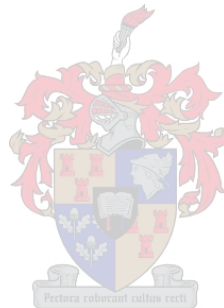


Chitin synthesis in response to environmental stress

by

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Declaration

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Summary

Previous studies have indicated that fermentation with yeast strains whose cell walls contain higher chitin levels may lead to reduced wine haze formation. In order to adjust cell wall chitin levels, more information on the regulation of chitin synthesis in wine-relevant yeast is required. Yeast cells are known to increase chitin levels when subjected to certain environmental changes such as an increase in temperature. The main aim of this project was to investigate chitin accumulation and synthesis in wine yeast strains when exposed to environmental change. This was achieved by subjecting the strains to various environmental conditions and comparing chitin levels. The information gained may aid future selection and/or manipulation of yeast strains for the production of higher chitin levels. Three *Saccharomyces cerevisiae* strains and two *Saccharomyces paradoxus* strains were subjected to conditions that had been linked to a change in chitin synthesis in past studies in laboratory yeast strains. Of the conditions used in this study, the addition of calcium to a rich media led to the highest cell wall chitin levels. The data also show that chitin synthesis is largely strain dependant. Two conditions which resulted in increased chitin deposition were chosen for gene expression analyses, using strains with strongly diverging average chitin levels. Results showed that an increase in chitin levels correlates with an increase in expression of *GFA1*, the gene encoding for the first enzyme of the chitin synthesis pathway. Overall, this study provides novel insights into chitin synthesis in *Saccharomyces cerevisiae* wine yeast strains as well as *Saccharomyces paradoxus* strains, with possible future implications on haze prevention studies.

Opsomming

Vorige studies het aangetoon dat fermentasie met gisrasse waarvan die selwande hoë chitienvlakke bevat, kan lei tot verminderde wynwaasvorming. Om selwandchitienvlakke aan te pas, word daar meer inligting rakende die regulering van chitienvlakke in wyn gisrasse verlang. Dit is bekend dat gisselle chitienvlakke verhoog wanneer die selle onderwerp word aan sekere veranderinge in die omgewing soos 'n verhoging in temperatuur. Die hoofdoel van hierdie projek was om die chitiënopbou en -sintese in wyngisrasse te ondersoek waar gis blootgestel word aan omgewingsveranderinge. Dit is bereik deur die selle aan verskeie omgewingstoestande bloot te stel en chitienvlakke met mekaar te vergelyk. Die inligting hieruit verkry kan toekomstige gisrasseuses asook die manipulering van gisrasse met die oog op hoër vlakke van chitiënproduksie vergemaklik. Drie *Saccharomyces cerevisiae* rasse en twee *Saccharomyces paradoxus* rasse is onderwerp aan toestande wat in vorige studies gekoppel is aan 'n verandering in chitiënvorming in laboratorium-gisrasse. Van die toestande toegepas in hierdie studie, het die toevoeging van kalsium tot 'n nutriëntryke medium gelei tot die hoogste chitienvlakke in selwande. Die data toon ook aan dat chitiënsintese hoofsaaklik rasverwant is. Twee toestande wat gelei het tot verhoogde chitiënafsetting is gekies vir geen-uitdrukkingsanalise, terwyl rasse gebruik is met gemiddelde chitienvlakke wat wyd uiteenlopend is. Die resultate het getoon dat 'n verhoging in chitienvlakke ooreenstem met 'n verhoging in die uitdrukkingsvlakke van *GFA1*, die geen wat kodeer vir die eerste ensiem in die chitiënsintesebaan. Oor die algemeen verskaf hierdie studie nuwe insigte oor chitiënsintese in *Saccharomyces cerevisiae* wyngisrasse en *Saccharomyces paradoxus* rasse en verskaf dit belangrike inligting vir moontlike toekomstige studies oor waasvoorkoming.

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Preface

This thesis is a compilation of four chapters. Each chapter is introduced separately and is written according to the style of *Yeast*.

Chapter 1 Introduction and project aims

Chapter 2 Literature review: Regulation of *Saccharomyces cerevisiae* and *S. paradoxus* cell wall chitin in response to environmental changes

Chapter 3 Research results: Chitin synthesis in response to environmental stress

Chapter 4 General discussion and conclusion

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Chapter 1

Introduction and project aims

Chapter 1: Introduction and project aims

1.1 General introduction

Clarity of wine is used as quality parameter by consumers and professional wine judges alike, and hazy wine is considered of poor quality. The most common cause of haziness in wine is linked to wine protein precipitation and is referred to as protein haze. Other, less common causes include microbial or acid instability. Currently, the most frequently used wine clarifying agent to eliminate or reduce protein haze formation is the clay bentonite. However, this method, besides being costly, is non-specific and can result in a loss of aromatic character and of up to 10% of total wine volume. These negative aspects of bentonite create a demand for an alternative haze protection method, whilst motivating studies on the proteins responsible for haze formation (Tattersall *et al.*, 1997).

Several studies have shown that pathogenesis-related (PR) proteins such as thaumatin-like proteins and chitinase are likely to constitute the majority of proteins responsible for haze formation (Waters *et al.*, 1996; Pocock *et al.*, 2000). Previous reports have more specifically implicated grape chitinase in haze formation and suggested that these proteins might be primarily responsible for a majority of haze problems encountered in the industry (Marangon *et al.*, 2010; Marangon *et al.*, 2011; Pocock *et al.*, 2000).

Data from a recent study by our group suggested a direct correlation between the level of cell wall chitin of a fermenting yeast strain and the haze formation potential of wine (Ndlovu, 2012). The study furthermore showed that the cell walls of strains with higher chitin levels were able to bind higher levels grape chitinase, providing a possible explanation for the observed effect. The principle of using yeast cells with high chitin levels as haze prevention mechanism was patented by our group (Patent P2355ZA00). Such data are furthermore supported by the finding that the addition of pure chitin to wine can result in less haze formation (Vincenzi *et al.*, 2005). Taken together, these observations motivated our study of chitin levels and chitin deposition in *Saccharomyces cerevisiae* wine yeast strains as well as strains of *Saccharomyces paradoxus*.

Chitin forms part of the inner layer of the yeast cell wall along with other structural components, $\beta(1,3)$ glucan and $\beta(1,6)$ glucan and provides rigidity and structural protection to the cell. Chitin is essential to the cell and the simultaneous deletion of two of the three chitin synthesis genes is lethal. The biopolymer is composed of N-Acetyl glucosamine monomers and the addition of the monomers to the growing chitin chain is orchestrated by one of three chitin synthase enzymes encoded for by *CHS1-3*.

Distribution of chitin is not equal throughout the cell wall. The majority of yeast cell wall chitin is present in the budscar, the scar which remains on the mother cell after cell division. Furthermore, the lateral cell wall has also been proven to contain chitin (Powell *et al.*, 2003). Chs3p is responsible for chitin synthesis in these extensions of the cell wall as well as the lateral cell wall. Chitin also plays a large role during budding, as the primary septum separating mother and daughter cell consists mainly of chitin. The cleavage of the primary septum by an endochitinase is the event responsible for the eventual separation of the daughter cell from the mother cell. Chs2p is responsible for chitin deposition in the septum and Chs1p is responsible for the repair of chitin that was excessively damaged by chitinase. Meanwhile, the birthscar, the result of budding on the daughter cell, has been proven to contain little to no chitin (Powell *et al.*, 2003). The only significant difference thus far reported between chitin located in different parts/ extensions of the cell wall is the linkage between chitin and β -glucans. In the lateral cell wall, the linkage between the compounds is $\beta(1,6)$ differing from the $\beta(1,4)$ linkage between the two compounds in the septum (Cabib, 2009).

The amount of chitin synthesised in the yeast cell wall has also been known to vary. In the yeast's natural environment of soil, tree barks, fruits, *Drosophila* guts, and grape juice or must, yeast cells have to adjust to environmental changes such as increase in sugar levels as fruit ripen or after rainfall, which would decrease osmolarity and availability of nutrients (Sampaio and Gonçalves, 2008; Schuller *et al.*, 2005; Sniegowski *et al.*, 2002). To adapt to such environmental changes, the yeast employs many adjustments and modifications of cell physiology, intracellular osmolite concentrations as well as an increase of the components of the inner layer namely, β -glucans or chitin (Aguilar-Uscanga and François, 2003; Lesage and Bussey, 2006; Shima and Takagi, 2009).

In this study the chitin deposition of yeast strains in response to various environmental changes was investigated. Cells were exposed to changes in temperature, osmolarity, high concentrations of calcium and subsequently chitin levels were measured. Chitin content during fermentation was also monitored. Information generated in this study can be used in further studies on yeast chitin levels as a possible haze protection factor.

1.2 Aims and Objectives

The main aim of this study is to evaluate cell wall chitin deposition and regulation in response to growth conditions.

There are two objectives of this study:

- Firstly, the effect of growth conditions on cell wall chitin synthesis is investigated to review conditions which have previously shown to result in high chitin levels in the context of *Saccharomyces cerevisiae* strains commonly used in the wine industry as well as *Saccharomyces paradoxus* strains. The results can then be evaluated to determine if changes can be applied to fermentation conditions, or applied to pre-inoculation growth conditions.
- Secondly, this study aimed to observe gene expression levels of chitin synthesis related genes for strains producing low or high chitin levels and in response to the environmental changes identified in the first objective.

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Chapter 2

Literature review

Regulation of *Saccharomyces cerevisiae* and *S. paradoxus* cell wall chitin in response to environmental changes

Chapter2: LITERATURE REVIEW

Regulation of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* cell wall chitin in response to environmental changes

2.1 Introduction

The adaptations of yeast cells to human-made surroundings such as large volumes of high-sugar fruit juices have been exploited by selecting for yeast strains displaying traits which are favourable for specific industries. The wine industry is no exception and uses industrially-produced *Saccharomyces cerevisiae* strains for the fermentation of wine due to the ability of these yeasts to produce high concentrations of ethanol (Bowyer and Moine-Ledoux, 2007). Recent findings in our group have shown that it may be possible to exploit yeast cell wall chitin levels to lower wine protein haze formation (Ndlovu, 2012).

Wine haze is unacceptable to consumers and is often seen as a sign of poor quality by consumers and panellists alike. For this reason, potentially haze causing proteins must be removed prior to bottling and there is currently a market for a cost-effective, biological haze removal method. Studies from our group indicated fermentation with yeast cells containing high cell wall chitin levels to result in less wine haze formation (Ndlovu, 2012). These findings are supported by previous studies where the addition of 20g/l chitin resulted in less wine haze formation, acting as an efficient fining technique (Vincenzi *et al.*, 2005).

The possible impact of yeast cell wall chitin on wine haze led us to propose a more in depth study on cell wall chitin in wine yeast strains. Knowledge acquired on chitin synthesis and cell wall modification with a specific focus on cell wall chitin may be of use for the manipulation of commercial wine yeast strains in order to improve their impact on wine clarity or in studies on the possible addition of yeast strains after fermentation as a fining technique. There is also need for quantitative studies on yeast chitin synthesis not only focussing on *Saccharomyces cerevisiae* laboratory strains but also in wine yeast strains as well as *Saccharomyces paradoxus* strains. This literature review will focus on the role of chitin in the yeast cell wall, chitin biosynthesis and its regulation in response to growth conditions.

2.2 Chitin and the cell wall

The cell wall of *S. cerevisiae* constitutes 10 – 30% of the dry weight of the cell and consists of about 60 - 90% polysaccharides and 10 – 15% proteins (Pérez and Ribas, 2013). It consists of two layers (Fig 2.1). The electron dense outer layer contains mannoproteins (Shreuder *et al.*, 1996) and is responsible for cell-to-cell interactions and attachment, cell-to-surface attachment and mating (Aguilar-Uscanga and François, 2003; Klis *et al.*, 2001). The inner layer is responsible for the integrity and shape of the cell during growth and cell division and consists of $\beta(1,3)$ and $\beta(1,6)$ linked glucose polymers and chitin. $\beta(1,3)$ -glucan branches are anchored to the plasma membrane and branches are either linked to the reducing end of $\beta(1,6)$ -glucan branches or to the reducing GlcNAc of the chitin chain with a $\beta(1,4)$ linkage as represented in Figure 2.1 (Kollár *et al.*, 1995).

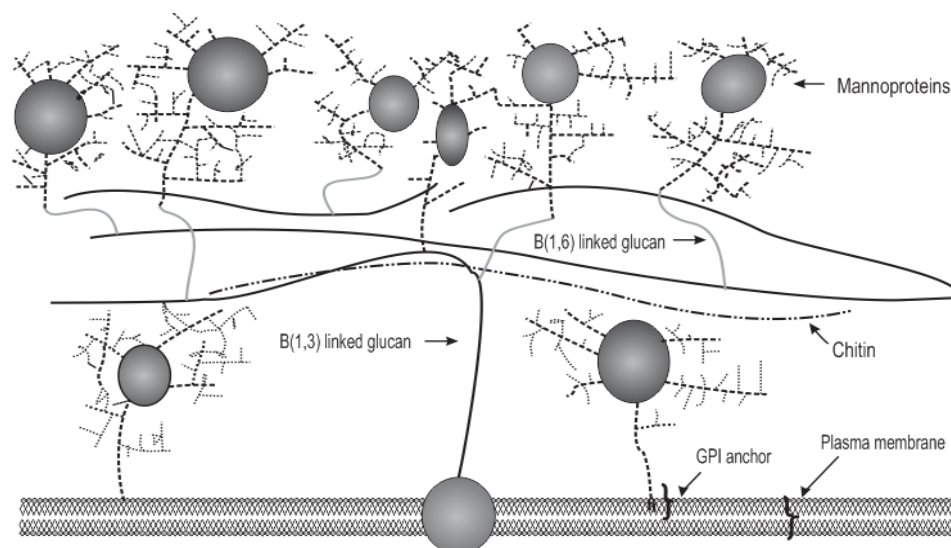


Figure 2.1: Simplified representation of the layered structure and composition of the yeast cell wall as adapted and modified from Schreuder *et al.*, (1996).

Chitin is a bio-polymer that occurs in a yeast cell wall as an insoluble chain of 120-170 $\beta(1,4)$ linked *N*-acetyl-glucosamine molecules with an α configuration consisting of approximately twenty single chains occurring in antiparallel. The antiparallel configuration allows the formation of hydrogen bonds both intra- and intersheet, ensuring the tight packaging of the molecule (Kameda *et al.*, 2005; Rudall and Kenchington, 1973). The biopolymer provides the yeast cell wall with strength and rigidity. As a consequence, cells which have mutations resulting in a decrease in chitin synthesis exhibit osmotic and temperature sensitivity,

sensitivity to the cell wall perturbing agent Calcofluor, abnormal cell shape, aggregation, and growth arrest with abnormal buds (Bulawa and Osmond, 1990; De Nobel *et al.*, 1991; Shaw *et al.*, 1991).

A relatively small (5-10%) percentage of total chitin is distributed in a fairly uniform manner in the lateral cell wall. The majority of chitin, however, is found in bud scars. These scars on the cell wall surface constitute the result of budding on the mother cell. During budding chitin is deposited at the site of bud emergence on the mother cell, and some chitin is also present in the septum separating mother and daughter cell (Powell *et al.*, 2003). However, little to no chitin is found in the birth-scar on the daughter cell (Cabib and Durán, 2005; Cabib 2009).

