the analyses.

Chapter 4

Results and Discussion

The effects of four different secretion signals on the secretion titer and processing of lysozyme by *Saccharomyces cerevisiae* under conditions simulating winemaking was evaluated in two genetic backgrounds (FY23 and VIN13). Previous studies have shown that the choice of secretion signal affects both the quantity and proteolytic maturation of many proteins, including lysozyme, secreted by *S. cerevisiae* (Bitter *et al.*, 1984; Marten & Jin-Ho, 1991; Harmsen *et al.*, 1993; Hashimoto *et al.*, 1998; Koganesawa *et al.*, 2001; Kawamura *et al.*, 2003). In the current study we investigate the effect of secretion signal choice on authenticity of proteolytic processing during protein maturation in *S. cerevisiae* FY23. This study attempted to quantify lysozyme produced from single copy integrants and multicopy episomal transformants in an artificial grape juice under anaerobic fermentative conditions.

4.1 Generation of *S. cerevisiae* transformants

4.1.1 Construction of vectors and expression cassettes

Four expression cassettes were constructed, each of which contained a structural gene for *Gallus gallus* hen egg white lysozyme (*HEWL*) under the control of the *S. cerevisiae* phosphoglycerate kinase 1 promoter (*PGK*1p) and terminator (*PGK*1t). Expression cassettes differed in terms of secretion signal.

These cassettes were incorporated into three vectors (pHVX2, YIpLac211 and pDMPOF1b). The first is a 2μ-based multicopy yeast episomal vector complementing *LEU* auxotrophy. YIpLac211 is an integrating vector complementing *URA* auxotrophy and pDMPOF1b contains sequences flanking the expression cassette

allowing integration into the VIN13 *PAD*1[1] locus.

Constructed vectors were analysed by sequencing. Plasmids based on pDrive® were sequenced with primer pair SP6 and T7. Plasmids based on YIpLac211 or pHVX2 were sequenced with primer pair M13 R and M13 F. Plasmids based on pDMPOF1b were sequenced with pDMPOF-F and pDMPOF-R.

4.1.2 Integration of Hen Egg White Lysozyme expression cassettes in yeast

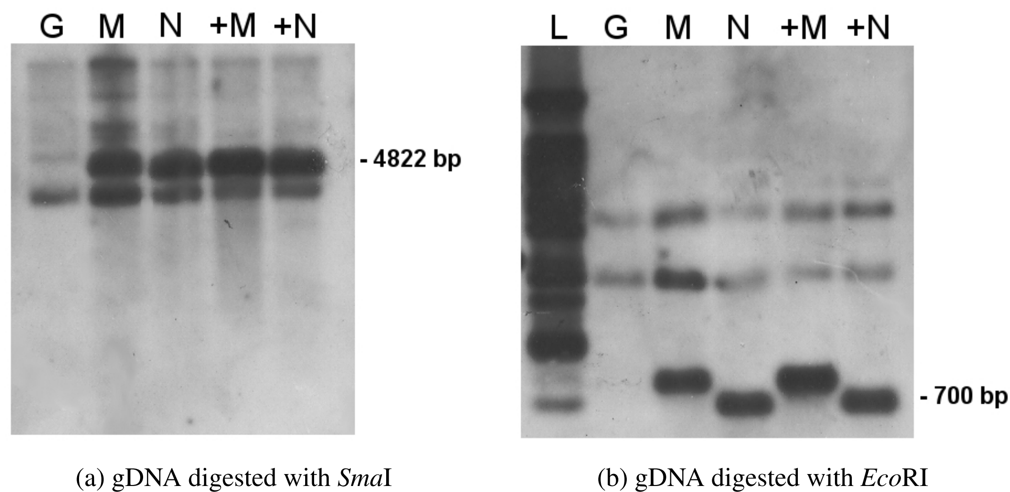
Integration of the various lysozyme YIpLac211 expression cassettes into the FY23 genome[2] was confirmed with Southern hybridization. The DIG hybridization probe used is homologous to a 300 bp section of the chicken *HEWL* gene. The results of the hybridization analyses are shown in Figure 4.1. Restriction enzyme *Sma*I cut within the yeast genome flanking the insertion and within the expression cassette. Expected sizes of restriction fragments containing the *HEWL* gene for transformants M, N, RM, and RN were 4920, 4725, 4926 and 4729 bp respectively. The presence of hybridization signals of these sizes for the transformants on Figure 4.1a and absence of the signal in lane G confirms the integrations.

The presence of the unexpected smaller band in all five lanes may be explained as follows: The DIG hybridization probe was created by PCR of plasmid “pDrive M” which contained the *HEWL* gene with the *PGK1* promoter and terminator sequences, with primers *HEWL*probe-F and *HEWL*probe-R. The PCR reaction mixture was used for hybridization directly, instead of undergoing gel electrophoresis and subsequent extraction of the probe from the gel (separating the probe from the template DNA). This means that template DNA was present in the hybridization mixture. It is therefore likely that single stranded template DNA hybridized with genomic *PGK1* sequences. The DIG probe would therefore have hybridized with both the genomicaly integrated target gene as well as the plasmid template DNA that had hybridized to genomic *PGK1* sequences, as it shared homology with both.

This hypothesis is further supported by examination of Figure 4.1b in which restriction digestion occurred close to the *HEWL* gene, within the integration cassette. The expected sizes of restriction fragments containing the *HEWL* gene for transformants M, N, RM, and RN were 877, 676, 883 and 682 bp respectively. The two

[1]Note that *PAD1* and *POF1* describe the same gene

[2]Please refer to Chapter 3 for details



(a) gDNA digested with *Sma*I (b) gDNA digested with *Eco*RI

Figure 4.1: Southern hybridization analyses of FY23 integrants. Lane G is untransformed FY23 genomic DNA, lane L is λ-phage DNA digested with *Bst*EII. Lanes M, N, +M and +N contain gDNA from individual transformants

other bands present in all lanes, including lane G, do not correspond to any possible fragments resulting from complete or partial digestion of any of the plasmids used in the transformation. They likely correspond to the sizes of restriction fragments containing the *PGK1* promoter and terminator generated when gDNA is digested with *Eco*RI, as described above.

Single copy integration of pDMPOF1b derived expression cassettes into VIN13 was confirmed by PCR with primer pair *HEWL*probe-F and ExPOF, priming within the yeast genome and the heterologous *HEWL* gene. Absence of PCR product in the negative control template lanes (VIN13 gDNA and pDMPOF-RM) and presence of products of the anticipated sizes in all four transformed VIN13 strains confirmed integration [results not shown].

4.2 Selection and evaluation of lysozyme secreting yeast

4.2.1 Radial diffusion assays

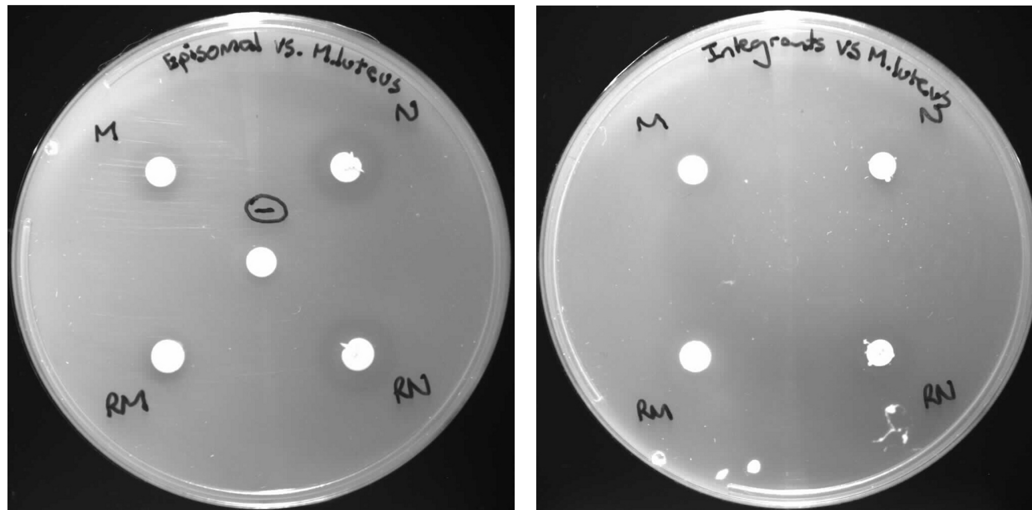
Radial diffusion assays were employed in order to confirm lysozyme secretion by yeast episomal and integrative yeast transformants. Lytic action of lysozyme se-

creted by FY23 episomal and integrated transformant colonies against overlaid suspensions of lyophilised *M. luteus*cell walls is illustrated in Figure 4.2a and Figure 4.2b respectively. The absence of a zone of lysis around the FY23 containing the pHVXII control plasmid in Figure 4.2a and presence of lytic zones around colonies of lysozyme-expressing transformants confirms expression and secretion of functional lysozyme under plate culture conditions.

