Stationary phase-specific expression of dominant flocculation genes for controlled flocculation of yeast

by

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DECLARATION

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

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SUMMARY

Flocculation can be defined as the asexual aggregation of yeast cells in a liquid environment. This aggregation of cells, also referred to as "floc formation", will in most cases lead to rapid settling or sedimentation. However, in so-called top-fermenting yeast strains, the flocs can move to the surface of the liquid growth substrate to form a thin layer, called a "velum", that has been compared to other microbial biofilms.

The factors that trigger flocculation can be divided into two groups, physical/chemical (e.g. sugar content, the presence of inorganic salts, organic solvents, ethanol concentration, pH, agitation etc.) and genetic factors (genes that encode for proteins that are either directly or indirectly involved in flocculation). In top-fermenting yeast strains, several physical and chemical factors that trigger the process have been described, including ethanol concentration, the presence of organic solvents, the absence of molecular oxygen and the presence of inorganic salts (Ca^{2+} and Mg^{2+}). These factors appear to affect the cell hydrophobicity and the cell surface charge. As for genetic factors, no specific genes have thus far been associated with flocculation in top fermenting yeast strains.

In bottom-fermenting yeast strains, the physical and chemical factors that affect the process are similar to the ones described for top-fermenting yeast strains, but include, more specifically, the concentration of hexoses in the media (mannose or glucose), which may inhibit the process. Indeed, flocculation in bottom-fermenting yeast strains has been divided into the NewFlo type (inhibited by both mannose and glucose) and the Flo1 type (inhibited by mannose) on the basis of the inhibitory effect of specific sugars. Various genes have been associated with the flocculation of bottom-fermenting yeast strains. Through genetic analysis, the genes have been categorised into dominant genes, semi-dominant genes and recessive genes.

In order to better understand the role of some of the proteins responsible for flocculation in *S. cerevisiae*, and to create strains whose flocculation properties would correspond to those wanted in the wine and beer industries, three of the dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, were placed under the control of the promoters of the stationary phase-induced genes, *ADH2* and *HSP30*. This was achieved by replacing the native promoters of the flocculation genes with the heterologous promoters through homologous recombination. The laboratory strain FY23, which is non-flocculent due to the absence of the transcription factor that is required for flocculation, Flo8p, was used as a model system.

Some of the transformed strains showed high flocculation, especially when the genes were placed under control of the *ADH2* promoter. In addition to this, the strains carrying a modified *FLO11* gene showed increased adhesion to solid agar media and were able to invade the growth substrate. These strains also showed an increased velum-forming ability when grown in media containing only non-fermentable carbon sources.

OPSOMMING

Flokkulasie kan gedefinieër word as die ongeslagtelike aggregasie van gisselle in 'n vloeibare medium. Hierdie aggregasie van selle, kan ook na verwys word as flok formasie, en in meeste gevalle lei dit tot 'n vinnige sedimentering. In oppervlak-fermenterende giste, beweeg die flokke na die oppervlakte van die vloeibare medium om sodoende 'n flor -lagie te vorm. Hierdie verskynsel was ook al gevind in ander organismes.

Verskeie faktore is verantwoordelik vir die effektiwiteit van flokkuklasie. Hierdie faktore kan in twee groepe verdeel word, nl. fisiese en chemiese faktore (byv. suikerkonsentrasie, die teenwoordigheid van anorganiese soute, organiese oplossings, etanol konsentrasie, pH, ens.) en genetiese faktore (gene wat kodeer vir die proteïene wat of direk of indirek betrokke is by flokkulasie). In oppervlak-fermenterende giste is daar al heelwat informasie beskikbaar omtrent fisies en chemiese faktore se effekte op flokkulasie. Van die faktore waarvan heelwat informasie beskikbaar is sluit in, etanol konsentrasie, die teenwoordigheid van organiese oplossings, die afwesigheid van molekulêre suurstof en die teenwoordigheid van anorganiese soute (Ca^{2+} en Mg^{2+}). Hierdie faktore toon 'n effek of hidrofobisiteit en elektriese lading op die seloppervlakte. Geen genetiese faktore kon tot dusver gekoppel word aan flokkulasie in oppervlak-fermenterende giste nie.

Benede-oppervlak fermenterende giste se fisies en chemiese faktore wat effektiwiteit van flokkulasie beïnvloed is dieselfde as die van oppervlak-fermenterende giste, maar sluit in meer spesifiek, die konsentrasie van heksoses in die media (nl. mannose en glukose), wat 'n inhiberende effek het op flokkulasie. Die benede-oppervlak fermenterende giste se flokkulasie kan in twee segmente verdeel word nl. die NewFlo tipe (word geïnhibeer deur die teenwoordigheid van mannose en glukose) en die Flo1-tipe (word geïnhibeer deur slegs die teenwoordigheid van mannose). Verskeie gene was ook al geidentifiseer wat die effektiwiteit van flokkulasie beïnvloed in benede-oppervlak fermenterende giste. Hierdie gene kan in drie kategorieë opverdeel word, nl dominante-, semi-dominante- en ressessiewe flokkulerende gene.

Ten orde 'n beter begrip te kry rondom die proteïene verantwoordelik vir die meeste effektiwiteit ten opsigte van flokkulasie in *S. cerevisiae*, asook om giste te manipuleer om spesifieke flokkulasie eienskappe te toon volgens die belange van die wyn en bier-industrieë, was drie dominante flokkulerende gene, nl. *FLO1*, *FLO5*, en *FLO11*, onder regulering van stationêre fase-geïnduseerde promotors, P_{ADH2} en P_{HSP30}, geplaas. Dit was verkry deur die vervanging van die wilde tipe promotors van die drie gene met die stationêre fase-geïnduseerde promotors deur middel van homoloë rekombinasie. Die laboratorium gisras, FY23, wat 'n nie-flokkulerende gisras is vanweë die afwesigheid van 'n transkripsionele faktor, Flo8p, wat verantwoordelik is vir die aktivering van belangrike gene in flokkulasie, was gebruik as 'n wilde tipe ras.