Chitin from different parts of the cell wall together is believed to constitute 1 - 2% of the dry weight of the cell wall or 0.1 - 0.2% if only chitin in the lateral cell wall is taken into account and bud scars excluded from measurement (Klis *et al.*, 2006; Nguyen *et al.*, 1998). However, these values can vary with changes in the cells surroundings, such as changes in carbon source, nitrogen limitation, pH, temperature, osmotic stress, aeration and mode of cell cultivation (Aguilar-Uscanga and François, 2003). At the level of the cell wall, these conditions elicit three main responses. The first is a change in the ratio of different cell wall polymers, most dramatically, a large increase in cell wall chitin levels. The second is the association between $\beta(1,3)$ glucans, mannoproteins and chitin, brought on by the decreased ratio of $\beta(1,6)$ glucans to other polysaccharides, which results in a large amount of cell wall proteins which are directly linked to $\beta(1,3)$ glucans and chitin (Kapteyn *et al.*, 1999). The third identified response is a redistribution of synthesis and repair proteins of cell wall components such as chitin, that under normal conditions are focused to active growth regions in the cell (For review see Klis *et al.*, 2001). Chitin is therefore crucial to the structure and integrity of the cell wall also under conditions of stress (Aguilar-Uscanga and François 2003; Shima and Takagi 2009).

2.3 Chitin distribution

2.3.1 Chitin in budding

In the early stages of budding, the late G1 phase, chitin is synthesized in the bud-neck region linking the mother and daughter cell. The main chitin synthase enzyme of the mother

cell, Chs3p, is responsible for this synthesis (Schmidt *et al.*, 2003). The chitin forms a ring around the neck separating the two cells, ensuring the rigidity of the bud-neck for the duration of budding (Shaw *et al.*, 1991). Similar to the rest of the cell wall, the bud neck ring is comprised of highly crystallized chitin linked to β -glucans, along with a reinforced glucan mannan layer. The synthesis of chitin in the ring is believed to be orchestrated post-transcriptionally by localization of Chs3p to the site of bud emergence at the beginning of the cell cycle. Bud scars are never lost or healed throughout the lifespan of the cell, and every new instance of budding forms a new scar. Therefore, the number of bud scars and consequently chitin levels are often used to estimate the age of a cell.

Near the conclusion of budding, chitin is deposited into the primary septum. The synthesis of the primary septum is initiated at the start of cytokinesis by the contraction of the actomyosin ring causing the plasma membrane at the bud neck to fold inward toward the center of the neck (Fig 2.2(A)) (Fang *et al.*, 2010; Pollard *et al.*, 2010). Thereafter Chs2p, which is now localized to the plasma membrane at the bud neck, synthesizes chitin and extends the chitin chain into the growing septum toward the middle of the channel (Fig 2.2 (B)) (Cabib 2004; Verplanck and Li, 2005). This results in an uninterrupted chitin layer as the plasma membranes of the two cells separate to form the primary septum (Fig 2.2(C)). Subsequently, the secondary septum is synthesized by both cells on either side of the primary septum forming a strong triple layered separation between the cells (Fig 2.2 (D)) (Cabib, 2004; Cid *et al.*, 2001).

Upon the completion of budding, when cells are ready to separate, the primary septum is dissociated by *S. cerevisiae* chitinase encoded for by *CTS1* (Kuranda and Robins, 1991). This disruption is initiated from the daughter side of the cell resulting in an asymmetric distribution of chitin leaving almost all chitin on the mother cell side. This part of the cell wall on the mother cell will later become part of the bud scar (Cabib, 2004; Cabib *et al.*, 1992; Baladrón *et al.*, 2002).

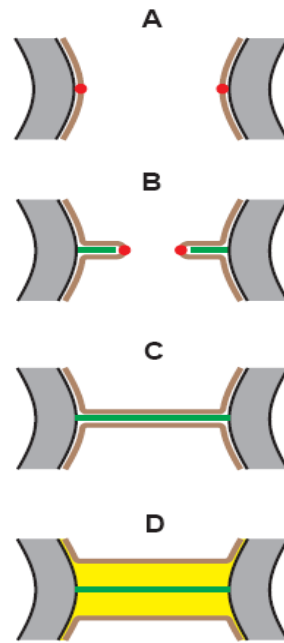


Figure 2.2: Schematic illustration of septation in budding yeast cells. The cell wall of the neck region connecting the mother and daughter cells is represented in grey, with the plasma membrane as the brown line. The red spots represent Chs2p and the green line formed in (B) represents chitin. In (B) the membrane folds inward and chitin is extended by the Chs2p complex. When the chitin layer is complete and the plasma membranes of the two cells are fully separated, the primary septum is complete (C). Thereafter the secondary septa are synthesized by both cells creating the triple layer septum (D) (Adapted from Schmidt *et al.*, 2002).

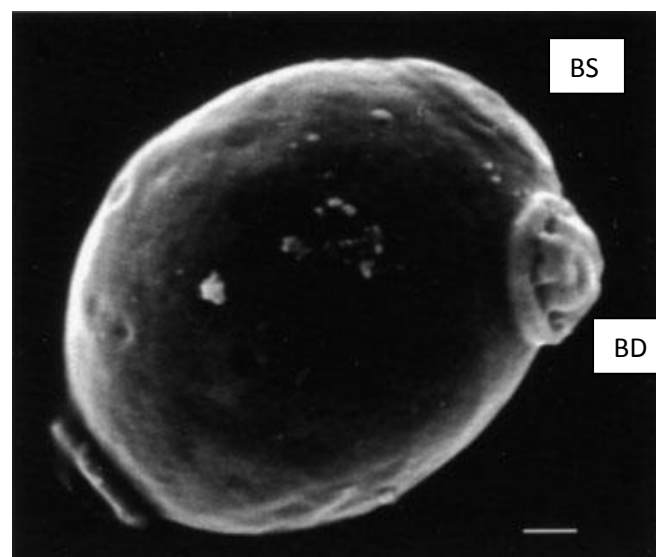


Figure 2.3: Depiction of the different morphology of the budscar (BD) and birthscar (BS). (Adapted from Powell *et al.*, 2003)

The chitinase-orchestrated separation which resulted in the bud scar on the mother cell also leaves a scar on the daughter cell – the birth scar. Birth scars can be distinguished from bud scars by their larger size as these scar rings were formed before bud scars and continue growing during the lifespan of the cell (Fig 2.3) (Powell *et al.*, 2003). The scars can also be distinguished by their chitin levels, because while the budscar contains the majority of cell wall chitin, birth scars contain little to no chitin (Shaw *et al.*, 1991; Powell *et al.*, 2003).

2.3.2 Chitin in the lateral cell wall

Very little information is known of differences between chitin in the lateral wall and chitin in the budscar. Recent studies show that chitin at the budneck region is more often linked to $\beta(1,3)$ glucan whereas in the lateral cell wall chitin is attached to $\beta(1,6)$ glucan (Cabib, 2009).

2.4 Chitin synthesis

Chitin synthesis uses fructose-6-phosphate as precursor and consists of 5 reactions as indicated in Figure 2.4. The synthesis of the UDP-GlcNAc monomer occurs in the cytosol (Orlean, 1997). The first reaction from the precursor is the synthesis of glucosamine-6-phosphate from the compounds fructose-6-phosphate and glutamine by the enzyme glutamine-fructose-6-Phosphate amidotransferase (Gfa1p) encoded for by the gene *GFA1* (Watzele and Tanner, 1989). Lagorce and his colleagues (2002) found the increase of chitin in response to cell wall damage correlates with an increase in *GFA1* expression. The authors also showed that the overexpression of the gene resulted in a three-fold increase in cell wall chitin levels and hypersensitivity to chitin targeting cell wall agent, Calcofluor white.

The next step combines glucosamine-6-phosphate, glutamate and Acetyl-CoA to form N-acetyl-glucosamine-6-phosphate. The reaction is catalysed by glucosamine-phosphate-N-acetyltransferase (Gna1p) encoded for by *GNA1*. Thereafter, phosphoacetylglucosamine mutase (Pcm1p) encoded for by *PCM1* converts N-Acetylglucosamine-6-phosphate to N-Acetylglucosamine-1-phosphate. The next reaction involves the conversion of UTP to UDP and subsequent addition to and dephosphorylation of N-Acetylglucosamine-1-phosphate to form UDP-N-Acetylglucosamine (Fig 2.4) by UDP-N-Acetylglucosamine pyrophosphorylase (Qri1p) encoded for by *QRI1*.

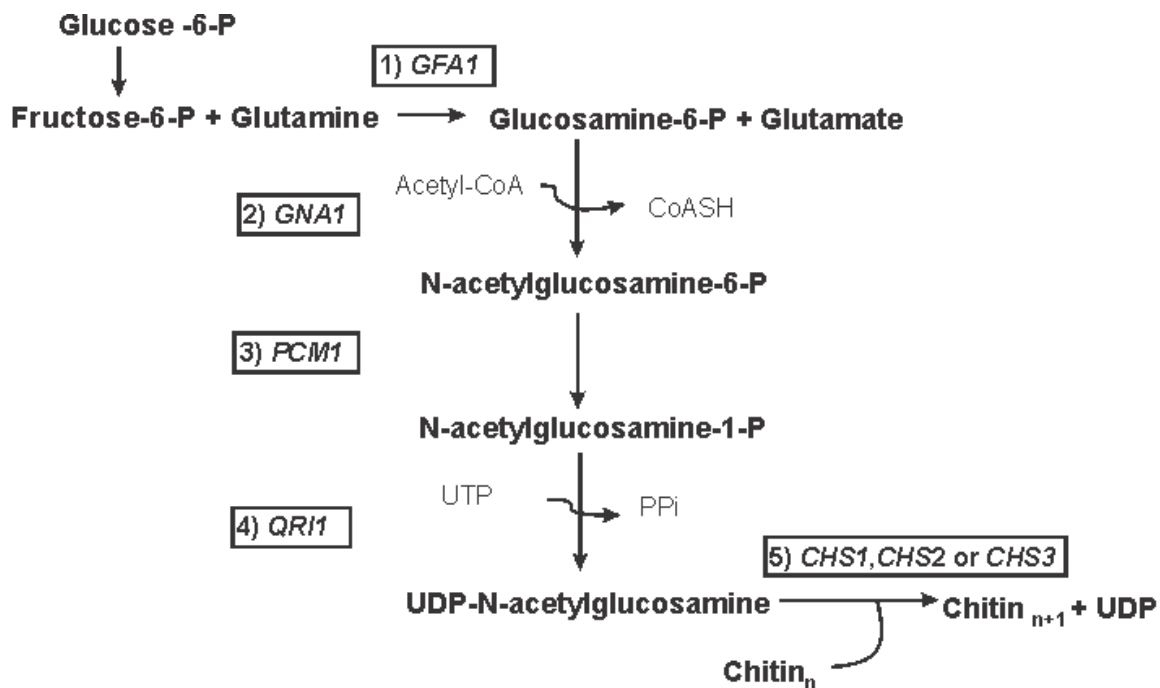


Figure 2.4: The chitin synthesis pathway from glycolytic intermediates to cell-wall synthesis. The numbers 1-4 represent the different steps performed by the enzymes glutamine-fructose-6-phosphate amidotransferase (Gfp1p), glucosamine-phosphate-N-acetyltransferase (Gna1p), phosphoacetylglucosamine mutase (Pcm1p), and UDP-N-Acetylglucosamine pyrophosphorylase (Qri1p) (Adapted from Lagorce *et al.*, 2002).

The last step of the synthesis pathway is catalysed by chitin synthase enzymes Chs1p, Chs2p and Chs3p. These enzymes act as transmembrane proteins building a chain of N-Acetylglucosamine monomers and extruding the resulting set of chitin on the outside of the membrane where Crh1p or Chr2p binds the polymer to $\beta(1,3)$ glucosidase or $\beta(1,6)$ glucosidase.

2.5 Regulation, transportation and function of genes and proteins involved in chitin synthesis

2.5.1 CHS1

The first chitin synthase enzyme discovered is encoded by *CHS1*. The enzyme was identified as a repair enzyme, chiefly responsible for repairing chitin hydrolysed by excessive action of *S. cerevisiae* endochitinase at the separation of mother and daughter cell at

cytokinesis. In this manner *CHS1* prevents a loss in cell wall chitin and subsequent lysis of daughter cells (Cabib *et al.*, 1989; Cabib *et al.*, 1992).

The enzyme was shown to be activated by zymogenic lysis and is believed to be under temporal and spatial regulation (Cabib, 2004; Pammer *et al.*, 1992; Spellman *et al.*, 1998). Chs1p is synthesized in the endoplasmic reticulum and at the M/G1 phase, transported in specialized endosomes (chitosomes) to the plasma membrane where the protein acts as a transmembrane protein (Choi and Cabib, 1994; Sanchez-Leon *et al.*, 2011; Ziman *et al.*, 1996). Although the disruption of *CHS1* is not fatal, it does result in lysing buds when grown in minimal medium (Bulawa *et al.*, 1986). This lysis phenotype can be rescued by the simultaneous disruption of chitinase 1 (*CTS1*) (Cabib *et al.*, 1992).

2.5.2. *CHS2*

The second enzyme to show chitin synthase activity (encoded by *CHS2*) is believed to be regulated by phosphorylation as well as possibly undergoing transcriptional regulation during the cell cycle (Pammer *et al.*, 1992; Lesage and Bussey, 2006; Choi and Cabib, 1994). Synthesis of the enzyme is believed to occur in the endoplasmic reticulum (ER) where the enzyme is phosphorylated by the protein Cdk1 at four N-terminal sites and this phosphorylation ensures the enzymes localization in the endoplasmic reticulum until septum formation (Teh *et al.*, 2009). Chs2p is triggered to exit the ER by the mitotic exit network (MEN), a network consisting of a Ras-related GTP-ase and other protein kinases (Verplanck and Li, 2005). The result of the MEN is the removal of the earlier Cdk1p phosphorylation of Chs2p, enabling Chs2p to leave the ER (Chin *et al.*, 2011). The enzyme then travels through the Golgi network where it is packaged into a secretory vesicle to be transported to the plasma membrane at the budneck region at the end of mitosis with the help of the vesicle transport protein Sec1 (Cid *et al.*, 2001).

Chs2p is then incorporated in the cleavage of the plasma membrane in the middle of the two septin rings (Fig 2.5) as a membrane-bound enzyme to actively assemble the chitin chain (Chuang and Schekman, 1996). After cytokinesis, the enzyme is believed to undergo endocytosis into endosomes and is then transported to a degradation vacuole where the enzyme is degraded by the vacuolar protease, Pep4. The enzyme must now be resynthesised at the next budding (Chuang and Schekman, 1996; Lesage and Bussey,

2006). This correlates with studies by Pammer *et al.*, (1992), who found transcription levels of *CHS2* to peak shortly before cell division, indicating that this enzyme differs from *CHS1* and *CHS3* in that it is degraded, and, through transcriptional regulation, resynthesised before cell division. Disruption of *CHS2* results in cells with clumpy growth and abnormal shape and size (Shaw *et al.*, 1991).