The amount of lysozyme secreted by the episomal FY23 transformants was greater than that of the integrants in the case of three of the secretion signal-lysozyme combinations, with zone diameters exceeding 12 mm for the episomals (the exception being the RM transformant, with a zone diameter not exceeding 8 mm) compared to a maximum of 9 mm for the best performing of the single-copy integrants (RN). This said, radial diffusion assays provide at best a semi-quantitative means of comparison between secreting strains and could not be used to predict strain performance in liquid media. There are two reasons for this. Firstly, secretion titer has been shown to be dependant on environmental factors such as growth conditions, pH and media composition (Rossini *et al.*, 1993; Choi *et al.*, 2004). As the strains were to be used in anaerobic fermentation of a grape juice-like medium no prediction as to how the strains would perform could be made from the plate assay results as the conditions of growth were completely different. Secondly, small variations in the volume of the agar on each plate would have a significant effect on the estimated titer. These variations would be difficult to avoid.

Three conclusions could be drawn from these results. Firstly, all of the transformants secreted active lysozyme. Secondly, the differences in zone sizes (zone sizes not reported, see Figure 4.2) were large enough to conclude that the episomal transformants secreted more lysozyme than the integrants under the tested conditions. Finally, while the addition of arginine residues to the N-terminal of the native lysozyme secretion signal apparently did not detectably influence secretion titer, the same alteration to the MF-α signal clearly affected secretion negatively. It was expected that alteration of the native signal would significantly increase secretion efficiency (Tsuchiya *et al.*, 2003), our result therefore contradicts previous findings. While the most chicken lysozyme secretion signal was used, human lysozyme was expressed in the previous study. It should also be noted that a different yeast strain was used. The effect on secretion with the MF-α signal was unexpected and could not be explained.

VIN13 integrants tested in the same way as the FY23 strains performed poorly



(a) Episomal transformants (pHVX2) (b) Chromosomal integrants (YIpLac211)

Figure 4.2: FY23 transformants lawned with *M. luteus*, zones of clearing indicate secretion of active lysozyme three days after lawning.

in comparison, with zone formation against *M. luteus*only becoming apparent after 9 days incubation for the best performing strain (VIN13-M). Zones were clearly apparent after three days for the FY23 strains. For this reason it was decided to proceed with fermentations with the FY23 strains instead of the VIN13 strains, as it was assumed that secretion in liquid medium would mirror that seen with the plate assays. It would also allow for comparison not only between the various secretion signals but also between expression from single copy integrants and multicopy expression from episomal transformants. VIN13-M was ultimately used for one experiment, the results of which are reported due to the interesting growth effect observed. These results are presented at the end of the chapter.

The proposed course of investigation was co-inoculation of the most lysozyme-sensitive LAB strain available with the best yeast secretor strain in a grape juice-like medium. As the episomal FY23 transformants were found to be the best producers and secretors of lysozyme under the described plate-assay conditions, colonies were overlaid with agar suspensions of wine-associated LAB in order to identify the LAB strain most susceptible to the producer yeast. Seventeen LAB strains were tested and are listed in Table 3.2. The result of this experiment with regards to *L. hilgardii* ATCC 8290 is presented in Figure 4.3. This result is representative of the results obtained for all the tested strains. No activity against any of the tested strains was

detected during a period of 14 days.

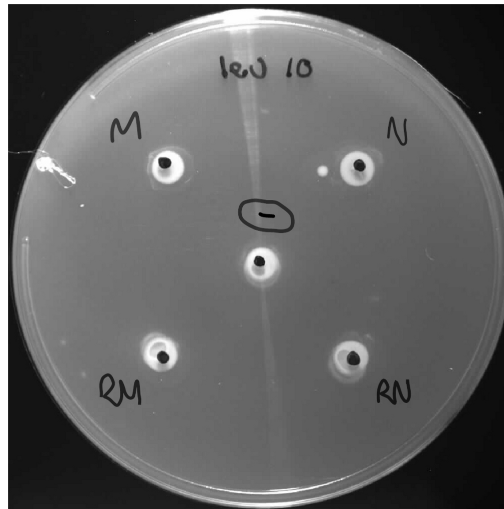
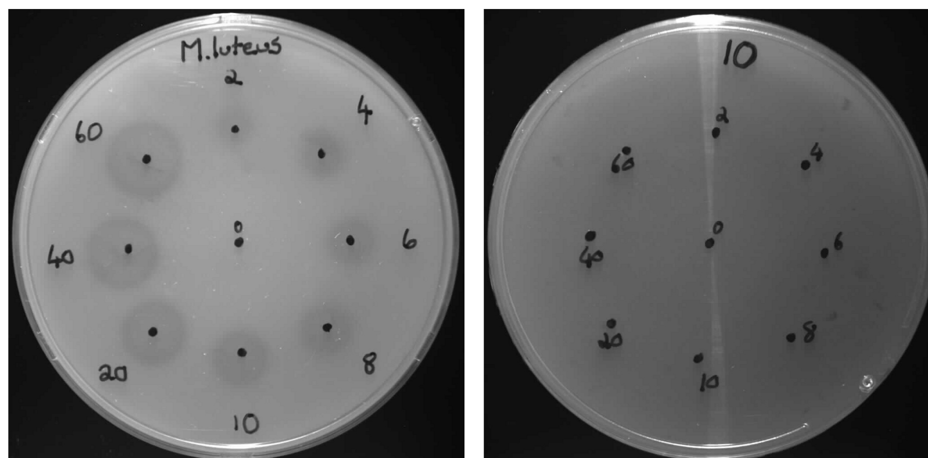


Figure 4.3: FY23 episomal transformants overlaid with *L. hilgardii* ATCC 8290

It has been observed that Alexander Flemming was fortunate in his choice of the bacterium *M. luteus* as substrate in his experiments with lysozyme, due to the extraordinary sensitivity of this organism's cell wall to the enzyme (Canfield *et al.*, 1972). *M. luteus* is still the organism of choice for assays involving lysozyme. On the other hand, LAB are generally resistant to lysozyme concentrations up to and frequently exceeding 100 mg/L in liquid culture, irrespective of the medium in which they are cultured (Kozakova *et al.*, 2005). The complete lack of inhibition against any of the LAB strains by even the best secreting yeast strains under plate assay conditions in the current study was therefore to be expected.

It was decided to test whether the quantities of lysozyme being secreted by the yeast were below those required to inhibit growth of LAB under the conditions of the plate assays. A lysozyme dilution series was prepared, spotted onto SC agar and overlaid *M. luteus* and LAB. The results of these experiments are presented in Figure 4.4. *L. hilgardii* ATCC 8290 is once again representative of all the tested LAB. No inhibition of LAB growth was detected at the highest concentration spotted (10 μL of 60 mg/L) while zone formation against *M. luteus* is clear even at the lowest lysozyme concentration. The largest zones of lysis against *M. luteus* produced by secreting strains of FY23 (Figure 4.2) were all smaller than those formed by 20 mg/L spots after equal incubation periods.



(a) Dilution series overlaid with *M. luteus* (b) Dilution series overlaid with *L. hilgardii* ATCC 8290, no zones are visible at 14 days after lawning

Figure 4.4: Lysozyme dilution series (10 μL of 2–60 mg/L) overlaid with sensitive organisms. *L. hilgardii* was found to be insensitive to the highest concentration tested.

As a result of these findings the proposed course of investigation (described above) was found to be unfeasible and was abandoned. Instead it was decided to proceed with concentration and quantification of lysozyme secreted by yeast fermenting grape juice-like medium under anaerobic conditions.

4.3 HPLC-FLD of lysozyme

The HPLC-FLD of Riponi *et al.* (Riponi *et al.*, 2007) was used to determine lysozyme concentrations in both the raw culture supernatants as well as the cation-exchange concentrates produced from these supernatants. Riponi's method is an improvement on a previous method (Delfini *et al.*, 2004).

The authors did not report the effect of acidification of the lysozyme standards with HCl on fluorescent response. Their reported LOD (0.18 mg/L) and LOQ (0.59 mg/L) for fluorometric detection are not approachable without acidification, indicating that they acidified their standards but did not report this in their publication. As acidification has a clear effect on the enzyme's fluorescence it is not possible to compare the amount of lysozyme in acidified vs. unacidified wines or musts using the same standard curve. Riponi *et al.* report only the use of an unacidified standard curve. As they did not report lysozyme concentration comparisons of acidified

and unacidified samples of the same wine determined with acidified and unacidified standards they could not have taken the effect of acidification into consideration.

With acidified standards within the range of 1-6 mg/L, fluorometric detection had excellent linearity with a minimum square regression coefficient (R^2) of 0.9991. As lysozyme peaks were integrated manually, the introduction of operator bias was offset by randomizing the order in which chromatograms were interpreted. Filtration of lysozyme standards through 0.45 μm and 0.22 μm filters was founf to result in significant decreases in lysozyme concentration [results not shown]. For this reason all samples were centrifuged to remove particulate material and not filtered.