Sommige van die transformante het 'n hoë mate van flokkulasie getoon, veral wanneer onder die regulering van die P_{ADH2}. Tesame met laasgenoemde verskynsel, was daar

gevind dat *FLO11*-transformante 'n verhoging in hul vermoeë het om te bind aan die agar en ook om die agar te penetreer. Laasgenoemde gisrasse het ook die vermoë getoon om 'n flor-lagie te vorm bo-op die oppervlakte van die medium, maar slegs wanneer dit in nie-fermenteerbare koolstofbronbevattende media opgegroei word.

This thesis is dedicated to my parents, Edgar and Eleanore Domingo. Thanks for believing in my abilities and always know that you hold a special place in my heart.

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BIOGRAPHICAL SKETCH

Jody Domingo was born in Mossel Bay, South Africa on the 8th of April 1976. He matriculated at Hillcrest Secondary School, Mossel Bay in 1993. In 1994 he enrolled at the University of Stellenbosch for a BSc degree, which he completed in 1997, majoring in Microbiology and Genetics. In 1998, he enrolled for a HonsBSc in Wine Biotechnology, completing it the following year. He enrolled for an MSc in Wine Biotechnology in 2000.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **LITERATURE REVIEW**
Flocculation in *Saccharomyces cerevisiae*

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The controlled expression of dominant flocculation genes in *Saccharomyces cerevisiae*

Chapter 4 **General Discussion and Conclusions**

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CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The term flocculation describes the non-sexual, calcium-dependent aggregation of yeast cells in liquid environments. In most cases, the formation of flocs results in rapid sedimentation of the cells (Stratford and Assinder, 1991). The phenomenon has been studied for many years and various hypotheses have been proposed regarding the mechanisms involved (Miki *et al.*, 1982). It has been shown that cellular flocculation is due to both specific (molecular recognition) and non-specific (double layer interactions, Van der Waals' forces, hydrophobic interactions, salt bridges and steric repulsion) interactions (Stratford and Assinder, 1991). Nevertheless, numerous questions regarding the regulation and the mechanism of the process remain unanswered.

Flocculation is regarded as a very important process in the brewing and winemaking industries (Straver *et al.*, 1993; Verstrepen *et al.*, 2001), and is seen as a cost-effective way of clarifying the beer and wine after fermentation. However, yeast strains should never flocculate before the end of the fermentation process, since flocculation may lead to premature fermentation arrest and may result in products that are too sweet and susceptible to spoilage (Verstrepen *et al.*, 2001). The risk of losing or spoiling the product is therefore high when yeast strains with an intrinsic ability to flocculate are employed, and most currently-used industrial yeast strains are unable to flocculate or flocculate inefficiently (Carstens *et al.*, 1998).

The ability of a yeast population to flocculate is affected by environmental and genetic factors. Environmental factors include physical parameters, i.e. temperature and agitation, and chemical parameters, i.e. the composition of the growth media (Dengis *et al.*, 1995). Genetic factors, on the other hand, refer to genes that encode proteins that are essential for flocculation to occur (Teunissen and Steensma, 1995).

Physical and chemical factors have been studied over a long period and are relatively well-understood. However, the genetic regulation of flocculation has only been partially elucidated. Indeed, not all of the genes that play a role in the process have been identified, while the exact function of many of the genes that are known to be involved in the process remains to be determined.

Some of the best characterised genes that are involved in flocculation are the so-called dominant floculation genes, which include *FLO1*, *FLO5* and *FLO11*. These genes encode structurally similar, membrane-anchored cell wall proteins. It has previously been shown that increased expression of any of these genes can lead to increased flocculation. However, it is also apparent that each gene plays a specific role in various cellular processes, including mating, pseudohyphal differentiation, invasive growth and flocculation.

This M.Sc. project aims at contributing to a better understanding of the specific roles of *FLO1*, *FLO5* and *FLO11* in cellular attachment processes. We furthermore investigate the suitability of two promoters for the induction of stationary phase-specific gene expression. For this purpose, the two promoters of the *HSP30* and *ADH2* genes were fused to the chromosomal copies of *FLO1*, *FLO5* and *FLO11* in a non-flocculent laboratory strain, FY23. Both promoters have previously been shown to be induced during the stationary phase.

Our data show that the adopted strategy has the potential to lead to the development of new commercial wine yeast strains with desirable flocculation properties. The transformed strains were indeed able to flocculate efficiently and in a manner that was dependent on the expression levels conferred by the heterologous promoters.

1.2 PROJECT AIMS

The specific aims of this study were to:

(i) Construct promoter replacement cassettes that would allow the replacement of the chromosomal promoters of *FLO1*, *FLO5* and *FLO11* by stationary phase-inducible promoters

(ii) Replace the promoters of *FLO1*, *FLO5* and *FLO11* by P*ADH2* and P*HSP30* in a non-flocculent Δ*flo8* strain, FY23.

(iii) Assess the suitability of the two stationary phase inducible promoters for induction of flocculation.

(iv) Assess the contribution of the three proteins to the processes of flocculation, invasive growth and cell adhesion.

1.3 LITERATURE CITED

Carstens, E., Lambrechts, M. G. and Pretorius, I. S. (1998). Flocculation, pseudohyphal development and invasive growth in commercial wine yeast strains. *S Afr J Enol Vitic* **19,** 52-61.

Dengis, P. B., Néllisen, L.R. and Rouxhet, P.G. (1995). Mechanisms of yeast flocculation: Comparison of Top- and Bottom-Fermenting Strains. *Appl Environ Microbial* **61,** 718-728.

Miki, B. L. A., Poon, N. H., James, A. P. and Seligy, V. L. (1982). Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. *J Bacteriol* **150,** 878-889.

Stratford, M. and Assinder, S. (1991). Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. *Yeast* **7,** 559-574.

Straver, M. H., Kijne, J. W. and Smit, G. (1993). Cause and control of flocculation in yeast. *Trends Biotechnol* **11,** 228-232.

Teunissen, A. W. and Steensma, H. Y. (1995). Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast* **11,** 1001-1013.

Verstrepen, K. J., Derdelinckx, G., Delvaux, F., Winderickx, J., Thevelein, J. M., Bauer, F. F. and Pretorius, I. S. (2001). Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59,** 69-76.