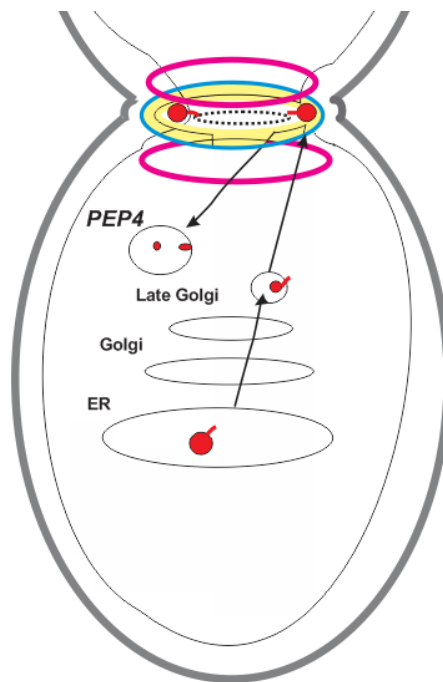


Figure 2.5: Synthesis transport and action of Chs2p. The enzyme is represented in red, the actin myosin ring is represented as a hatched line, the septin rings are represented in pink, the bud neck ring is represented in blue and the primary septum is represented in yellow. (Adapted from Lesage and Bussey, 2006)

2.5.3 *CHS3*

The chitin synthase gene that has been studied to the greatest extent regarding transcription, transport and regulation is *CHS3*. The enzyme encoded by this gene is responsible both for the synthesis of chitin in the lateral cell wall and in the neck separating mother and daughter cells during budding. Although none of the three chitin synthase genes are essential for growth or survival of the cell, a simultaneous defect in Chs2p and Chs3p cannot be compensated for by the activity of Chs1p and has lethal consequences for the cell (Shaw *et al.*, 1991).

Chs3p is synthesized in the endoplasmic reticulum and not believed to be under transcriptional regulation, due to similar levels of the enzyme being present at different stages of the cell cycle (Choi and Cabib, 1994; Lesage and Bussey, 2006; Ziman *et al.*, 1996). In the early stages of the daughter cell formation, as well as occasionally at the end of mitosis, the enzyme exits the endoplasmic reticulum with the aid of Chs7p (Lesage and Bussey, 2006). In the absence of a functional Chs7p, Chs3p does not leave the ER and consequently the activity of Chs3p is greatly diminished (Lesage and Bussey, 2006).

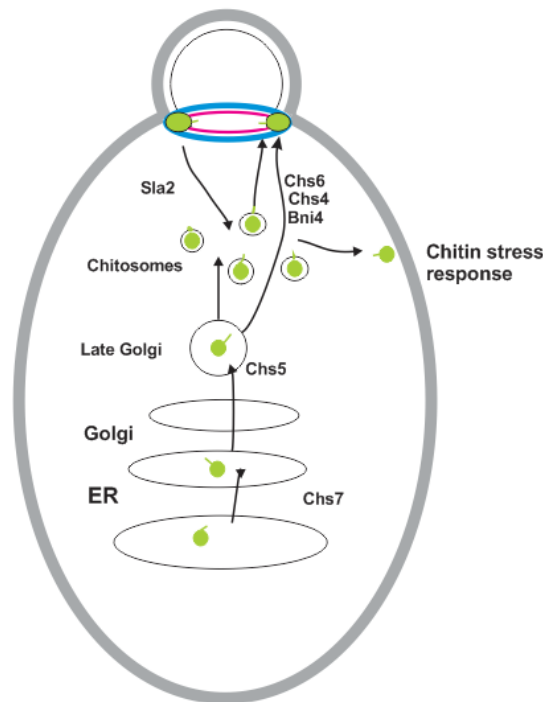


Figure 2.6: The lifecycle of enzyme chitin synthase 3 and synthesis of chitin (blue line) in the budscar region. (Adapted from Lesage and Bussey, 2006).

Similar to Chs1p, Chs3p is then transported in specialized endosomes known as chitosomes to the plasma membrane (Chuang and Schekman, 1996). The transportation is believed to occur with the help of Chs5p and Chs6p. If *CHS5* is disrupted in *S. cerevisiae*, Chs3p fails to localise to the bud scar and accumulates in an active form in internal compartments, resulting in a decreased amount of chitin synthesis (Ortiz and Novick, 2006; Osmond *et al.*, 1999; Santos and Snyder, 1997). Chs6p is required for chitin synthesis *in vivo*, but not for chitin synthase III activity *in vitro* and the deletion of this gene results in the localization of Chs3p almost exclusively in the trans Golgi network. Very little enzyme can be found at the plasma membrane or bud neck region, indicating that the protein is responsible for the movement of Chs3p to the plasma membrane (Valdivia *et al.*, 2002).

At the plasma membrane Chs3p is believed to function as a trans-membrane protein (Bulawa 1992; Chuang and Schekman, 1996). Sec6p, a protein related to fusion of secretory vesicles to the plasma membrane, was proven necessary for the fusion of chitosomes to the plasma membrane for the subsequent incorporation of Chs3p into the membrane (Ortiz and Novick, 2006). The deletion of *SEC6* does not result in any difference in phenotype under the permissive temperature of 25°C, however, after 90 minutes of exposure to an elevated temperature (37°C), the mutants revealed a severe loss of Chs3p localisation to the plasma membrane (Ortiz and Novick, 2006). Chs3p was also shown to interact with Dnf1p and Drs2p, aminophospholipid translocases. This interaction could possibly provide further insight on the incorporation of Chs3p in the plasma membrane (Trassov *et al.*, 2008).

Once Chs3p is no longer needed at the membrane, *END3/END4* aid in the endocytosis of Chs3p into chitosomes. These vesicles are where 30-50% of the cell's Chs3p enzyme resides when the daughter cell growth is complete (Chuang and Schekmann, 1996; Holthuis *et al.*, 1998; Ziman *et al.*, 1996). The deletion of *END3/END4* results in the redistribution of Chs3p to the plasma membrane and a failure of the enzyme to re-enter chitosomes for storage after the enzyme has performed its function (Chuang and Schekman, 1997; Raths *et al.*, 1993; Ziman *et al.*, 1996).

Localisation of Chs3p and Chs2p to the budneck region specifically occurs with the aid of Chs4p, Bni4p and septin proteins. *CHS4*, also known as *SKT5*, encodes for Chs4p, a protein which, when activated, binds to Chs3p, forming an active protein complex that can now be recruited by Bni4p, a scaffold protein, that binds to the septin ring before budding and remains on the mother cell side until late in the cell cycle (Longtine *et al.*, 1996; Cabib, 2009). In vegetative cells, the amount of Chs4p was found to be limiting, thereby impacting chitin synthesis by the availability of the protein to form an active complex with Chs3p (Ono and Han, 2000). A cell with a disrupted *SKT5* gene lacks in the formation of chitin in the bud neck region and displays decreased chitin deposition (Lesage *et al.*, 2005; Lam *et al.*, 2006). Deletion of *BNI4* is not lethal, in fact mutants with this deletion contain normal chitin levels and show no significant difference in Chs3p activity; however, the chitin ring shows abnormal structure and consequently the cell was found to have an aberrant morphology (DeMarini *et al.*, 1997; Sanz *et al.*, 1994).

Chs3p transport to the active site is increased under conditions of cell wall stress (e.g. heat shock at 37°C). Chs3p exits the trans-Golgi network and translocates to the plasma membrane (Santos and Snyder, 1997; Trilla *et al.*, 1999; Ziman *et al.*, 1996) and within 30 minutes a radical increase in Chs3p mobilized from storage to the plasma membrane can be observed (Valdivia and Schekman, 2003). This correlates with a study performed by Pammer and colleagues (1992) which indicated that *CHS3* transcript levels did not change significantly throughout the cell cycle, suggesting that regulation of the enzyme is not transcriptional.

2.6 Chitin synthesis in response to stress conditions

Eukaryotic organisms have developed sensing mechanisms and signal transfer pathways that allow organisms to detect and respond to a change in the environment. Several different types of responses exist, including gene expression alterations, up- or down regulation of metabolism, changes to protein homeostasis and activity, cytoskeletal reorganization and cell cycle modification or arrest (Chen and Thorner, 2007; Gehart *et al.*, 2010; Hohmann *et al.*, 2002; Richter *et al.*, 2010).

In response to many conditions that have a negative impact on the yeast, cells tend to increase the synthesis of components which are responsible for rigidity in the cell wall, such as chitin and β -glucans (Valdivia and Schekman, 2003; Aguilar-Uscanga and François, 2003). The best known example would be the exposure of cells to elevated temperatures, which results in the transportation of Chs3p from the trans-Golgi network to the plasma membrane (Ortiz and Novick, 2006; Munro *et al.*, 2007; Yoshimoto *et al.*, 2002). Table 2.1 illustrates the different conditions which have been shown to result in elevated cell wall chitin levels, increased activity of the chitin synthase enzymes or an increase in transcription of chitin synthesis genes.

Table 2.1: Different stress conditions that result in yeast increasing chitin synthesis, increasing activation or transportation of chitin synthesizing enzymes or increasing transcription of chitin synthesizing enzymes.

| Conditions that have shown to have an impact on cell wall chitin levels | The change in chitin level | Citations |
|---|--|--|
| Elevated extracellular Ca ²⁺ levels | Increase in transcription of <i>CHS1</i> , <i>CHS2</i> , <i>CHS3</i> | Yoshimoto <i>et al.</i> , 2002; Munro <i>et al.</i> , 2007 |
| Elevated temperature (37°C) (compared to 30°C) | Increase in chitin dry weight, stimulation of promoters of <i>CHS1</i> , <i>CHS2</i> and <i>CHS3</i> | Aguilar-Uscanga and François, 2003; Munro <i>et al.</i> , 2007 |
| Hypo-osmolarity | Increase in chitin synthase enzyme activity | Deshpande <i>et al.</i> , 1997 |
| Hyper-osmolarity | Increase in chitin synthase enzyme activity | Deshpande <i>et al.</i> , 1997 |
| Addition of cell wall perturbing agents | Addition of Calcofluor white both stimulated the transcription of <i>CHS1</i> , <i>CHS2</i> and <i>CHS3</i> and resulted in elevated cell wall chitin levels; Addition of Congo-Red also induced elevated synthesis of cell wall chitin | Munro <i>et al.</i> , 2007; Roncero and Durán 1985; Roncero <i>et al.</i> , 1988 |
| Glucosamine addition to the media | Increase in cell wall chitin content (measured in GlcNAc) | Bulik <i>et al.</i> , 2003 |

A number of general repair pathways have also been implicated in aiding the cell's global response for adaptation and recovery. The cell wall integrity pathway for instance is believed to be activated upon exposure to cell wall perturbing agents or a change in the mechanical properties of the plasma membrane and the pathway can also be activated under conditions of heat stress, a hypo-osmotic environment or nutrient limitation (Lesage *et al.*, 2005). Through such pathways, the cell is able to increase its chances of survival by making a number of different changes simultaneously, allowing a more rapid recovery and homeostasis.

2.7 Pathways suspected to be involved in chitin synthase regulation:

2.7.1 High Osmolarity Glycerol pathway (HOG)

The High Osmolarity Glycerol pathway is a MAPK (Mitogen Activated Protein Kinase) signal transduction pathway that controls the adaptation of yeast cells to increased osmolarity of the surrounding environment. In a hypertonic medium, water would naturally diffuse from the higher water potential (inside the cell) to the lower water potential (surrounding medium) resulting in cell water loss, shrinking, temporary arrest of cell growth, disassembly of actin cytoskeleton, changes in membrane permeability and loss of cell polarity (Millar *et al.*, 1995; Miermont *et al.*, 2011; Hohmann *et al.*, 2002). *S. cerevisiae* responds by activating the High Osmolarity Glycerol (HOG) pathway. The pathway is believed to be initiated at low temperatures, oxidative stress and turgor pressure changes.

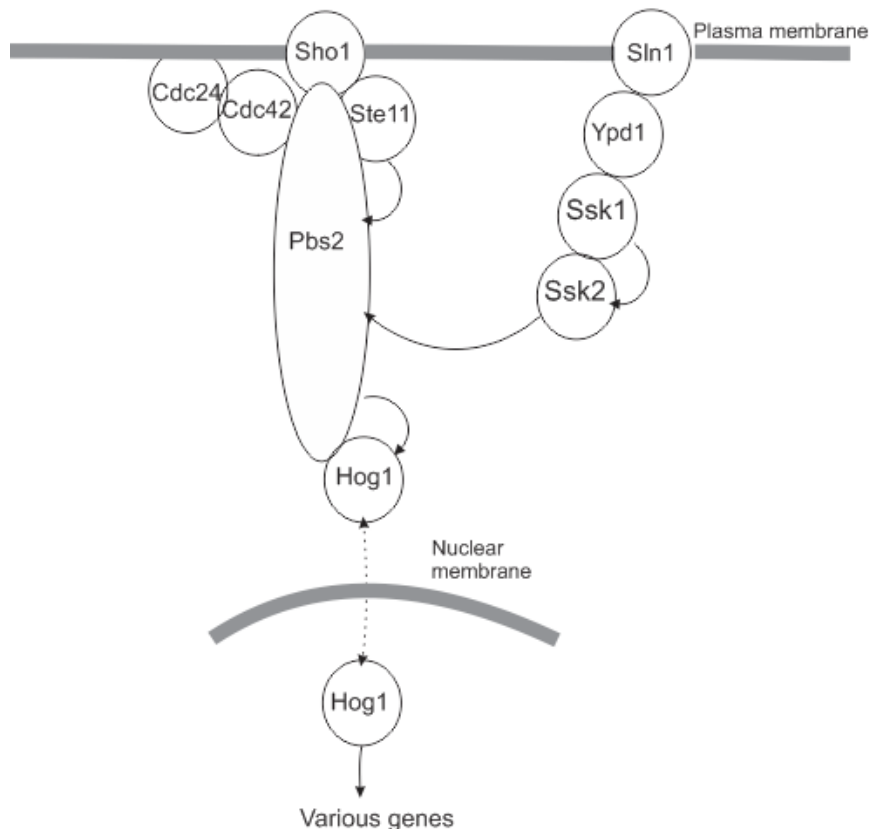


Figure 2.7: Representation of the HOG pathway adapted from Hohman (2002). Two branches are incorporated in the pathway, the Sln1p branch and the Sho1p branch. In Sln1p the protein is inactivated under conditions of cell stress, resulting in an inability to start a chain of phosphorylation which now leaves Ssk2 able to phosphorylate Pbs2. The Sho1p branch is a network of proteins binding to one another and also to Pbs2. Once environmental stress activates Sho1p these proteins are able to phosphorylate Pbs2. Pbs2 in turn phosphorylates Hog1p which can locate into the nucleus to activate the transcription of various genes by binding to their promoter region.

The HOG pathway is also believed to play a role in adjusting the rigidity of the *S. cerevisiae* cell wall via controlling β -glucan and chitin synthesis (Jiang *et al.*, 1995). Munro and colleagues (2007) found that *hog1* Δ strains show a significant decrease in *CHS1*, *CHS2* and *CHS8* expression in *Candida albicans*. However, *CHS3* expression was increased significantly, implicating Hog1p in the suppression of *CHS3* and the expression of *CHS1* and *CHS2*. The authors did, however see an increase in chitin synthesis.