Figure 4.5 illustrates the effect of acidification on the fluorescence of lysozyme. With acidification with $1/10$ N HCL the fluorescence of the native protein in both the artificial wine matrix and the IEX elution buffer increased substantially. No mention of this effect was made in any of the publications using this technique (Riponi *et al.*, 2007; Rinpoi *et al.*, 2008). Additionally, without acidification the detection limits reported are not approachable with the instrumentation used in this study. With acidification the limits of detection and quantification found in this investigation are in good agreement with those reported by the authors. While disassociation from phenols and other compounds may well account for better detection, it is possible that the increased recovery of lysozyme was in part due to the effect of acidification. The apparent increase in detectable lysozyme is likely due to the increased florescence and not disassociation from phenols, tannins or other compounds exclusively.

Lysozyme was undetectable in the unconcentrated fermentation supernatants. Concentration of the samples was necessary as lysozyme was undetectable by HPLC-FLD in the raw culture supernatants. Even with 10x concentration lysozyme concentrations were close to the limit of quantification for the HPLC-FLD, which was considerably more sensitive than UV detection [results not shown].

Isolated lysozyme eluted at 22 minutes and approximately 55% ACN. The shoulder on the left of the lysozyme peak in Figure 4.6 was also present in the lysozyme standards. This shoulder was therefore included in the integration of the lysozyme peak. The broadness of the peak could be accounted for by diffusion of the analyte during the separation. The extremely low concentration of the analyte would make this more noticeable.

The native/authentic lysozyme peak in Figure 4.7 was preceded by a peak of similar area eluting at 20.6 minutes. This peak was present in all supernatant con-

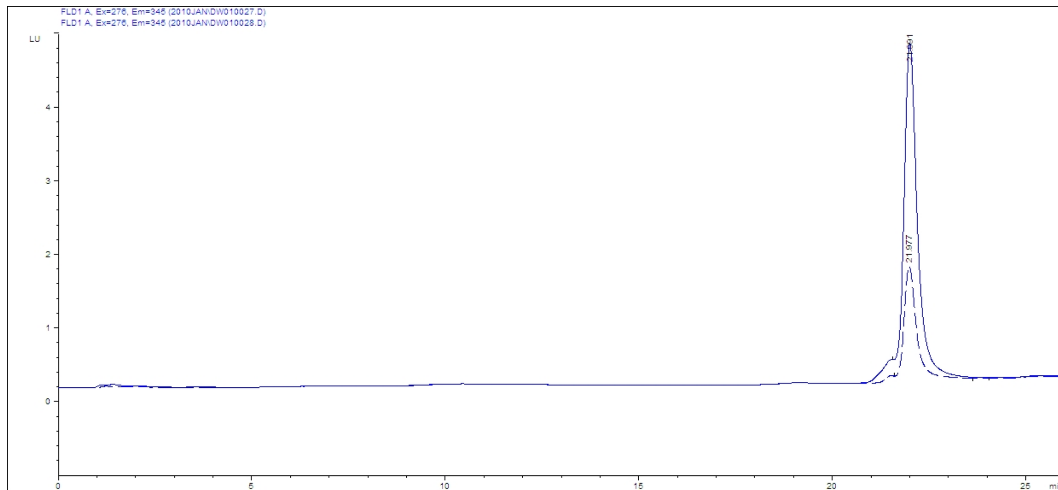


Figure 4.5: Chromatogram illustrating the effect of acidification on lysozyme fluorescence as determined by HPLC-FLD. The acidified lysozyme standard is represented by a solid line whereas unacidified lysozyme is represented by a broken line

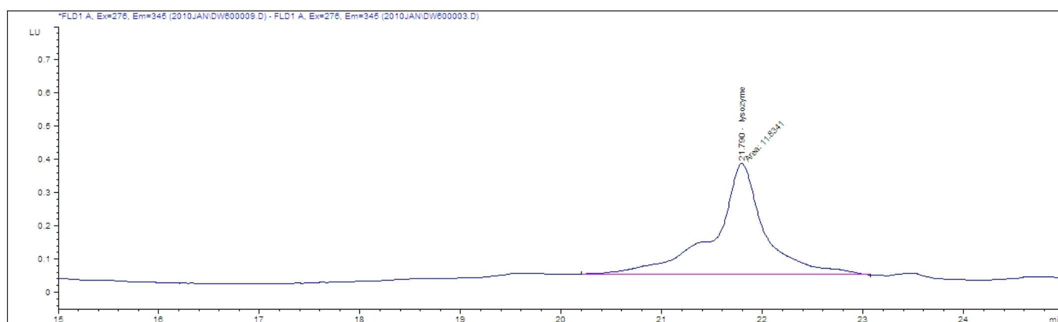


Figure 4.6: HPLC-FLD chromatogram of purified lysozyme from episomal FY23 transformant "N" fermenting 160 g/L hexoses to dryness

centrates where mating factor alpha's secretion signal was used by the fermenting strain and could represent a misprocessed form of lysozyme in which the secretion signal has not been properly cleaved. This has been observed previously (Kawamura *et al.*, 2003; Hashimoto *et al.*, 1998) when chicken and other c-type lysozymes were produced heterologously in *S. cerevisiae* with MF-α as the secretion signal. The proximity of this peak to the authentic lysozyme's peak complicated lysozyme concentration estimation. A dropline was placed at the lowest point between the peaks, resulting in a possible underestimation of the authentic lysozyme as the peak's shoulder (see Figure 4.6) could not be factored into the estimation. The misprocessed lysozyme was not quantified as the nature of the cleavage anomaly was not determined and no standard was available. With adequate purification of

the protein, either N-terminal sequencing or HPLC-MS could have been used to identify this compound.

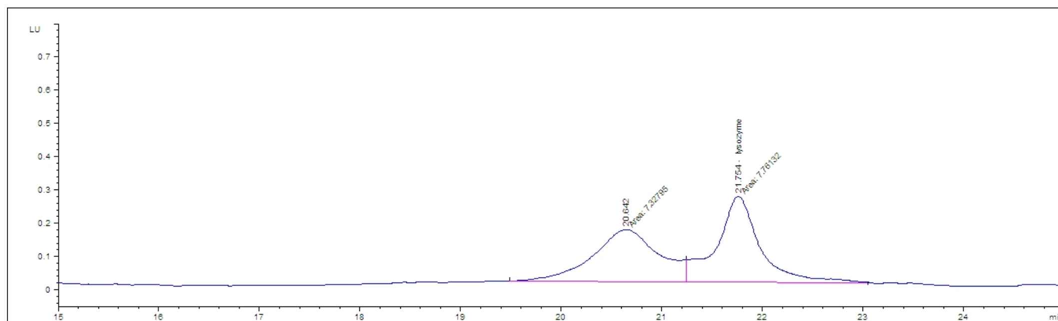


Figure 4.7: HPLC-FLD chromatogram of purified lysozyme from episomal FY23 transformant "M" fermenting 160 g/L hexoses to dryness

4.4 Lysozyme produced during alcoholic fermentation

Alcoholic fermentations were conducted anaerobically by FY23 transformed with multicopy episomal plasmids as well as FY23 integrants containing a single copy expression cassette. An artificial grape juice-like medium reflecting the nutrient status of natural grape juice, with particular attention paid to assimilable nitrogen and anaerobic growth factors, was devised in this study and used for all fermentations. It's composition is given in Table 3.7. Existing recipes for artificial grape juices were considered inappropriate for the study as, for various reasons dependant on the nature of the studies for which they were devised, they do not accurately reflect said nutrient status. For example, the AWRI medium recipe based on (Jiranek, 1995) states: "The concentrations of nitrogen sources (amino acids and ammonium) shown in this recipe have been chosen to provide an excess of each... under aerobic growth conditions (headspace exposed to air)". Higher amino acid concentrations than would normally be found in juice would potentially have led to higher production of secreted protein than could be expected of the same strains from a natural juice.

Three concentrations of fermentable sugar were used (50, 100 and 160 g/L) as the auxotrophic strains were not expected to be capable of completing fermentation

at higher sugar concentrations. The strains had previously been used to ferment both AWRI and MS300 media with 160 and 200 g/L hexoses, both media resulting in stuck fermentations [results not shown]. The yeasts were able to ferment the new media to dryness (less than 0.5 g/L residual sugar) within 32 days for the highest hexose concentration tested (160 g/L).

Lysozyme was purified from supernatants of finished fermentations by means of cation exchange, resulting in ten-times concentrates. The combination of lysozyme's high pI value (10) and the fact that *S. cerevisiae* does not naturally secrete many proteins meant that lysozyme of acceptable purity could be extracted by means of cation exchange in a single step. This is supported by the silver stained SDS-PAGE gel of the cation exchange concentrates in Figure 4.11. With the exception of a single extra band in lane 4, no protein bands other than ones corresponding to the lysozyme standard in lane 1 may are seen. There are also no protein bands in the lane containing the sample from the fermentation with wild-type FY23 (lane 5). The amount of lysozyme in both the concentrates and the raw supernatants was quantified by HPLC-FLD and turbidometric enzyme assay according to published methods. Lysozyme in the raw supernatants was in all cases present in quantities lower than the detection limit for the HPLC-FLD method.