CHAPTER 2

LITERATURE REVIEW

Flocculation in *Saccharomyces cerevisiae*

LITERATURE REVIEW

2.1 INTRODUCTION

The aggregation of microorganisms (i.e. the process leading to the formation of groups of microbial cells in direct physical contact) is of particular importance in many industrial processes, such as brewing, winemaking, waste water treatment and bioconversion. One such form of aggregation is flocculation, a complex process that is currently only partly understood. The process has been observed for many *Saccharomyces* species (Miki *et al.*, 1982b) and for members of other yeast genera.

Over the years, biochemical and molecular studies of the mechanism of yeast flocculation led to three hypotheses: (a) the early colloidal theory of flocculation was based on the assumption that, in an aqueous suspension, cells behave as negatively charged colloids. The observation that inorganic salts promote flocculation of yeast cells was explained as surface charge neutralisation (Stewart *et al.*, 1975; Beaven *et al.,* 1979), leading to aggregation and sedimentation of the cells. However, the specific requirement of most yeast strains for calcium to promote floc formation discredited the colloidal theory and led to (b) the calcium-bridging hypothesis. This theory proposed that Ca^{2+} ions act as bridges between the yeast cells, coupling carboxyl groups on the surfaces of adjacent cells (Stewart *et al.,* 1975; Jayatissa and Rose, 1976; Beaven *et al.,* 1979). However, given the observation that flocculation can be inhibited specifically by sugars such as mannose, various authors have suggested over the past decades that (c) a specific lectin-carbohydrate interaction may be involved in flocculation. This third hypothesis proposed that the interaction between a sugar-binding protein (lectin-like protein) and a mannose chain present on the yeast cell surface is responsible for flocculation (Miki *et al.,* 1982a,b). Today, this hypothesis is generally accepted for the following reasons: (i) flocculation is proteinase-sensitive, suggesting the contribution of a protein; (ii) flocculation is inhibited by the presence of saccharides such as mannose, suggesting the existence of a protein that recognises saccharides; and (iii) flocculation is Ca^{2+} dependent, suggesting the existence of a lectin-like protein, such as the C-type animal lectins and lectins of the plant group *Leguminosae*, which require a Ca^{2+} ion for binding activity (Sharon and Lis, 1990).

Another interesting characteristic of the flocculation process is that it is reversible by water washing or EDTA chelation, which remove divalent ions such as calcium and zinc. Flocculation can, therefore, be defined as the asexual, calcium-dependent and reversible aggregation of cells to form flocs, which are rapidly separated from the bulk medium by sedimentation (Bony *et al.,* 1997).

Yeast flocculation is often exploited in the production of lager beer and wines (especially bottle-fermented sparkling wine). The flocs that settle to the bottom of the fermenter by the end of the primary fermentation can easily be removed from the

fermentation product, thereby allowing for rapid and efficient clarification and reduced handling of the wine (Pretorius, 2000). The problem with flocculation, however, is its variability. Premature flocculation hampers complete fermentation of the hexoses (stuck fermentation), whereas failure of the cells to flocculate at the end of the fermentation process requires the use of expensive centrifugation and/or filtration techniques to remove cells (Straver *et al.,* 1993a,b). Stuck fermentation occurs because the fermentation process is severely slowed down due to insufficient contact between yeast cells and the medium. This results in final products with high residual sugars, a low alcohol content and unsatisfactory aromatic characteristics due to off-flavours (Verstrepen *et al.,* 2001).

Several factors, such as cell surface charge and hydrophobicity, have been implicated in a primary or complementary role with lectins to facilitate the onset of flocculation (Smit *et al.,* 1992). Environmental factors that may influence the level of flocculation by *Saccharomyces cerevisiae* strains include temperature and pH, as well as the concentrations of calcium, zinc, molecular oxygen, sugar and inositol. Furthermore, the growth phase and the cell density also influence the process (Pretorius, 2000).

The genetic background of the strain also determines its ability to flocculate and several dominant, semi-dominant or recessive genes are known to be involved in flocculation. Distinct flocculation phenotypes have been identified, based on their sensitivities to sugar inhibition and proteolytic enzymes. These phenotypes, designated Flo and NewFlo, also display different sensitivities to yeast growth conditions, most notably temperature, acidity of the culture medium and glucose availability (Stratford and Assinder, 1991; Pretorius, 2000).

The importance of the yeast flocculation process to the wine, brewing and related beverage industries as the most cost-effective method of product clarification cannot be overemphasised. Therefore, before describing the conceptual basis of the present research project, some specific aspects related to this phenomenon will be reviewed below.

2.2 PHYSICAL AND CHEMICAL FACTORS INFLUENCING FLOCCULATION

Physical and chemical factors greatly influence the process and the degree of flocculation. In the next section of the literature review, we will be focusing on the following aspects:

1. Characteristics of the cellular surface, in particular cell wall composition, cell surface charge and cell surface hydrophobicity.
2. Media composition and pH.
3. Physical aspects of culture conditions, in particular agitation and aeration.

2.2.1 FACTORS THAT ARE LINKED TO THE COMPOSITION OF THE CELLULAR SURFACE

2.2.1.1 Cell wall composition

The cell wall of *S. cerevisiae* is a complex carbohydrate structure (100-200nm thick) that surrounds the periplasmic space and the plasma membrane. It is composed largely of β-glucans and α-mannans, together with smaller proportions of chitin and proteins. However, the exact composition and proportions are found to vary with yeast strain and culture conditions. The inner structural layer of the cell wall consists of β-glucans arranged in an insoluble network of crystalline fibrils (Kopecka, 1985). These are formed by glucose residues that are linked by β-(1-3) bonds, with occasional β-(1-6)-linked side branches. Chitin, on the other hand, is composed of β-(1-4)-linked *N*-acetylglucosamine residues in linear chains and is found mainly in primary septa and bud scars.