These findings may agree with other results (Chuang and Schekman, 1996, Cos *et al.*, 1998), which show that *S. cerevisiae* chitin synthase genes are mostly post-translationally regulated. The effect of the HOG pathway on chitin synthesis is however disputed, with several studies linking the HOG pathway to increased chitin synthesis (Walker *et al.*, 2008, Lenardon *et al.*, 2010, Seet and Pawson, 2004), while other results suggest no involvement of this pathway in chitin synthesis (García-Rodríguez *et al.*, 2000). García-Rodríguez *et al.*, (2000) proposed with this pathway, as with their hypothesis on the discrepancies of the cell wall integrity pathway, that an unknown pathway or linkage between the different pathways may be behind these different findings.

2.7.2 Cell Wall Integrity (CWI) pathway

The cell wall integrity pathway is regulated throughout the cell cycle and pseudohyphal development, as well as being activated when cell receptors pick up external stimuli such as hypo-osmotic extracellular conditions, heat stress, nutrient limitation, pheromone induced morphogenesis, the presence of cell wall stressing agents and oxidative stress (For review see Levin, 2005). As illustrated in Fig 2.8; cell wall receptors such as *Wsc1-3*, *Hcs77*, *Mid2* and *Mtl1* observe stimuli which lead to activation of the pathway (Green *et al.*, 2003; Ketela *et al.*, 1999). A MAPK-pathway results in the conversion of Rho1p-GDP to the active Rho1 – GTP (Audhya and Emr, 2002; Ozaki *et al.*, 1996). Rho1p then continues to phosphorylate one of a number of different effector proteins such as β (1,3)glucan synthase, Bni1p and Bnr1p formin proteins, Skn7p transcription factor, Sec3p the most important protein for chitin synthesis and protein kinase C (Pkc1p) (Martin *et al.*, 2000).

Previous studies have proven that overexpression of Pkc1p or Rho1p correlates significantly with the transposition of Chs3p from the Golgi network to the plasma membrane and subsequent chitin synthesis (Valdivia and Schekman, 2003). However, overexpression of neither Bck1p nor MAPK proteins had a significant impact on the expression of *CHS3*. The mechanism with which Pkc1p achieves this translocation is unknown and overexpression of this protein did not lead to an increased amount of phosphorylated Chs3p. Several other studies have shown stimuli of the cell wall integrity pathway to result in increased levels of chitin synthesis (Choi and Cabib, 1994, Yoshida *et al.* 1994). Overexpression of the protein Rlm1 does, however, lead to significant increase in chitin content presumably through activation of the *CHS1* and *CHS2* genes (Valdivia *et al.*, 2002; Igual *et al.*, 1996).

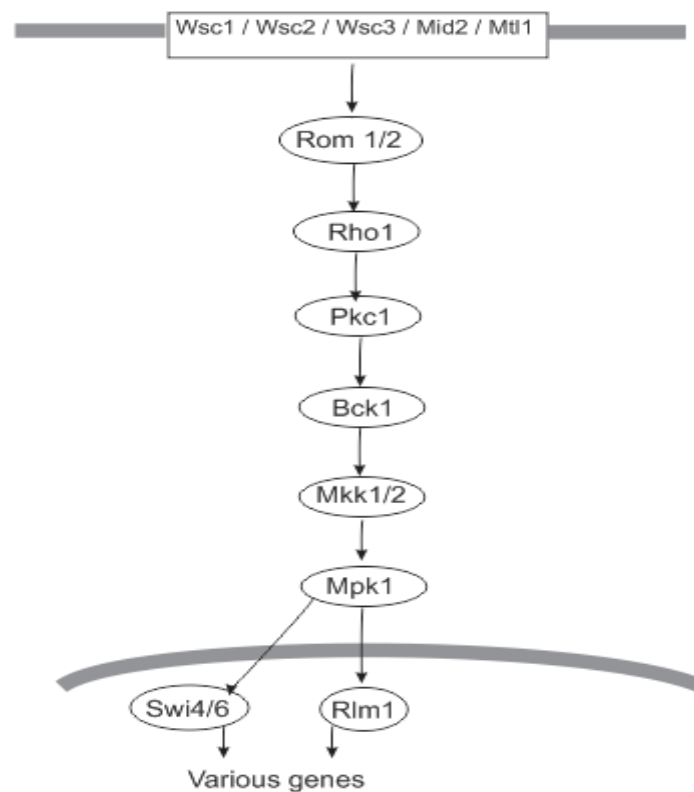


Figure 2.8: Representation of cell wall integrity pathway (as adapted from Levin 2005)

The exact relationship between the CWI pathway and chitin synthesis is, however, still not entirely clear as several studies have shown that chitin synthesis is mostly controlled through post-translational modifications (Chuang and Schekman, 1996; Cos *et al.*, 1998). Although Pkc1p was shown to be able to orchestrate *in vivo* phosphorylation of Chs3 (Valdivia and

Schekman, 2003), the level of phosphorylated Chs3p did not correlate with the stress-induced transport of this protein to the plasma membrane. Therefore the regulatory function of the CWI pathway in Chs3 function remains unclear. García – Rodriguez and colleagues (2000) proposed that another, yet unknown pathway or MAP cascade may be involved in these discrepancies. It should also be noted that the *GFA1* gene contains two sites for the binding of Rlm1p (a transcription factor often associated with this pathway, as can be seen in Fig 2.10) in its promoter. Therefore, in the event of cell wall stress and in response to mating pheromones, the expression of *GFA1* is induced several fold (Lagorce *et al.*, 2002).

2.7.3 Calcium-calcineurin pathway

Under standard laboratory growth conditions, cytosolic calcium ion levels remain relatively low, however, if the yeast cell experiences stressful conditions, the cytosolic levels of Ca^{2+} increase, which in turn activates the protein phosphatase calmodulin which activates calcineurin. The calcineurin protein molecule consists of a catalytic subunit and a regulatory subunit. At the catalytic subunit, the activated form of calcineurin dephosphorylates the transcription factor Crz1p. This contains a zinc-finger motif for binding specifically to the calcineurin dependent response element (CDRE) region in DNA (Stathopolous-Gerontides *et al.*, 1997). Several genes have been identified that contain the CDRE region and are believed to be under Crz1p control, including genes encoding for ion- and small molecule transport proteins and for chitin synthesis such as *CHS1* (Haro *et al.*, 1991; Cunningham and Fink, 1994; Mazur *et al.*, 1995; Mendoza *et al.*, 1994). Under optimal conditions the Crz1p resides in the cytoplasm, but under stressful conditions it is activated by calcineurin and binds to Nmd5p to be transported into the nucleus where it can bind to the promoter of target genes (Matheos *et al.*, 1997). It is hypothesized that the increase in cytosolic Ca^{2+} levels in the yeast cell during stress conditions is due in part to the stimulation of Ca^{2+} import channels and in part to the efflux of Ca^{2+} from storage vesicles. Under certain conditions, Ca^{2+} can be released from storage vacuoles via the stimulation of transporter molecules on the membrane of these vacuoles (Tanida *et al.*, 1995; Groppi *et al.*, 2011).

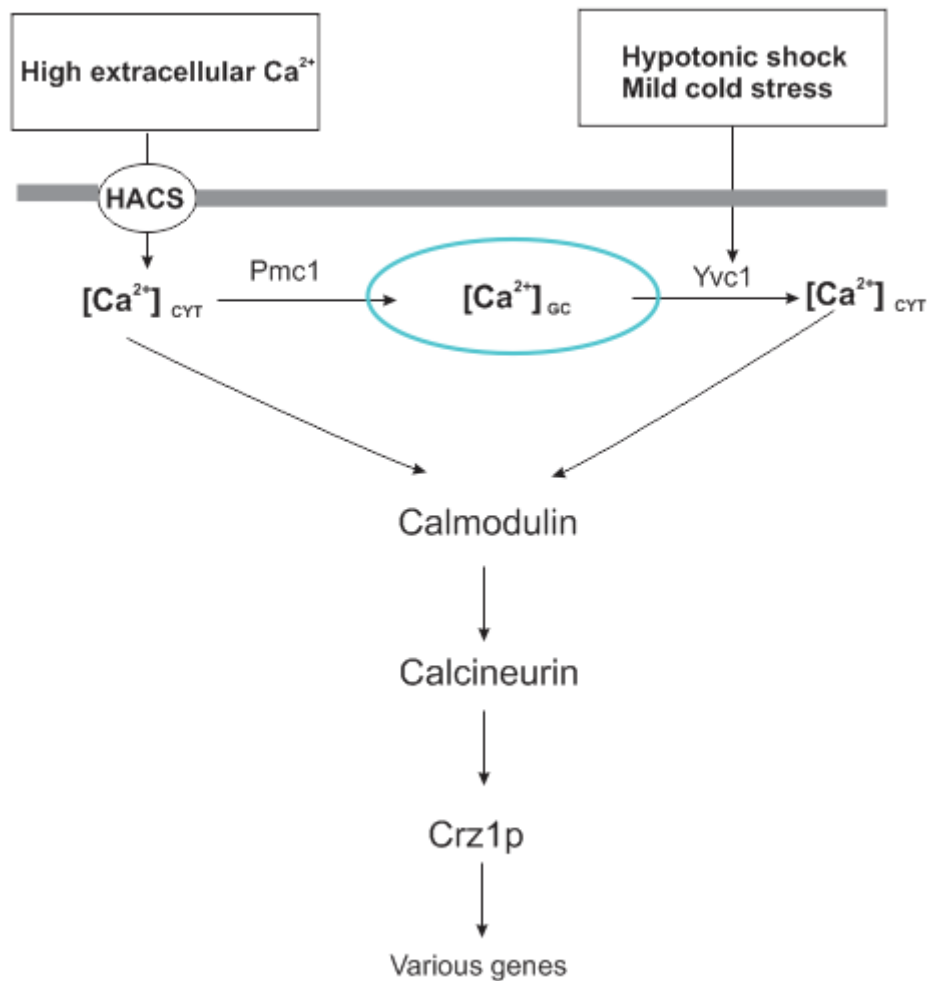


Fig 2.9 Calcium-calcineurin pathway as adapted from Cunningham *et al.*, (2011). High extracellular calcium results in the influx of calcium via a high affinity calcium influx system (HACS) into the cytosol that is both incorporated into vesicles by Pmc1p and results in the activation of calmodulin. Hypotonic shock and mild cold shock result in the release of Calcium ions from vesicles by Yvc1, increasing cytosolic calcium levels which activates calmodulin. Activated calmodulin now activates calcineurin, which in turn dephosphorylates Crz1p which can now bind to the CDRE regions of various genes, enabling their transcription.

2.8 Concluding remarks

Chitin forms a very important component of the yeast cell wall, providing rigidity and stability to this protective structure. Our recent studies have linked fermentation with yeast strains with high chitin levels to a reduction in haze formation in wine. These on-going studies have also shown that grape chitinases, the proteins that have been suggested to be primarily responsible for haze formation, can bind to yeast cell walls, and that the amount of chitinase bound to the cell wall is linearly correlated with chitin levels. These findings motivated this study on chitin and the impact of environmental changes on cell wall chitin deposition in wine yeast strains.

Indeed, the data described in the literature review are almost exclusively based on studies of a limited number of laboratory strains. It has by now been well established that these laboratory strains are not always representative of the genetic diversity and phenotypic plasticity of industrial strains. The subject of the research that is presented in the following chapter was therefore to study the effect of environmental conditions on chitin deposition in wine yeast strains, and evaluating whether the data generated in laboratory yeast were applicable to industrial *S. cerevisiae* wine yeast strains and to the closely related species *S. paradoxus*. Our initial studies had indeed suggested that the cell wall of this species, which is frequently encountered in wine environments, and which displays good fermentative activities, might contain higher levels of chitin than *S. cerevisiae*.

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Chapter 3

Research results

Chitin synthesis in response to
environmental stress

Chapter 3: RESEARCH RESULTS

Chitin synthesis in response to environmental stress

3.1 Introduction

Haziness / turbidity in wine is most often the result of protein denaturation and aggregation. Recently, findings from our own group showed a link between chitin levels and wine haze prevention (Ndlovu, 2012). These results are also supported by studies where chitin addition to wine was reported to result in reduced haze formation (Vincenzi *et al.*, 2005). Such data may also link to observations that suggest that grape chitinase are primarily responsible for haze formation in many, if not most cases of protein precipitation (Marangon *et al.*, 2010; Marangon *et al.*, 2011).

Preliminary data indicate the addition of yeast cells with high cell wall chitin levels to wine may result in lower haze production (Ndlovu, 2012). Knowledge on chitin synthesis in response to fermentation conditions, as well as environmental conditions is therefore important for future studies on the subject. This study therefore aimed to provide insights into the regulation of cell wall chitin levels in various environmental conditions in *S. cerevisiae* and *S. paradoxus* strains that are either commercially used in the wine industry, have been isolated from natural wine fermentation or have previously shown elevated chitin levels as compared to other loosely related strains. Some data is available on chitin levels of laboratory yeast strains and on chitin deposition in response to various extracellular conditions. However, very little data is available on the response of yeast strains commonly used in the wine industry, and about the impact of fermentative conditions on chitin levels in yeast. Furthermore, preliminary data had suggested that levels of cell wall chitin differed significantly between industrial wine yeast strains and *Saccharomyces paradoxus* strains (Ndlovu, 2012).

We investigated the impact of environmental conditions on chitin levels in several yeast strains, and the relationship between chitin levels and the expression of genes that are associated with chitin biosynthesis. The genes chosen for this study encode for enzymes that are part of the chitin biosynthesis pathway from the precursor fructose-6-phosphate, *CHS1-3*, as well as the *CHS 4-7* which aid in the transportation of Chs1-3p. Chs1 and 3p are known to be under spatial and temporal regulation rather than genetic regulation (Pammer *et al.*, 2002). The expression levels of *GFA1*, the gene encoding glutamine-fructose

amidotransferase, has previously been shown in laboratory strains to increase under conditions that result in high chitin levels such as exposure to elevated temperature (Munro *et al.*, 2007). This indicates that the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate by GFA1p is an important step in chitin synthesis for chitin regulation and that transcriptional regulation of *GFA1* plays a very important role in regulation of chitin synthesis upon exposure to changes in external conditions. Very little is known about the regulation of other *CHS* genes and the other genes in the chitin synthesis pathway. *GNA1* encodes for glucosamine-phosphate-N-acetyltransferase, the enzyme responsible for the second step in the synthesis of chitin from fructose-6-phosphate. Literature on this gene, obtained from laboratory strains is divided as to the degree to which *GNA1* is transcriptionally regulated, indicating the importance of including strains other than laboratory strains in a study such as this.

The strains used in this study were chosen either because they are commonly used in the wine industry (EC1118; BM45) or because of previous reports of high chitin levels for these strains (PO146; PO208) (Ndlovu, 2012). In addition, the common laboratory strain BY4742 was included as control for future comparison to strains selected from the deletion libraries. Cells were subjected to conditions previously shown to impact chitin levels in laboratory strains and cell wall chitin levels were determined by staining cells with Calcofluor white and measuring the resulting fluorescence. The data show several conditions such as temperature changes as well as hypo-osmotic conditions and most notably the exposure to high calcium levels in media resulted in increased chitin levels in *Saccharomyces cerevisiae* strains used in the wine industry and *Saccharomyces paradoxus* strains included in this study, while the lab strain, BY4742 only showed a significant increase in chitin synthesis upon exposure to low temperatures. Chitin levels are highly strain dependant, and strains responded differently to the different environmental parameters investigated.