Lysozyme is produced gradually as fermentation proceeds under the conditions tested, with half of the lysozyme present at the end of the fermentation being present in the supernatant within the first week. Figure 4.8 is a dot plot of lysozyme concentrations in culture supernatants 7 days after inoculation and upon completion of fermentation (32 days). This result illustrates that, with the described secretion system and ignoring the low titers, lysozyme would not be produced rapidly enough to have any effect on microbial populations present at the beginning of fermentation. Lysozyme (when it is used) is commonly added at concentrations exceeding 100 mg/L at the beginning of industrial fermentations, when a fermentation is considered to be at risk from LAB spoilage. There was no significant difference between FY23 yeast biomass present in finished fermentation and those harvested after seven days [results not shown]. Furthermore, no differences in terms of biomass production were observed for any of the FY23 transformants expressing lysozyme when compared to FY23 negative control (transformed with pHVX2 minus the lysozyme expression cassette).

Figure 4.9 illustrates the effect of initial sugar concentration on the amount of lysozyme secreted into the culture supernatants by FY23 episomal transformants.

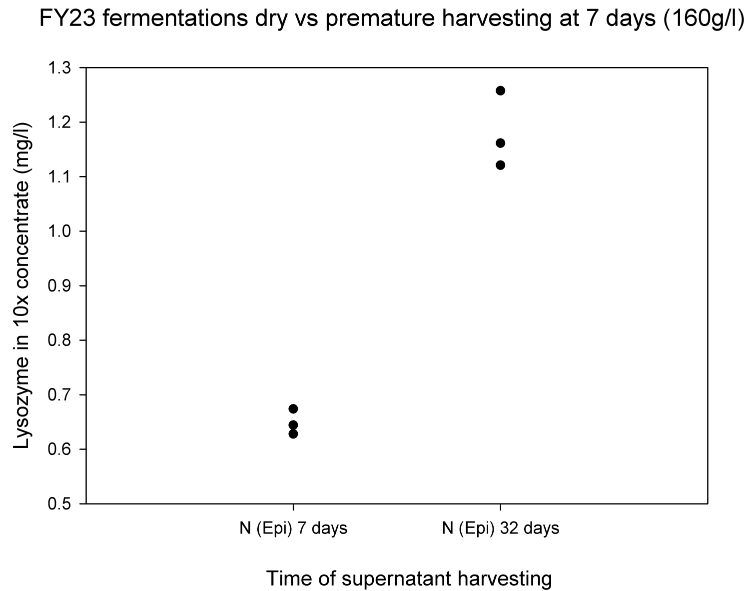


Figure 4.8: Six flasks inoculated with FY23 transformed with episomal plasmid pHVX2-N. Three were harvested after 7 days, three allowed to ferment to dryness. The concentration of fermentable sugars was 160 g/L

Lysozyme concentration in fermentation supernatants was related to fermentable sugar concentration. Differences in FY23 biomass at the end of fermentation (as determined by OD_{600} measurement) for all fermentation conditions were statistically insignificant [results not shown]. In combination these findings indicate that neither nitrogen availability nor biomass formation were the main limiting factors in lysozyme production, even though FY23's tryptophan auxotrophy was not compensated for in the media's formulation. Grape juice concentrations of tryptophan are sometimes too low to quantify, although in American musts may be as high as 50 mg/L (Bell & Henschke, 2003).

4.4.1 SDS-PAGE of lysozyme in fermentation concentrates

Supernatants (and 10x ion-exchange concentrates thereof) from fermentations of grape juice-like medium conducted anaerobically by FY23 transformants were analysed by SDS-PAGE. The concentrations of lysozyme in both the culture supernatants and the 10x ion-exchange concentrates were too low for visualization on SDS-PAGE gels with Coomassie staining. This is illustrated in Figure 4.10. Lane L is PAGE-ruler protein ladder. Consequently, silver staining was employed. The concentration of lysozyme in all of the culture supernatants was too low to be de-

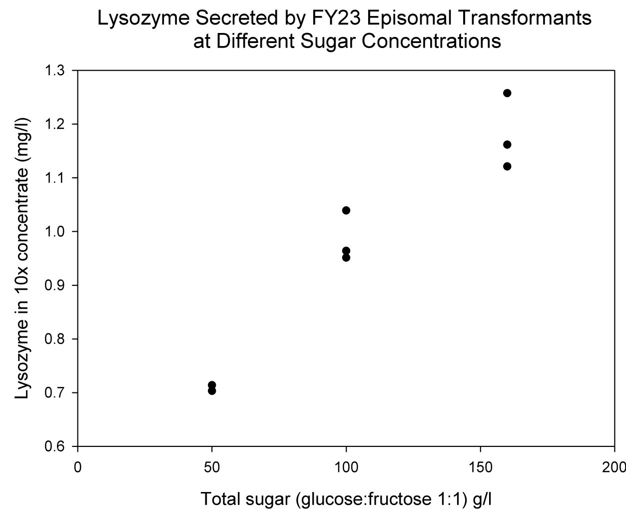


Figure 4.9: Effect of initial fermentable sugar concentration (50, 100 and 160 g/L hexoses) on secreted lysozyme recovered from finished fermentations by FY23 transformed with pHVX2-N

tected by this method. No glycosylated proteins were detected in any of the cation exchange concentrates when gels were stained with Pro-Q Emerald 300 glycoprotein gel stain.

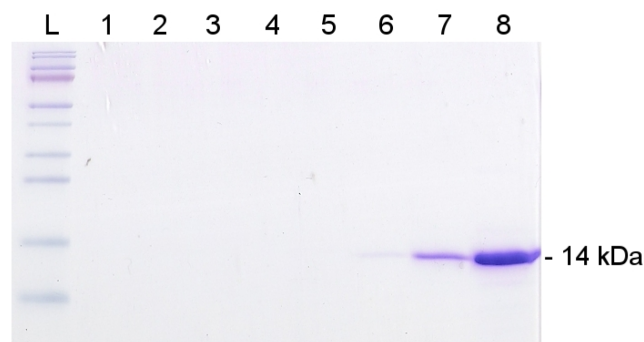


Figure 4.10: SDS-PAGE of ion-exchange concentrates stained with Coomassie brilliant blue. Lanes 1 – 5 are 10x concentrates of supernatants from fermentation of artificial juices containing 160 g/L hexose with FY23 episomal transformants M, N, RM, RN and the control plasmid. Lanes 6, 7 and 8 are 1, 10 and 100 mg/L lysozyme standards, respectively.

Figure 4.11 compares a lysozyme commercial standard with ion-exchange concentrates of FY23 fermentation supernatants. Three strains were used, one without a *HEWL* expression cassette and two expressing lysozyme with either the MF-α or native-lysozyme secretion signal. Lane 1 contains a commercial lysozyme stan-

dard. The 14 kD band in lane 2 is lysozyme spiked into the fermentation medium before inoculation. There is no noticeable smearing of this band, indicating that degradation of the enzyme did not take place during fermentation. The presence of a single band of the correct size in lane 3 indicates that lysozyme secreted with its own secretion signal is correctly processed by FY23. In lane 4 two bands are present, one corresponding to correctly processed lysozyme and a larger band possibly representing a form of lysozyme in which the MF-α signal was not cleaved correctly. The absence of a protein band at the same position in lane 5 indicates that the wild-type FY23 produced no lysozyme.

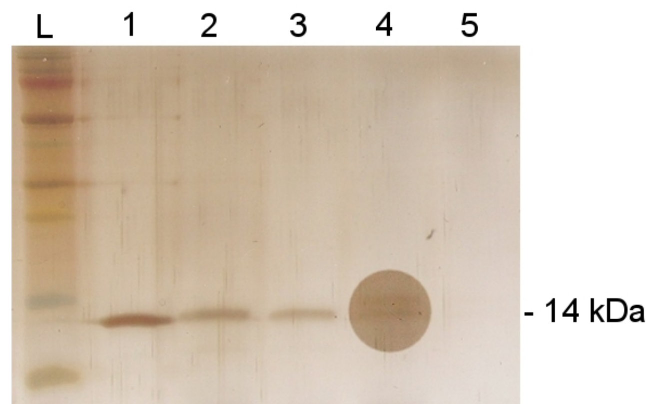


Figure 4.11: Silver stained SDS-PAGE of ion-exchange concentrates. Lane L is PAGE-ruler marker. Lane 1 contains 1 mg/L lysozyme standard solution. Lane 2 contains the 10x concentrate of a fermentation spiked with 10 mg/l lysozyme, diluted to 1 mg/L. Lane 3 contains nativeSS-lysozyme. Lane 4 contains MFα-lysozyme, the circle representing a region in which image-enhancement was performed to make protein bands visible. Lane 5 contains CEX concentrate of WT FY23 fermentation supernatant. For full explaination please refer to the text.

In Figure 4.12 the effect of arginines introduced to the N-terminals of the native-lysozyme and MF-α signals on proteolytic processing is shown. Lysozyme secreted with both its native secretion signal and the mutated variant thereof appears to be present in a single band which corresponds to the expected size of mature lysozyme (14 kDa). When lysozyme is secreted with the MFα secretion signal and its mutated variant two bands are present, one corresponding to correctly processed lysozyme and the other a larger (approx. 14.4 kDa) incompletely processed form. From these results it may be concluded that the mutations introduced into the secretion signals probably did not affect their proteolytic maturation.