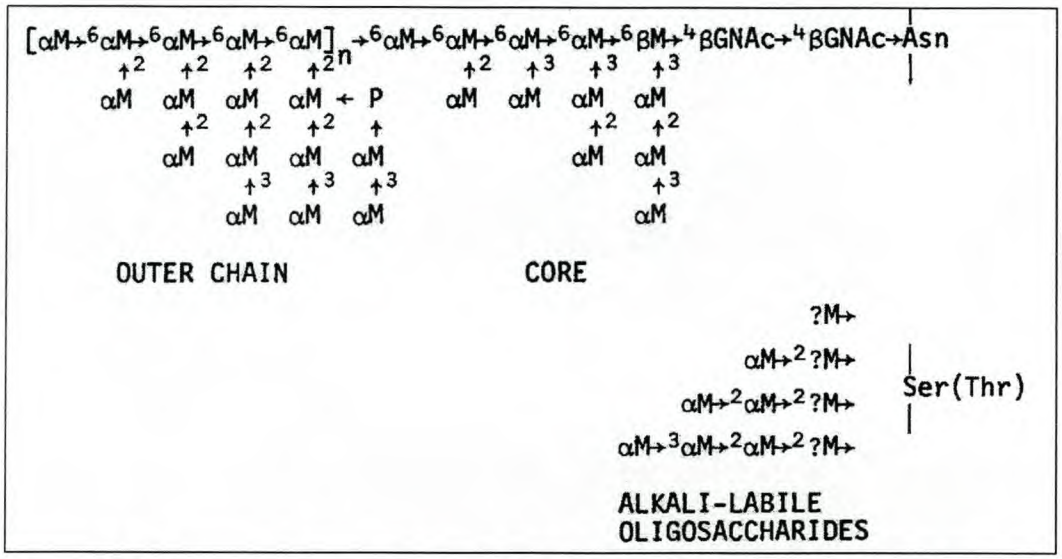

Figure 2.1. Representative structures for *S. cerevisiae* mannoprotein carbohydrate components. (M) mannose; (GNAc) *N*-acetylglucosamine; (Asn, Ser, Thr) amino acids (according to Ballou, 1990).

The mannan structure is made of long, branched chains of mannose residues that are linked to proteins, forming so-called mannoproteins. The mannose residues form an outer chain and an inner core, which is attached to an asparagine residue of the protein component via 2 *N*-acetylglucosamine molecules linked by β(1-4) glycosidic bonds. The inner core consists of 11 mannose residues with four side chains that vary between one and three mannose residues in length. These side chains are attached to the backbone structure via α(1-2) and α(1-3) linkages. The outer chain contains up to 150 mannose residues, and its side chains can vary between one to three mannose residues in length. These side chains are all attached to the main α(1-6)-linked backbone structure via α(1-2) glycosidic bonds (Figure 1). Cell wall mannans and proteins are inextricably linked together in covalent complexes (Figure 2). It thus is possible to regard the mannan outer

layer of the cell wall as a coating of secreted mannoproteins that are fixed onto a β-glucan framework.

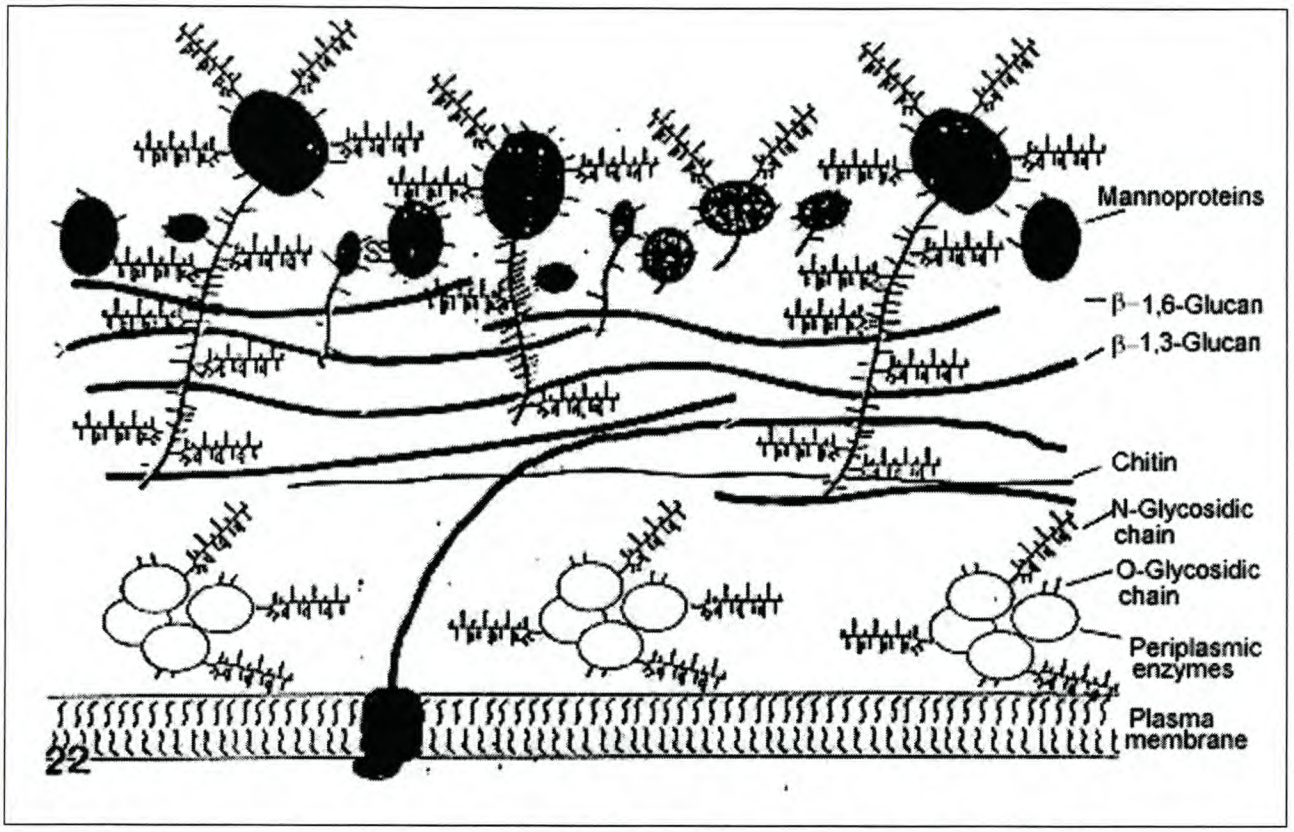

Figure 2.2. Composition and structure of the cell wall of *Saccharomyces cerevisiae* (Klis, 1994).

In addition, and apart from inner core and outer core mannans, short mannan-oligosaccharide chains exist that are directly attached to serine and threonine residues present in the proteins (Stratford, 1992; Fleet, 1991). However, wide variations in mannan content have been found in different strains.