Conditions that have previously been reported to impact chitin levels in laboratory strains were investigated for their impact on wine yeast strains as well as *Saccharomyces paradoxus* strains that have previously shown relatively high chitin levels (Ndlovu 2012). Such conditions included changes in temperature, changes in external calcium conditions and changes in osmolarity. Strains that differed significantly from one another regarding chitin synthesis in response to these conditions were chosen for gene expression analysis.

3.2 Materials and Methods

3.2.1 Strains, media and culture conditions

Table 3.1 lists the strains employed in this study. Yeast cultures were grown at 30°C except for experiments which involve the evaluation of yeast adaptation to various temperatures. Yeast peptone dextrose (YPD) was used as rich media. Minimal media contained 0.67% yeast nitrogen base (YNB) without amino acids, with 2% added glucose (w/v) supplementation.

Table 3.1: Strains used in this study.

| Strain | Relevant Genotype | Source or reference |
|---------------------------------|---|-----------------------------------|
| <i>Saccharomyces cerevisiae</i> | | |
| EC1118 | Commercial wine yeast strain | Lallemand Inc. (Montreal, Canada) |
| BM45 | Commercial wine yeast strain | Lallemand Inc. (Montreal, Canada) |
| BY4742 | <i>Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i> | Brachmann <i>et al.</i> , 1988 |
| <i>Saccharomyces paradoxus</i> | | |
| P01-146 | Unknown origin | Phaff collection |
| P02-208 | Olive brine isolation | Phaff collection |

For fermentation, synthetic grape medium (MS300) was used as described by Bely and colleagues (1990).

3.2.2 Staining and flow cytometry

Cells were stained with Calcofluor white according to an adapted method from De Groot *et al.* (2001). 2ml of the cell culture of each different condition was centrifuged 11000 RPM, 2min and the cells were washed with Phosphate Buffered Saline (PBS) (pH 7.4). PBS was made by adding the following (g/l): 8.01 NaCl, 0.20 KCl, 1.78 Na₂HPO₄·2H₂O and 0.27

KH_2PO_4 then resuspended in 2ml PBS buffer. Cells were stained with 20 μl of Calcofluor white stain after adding 20 μl KOH (10%), following the manufacturer's instructions (Fluka Analytical, Sigma-Aldrich). Fluorescence of Calcofluor white was quantified for the measurement of chitin-levels with flow cytometry, using a BD FACS Aria flow cytometer. BD FACS Diva v.6.1.3 software was used for the data capture. 50, 000 cells were used for the quantification of chitin levels and the treatment to each condition was done in triplicate for each strain.

To assess chitin level at different temperatures, cells from overnight precultures in YPD broth (2% glucose) were grown at 15°C, 30°C or 37°C for duration of 5 hours. Thereafter, 2ml of the culture was sampled and cells were harvested by centrifugation.

In order to take into account changes in nutrient content after media transfers, hypo-osmotic growth conditions were created through a two-step protocol. Cells grown from an overnight pre-culture in YPD media were first transferred to a medium containing YPD diluted to 20% the original strength with 1M sorbitol. After 5 hours, they were transferred back to a medium containing 20% YPD to create a hypo-osmotic stress. As controls, cells were either grown continuously in high osmolarity (20% YPD (0.4% glucose) with 1M sorbitol) or low osmolarity, 20% YPD (0.4% glucose) without transfers.

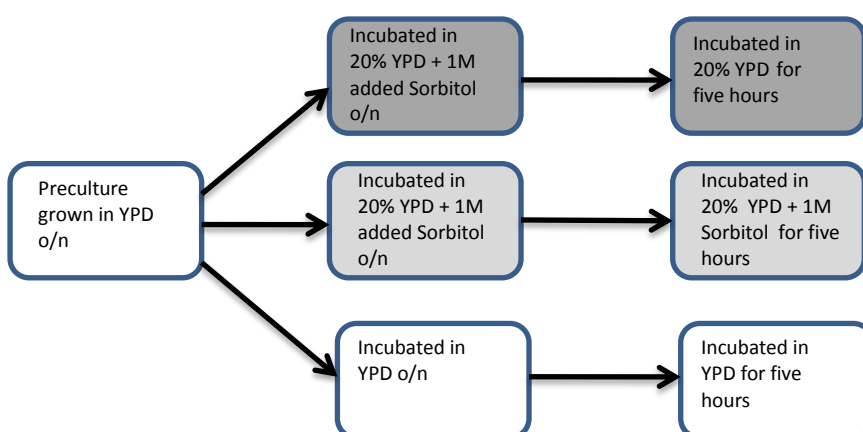


Fig 3.1: Depiction of the steps employed to create a hypo-osmotic environment as well as creating the two control environments. The top path represents the hypo-osmotic treatment condition, the second path represents the first control and the bottom path represents the last control.

A washing step was incorporated after staining with Calcofluor white to determine the necessity of washing cells after staining with the chitin-binding fluorescent dye Calcofluor white. Cells from overnight preculture were grown for 5 hours in YPD broth with 0.1M added calcium. 2ml of cells were harvested at 11000RPM for 2mins, washed with PBS buffer. Cells were then stained with 20ul Calcofluor white and 20ul KOH (10%) and left for 10 mins at room temperature (approx. 25°C). Thereafter, cells were washed with PBS again, resuspended in PBS and fluorescence measured by flow cytometry.

3.2.3 Fermentation conditions and monitoring

Yeast strains were grown to stationary phase in rich media (YPD 2% glucose). Cells were harvested, washed and inoculated into fermentation media to an optical density of 0.1, as determined by spectrophotometric analyses at 600nm. Fermentations were carried out at 30°C in 250-ml Erlen Meyer flasks with a 100 ml working volume with air traps in triplicate. Strains of table 3.1 were used to ferment chemically defined synthetic MS300 media (Bely *et al.*, 1990) for 21 days. Every two days flasks were measured and weight loss calculated. On day 0, 3, 7, 14 and 21, 2ml of cells were harvested, stained with 10% KOH and Calcofluor white and fluorescence was measured.

3.2.4 Preparation of yeast total RNA

Yeast starter cultures were grown o/n in YPD at 30°C then triplicate flasks of 100ml YPD broth was inoculated to an optical density of between 0.01 - 0.1 as determined by spectrophotometric absorbance at 600nm.

In the case of the temperature study, cells were then subjected to 37°C for between 0 to 60mins, cells were then harvested, washed with dH₂O and re-suspended in AE buffer (50mM sodium acetate, 10mM EDTA, pH5.0). A control was included where cells were simply left at 30°C and cells were then harvested at timepoints 0, 2, 15, 30 and 60mins.

In the case of the evaluation of genes under conditions of high calcium, after OD₆₀₀ 0.01-0.1 was reached, calcium was added to cells at the OD₆₀₀ of 0.5 to create a final concentration of 0.1M. Cells were then re-incubated at 30°C for 0-60min. Cells were then harvested, washed with 0.1M EDTA and re-suspended in AE buffer (50mM sodium acetate, 10mM EDTA, pH5.0). A control was included where no calcium was added and cells were simply left at 30°C and cells were then harvested at 0-60mins.

Total RNA was isolated from all samples as described previously (Schmitt *et al.*, 1990).

3.2.5 Quantitative PCR

All individual qPCR reactions were performed in duplicate. RNA was treated with DNase I (Roche diagnostics, Basel, Switzerland). Approximately 1µg total RNA was used as template for cDNA synthesis using ImProm-II™ reverse transcriptase system according to the manufacturer's instructions (Promega). Primers used for QRT-PCR analysis are listed in Table 3.3. Primer designing was performed using Primer Express software ver. 3 (Applied Biosystems, CA, USA). Regions of the open-reading frame of *S. paradoxus* P02-208 were sequenced for the design of qPCR primers. Reagents were obtained from Applied Biosystems and Kapa Biosystems (Cape Town, South Africa). The dye SYBR Green was used for amplicon detection and primers were diluted to a final concentration of 100nM. QRT-PCR runs were performed using the 7500 cycler (Applied Biosystems, CA, USA)

The runs consisted of the following conditions: 50°C for 2minutes, 95°C for 10 minutes, 95°C for 15 seconds repeating for 40 cycles and lastly 60°C for 1 minute and a dissociation curve analysis was added for confirmation of primer specificity. Signal Detection Software (SDS) vs 1.3.1 (Applied Biosystems) was used for initial analysis of data where the relative expression value for each sample was defined as $2^{-Ct(target)}$ with Ct(target) representing the cycle number at which a sample reaches the threshold value for the specific gene. The relative expression data was normalised to the value of the normalisation gene *PDA1* due to stability of this gene compared to other genes evaluated (TOP1; 18S). This gene has also previously shown to be a stable housekeeping gene (Wentzel *et al.*, 1995). In each respective sample with the resulted normalised relative expression for a target gene calculated by $2^{Ct(PDA1) - Ct(target)}$.

Table 3.2: Sequences of primers used to sequence regions of *S. paradoxus* P02-208 genes

| Sequencing primers used in this study | Primer sequence 5'-3' |
|---------------------------------------|-----------------------|
| CHS1_F_Spar_seq | ACCAATCAAACCGAGAGGAC |
| CHS1_R_Spar_seq | CTCTTTGATCGATGTAACCGC |
| CHS2_F_Spar_seq | CGAGAAATCCATTCATGGTGG |
| CHS2_R_Spar_seq | GGCAAGTCTGAGACCATTCT |
| CHS3_F_Spar_seq | GACCGGTTTGAATGGAGATG |
| CHS3_R_Spar_seq | CCAAATGTCAGGAAAGCCAC |
| CHS5_F_Spar_seq | CAGTAGGTAAGTTGGATGCC |
| CHS5_R_Spar_seq | GTGCTTTGCCTTTATCAGCTC |
| CHS6_F_Spar_seq | CACCTGGTGGCGATTATAC |
| CHS6_R_Spar_seq | GTAGAATCCCATCCGCAGTC |
| CHS7_F_Spar_seq | CCATATGCTCGAAGACTCCG |
| CHS7_R_Spar_seq | GCCCACAAGTTTCGAAGTAC |
| GFA1_F_Spar_seq | CTGGTGGAAAGATCCAGAGG |
| GFA1_R_Spar_seq | GGCCCAAGCCAAATGATTTG |
| GNA1_F_Scer_seq | GGATTTGGAACAGGTCCTGAG |
| GNA1_R_Spar_seq | CACGCCTGCATTACTAAACC |
| PCM1_F_Spar_seq | GCTATTGCGCAAACCTCTACG |
| PCM1_R_Spar_seq | CGGACCACCGATACCATTAG |
| QRI1_F_Spar_seq | GGAAAGCTTGTCTAGCCAAG |
| QRI1_R_Spar_seq | TCCACCATTTCCATCTGGAG |
| SKT5_F_Spar_seq | CAAGTTCACCGCAGATACATC |
| SKT5_R_Spar_seq | CGTACTGGAACAACACCTCC |

Table 3.3: Sequences of primers used in quantitative real-time PCR analysis of both *S. cerevisiae* EC1118 and *S. paradoxus* P02-208

| Quantitative real-time PCR oligonucleotide primers used in this study | Primer sequence (5'→3') |
|---|---------------------------|
| CHS1_F_QPCR | CGTCCCCCACCAGCAA |
| CHS1_R_QPCR | CGACGGTAGCTGTTGACTATTCG |
| CHS2_F_QPCR | AGTTGCTCCGAACAGGTATGCT |
| CHS2_R_QPCR | ACAGCCTCGCCTGCTCTTT |
| CHS3_F_QPCR | TCACCTGGATGTTTTACCATCAAG |
| CHS3_R_QPCR | CCACTCCGACGAGTTGCAT |
| CHS5_F_QPCR | GGTAATTCAAATCCATTCAAAGTG |
| CHS5_R_QPCR | GTATGGCGTATCAACGGAAAGAC |
| CHS6_F_QPCR | ACGATGCTGACGATACTTTCCA |
| CHS6_R_QPCR | CAGGTCAGTGGTTTTCTTGTTACA |
| CHS7_F_QPCR_2 | CATGCTAGGATTCATAGCCAATTTT |
| CHS7_R_QPCR_2 | TGGTCGGTGATCCATGCTT |
| GFA1_F_QPCR_3 | CAATCCAGGGCTTTCCTATCA |
| GFA1_R_QPCR_3 | CGCATCCGAAGAAACAAAAA |
| GNA1_F_QPCR_2 | CGCACCGAGACGATTGC |
| GNA1_R_QPCR_2 | CCACATAGCCCCAATTCATGA |
| PCM1_F_QPCR | TCACGGCGTCGCACAA |
| PCM1_R_QPCR | TCCGGTTCACGATCTTGAC |
| QRI1_F_QPCR | ACTGGCGTTTAGGCCTTGAA |
| QRI1_R_QPCR | CCGCCAGCCATTAAAATCAC |
| SKT5_F_QPCR | GGAAAACAGGGACAATGAAGACTT |
| SKT5_R_QPCR | TGGTTTGCCGACCGCTTA |

3.2.6 Statistical analysis

Standard deviations from the mean are calculated as deviation from three biological replicates and in the case of gene expression data, two technical replicates. Statistical comparisons between values and the corresponding controls were performed by T-tests and in the case of comparison of a specific strain compared to other strains exposed to the same condition, ANOVA using Statistica vs. 10 ($p > 0.05$).

3.3 Results

3.3.1 Evaluation of chitin measurement through fluorescence: Method optimisation

The validity of flow cytometry data was investigated. Two issues arose: The first was that data sets showed absolute values that tended to differ for each run, a run referring to a set of data comparing all strains or conditions. However, the trends and relative values were consistent between independent biological repeats. The data are therefore shown in arbitrary units to reveal these consistent trends. Secondly, the impact of a washing step after staining cells was investigated (Fig 3.2). The trends seen between different strains remained the same for washed and unwashed samples (Fig. 3.2). In this figure, it is apparent that upon exposure to 37°C the two *S. paradoxus* strains show the highest chitin levels, with P01-146 displaying the highest fluorescence. However, higher values were observed for the washed cells when compared to unwashed cells. This difference can be explained by the increased background fluorescence in samples of unwashed cells. However, the evaluation clearly shows that a washing step is not essential as the values of different strains were equally affected by the incorporation of the washing step.

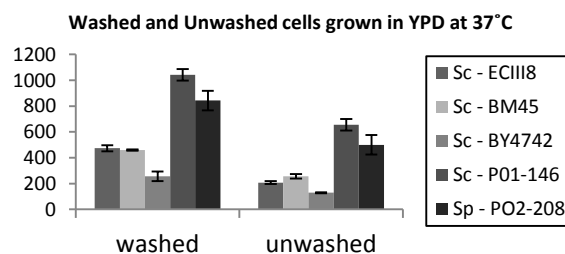


Figure 3.2: Cell wall chitin levels as calculated by measuring the fluorescence of chitin-binding dye Calcofluor white for *S. cerevisiae* (EC1118; BM45 and BY4742) or *S. paradoxus* (P01-146; P02-208) strains to distinguish cells washed or not washed after dying with the fluorescent dye Calcofluor. Error bars indicate variations between biological repeats.