Figure 4.12: Silver stained SDS-PAGE showing effect of secretion signal on protein maturation. Lanes $1-4$ are concentrates of fermentations performed with FY23 episomal transformants N, RN, M and RM. Lane 5 contains 1 mg/L lysozyme standard solution

4.4.2 HPLC-FLD determination of lysozyme concentrations in fermentation concentrates

In order to determine the effect of secretion signal choice on secretion of authentic, correctly-processed lysozyme under simulated winemaking conditions, an artificial grape juice containing 50, 100 and 160 g/L hexoses (glucose:fructose 1:1) was fermented by FY23 single-copy integrative and multicopy episomal transformants. These transformants contained expression cassettes containing the *Gallus gallus* lysozyme gene under the control of the *PGK*1 promoter and terminator. Four secretion signals were used, namely the lysozyme native signal, the *S. cerevisiae* MFα signal and corresponding mutated variants of these containing two arginines inserted on their N-terminals proximal to the N-terminal methionine. Fermentations were performed anaerobically, in triplicate, to dryness (< 0.5 g/L sugar). Lysozyme in the resulting supernatants was purified and concentrated by cation exchange and the resulting concentrate analysed by HPLC-FLD[3]. Only peaks corresponding to authentic lysozyme were taken into account.

The results of HPLC-FLD determination of lysozyme concentrations in supernatant concentrates from fermentations of artificial grape juice containing 100 g/L fermentable sugars are presented in Figure 4.13 and may be summarized as follows: expression from the episomal plasmid containing strains resulted in significantly higher secretion than the integrants in the case of the MF-α secretion signal. The opposite was found in the case of the native lysozyme signal, with the integrant secreting significantly more lysozyme than the episomal transformant. There were no statistical differences between the amounts of secreted lysozyme for any of the episomal fermentations. There were however differences between the integrants, with secretion from M being significantly higher than that from RM. Secretion by both N and RN integrants was greater than that from M and RM integrants. Unexpect-

[3] Please note that RM and RN lysozymes are represented as M+ and N+ in Figures 4.13 and 4.14

edly, the additional arginines in the RM secretion signal had an apparent negative effect on secretion of authentic lysozyme when compared to the unaltered signal. The reason for this is unclear. The additional arginines in the mutated native signal (RN) did not appear to result in an increase in secretion when compared to the unaltered signal. This contradicts the results of (Tsuchiya *et al.*, 2003), who found an almost three-times increase with the same altered sequence. It should be noted that different growth conditions and *S. cerevisiae* strains were used in this study and that human (and not hen) lysozyme was expressed. It should also be noted that, in every case where no statistical difference in lysozyme secretion was found between groups, sample sizes ($n = 3$) in the current study were too small to exclude the possibility that a significant difference did exist but was not detected.

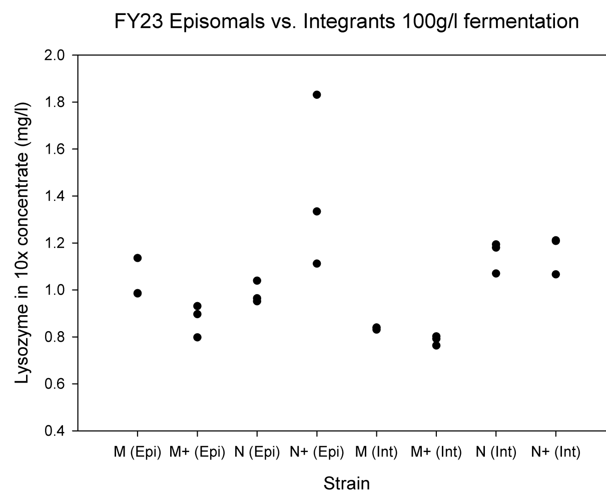


Figure 4.13: Comparison of integrated (Int) vs. episomal (Epi) secretion by FY23 transformants fermenting 100 g/L sugar, as determined by HPLC-FLD of cation exchange concentrates. “M” is MFα, “N” is native lysozyme secretion signal. “+” denotes constructs incorporating additional arginine residues on the secretion signal N-terminals

Results of HPLC-FLD determination of lysozyme concentrations in supernatant concentrates from fermentations containing 160 g/L fermentable sugars are presented in Figure 4.14. In the case of the FY23 episomal transformants, RM secreted significantly less lysozyme than episomal transformant RN. Transformant N secreted significantly more lysozyme than M. In the case of the integrants, only one significant difference in secretion was found, with N secreting more lysozyme than M. There was no significant difference between titers of secreted lysozyme when the native signal and its mutated counterpart are compared. This agrees with the re-

sult of the 100 g/L fermentations and once again contradicts expectations based on published results (Tsuchiya *et al.*, 2003). When integrants were compared to episomal transformants, no difference in secretion titer between M (epi) and M (int) or between N (epi) and N (int) was found. There was, however a difference between RM (epi) and RM (int)[4] with significantly less lysozyme being secreted by the episomal transformant. This result was not found with the 100 g/L fermentations.

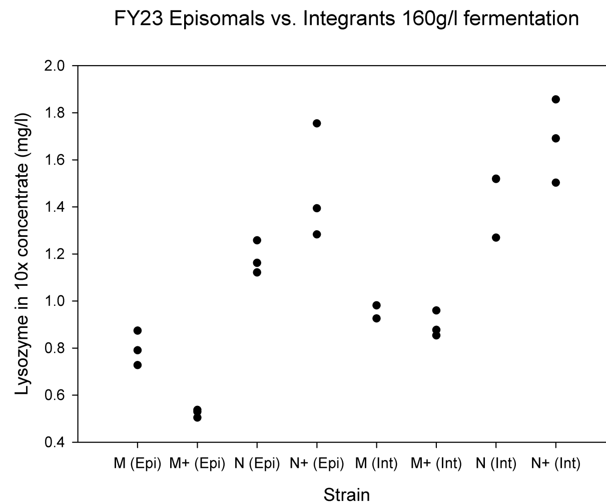


Figure 4.14: Lysozyme secretion by integrated (Int) and episomal (Epi) FY23 transformants fermenting 160 g/l sugar, as determined by HPLC-FLD of cation exchange concentrates. "N" is lysozyme with its native secretion signal, "M" is lysozyme with the MFα signal. "+" denotes constructs incorporating additional arginine residues on the secretion signal N-terminals

When HPLC-FLD determined lysozyme concentrations in concentrates from the 100 and 160 g/L fermentations (Figures 4.13 and 4.14) are compared the following observations were made. Firstly, the only groups differing significantly in both the 100 and 160 g/L fermentations are those of the M and N FY23 integrants. In both cases N was secreted to a higher titer than M. Secondly, the amount of lysozyme secreted by FY23 episomal transformants was higher in fermentations with 160 g/L sugar than in those with 100 g/L when its native secretion signal and the mutant variant thereof were used (means of N and RN: 0.985 and 1.425 mg/L lysozyme with 100 g/L sugar versus 1.180 and 1.477 mg/L lysozyme with 160 g/L

[4]FY23 transformed with either episomal plasmids or containing integrated expression cassettes are here indicated, for the sake of brevity, by (epi)and (int), respectively.

sugar, respectively). Unexpectedly, the opposite was found when MF-α and its mutated counterpart were used (means of M and RM: 1.035 and 0.875 mg/L lysozyme with 100 g/L sugar versus 0.797 and 0.523 mg/L lysozyme with 160 g/L sugar, respectively).

When the same comparison was made between FY23 integrants fermenting 100 and 160 g/L sugars, the same trend seen with the episomal transformants is observed when the native and mutant-native secretion signal is used (means of N and RN: 1.147 and 1.162 mg/L lysozyme with 100 g/L sugar versus 1.435 and 1.683 mg/L lysozyme with 160 g/L sugar, respectively). In the case of M and RM FY23 integrants the trend seen with the episomal transformants is not repeated (means of M and RM: 0.835 and 0.785 mg/L lysozyme with 100 g/L sugar versus 0.953 and 0.896 mg/L lysozyme with 160 g/L sugar, respectively).

Due to the complex nature of protein production and secretion it is difficult to speculate as to the reasons for these effects. One possibility is that during the purification process channel formation during packing of the ion-exchange media in the SPE cartridge resulted in substantial loss of protein in the case of the 160 g/L MF-α samples. This seems unlikely as the effect is only observed in the MF-α variants from the 160 g/L fermentations and nowhere else.