Experimental data have highlighted the importance of mannans in the process of flocculation. Jayatissa and Rose (1976) showed that flocculating cells contain higher levels of polysaccharides, specifically mannans, than non-flocculent cells. Furthermore, mannose inhibits flocculation, and the authors suggested that the mannans on the cell wall may be responsible for interaction with lectin-like proteins. This was investigated through various mutant strains with mutations in genes required for the synthesis of specific sections of the structure of cell wall polysaccharides (Ballou, 1990). These strains were treated with protein denaturants in order to destroy the cell wall proteins. The ability to co-flocculate and form aggregations with flocculent cells of *Saccharomyces cerevisiae* was then tested for these cells. Results obtained from this study suggested that the outer-chain mannan side branches, which are two to three mannose residues in length, are required for the interaction with adjacent cells (Stratford, 1992).

The same studies, while highlighting the importance of mannans in the process of flocculation, also established the importance of another, quantitatively minor component of

the cell wall; the so-called lectin-like proteins. These proteins will be described in greater detail in a latter section of this review. In general terms, the hypothesis proposes that the mannan layers and the lectin-like cell surface proteins in the cell walls of adjacent cells interact at multiple contact areas distributed all over the cell surface (Figures 3 and 4).

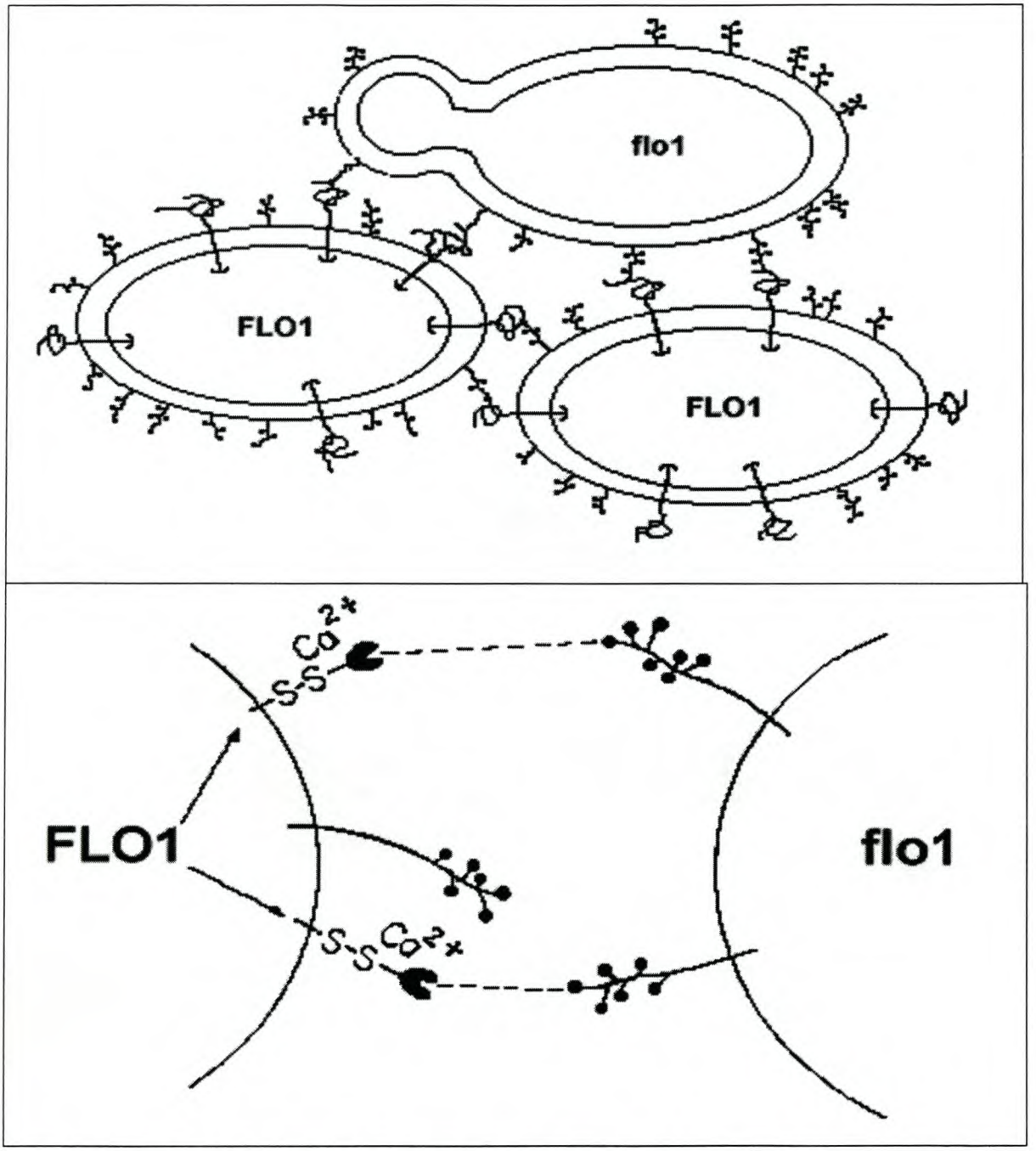

Figure 2.3. Model for the possible mechanism of flocculation interactions by the gene *FLO1*. Unilateral interactions between flocculent and non-flocculent cells (Teunissen and Steensma, 1995).

Cells that lack α-mannans in their cell walls cannot flocculate. However, when mixed with flocculent yeast strains, these cells are able to co-flocculate efficiently (Stratford, 1992). On the other hand, cells that do not contain lectin-like proteins, and which therefore

are not able to flocculate in a pure culture, can also aggregate with flocculent cells in a heterogenous suspension (Figure 3). The authors suggest that this is due to the interaction of the lectins with the flocculent cells that can bind to the mannan receptors on the non-flocculent cells. This hypothesis is furthermore strengthened by the fact that the presence of non-flocculent cells in large numbers interferes with flocculation (Miki *et al.,* 1982a).