3.3.2 Fermentation conditions in synthetic wine do not significantly impact chitin synthesis

To evaluate chitin levels in the five selected yeast strains during fermentative growth, strains were grown in synthetic grape must MS300 and chitin measurement was performed. Weight loss, reflecting CO₂ release and therefore fermentation progress was measured every two days starting from day 1 to day 21 when no more significant weight loss could be detected. Weight loss was similar for all strains, with the haploid laboratory yeast BY4742 fermenting at slightly slower speed than the other strains. However, the total weight loss over 21 days was similar in all strains (Fig 3.3). Samples were taken on day 0, 3, 7, 14, and 21 and chitin levels of these samples were measured (Fig 3.4 A; B; C; D; E). It should again be mentioned that flow cytometry arbitrary values obtained in different runs cannot be compared, so a judgement cannot be made on chitin level changes for each strain over a period of time. Rather values of different strains can be compared to one another. Significant differences were observed between strains, with *S. cerevisiae* EC1118 consistently showing the lowest chitin levels and *S. paradoxus* strain P02-208 the highest chitin levels of all the strains throughout the fermentation. Interestingly, chitin levels of the strains did not appear to vary significantly from one another throughout the fermentation. Of the *S. cerevisiae* strains, the strain BM45 showed the highest levels of fluorescence from the third day onward. From the graph it can be observed that day 3 is in the exponential phase of weight loss during fermentation.

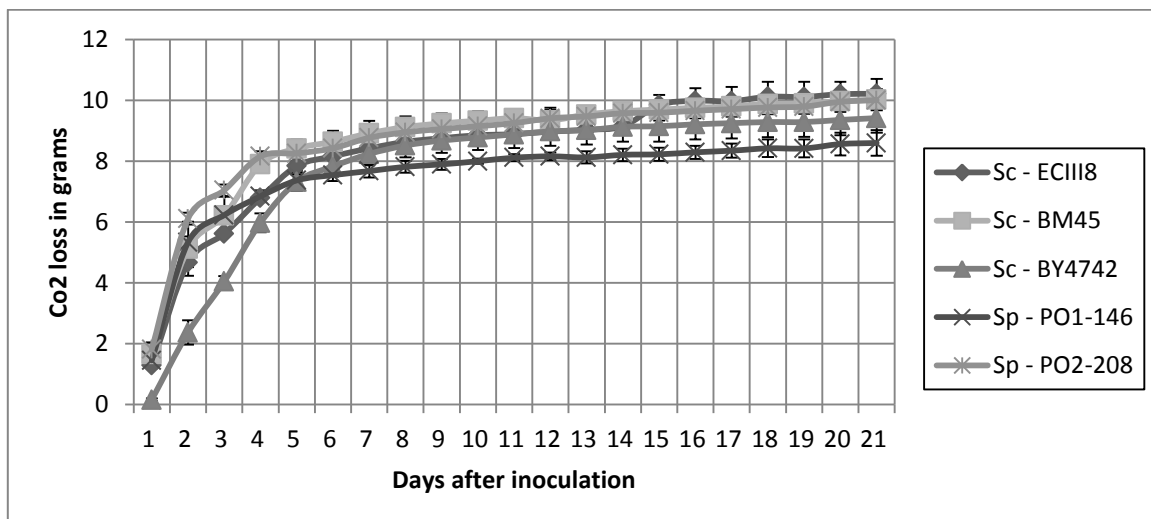


Figure 3.3: Weight loss curve of fermentations performed in synthetic wine MS300. Fermentations were weighed every two days until day 21 when no significant weight loss could be detected. Error bars indicate the standard deviations between biological repeats.

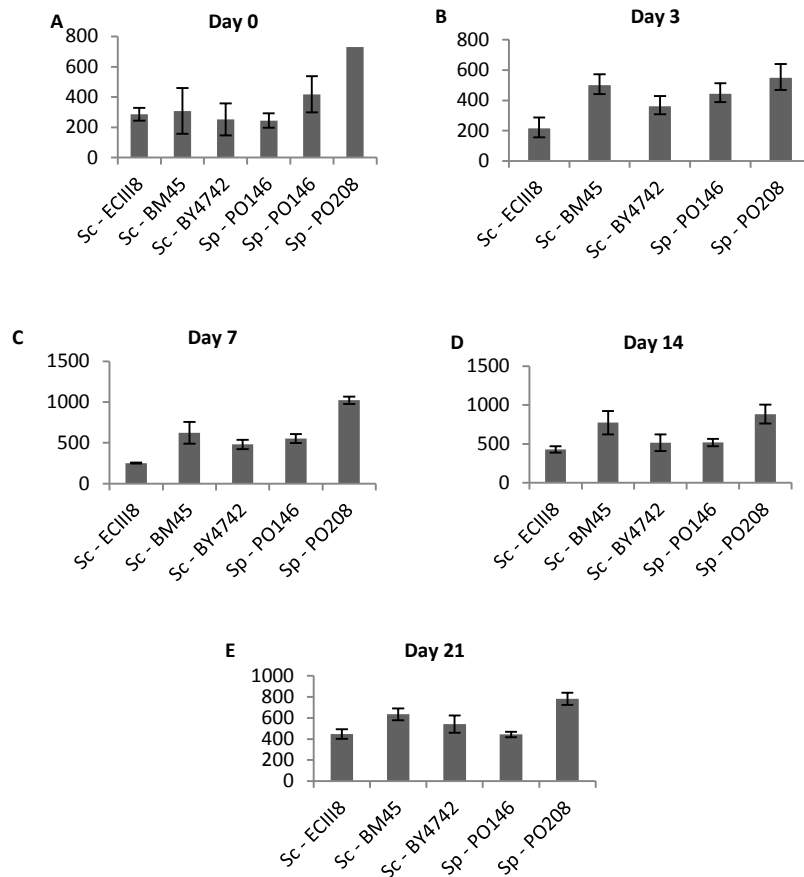


Figure 3.4: Fluorescence intensities of Calcofluor white throughout fermentation in MS300 synthetic wine on (A) Day 0; (B) Day 3; (C) Day 7; (D) Day 14 and (E) Day 21. Error bars indicate standard deviations between biological repeats.

3.3.3 Temperature changes

All strains grown in rich media of YPD (2% glucose), with the exception of the laboratory strain *S. cerevisiae* BY4742, showed an increase in chitin levels when grown at 37 degrees temperatures for five hours (Fig 3.5A) when compared to the control grown at 30 degrees. At this elevated temperature, two *S. paradoxus* strains showed a significantly higher increase of chitin levels when compared to the three *S. cerevisiae* strains, with P01-146 showing the highest increase with EC1118 showing barely any increase. In all strains, growth at a lower temperature of 15 degrees also led to significant increases in chitin levels. In this case, it was *S. paradoxus* P02-208 that exhibited the highest increase in fluorescence. Interestingly, strain P01-146, increased chitin in line with the *S. cerevisiae* strains, indicating that chitin deposition of the strains responded differently and in a strain-specific manner to changes environmental conditions. Notably, the laboratory strain, *S. cerevisiae* BY4742, showed a larger increase in chitin levels upon exposure to decreased

temperature than wine fermentation strain *S. cerevisiae* EC1118. Once again, the strain dependence of chitin synthesis in response to stress conditions is highlighted, as well as the importance of a study employing not only laboratory strains, but also strains isolated from nature. For all *S. cerevisiae* strains, higher chitin levels were synthesised upon exposure to lower temperature of 15°C than when exposed to elevated temperature of 37°C. For both *S. paradoxus* strains the average chitin levels were not significantly different between 15°C and 37°C. Similar to fermentation conditions at 30°C (after day 3); *S. cerevisiae* BM45 showed significantly higher chitin levels than *S. cerevisiae* EC1118 upon exposure to decreased temperature of 15°C, however, the difference between the chitin levels of the two strains was not as large as was seen under fermentation conditions. At an increased temperature of 37°C, the apparent difference between chitin levels of BM45 and EC1118, did not prove significant in analysis.

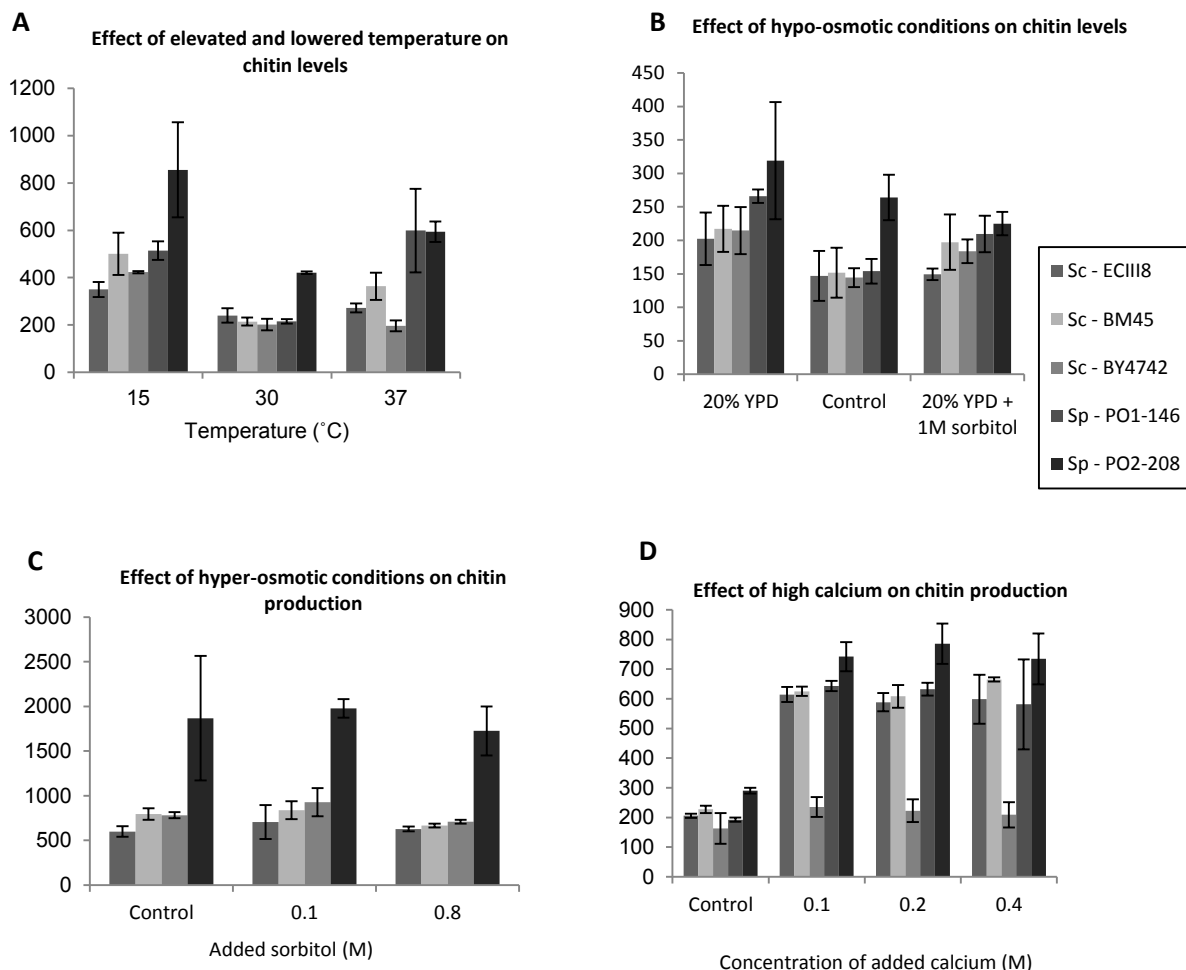


Figure 3.5: Different cell wall chitin levels in cell wall of *S. cerevisiae* (EC1118; BM45 and BY4742) or *S. paradoxus* (P01-146 and P02-208) strains. Intensity of chitin-binding dye, Calcofluor white fluorescent signal was used as measurement of chitin levels. Error bars indicate the differences between the three biological repeats.

3.3.4 Osmolarity changes

The results show that chitin levels increased significantly from both controls when cells are exposed to hypo-osmotic conditions for all strains except P02-208 (Fig 3.5B). For this strain the apparent increase was not significant. An overall increase in the majority of strains could be seen from nutrient-poor, high sorbitol control (hereafter referred to as control A) to undiluted YPD (2% glucose) without added sorbitol (hereafter referred to as control B), which may possibly be related to nutrient starvation. However, chitin levels of P02-208 decreased from control A to control B and *S. cerevisiae* EC1118 showed no significant difference in chitin levels between the two controls. *S. paradoxus* P02-208 showed the highest chitin levels in both controls. However, despite the overall increase in chitin upon exposure to hypo-osmotic conditions there was no significant difference between chitin levels of any of the strains. The *S. paradoxus* strain P02-208 showed the lowest increase in chitin levels upon exposure to hypo-osmotic conditions as compared to control A. Due to time limitations the experiment was not repeated, however even with the strong deviation of P02-208 the data remains clear.

In order to rule out the possibility that the increase of chitin levels from control A to control B was due to an increase in osmolarity the effect of adding different concentrations of sorbitol was studied. In our study, hyper-osmotic conditions were created by the addition of sorbitol (0.1M and 0.8M respectively) to nutrient-rich media, for two *S. cerevisiae* and two *S. paradoxus* strains respectively. The results from this study indicate no notable increase in chitin levels between different concentrations of sorbitol (Fig 3.5C). *S. paradoxus* P02-208 showed the highest levels of chitin throughout the different concentrations of added sorbitol, with chitin levels of other strains portraying similar values to one another. These results indicate that none of the strains adjusted chitin levels in the cell wall upon exposure to hyperosmotic conditions.

3.3.5 Extracellular calcium

In order to determine the impact of a change in extracellular calcium concentrations on cell wall chitin levels, cells were incubated in rich media (YPD 2% glucose) containing different concentrations of calcium (0.1M; 0.2M and 0.4M) (Fig 3.5 D). Results show a significant increase in chitin levels when 0.1 M of calcium was used compared to the control conditions (30°C YPD, no added calcium) for all strains except BY4742, but chitin levels did not increase further when higher Ca^{2+} concentrations were applied. *S. paradoxus* P02-208

showed the highest chitin levels throughout the study. With the exception of lab strain *S. cerevisiae* BY4742, all strains appear to have increased chitin levels by equal amounts. This indicates the necessity of confirming data obtained from laboratory strains on strains isolated from elsewhere.

The possibility of calcium dichloride affecting the fluorescence of Calcofluor white was also examined. Cells grown in nutrient rich media were briefly exposed to 0.1M calcium before harvesting cells. Total exposure to calcium from the addition of the media to centrifuging was approximately 1min. The results showed no significant increase in fluorescence (Fig 3.6B) and trends between strains remained the same, indicating that the increase in fluorescence observed in the treated cells was indeed due to increased deposition of chitin in the cell wall.