4.4.3 Lysozyme concentration in fermentation concentrates as determined by enzyme assay

In order to determine the effect of secretion signal choice on secreted lysozyme activity under simulated winemaking conditions, an artificial grape juice containing 50, 100 and 160 g/L hexoses (glucose:fructose 1:1) was fermented by FY23 single-copy integrative and multicopy episomal transformants. These transformants contained expression cassettes containing the *Gallus gallus HEWL* gene under the control of the *PGK*1 promoter and terminator. Four secretion signal were used, namely the lysozyme native signal, the *S. cerevisiae* MFα signal and mutants of these containing two arginines inserted on their N-terminals proximal to the terminal methionine. Fermentations were performed anaerobically, in triplicate, to dryness (< 0.5 g/L sugar). Lysozyme in the resulting supernatants was purified and concentrated by cation exchange and the resulting concentrate assayed for activity against *M. luteus*. Lysozyme concentration was estimated by comparison of activity in the cation-exchange concentrate with that of a lysozyme standard curve prepared

in cation-exchange elution buffer[5].

Results of active lysozyme concentration as determined by measurement of enzymatic activity in the supernatant concentrates resulting from fermentation of artificial grape juice containing 100 g/L hexoses is presented in Figure 4.15. Amongst the episomal transformants, M produced significantly more lysozyme activity than the RM variant. No significant difference in activity was found between any of the integrative transformants. When lysozyme activity derived from the episomal transformants was compared to that of the integrants, both M and N episomals displayed significantly higher lysozyme activity than that of their integrative counterparts.

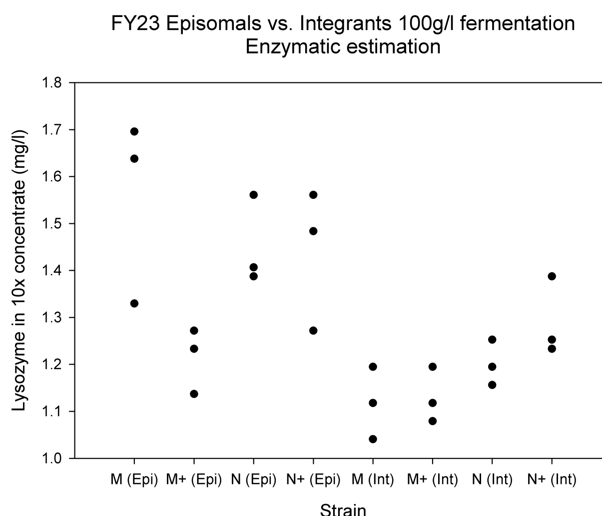


Figure 4.15: Secretion of active lysozyme by integrated (Int) and episomal (Epi) FY23 transformants fermenting 100 g/L sugar, as determined by enzymatic assay of cation exchange concentrates. "M" is MFα, "N" is native lysozyme secretion signal. "+" denotes constructs incorporating additional arginine residues on the secretion signal N-terminals

The results of enzyme assay estimation of lysozyme concentrations in supernatant concentrates from fermentations of artificial grape juice containing 160 g/L hexoses are presented in Figure 4.16. Of the episomal transformants, a significant difference between M and RM exists, with M possessing more activity than RM. With reference to the integrants, more secreted activity was found in the RN than in the N concentrate. The N integrant produced significantly more secreted activity than the M integrant. Secretion of activity by the episomal M transformant was greater than that of the corresponding M integrant. No other significant differences

[5]Please note that RM and RN lysozymes are represented as M+ and N+ in Figures 4.15 and 4.16

between secretion by the remaining episomal and their corresponding integrative transformants were found.

Comparison of the 100 and 160 g/L fermentations (Figures 4.13 and 4.14) in terms of secreted lysozyme activity allows for the following conclusion to be drawn. For each transformant more activity was secreted at the higher sugar concentration.

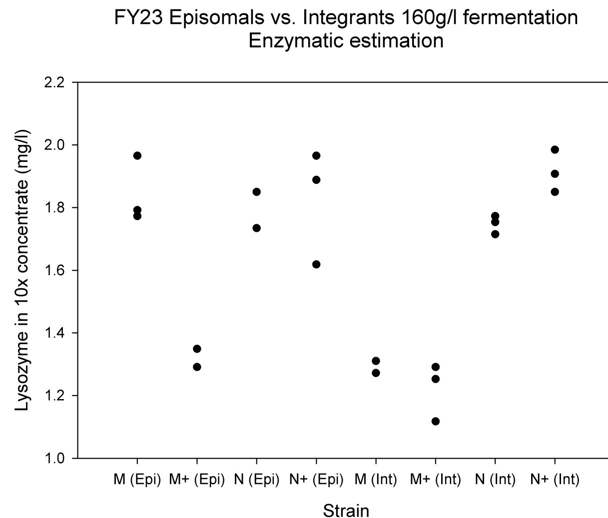


Figure 4.16: Lysozyme secretion by Integrated (Int) and episomal (Epi) FY23 transformants fermenting 160 g/L sugar, as determined by enzymatic assay of cation exchange concentrates. “M” is MFα, “N” is native lysozyme secretion signal. “+” denotes constructs incorporating additional arginine residues on the secretion signal N-terminals

4.4.4 Comparison of lysozyme concentration estimations by HPLC-FLD and enzymatic activity

When lysozyme is secreted with the MF-α or KILM signal in *S. cerevisiae*, signal cleavage is not always correctly carried out. More than one secretion product is therefore seen, corresponding to authentic lysozyme and one or more misprocessed products (Hashimoto *et al.*, 1998). This misprocessing does not necessarily abolish lysozyme’s enzymatic activity (Hashimoto *et al.*, 1998). Therefore HPLC quantification of the elution peak corresponding to authentic lysozyme may result in underestimation of total lysozyme activity. As lysozyme’s native signal is known to be correctly cleaved in *S. cerevisiae* (Arima *et al.*, 1997) while use of the MF-α signal has been shown to result in misprocessing, it was decided to compare the

concentration of authentic lysozyme as determined by HPLC-FLD and the apparent lysozyme concentration estimated from its enzymatic activity against *M. Luteus*[6].

Figure 4.17 compares lysozyme concentrations for FY23 integrants fermenting artificial grape juice containing 100 g/L of hexose as determined by HPLC-FLD to lysozyme concentrations in the same samples as determined by enzyme activity. The HPLC determinations reflect the absolute quantity of correctly processed lysozyme whereas the microtiter assay estimates lysozyme concentration based on enzymatic activity. These results appear to indicate that the larger lysozyme in which the MF-α secretion signal has not been properly cleaved a significant proportion of the secreted lysozyme population is enzymatically active. This is because the enzymatic estimations of concentration for lysozyme secreted with its native signal agree with the HPLC-FLD estimations. The native-signal lysozyme appears to be correctly cleaved in both the HPLC (Figure 4.6) and SDS-PAGE (Figure 4.12) analyses.

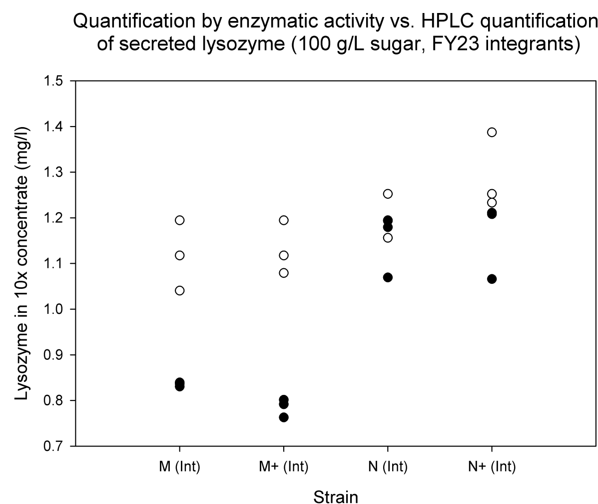


Figure 4.17: Comparison between *HEWL* concentrations as determined by HPLC (black circles) and enzymatic activity against *M. luteus* (white circles) for FY23 integrants fermenting artificial grape juice containing 100 g/L hexoses

In Figure 4.18 the concentrations of authentic lysozyme as determined by HPLC-FLD are compared to concentrations determined by assay of enzyme activity. Samples tested were cation-exchange concentrates of artificial grape juice containing 100 g/L hexoses, fermented to dryness (<0.5 g/L fructose and glucose) by FY23

[6]Please note that RM and RN lysozymes are represented as M+ and N+ in Figures 4.17, 4.18 and 4.19

episomal and integrative transformants. In the case of all the MF-α transformants (M (int), RM (int), M (epi) and RM (epi)) the concentration of lysozyme was significantly greater when determined by enzyme assay than when determined by HPLC-FLD. This indicates that the lysozyme derivative seen in both the HPLC chromatograms (Figure 4.7) and on the SDS-PAGE gels (Figure 4.12) retained at least some enzyme activity. This result appears to agree with the findings of (Hashimoto *et al.*, 1998), although in the current study only two products were seen as opposed to the three seen in their investigation.

By way of contrast, the lysozyme concentrations determined by the two methods were in agreement for three of the four transformants using the native and mutant-native lysozyme signals. In the case of FY23 transformed with pHVX2-N, the concentration of active lysozyme predicted by the turbidometric assay was significantly higher than that determined by HPLC-FLD. This is an unexpected result as only a single band was seen when the samples were separated by SDS-PAGE and only a single peak was detected by HPLC-FLD.