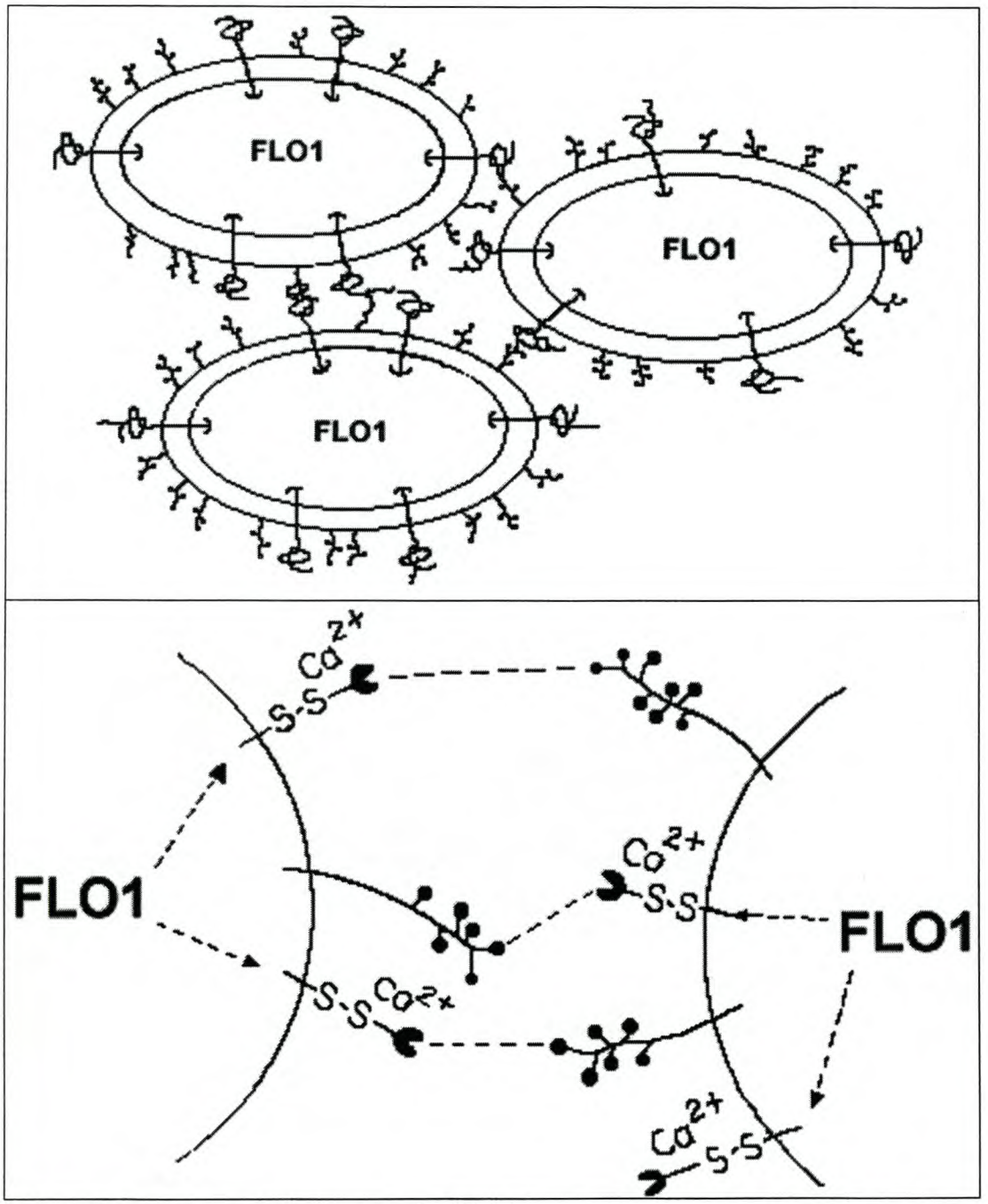

Figure 2.4. Bilateral interactions between flocculent yeast cells (Teunissen and Steensma, 1995).

2.2.1.2 Cell surface hydrophobicity

The hydrophobic properties of the microbial cell surface have been studied extensively and it has become increasingly clear that hydrophobic interactions play a crucial role in

various microbial adhesion phenomena. Such interactions facilitate the non-specific adhesion of microorganisms to inanimate surfaces, such as bioreactors, implants, contact lenses and teeth. They also appear to enhance the specific adhesion of *Candida albicans* to human epithelial cells, of *Streptococcus gordonii* to saliva-coated surfaces, of bacteria to plant cells and also of cells of *Saccharomyces cerevisiae* to each other (Straver *et al.*, 1993b).

Cell surface hydrophobicity has been shown to be a major factor in the flocculation of brewer's yeast cells. This conclusion is based on several observations: (i) cell-surface hydrophobicity increases shortly before the onset of flocculation; (ii) the growth of cells under magnesium-limited conditions results in non-flocculent cells and in a decrease in hydrophobicity, as judged by the cells' ability to adhere to polystyrene; and (iii) treatment of flocculating cells cells with polycations renders the cells more hydrophobic and reinforces flocculation (Straver *et al.*, 1993b). In all cases, the flocculation was still calcium dependent and magnesium-sensitive, indicating that hydrophobicity alone is insufficient for efficient flocculation. Furthermore, hydrophobicity appeared not to be influenced by the presence of EDTA, which means that calcium is most probably required for the specific lectin-sugar interaction.

2.2.1.3 Cell surface charges

Surface charges play important roles in biological attachment processes. Yeasts are electrostatically charged. The iso-electric point, at which the overall yeast cell charge is neutral, is between pH 2 and 3.2, with the negative charge increasing at higher pH values. Yeast cells are kept dispersed by repulsion between like-charged cells, which may be regarded as an energy barrier preventing cells approaching each other sufficiently closely to bond together (Stratford and Keenan, 1987). The overall surface charge therefore has a negative effect on cellular association at the average pH of yeast growth substrates. However, Van Hamersveld *et al.*, (1994) found that specific electrostatic interactions may also favour the association of cells when positioned at specific distances from each other. This highlights the fact that the involvement of specific molecular recognition mechanisms appears to be the main factor responsible for yeast flocculation. These specific interactions, however, do not eliminate the need for non-specific interactions in the process of aggregation. Indeed, specific interactions take place at a very short distance (less than 1.5 nm), whereas non-specific interactions can occur over a long distance (Dengis *et al.*, 1995). In order for specific interactions to assert themselves, the cells therefore first have to be brought into close proximity with one another.

Investigations of the importance of physical and chemical properties that are responsible for non-specific interactions in brewer's yeast revealed a correlation between the isoelectric point of yeast cells and the N/P concentration ratio measured at the surface by X-ray Photoelectron Spectrophotometry (XPS) (Van Haecht *et al.*, 1982).