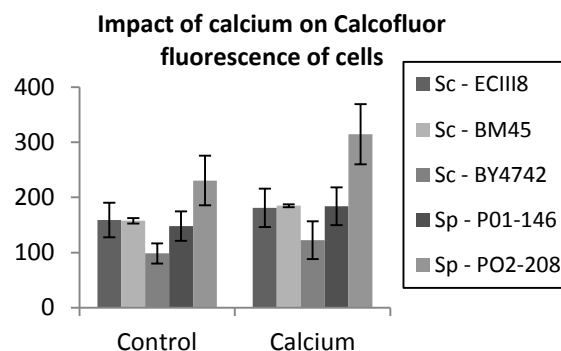


Figure 3.6: Cell wall chitin levels as calculated by measuring the fluorescence of chitin-binding dye Calcofluor white for *S. cerevisiae* (EC1118; BM45 and BY4742) or *S. paradoxus* (P01-146; P02-208) strains to distinguish the fluorescence of Calcofluor white with cells only briefly exposed to calcium from media where cells have not been exposed to calcium. Error bars indicate variation between biological repeats.

3.3.7 Gene expression

The gene expression levels of chitin synthesis related genes were analysed for *S. paradoxus* strain P02-208 and *S. cerevisiae* EC1118 under two conditions which led to a high increase in chitin levels; 37°C and 0.1M added Ca²⁺, and using the two strains with the highest difference in fluorescence levels as observed in the previous data sets. The genes chosen for this targeted gene expression analysis were those encoding for enzymes of the chitin synthesis pathway from the precursor fructose-6-phosphate (*GFA1*; *GNA1*; *PCM1*; *QRI1*; *CHS1*; *CHS2*; *CHS3*). Additionally, expression levels of genes encoding for chitin synthase helper proteins (*SKT5*; *CHS5*; *CHS6*; *CHS7*) were analysed. RNA extraction was performed at time-points 0, 2, 15, 30 and 60 minutes. The expression data for qRT-PCR was normalised relative to the housekeeping gene *PDA1*. The first time-point is representative of

the exponential phase and was taken at optical density of 0.5 at 600nm. For both strains there was no change in expression for any of the genes involved in chitin synthesis, except *GFA1*. Significant increase could be observed in regulation of this gene during both conditions (Fig 3.6; 3.7), although with added calcium the increase could only be seen at the time-point 2 minutes. T-tests were performed to confirm the significant increase in expression levels for both strains at time-points 2 minutes and 15 minutes. The two strains showed similar gene expression levels throughout the study, perhaps indicating how closely related the two strains are to one another. The increase in *GFA1* expression levels was, however, more severe for P02-208 compared to EC1118 at 37°C. Although this may be an indication of the regulation behind the increase in chitin synthesis, the increase in gene expression is not proportionate to the increase in chitin levels. Upon the addition of 0.1M calcium, regulation of *GFA1* was remarkably similar for both strains despite different chitin levels for the different strains under these conditions. This may indicate that another regulation mechanism is involved.

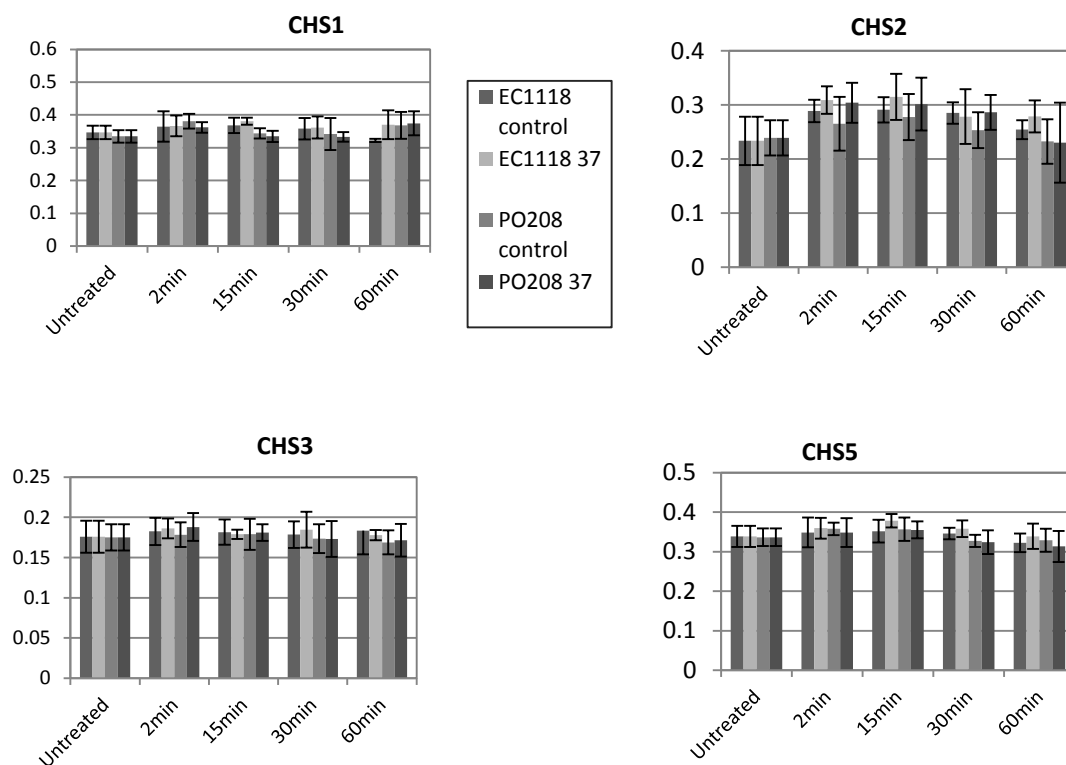


Figure 3.7: qRT-PCR relative expression levels of chitin synthesis related genes in *S. cerevisiae* EC1118 and *S. paradoxus* P02-208 after cells were incubated at 37°C. Expression data was normalised to the expression levels of *PDA1*. Error bars indicate the standard deviation between three biological repeats.

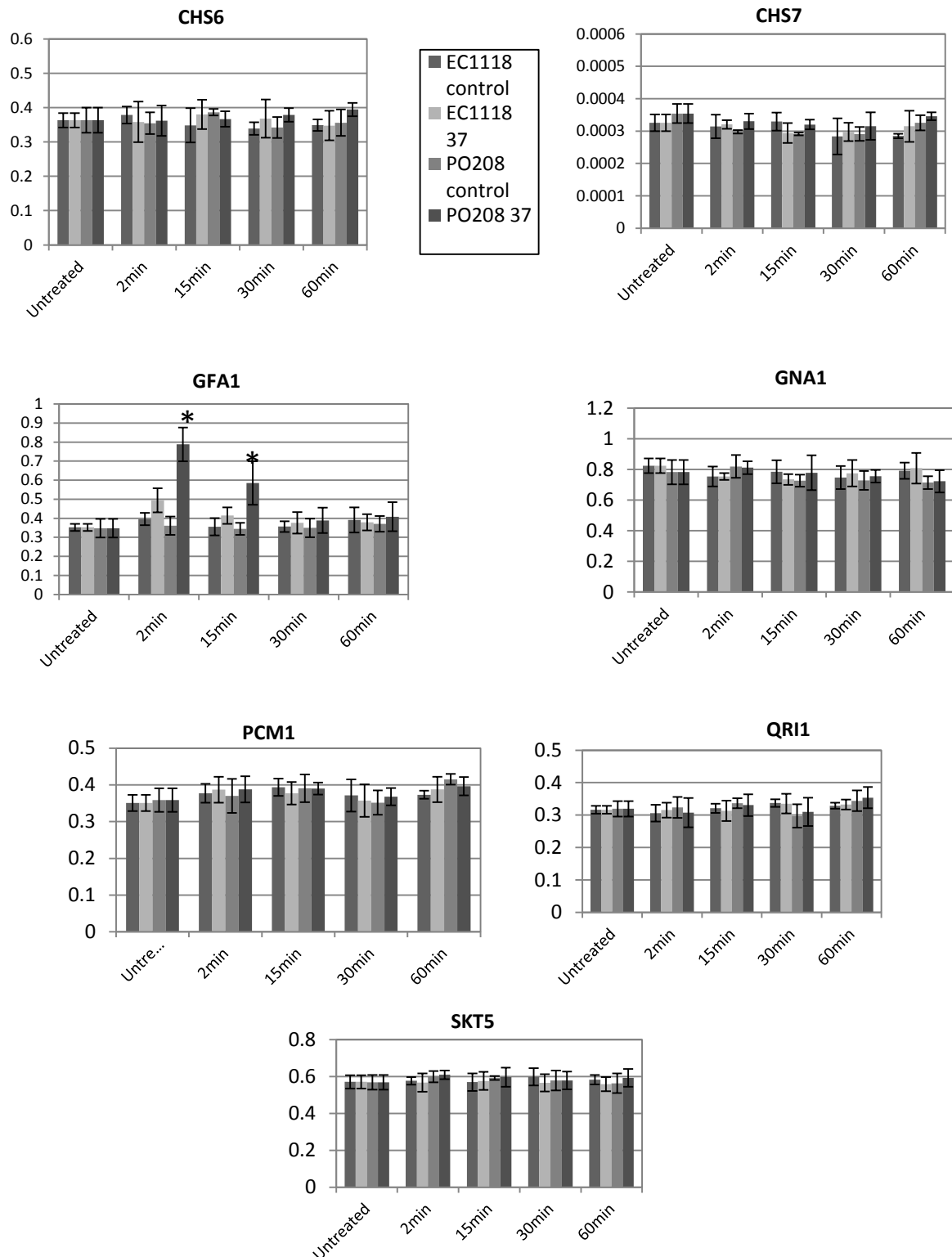


Figure 3.7 (continued): qRT-PCR relative expression levels of chitin synthesis related genes in *S. cerevisiae* EC1118 and *S. paradoxus* P02-208 after cells were incubated at 37°C. Expression data was normalised to the expression levels of *PDA1*. Error bars indicate the standard deviation between three biological repeats. * indicates values that is significantly different from untreated timepoint for that specific strain. The value of $P < 0.05$ was considered significant.

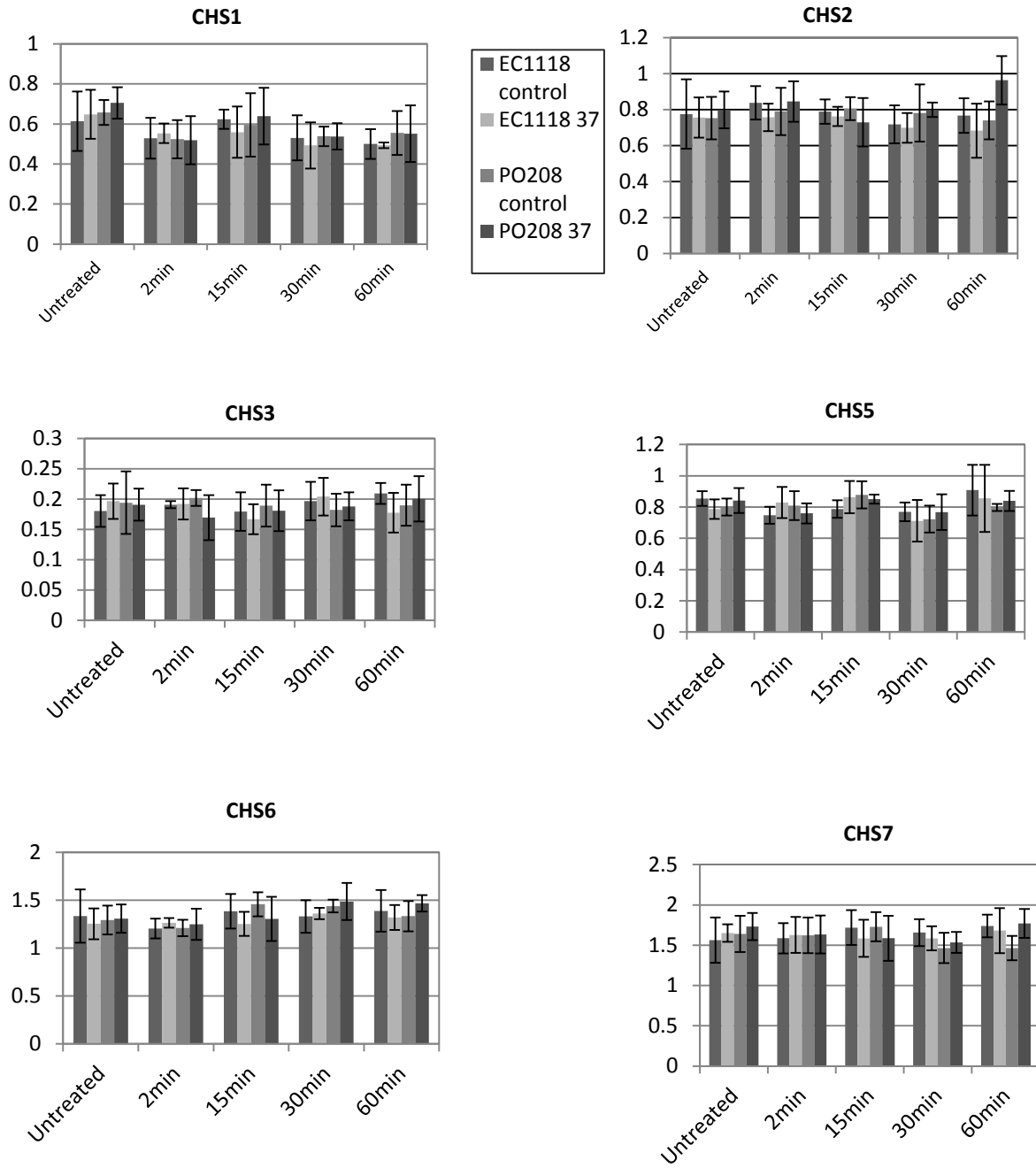


Figure 3.8: qRT-PCR relative expression levels of chitin synthesis related genes in *S. cerevisiae* EC1118 and *S. paradoxus* P02-208 after 0.1M calcium were added to cells. Expression data was normalised to the expression levels of *PDA1*. Error bars indicate the standard deviation between three biological repeats.