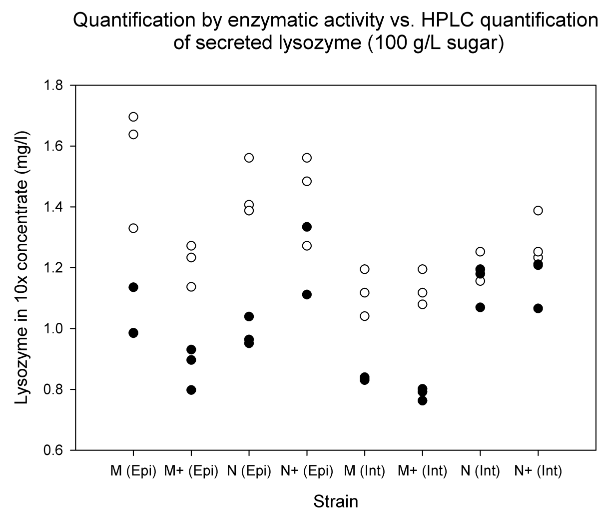


Figure 4.18: Comparison between lysozyme concentrations as determined by HPLC (black circles) and enzymatic activity (white circles). FY23 integrative (Int) and episomal (Epi) transformants fermented artificial grape juice containing 100 g/l hexoses."M" is MFα, "N" is native lysozyme secretion signal. "+" denotes constructs incorporating additional arginine residues on the secretion signal N-terminals

Figure 4.19 compares lysozyme concentrations in cation-exchange concentrates of artificial grape juice containing 160 g/L hexoses, fermented to dryness (<0.5 g/L fructose and glucose) by FY23 episomal and integrative transformants, as deter-

mined by HPLC-FLD and enzyme assay. Lysozyme concentrations determined by enzyme assay were significantly greater than those determined by HPLC-FLD in the case of M (epi), RM (epi), RM (int), N(epi) and N (int).

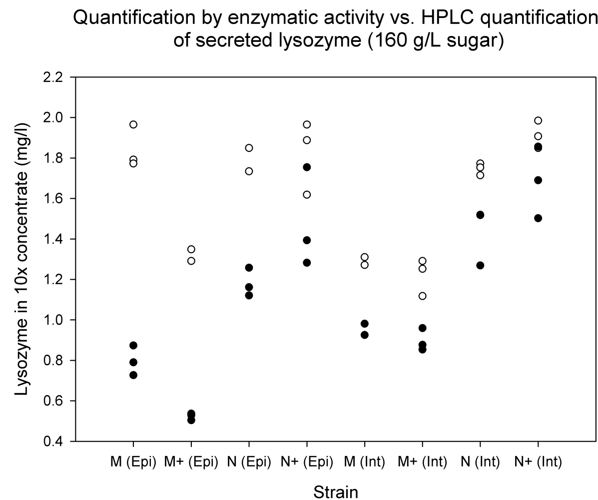


Figure 4.19: Comparison between lysozyme concentrations as determined by HPLC (black circles) and enzymatic activity (white circles). FY23 integrative (Int) and episomal (Epi) transformants fermented artificial grape juice containing 160 g/l hexoses."M" is MFα, "N" is native lysozyme secretion signal. "+" denotes constructs incorporating additional arginine residues on the secretion signal N-terminals

In every case where the native or mutant-native lysozyme signal was used a single peak was detected by HPLC-FLD and a single band was seen on the SDS-PAGE gels, both corresponding to authentic lysozyme (from commercial standards). Likewise, in every sample resulting from fermentations with transformants containing the MF-α and corresponding mutant varient were used, two peaks were detected by HPLC-FLD and two bands were seen on the SDS-PAGE gels. As has been discussed above, lysozyme titers were expected to be the same for both methods where the native lysozyme signals were used. The MF-α based signals should in all cases have resulted in a higher concentration estimation when determined by enzymatic assay than when determined by HPLC-FLD. When considered in isolation, the results seen in Figure 4.17 seem to fulfill this expectation and confirm previous findings (Hashimoto *et al.*, 1998). However, when the full data set, including all the data from fermentations of artificial grape juice containing both 100 and 160 g/L hexoses by all eight of the FY23 transformants, is examined a different picture emerges and it becomes impossible to support the conclusion suggested by

the data included in Figure 4.17. The reasons for these discrepancies may be errors introduced during the course of the experiments.

It should be noted that in every case where no statistically significant difference between groups was found, with the exception of the turbidometric concentration estimations for the 100 g/L fermentation concentrates, the power of the t-tests with alpha at 0.050 was less than the desired power of 0.800. This means that the likelihood of detecting a difference between groups where one actually existed was decreased. In other words, had the sample sizes been larger the expected differences in secretion titers as predicted from existing literature may well have been seen. The results illustrate the importance of considering all data generated during a study and not cherry-picking results that support a pre-conception.

In yeast transformed with episomal 2-micron based plasmids, plasmid copy number varies between individual cells in the producer strain population (Friehs, 2004). This has three important consequences for protein expression. Firstly, a varied plasmid population would result in varied growth rates and secretion titers for different cells within the population. This means that the titer is an average of total population production, with no data on the absolute copy number to protein secretion ratio being generated. Secondly, the copy number distribution may vary with experimental repeats resulting in greater variability between samples. Finally, if translation occurs at a sufficiently high rate it is possible to saturate secretory pathways and effectively block secretion (Wittrup *et al.*, 1995). If high copy number plasmids are used, and as it is possible for plasmids to be maintained at different copy numbers within a culture of host cells, it is possible for a percentage of the cells to translate at a level higher than that necessary to saturate those cell's secretory pathways. This would mean that secreted protein titer could not be accurately expressed as a function of total culture biomass, as a proportion of the culture is not contributing to product formation. It would be difficult to ascertain whether this was happening and to what extent.

When protein has to be secreted to high titers, gene dosage optimization is an important step in any engineering strategy. Methods do exist for stable chromosomal integration of different copy numbers of a gene at a specific locus and have been used successfully in the past (Lopes *et al.*, 1989; Sakai *et al.*, 1990; Choi *et al.*, 2002; Parekh *et al.*, 2008). While episomal expression is convenient from a transformation perspective, the data generated is not necessarily useful nor the yield optimal. Protein titers can usually be improved by proper gene dosage optimization

under the same growth conditions.

4.4.4.1 Lysozyme secretion by VIN13 integrant "M"

As can be seen in Table 4.1, when fermenting a grape juice-like medium containing 50 g/L of hexoses, VIN13-Mi secretes lysozyme but, unlike FY23 [results not shown], suffers a significant growth defect. This result is reported, not because of its presence, but because of the unexpected magnitude of this effect. Growth defects as a result of the metabolic burden of heterologous expression have been reported in numerous studies (Snoep *et al.*, 1995; Gorgens *et al.*, 2001; Martinez *et al.*, 1999). The effect of the burden of heterologous protein production on growth has been found to be more pronounced in industrial than in laboratory yeast strains (Krogh *et al.*, 2008). It has also been demonstrated that the presence of plasmid-borne copies of the *PGK1* promoter not coupled to any gene perturbs normal cell function, altering growth rate and flux through glycolysis (Snoep *et al.*, 1995). This is likely due to competition with the indigenous *PGK1* gene for a low-abundance transcription factor (Packham, 1996), altering the quantity of normally highly abundant PGK1p in the cell. Lysozyme expression under the control of a strong promoter such as *PGK*1 likely places VIN13 under stress as highly expressed genes in most organisms generally display bias with regard to codon usage (Kane, 1995; Bennetzen & Hall, 1981; Sharp & Cowe, 1991; Gustafsson *et al.*, 2004). Lysozyme from *Gallus gallus* has a very low codon bias (0.049) in regard to preferred *S. cerevisiae* codon utilization (Oberto & Davidson, 1985). This means that, especially in a gene whose product is expected to be translated at a high level, the need for large populations of peptidyl-tRNAs will not be met in the case of some of the codons used. This will result in a paucity of these tRNAs with global consequences for translation.

Synthesis of amino acids, an energetically expensive process, is upregulated as a stress-response when heterologous protein's are produced (Mattanovich *et al.*, 2004). As metabolically available nitrogen is usually limiting in grape juice the amount of amino acids available for production of cellular protein is decreased when additional protein has to be produced heterologously. The ratio of amino acid utilization in a heterologously produced protein as a function of that used in total wild-type yeast protein will also affect the organism's metabolism. Additionally, *S. cerevisiae* does not naturally secrete many proteins. This is often cited as a beneficial quality of *S. cerevisiae* secretion systems as it simplifies purification of secreted heterologous proteins. It must, however, be remembered that transport

Table 4.1: VIN13 results from grape juice-like culture experiments

Glu/Fru 25/25	raw supernatant	10x concentrate	OD_{600}[b]
	Turbidometric/HPLC	HPLC-FLD (mg/L)	
VIN13 strain			
VIN13 №1	undetectable	undetectable	10.77
VIN13 №2	undetectable	undetectable	10.27
VIN13 №3	undetectable	undetectable	10.25
VIN13-Mi[a]№1	undetectable	1.402	7.47
VIN13-Mi№2	undetectable	0.890	7.54
VIN13-Mi№3	undetectable	1.774	8.21

[a] VIN13-Mi as per table 3.1
[b] OD_{600}s were determined at the end of fermentation. For inoculation conditions please refer to Chapter 3.5

across the various organelle membranes during secretory protein maturation is energetically expensive. These various effects must necessarily be compensated for, the effect in the case of lysozyme expression by VIN13 apparently being a decrease in final biomass (under the tested conditions).