An interesting observation regarding the electrostatic properties of differently flocculating yeast cells was made by Dengis *et al.* in 1995. The authors compared a top-

fermenting strain (yeast strain used for sherry production) with a bottom-fermenting yeast strain (yeast strain used in sparkling wine production). Electrostatic differences between the two strains were found: the top-fermenting strain showed a higher isoelectric point and its electrophoretic mobility seemed to be strongly dependent on pH. Amory *et al.,* (1988) found a systematically lower electrophoretic mobility below pH 5 for top-fermenting strains when compared with bottom-fermenting strains. They related the more negative electrophoretic mobility of bottom strains at pH 4 to a higher surface concentration of phosphate groups, as measured by XPS.

2.2.2 THE INFLUENCE OF MEDIA COMPOSITION

2.2.2.1 Growth phase and media composition

Yeast cells, in the exponential growth phase, do not flocculate when placed in just any media and neither do stationary phase cells when resuspended in fresh media (Dengis *et al.*, 1995). However, stationary phase cells flocculate partially in media separated from exponentially-growing cultures but flocculate completely in media separated from stationary phase cultures. This suggests that yeast growth leads to modifications in the growth substrate that favour cellular aggregation. Interestingly, stationary phase cells of a bottom-fermenting strain only flocculated in a medium separated from a stationary phase culture.

It can be suggested that several of the factors that are listed below contributed to the observed phenomena.

2.2.2.2 Sugars

Simple hexoses, especially mannose and glucose, can prevent the formation of aggregates (Smit *et al.,* 1992; Speers *et al.,* 1992; Stratford, 1992). Sugar alcohols and sugars of L-configurations, however, do not inhibit flocculation (Stratford and Assinder, 1991). The same authors reported that the amount of mannose required to inhibit flocculation in cells that flocculate poorly is significantly lower than that needed in cells that flocculate strongly. This suggested the presence of a saturable component that was directly inhibited by the presence of specific hexoses. The authors suggested that sugar-binding proteins might be inhibited by the hexose, and that fewer of these proteins were present on the cell wall of poorly flocculating yeast. Other data suggested that the binding of proteins to the ligands is inhibited more strongly by longer chain mannans, possibly due to the higher molecular flexibility of longer chains (Stratford and Assinder, 1991).

Of all hexoses, mannose exerts the strongest inhibitory effect. This inhibition was shown to be fully reversible and is due to the competition of mannose with cell wall mannans for binding to lectin-like proteins (Stratford, 1992). The data clearly indicate that cell wall mannans do indeed act as receptors of lectin-like proteins (Mota and Soares, 1994).

The inhibitory effect of glucose is in most cases due to a regulatory cascade that represses the synthesis of lectin-like proteins. This phenomenon will be discussed at a later stage in this literature review. However, in some specific strains that display a phenotype referred to as NewFlo, glucose may have a direct inhibitory role similar to that of mannose. This phenotype has been described in several bottom-fermenting lager brewing yeasts, and was found to be associated with a specific version of the *FLO1* gene, Lg*FLO1*. The more abundant, classical phenotype, which refers to flocculation that is inhibited by mannose alone, is therefore referred to as the Flo phenotype.

The above-mentioned effect of sugar on flocculation is only relevant for bottom-fermenting strains. The only factors that affect the flocculation ability of top-fermenting strains are ethanol concentration, inorganic solvents, pH and the magnesium concentration

2.2.2.3 Ethanol

In 1995, Dengis *et al.* studied the effect of ethanol on flocculation in top- and bottom-fermenting yeast strains. Cells in the exponential growth phase did not flocculate upon ethanol addition to both types of strains. In the case of stationary phase cells, however, the results differed between the strains. While the bottom-fermenting strain was not influenced by ethanol at all, regardless of the calcium concentrations in the medium, flocculation was induced at 5% (vol/vol) ethanol when no calcium was added and at a slightly lower ethanol concentration when calcium was added in the case of the top-fermenting strain. If the pH was adjusted to 5.2 with HCl instead of buffer, maintaining a low ionic strength, flocculation in 10% (vol/vol) ethanol was observed only when $CaCl_2$ was added at 1mM or more.

2.2.2.4 pH

The influence of pH on flocculation was also evaluated for top- and bottom-fermenting yeast strains. The results showed clearly that exponentially-growing cells appear not to flocculate at just any pH investigated for both strains. However, stationary phase cells flocculated in a narrow pH range. For top-fermenting yeast strains, the optimal pH range was found to be situated between pH 3.0 and 4.5, while a slightly larger range of pH 3.5 to 6.0 was found for the bottom-fermenting yeast strain (Dengis *et al.*, 1995).

A low pH (1.2-1.8) causes irreversible inhibition of flocculation, which may be ascribed to the distortion of proteins at low pH (Stratford and Assinder, 1991). Flocculation does not occur at an extremely high pH, irrespective of the presence of chelating agents (e.g. EDTA). This may be due to the changes in ionic strength and conformational changes in the flocculation receptors (Masy *et al.*, 1991).

A study of the combined effect of ethanol and pH on the flocculation of a top-fermenting yeast strain revealed that the addition of ethanol broadens the pH range in which flocculation can occur. At ethanol concentrations of 5% or 10% (v/v), the pH range in which flocculation could occur was extended to a pH of 5.2. This again clearly indicates

the requirement for a sufficient concentration of ethanol for the flocculation of top-fermenting strains (Dengis *et al.*, 1995).

2.2.2.5 Inorganic salts

Yeast lectin-like proteins only bind mannans in the presence of metallic cations, i.e. calcium (Shankar and Umesh-Kamar, 1994), manganese (Stratford, 1992) and magnesium (Smit *et al.,* 1992). According to Smit and co-workers (1992), a shortage of magnesium inhibits flocculation during any stage of growth, as it is essential for lectin production and the stability of the membrane. Tin (Sn^{4+}) is required for the flocculation of cells in the physiological saline (NaCl 150mM), but its effects appear rather non-specific, since it promotes flocculation of non-flocculating cells as well.