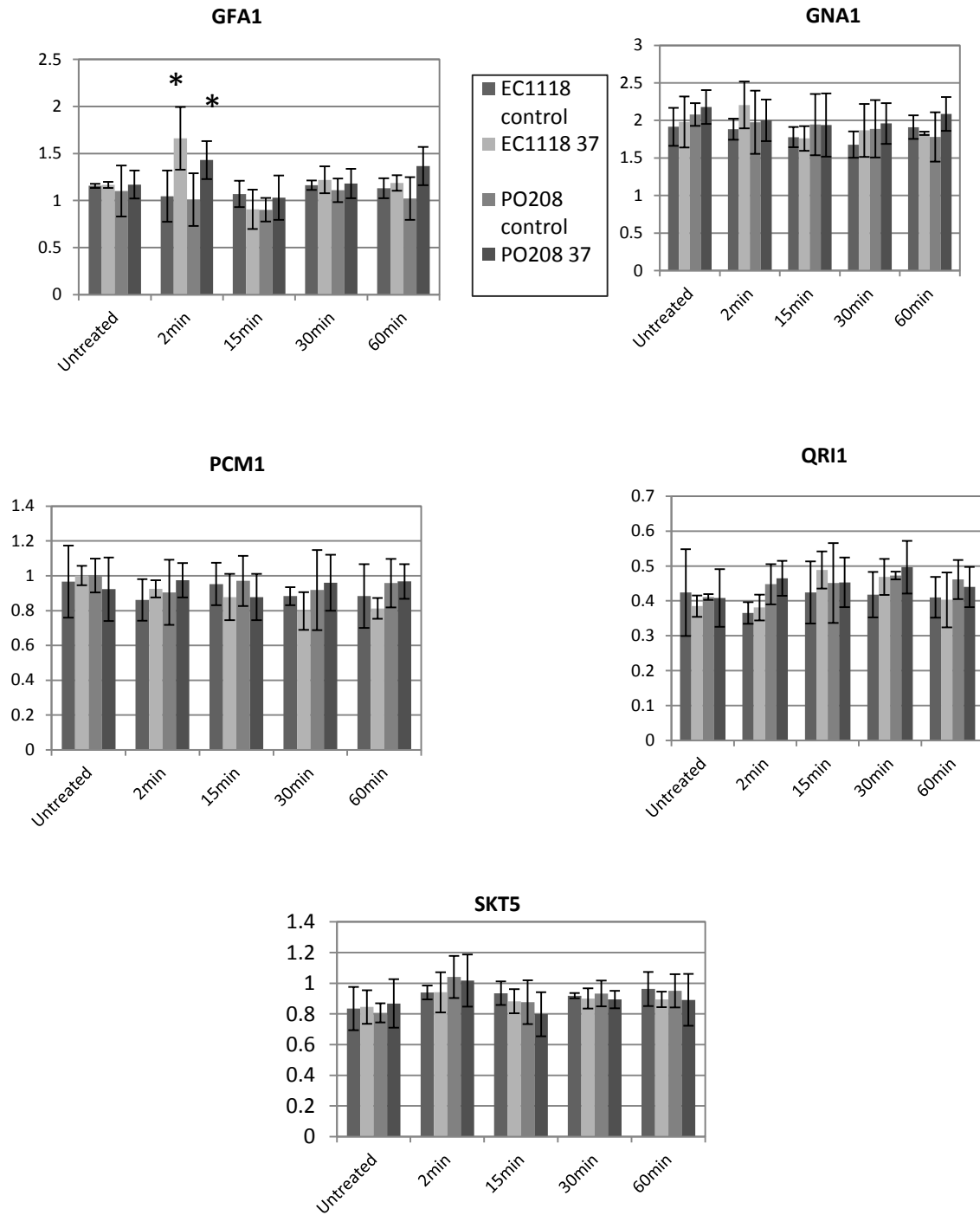


Figure 3.8 (continued): qRT-PCR relative expression levels of chitin synthesis related genes in *S. cerevisiae* EC1118 and *S. paradoxus* P02-208 after 0.1M calcium were added to cells. Expression data was normalised to the expression levels of *PDA1*. Error bars indicate the standard deviation between three biological repeats. * indicates values that are significantly different from the untreated timepoint for the same strain. The value of $P < 0.05$ was considered significant.

3.4 Discussion

This study is the first to evaluate the effect of environmental change on chitin deposition in commercial wine yeast strains of *Saccharomyces cerevisiae* whilst also including certain strains of *Saccharomyces paradoxus*. Only some of the conditions applied in this study resulted in a change in cell wall chitin levels and these chitin levels were strain dependant. The results suggest *GFA1* genetic regulation is partially, but not completely responsible for an increase in chitin levels due to environmental conditions in both *S. cerevisiae* EC1118 and *S. paradoxus* P02-208 irrespective of differences in chitin levels between the two strains. Our gene expression findings therefore do not contradict previous studies employing laboratory strains, which suggested that chitin synthesis is primarily regulated at a post-translational level.

Data on chitin levels indicate that chitin deposition is largely strain dependent and is not, as previously hypothesised, a species-dependent difference between *S. paradoxus* and *S. cerevisiae* (Ndlovu, 2012). From our results, it is also very clear that each condition is treated differently by the cell and that an increase in chitin levels is not simply a general stress response.

The data on the impact of environmental factors on chitin levels from *S. paradoxus* strains as well as from wine yeast strain broadly confirm previous datasets obtained in laboratory strains. The temperature response suggests that chitin deposition in *S. cerevisiae* wine yeast strains as well as *S. paradoxus* strains is also regulated by this parameter. This correlates to previous studies where mild heat shock (37°C) was shown to increase dry weight chitin levels in *S. cerevisiae* (Aguilar-Uscanga and François, 2003; Munro *et al.*, 2007). Valdivia and Schekman (2003), observed increased localisation of *CHS3* from chitosomes where the enzyme is stored, to the plasma membrane where it is active. Decreased temperature (10°C or 25°C) has also been linked to an increase in *CHS1*, *CHS3* and *GNA1* expression, which correlates to the results of this study showing high chitin levels when cells are incubated at 15°C (Gasch *et al.*, 2000; Sahara *et al.*, 2002).

Our results on hypo-osmotic conditions correlate with an association between hypo-osmolarity and elevated extracellular chitin levels in *Benjaminiella poitrassi* (Deshpande *et al.* 1997). The increase of cell wall chitin in response to hypo-osmotic conditions could be an

adaptation of the yeast cell to increase cell wall rigidity to avoid cell bursting if water enters the cell via osmosis. Despite several reports previously implicating the High-osmolarity glycerol (HOG) pathway (A pathway responsible for glycerol synthesis during hyper-osmolarity stress) genes to chitin synthesis (García-Rodríguez *et al.*, 2000); hyper-osmolarity in this study showed no increase in chitin synthesis, correlating to previous findings (Deshpande *et al.*, 1997).

Our data pertaining to the addition of calcium to media is corroborated by a study where elevated extracellular calcium levels were observed to increase cell wall chitin levels in *Candida albicans* as well as stimulating *CHS* promoters (Munro and colleagues, 2007). Yoshimoto and colleagues (2002), reported an increase in expression levels of *CHS1* and *CHS3* upon the addition of calcium to media for the yeast *S. cerevisiae*. In the same study the addition of 0.1M Mg^{2+} ions to growth media did not stimulate *CaCHS* promoters, indicating that the perhaps the impact of Ca^{2+} ions on chitin synthesis is not due to the presence of a divalent cation (Munro *et al.*, 2007). High extracellular calcium is a complex condition which simultaneously impacts pH level of the media, confers cation toxicity and activates the calcium-calcineurin pathway (Yoshimoto *et al.*, 2002; Munro *et al.*, 2007). The promoter region of *CHS1* has also been found to contain the Crz1p binding site, CDRE (Calcineurin-dependant-response-element) region (Yoshimoto *et al.*, 2002). This information suggests the calcium-calcineurin pathway may play a large role in chitin synthesis. However, although this pathway has been linked to mild heat shock, the expression of *CHS1* was not found to be increased at 37°C (Gasch *et al.*, 2000). Munro and colleagues (2007), also found an increase in *CHS1* expression and activity upon exposure to extracellular calcium, regardless of a deletion of *CRZ1*. These findings possibly indicate that cross-talking may play a significant role and that other factors besides the calcium-calcineurin pathway may be involved in increased chitin synthesis during these conditions. Further investigation is necessary regarding the effect of calcium on the cell wall to determine the mechanism by which calcium increases chitin levels. It would be interesting to include BY4742 in this study to determine why this strain did not synthesise increased amounts of chitin upon the addition of extracellular calcium.

Overall the chitin level data indicate that, although a large number of conditions do result in increased chitin deposition, changes in extracellular conditions are drastic and may be too extreme to apply to fermentation conditions. Excluding calcium addition, a drastic change in

environmental conditions applied in this study merely results in a minor increase in chitin deposition and may not have much practical value in the wine industry. Where calcium is concerned, the treatment of cells prior to inoculation may prove useful and further investigation on this treatment may prove worthwhile.

The transcriptional control of the chitin synthase genes have been under much debate. The results generated here did not show any change in the expression of *CHS1*, *2*, *3*, *4*, *5*, *6* or *7* during chitin synthesis under environmental change. This contradicts studies which report an increase in expression levels of *ScCHS3* at 33°C after 15 minutes (Gasch *et al.*, 2000) and *ScCHS1* at 43°C (Matsumoto *et al.*, 2005). Increased expression levels of *Candida albicans CHS3* at 37°C (Munro *et al.*, 2007); and *CaCHS1*, *CaCHS2* and *CaCHS3* upon 0.1M added calcium (Munro *et al.*, 2007) were also reported. However, other studies did not show a difference in expression levels of any of the *CHS* genes when cells were exposed to stress conditions such as 37°C (Lagorce *et al.*, 2002; Wang and Szaniszló, 2000). It is our belief that the different findings in gene regulation can possibly be attributed to strain and species differences. Additionally, the degree to which the *CHS* genes are under transcriptional control is still much debated. Previous studies have shown that during the cell cycle at 30°C in a rich medium, *CHS1* and *CHS3* expression levels do not change, but rather these enzymes are spatially regulated (Lesage and Bussey, 2006; Pammer *et al.*, 1992; Valdivia *et al.*, 2002).

The expression data of *CHS4* (*SKT5*) and *CHS7* in this study correlates with studies by Lagorce and colleagues (2002). Although in their study the overexpression of *CHS7* resulted in increased activity of *CHS3*, this had no effect on chitin content. Increased chitin synthesis due to cell wall mutants also did not correlate with an increase in the expression of *CHS4* or *CHS7*. This study also did not find an increase in the expression levels of *CHS5* or *CHS6*. This may indicate that these four genes are not transcriptionally regulated under the two conditions implemented in this study and that the increase in cell wall chitin synthesis at 37°C or 0.1M added calcium is not linked to an increase in the gene expression levels of these genes.

The expression of *GFA1* under conditions of added calcium indicates that the transcriptional regulation of this gene is possibly directly correlated to an increase in chitin synthesis. The

fact that the correlation between increased chitin levels and *GFA1* expression levels was not quantitative indicates that there may be additional methods in which the cell controls chitin synthesis. Lagorce and colleagues (2002), saw a similar increase in *GFA1* expression levels when chitin synthesis in cell wall mutants was increased. In the same study, overexpression of the gene *GFA1* resulted in a threefold chitin increase. However, overexpression of *GNA1*, *PCM1* or *QRI1* did not have a significant impact on chitin synthesis. These findings correlate with our study where we did not see an increase in the expression levels of any of the other genes of the pathway. Little is known of the synthesis, action or regulation of the genes in this pathway except for *CHS 1- 3* and it is possible that the other genes in this pathway are regulated in a manner other than transcriptional control.

3.5 Conclusion

The possible implication of chitin as a haze prevention mechanism has motivated research on chitin levels in wine yeast strains as well as *S. paradoxus* strains. Such research may aid to ultimately develop a cost-effective, yet specific method of treating wine which may become hazy (Ndlovu, 2012). This study has focussed on chitin deposition in *S. cerevisiae* wine yeast strains as well as two *S. paradoxus* strains upon exposure to environmental change. The data presented here must be investigated further to determine the impact of using cells which have undergone treatment prior to fermentation on both wine quality, fermentation kinetics and haze levels. Gene expression data provide an insight into chitin regulation of the yeast strains used in this study.

Future work might also include studying the spatial and temporal regulation of proteins involved in chitin synthesis under environmental conditions that result in increased chitin levels. Furthermore, very little information is known of the mechanism and logistics of chitin incorporation into the cell wall, especially the lateral cell wall and more information is necessary in this regard. As more advanced research tools for scientific study become available, scientists are able to study chitin synthesis more in-depth and a more complete picture may soon be formed.

3.8 References

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Chapter 4

General discussion and results

Chapter 4: GENERAL DISCUSSION AND RESULTS

4.1 General discussion

Several studies have implicated chitinase in haze formation (Waters *et al.*, 1996; Marangon *et al.*, 2010; 2011) and recent studies have linked yeast cells with high chitin levels to haze prevention (Ndlovu 2012). A patent was also registered on the principle of using yeast cells with high chitin as haze prevention mechanism (Patent P2355ZA00). Such findings motivated this study on yeast cell wall chitin levels upon exposure to different environmental conditions. The main aim of this study was to determine the effect of different physiological growth conditions on cell wall chitin synthesis in strains used in the wine industry as well as *S. paradoxus* strains that have previously shown high chitin levels. Additionally, the study investigated expression levels of genes involved in chitin synthesis under conditions that result in increased yeast cell wall chitin levels.

Chitin level data presented in this study indicate that for most conditions a relatively large change in conditions is required to obtain a minor increase in chitin levels, with the exception of calcium addition where the addition of 0.1M appears sufficient to induce an increase in chitin levels in the yeast strains used in this study. The application of this information in a cost-effective, yet practical manner which does not affect wine quality, therefore still needs to be examined. Gene expression data indicate that the only chitin synthesis related gene of which expression changes during the first hour of exposure to changing environmental conditions, is *GFA1*. However this increase in expression level is not proportionate, suggesting that other factors are involved.

This study is the first to analyse strains that are used in the wine industry, whereas past studies have focussed on laboratory strains. Additionally, chitin levels could be linked to gene expression data, providing valuable information relevant to chitin regulation. Thereby, the goal of investigating chitin synthesis in several yeast strains subjected to different environmental conditions was achieved.

The results clearly show that increasing cell growth temperature to 37°C or adding 0.1M calcium to growth media results in increased chitin deposition for the yeasts used in this study. However, these changes may be too drastic to implement in fermentation conditions and may have an impact on wine quality. Therefore, exposing cells to these conditions prior to inoculation may be a more feasible option. It may be worthwhile for future studies to investigate the impact that this will have on wine quality as well as wine haze levels.

The precise mechanism by which calcium increases cell wall chitin must also be explored further. More information on the effect of extracellular calcium on the yeast cell and perhaps specifically the cell wall would definitely be beneficial. The linkage of the calcium – calcineurin pathway to increased cell wall chitin does not explain previous findings of an increase in chitin levels despite the deletion of *CRZ1* (Munro *et al.*, 2007). This indicates that there are still factors concerning extracellular calcium and chitin synthesis that are yet unknown.

Cabib (2009) discovered chitin in the mother-budneck is linked mostly to $\beta(1,3)$ -glucan and in the lateral cell wall chitin is linked to $\beta(1,6)$ -glucan. It remains to be determined whether the linkage of chitin to any of the two glucans is more accessible to chitinase than the other. It would also be interesting to investigate if free chitin is more accessible to chitinase than bound chitin. Cabib (2009) found the proteins Crz1p and Crz2p to be responsible for chitin linkage to the cell wall. Additionally the study proved a $\Delta crz1p\Delta crz2p$ strain could not bind chitin to glucans. Therefore, such a double mutant where chitin is not bound to glucans, could be used to study the accessibility of chitinase to free chitin. This information could be vital in manipulating cells to increase the accessibility of chitin to chitinase for increased haze protection.

This study has provided valuable information for the wine industry. Rising global trends of consumers preferring natural, environmentally friendly and organic products to products with added chemicals indicate that the treatment of wine with yeast cells with high chitin levels rather than the addition of the harmful clay bentonite, should increase the appeal of the wine. Additionally, the costs of removing bentonite waste in an environmentally friendly manner, as well as the loss of wine volume when using bentonite adds financial stress to the winemaker that will ultimately negatively impact the consumer. It would ultimately be in the best interest

of both the consumer and the winemaker should, in the future, haze be preventable simply by yeast manufacturers producing cells with higher chitin levels or a treatment applied to cells by winemakers after rehydration.

4.2 References

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