This may not be important where growth effects are inconsequential but should be taken into account in the case of yeast responsible for food-industry fermentations. For winemakers any unintentional effects on growth resulting from genetic modification could be significant, as an engineered VIN13's performance would differ from that of the commercially available "wild type" strain upon which the GMO was based.

4.5 General conclusion

In this study the gene for hen egg white lysozyme was cloned into episomal and integrating plasmids under the control of the *PGK1* promoter and terminator. Four secretion signals were used to direct secretion of lysozyme into the culture medium. These signals were lysozyme's native signal, the secretion signal for mating factor alpha and two varients on these signals. These varients incorporated arginines into the N-terminals of the signals, increasing the secretion signal's positive charge, in an attempt to increase the titer of secreted lysozyme.

The four expression cassettes generated were introduced into multicopy episomal (pHVX2) plasmids as well as plasmids for chromosomal integration (YIpLac211 and pDMPOF1b). *S. cerevisiae* FY23 was transformed with pHVX2 and YIpLac211 based plasmids. VIN13 was transformed with expression cassettes excised from

pDMPOF1b based plasmids.

Lysozyme was successfully secreted by all the above-mentioned *S. cerevisiae* FY23 transformants when propagated by plate culture as demonstrated by secreted lysozyme's activity against overlaid *M. luteus*.

Lysozyme was also successfully secreted by all the above-mentioned *S. cerevisiae* FY23 transformants fermenting an artificial grape juice like medium under anaerobic conditions. Heterologously expressed lysozyme was identified based on its co-migration with a lysozyme standard (when analysed by SDS-PAGE), co-elution with the standard (during HPLC analysis) and the presence of lytic activity against *Micrococcus luteus*, mirroring the enzymatic activity of hen egg white lysozyme. Secretion of lysozyme from a strain of VIN13 containing the MF-alpha secretion signal – lysozyme fusion was confirmed.

In FY23, under the conditions tested, lysozyme appears to be properly processed during secretion when its own secretion signal is used. Significant amounts of what may be an incompletely cleaved lysozyme precursor appear when the MF-α signal is used. This supports Hashimoto *et al.*'s findings (Hashimoto *et al.*, 1998) and illustrates that choice of an appropriate secretion signal should be among the first steps in any protein secretion study. While it is possible that mutations increasing the N-terminal positive charge increase secretion in the case of the native lysozyme signal (and decrease it in the case of MF-α) the differences in titer were, for the most part, too small to be significant. Due to the small sample sizes ($n = 3$), necessity for sample purification and extremely low secreted lysozyme concentrations (close to the limit of quantification for the HPLC-FLD method employed) caution should be used in interpretation of this study's results as pertaining to the relative effectiveness of the different secretion signals. Secretion of lysozyme with the MF-alpha secretion signal was successful in VIN13. The transgenic strain produced significantly lower biomass when compared to the wild-type VIN13.

As has been discussed by previous authors (Gorgens *et al.*, 2001), auxotrophic yeast strains should be used for neither protein expression nor metabolic engineering studies, unless it is the auxotrophy itself being studied. It is not necessarily possible to predict how an industrial strain, or indeed any closely related strain, will perform based on the performance of an auxotroph.

Chapter 5

Final Discussion

The main aim of the current study was to investigate the effect of secretion signal choice on the secretion of hen egg white lysozyme in *S. cerevisiae*.

When lysozyme was secreted by FY23 under fermentative conditions, the MFα samples were misprocessed under all culture conditions used, with two products being observed. One corresponded to authentic lysozyme when analysed by both HPLC and SDS-PAGE, the other was a larger peptide. This larger peptide may represent a lysozyme-processing varient in which the MF-α secretion signal was not properly cleaved. Both of these products appeared to have lytic activity against *M. luteus*. This is in agreement with published literature as (Hashimoto *et al.*, 1998) found three forms of lysozyme, two of which displayed activity against *M. luteus*. It is well established that use the MFα secretion signal results in misprocessed product formation, especially with c-type lysozymes. The absence of the third form in the present study may have been a result of the cation-exchange isolation procedure. That the native signal produced a single peak corresponding to wild-type hen egg white lysozyme indicates that this secretion signal is a better choice for secretion of this enzyme by the strain of *S. cerevisiae* used.

Yields of secreted lysozyme were extremely low under all tested conditions, being undetectable by HPLC-FLD without isolation and concentration by cation exchange. Lysozyme's fluorescence was found to be affected by acidification when the HPLC-FLD method of Riponi *et al.* was used, with acidification causing a significant increase in fluorescence. Riponi *et al.* reported that lysozyme association with phenols or other compounds in especially red wines resulted in underestimation of it's concentration when previous HPLC methods were used. They postulated that acidification would free the enzyme from interfering substances and allow for

better detection. As they did not report lysozyme concentration comparisons of acidified and unacidified samples of the same wine determined with acidified and unacidified standards they could not have taken the effect of acidification into consideration. For this reason their study should be revisited.

Lysozyme secretion by VIN13 was found to cause a significant decease in final culture biomass, even though the concentration of protein secreted was comparatively low. This result is not unprecedented, as the effect of the burden of heterologous protein production on growth has been found to be more pronounced in industrial than in laboratory yeast strains Krogh *et al.* (2008).

The amounts of lysozyme required in any of it's winemaking applications are extremely high compared to published yields of either HEWL itself or indeed many other heterologous proteins secreted by *S. cerevisiae*. Factors such as gene-dosage optimization, integration locus, mRNA structure around the initiation codon, use of a strong promoter not linked to central glycolysis and optimization of codon usage are all known to affect yields of protein secretion by *S. cerevisiae*. While selection of an appropriate secretion signal is an important consideration in heterologous protein secretion, it is of lesser importance than these other factors, particularly in the context of the current project where high secretion titers are necessary.

Grape juice is usually regarded as a nitrogen-poor growth medium for yeast, hence the commonplace addition of diammonium hydrogen phosphate (DAP) to compensate for this deficiency (Boulton *et al.*, 1996). The extra demand for nitrogen to produce comparatively large amounts of secreted protein would potentially increase the risk of stuck fermentations. This is especially the case if the metabolic burden of expression results in lower than usual biomass formation[1], as it has been reported that the rate of fermentation in nitrogen-poor musts is dependant on yeast biomass (Valera *et al.*, 2004).

Should it be it possible to produce an oenological strain of *S. cerevisiae* capable of secreting oenologically useful quantities of lysozyme under winemaking conditions, the practical applications for such a yeast remain to be defined. How long it would take for the gradually-secreted lysozyme to reach effective concentrations would have to be determined under a realistically wide range of real-world conditions. Lysozyme is generally applied either before yeast inoculation as a means of preserving juice in storage, simultaneously with yeast inoculation[2] to either prevent

[1] As it did in the case of the VIN13's under tested conditions in the present study

[2] Lysozyme may be added in a double dose, at the start of and during fermentation

premature MLF or inhibit it completely. Finally, lysozyme may be added after fining, as a means of preventing MLF after bottling. If lysozyme is gradually produced throughout fermentation it cannot fulfill either of first two roles. If lysozyme were produced to adequate titers extremely rapidly immediately after inoculation it could be argued that certain growth defects[3] would be difficult to avoid. Finally, the fining of white wines with bentonite prior to bottling is a very common practice in the industry. As bentonite functions as a cation exchanger and is added to prevent protein haze formation, any lysozyme present in the wine would be removed immediately upon its addition (Achaerandio *et al.*, 2001). No application for the yeast appears to remain.

In addition, heterologous secretory protein yield by *S. cerevisiae* is dependant on many factors. The highly-variable conditions of winemaking and variable compositions of grape musts could consequently result in unreliable secretory performance. It may be impossible to know how much lysozyme had been produced by what stage of the fermentation without costly and time-consuming analysis, making the application of such a yeast impractical in the industry for this reason alone. The adoption of a lysozyme producing yeast could potentially introduce unacceptable complications to an already labour and expertise intensive endeavour, requiring unnecessary adaptation of established cellar practice.

5.1 Prospects for future research

Prospects for future research are as follows: of great interest would be thorough investigation of the fermentation and growth properties of existing GMO wine yeast strains in comparison to their parent strains. Additionally, careful evaluation by properly experienced experts of previously suggested wine-yeast modification projects, in terms of plausibility, should be undertaken. While previous studies have examined the impact of metabolic burden of heterologous protein secretion under aerobic growth conditions, no study has yet specifically been performed to determine the effects by anaerobically fermenting yeast. It would be interesting to compare the effects of these different growth conditions on both the magnitude and nature of any growth anomalies in yeast.

[3] Lower biomass formation and possibly a decreased growth rate

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