Inorganic salts within the growth medium affect the rate of flocculation; depending on the type and the concentration of salt, the time of inoculation, yeast strain, pH and the medium buffer strength (Straver *et al.,* 1993a). Chelating agents cause reversible inhibition of flocculation because of the removal of metallic ions from the medium.

For all inorganic salts that affect flocculation directly, three types of effects can be observed. Low salt concentrations, especially for calcium, are essential for flocculation to occur. At moderate concentrations, salts enhance both the rate and extent of flocculation and, at high concentrations, salts inhibit the flocculation process. A high concentration of either calcium or magnesium might indeed be regarded as the strongest inhibitor of flocculation (Stratford and Brundish, 1990).

Both calcium and manganese are prerequisites for flocculation (Speers *et al.,* 1992), even though they cannot induce flocculation in non-flocculating cells. Structural research on lectins revealed that these cations were acting close to, but not proximal to, the sugar-binding sites. In other words, cations were found to be essential for maintaining proper conformation of the sugar-binding proteins (lectins), rather than being directly involved in sugar binding (Hodgson *et al.,* 1985). Calcium is also needed throughout all growth phases in order to ensure the optimisation of yeast metabolism, cell wall structure and cell flocculation.

Cationic inhibition, on the other hand, was shown to be the result of protein dehydration. Lectin molecules are highly hydrophilic and a high water activity is required for mannan-protein interaction, as well as for the interaction with calcium. The dehydration of these proteins in media with high salt concentrations therefore would result in proteins that cannot be activated by the binding of calcium, leading to loss of flocculation ability. NewFlo-type surface proteins were found to be more susceptible for protein dehydration than the Flo1 type of surface proteins (Stratford and Assinder, 1991).

The flocculation threshold value can be described as the specific quantity of free calcium that is required by yeast cells in order to flocculate. This quantity of free calcium is regulated by complexing agents within the growth medium. The calcium threshold values necessary for flocculation correlate directly with the concentrations of chelating agents, while the strength of the bonds between flocculating cells correlates negatively with the

existing free calcium concentration. In the absence of free calcium in the growth medium, only loose bindings will occur between mannose and lectins (Shankar and Umesh-Kamar, 1994).

Observations made by Stratford and Brundish (1990) with regards to certain ions indicated that an increase in ion charge and ion concentration, together with a decrease in ion size, increases the degree to which flocculation is inhibited by salts. The ions Ba^{2+}(barium) and Sr^{2+}(strontium) were shown to inhibit flocculation by competing with Ca^{2+} (Nishihara *et al.*, 1982), whilst other metal ions, such as copper, manganese and iron, also inhibit flocculation. Zinc, an ion naturally present in yeast cells, is associated with the control of enzymatic functions and stimulates flocculation at concentrations of less than 0.04mM, but, above this concentration and especially in the presence of calcium, de-flocculation occurs in some strains. Sodium and potassium are the only monovalent ions that influencing flocculation (Smit *et al.,* 1992) at concentrations below 10mg/l, but they antagonise flocculation at concentrations above 50mg/l (Nishihara *et al.*, 1982). Above a certain minimal concentration, the rate of cell dispersion is correlated positively with the concentration of the ions in the medium. Nishihara *et al.* (1982) also found non-specific flocculation in the presence of heavy metals.

Some salts show additive effects on the inhibition of flocculation that cannot be ascribed to a change in cell metabolism. The salts may change the conformation of specific proteins on the surface of flocculating cells, possibly due to the dehydration of proteins, as mentioned earlier. In contrast to non-flocculating cells, salt-inhibited flocculating strains lose their ability to co-flocculate with other flocculent strains (Stratford and Brundish, 1990). At concentrations that are too low for inhibition, flocculation is usually enhanced. An increase in incubation time in the presence of inhibitory ions and extremely high or low pH values increases the degree of salt inhibition (Stratford and Brundish, 1990).

2.2.3 PHYSICAL ASPECTS OF CULTURE CONDITIONS

2.2.3.1 Agitation

The theory of the effects of mechanical agitation has its roots in the early colloidal theory of flocculation. Yeasts are electrostatically charged, as described earlier in the review, and are kept dispersed by repulsion between like-charged cells (Stratford and Keenan, 1987).

Mechanical agitation gives cells sufficient momentum (external energy) to overcome this energy barrier and to collide physically, enabling bonds to form between cells. Thus, without mechanical agitation, flocculation does not occur. The cell size also influences the effectiveness of the process, since larger cells will have a greater momentum.

The strength of the bonds between flocculating cells determines the size of the flocs that will be formed. At a uniform agitation rate, flocs will be of a uniform size and this size will be specific for each strain. Size uniformity is due to the equilibrium between the

strength of the bond between the cells and the strength of the mechanical shearing forces that will break away cells on the outside of the floc.

For the same reasons, the agitation speed for optimal flocculation to occur is strain-dependent. Increased agitation increases the frequency of interactions between particles as well as the force of the collisions. The agitation speed needed for the induction of flocculation can also decrease with a decrease in pH, which leads to a lower negative cell surface charge (Stratford and Keenan, 1987).

The steady state in yeast flocculation is a dynamic equilibrium between flocculated and dispersed yeast cells. In these conditions, the free cell concentration is directly proportional to the total cell concentration and may be expressed as an equilibrium constant. Furthermore, at specific critical cell densities, no additional flocculation and cell aggregation will take place, as a dynamic equilibrium has been reached (Stratford *et al.*, 1988).

2.2.3.2 Aeration

Flocculation is not influenced by the amount of aeration during the exponential growth phase (Miki *et al.,* 1982b). However, in the stationary phase flocculation appears to be de-repressed upon aeration (Miki *et al.*, 1982a). According to Mota and Soares (1994), flocculation in the stationary phase can be controlled by the amount of aeration of the growth medium, since a minimum of oxygen is necessary for the biosynthesis, transport and/or anchorage of flocculation proteins. The same authors found a correlation between the effect of the initial sugar concentration and the amount of aeration on flocculation. With too much or too little aeration, the inhibition of flocculation is independent of the sugar concentration, while an increase in glucose concentration increases flocculation with intermediate aeration.

2.3 THE GENETIC FACTORS INVOLVED IN FLOCCULATION

Several genes that are required for flocculation have been cloned and characterised. These include the dominant genes *FLO1* (allelic to *FLO4*), *FLO5*, *FLO8*, *FLO9*, *FLO10* and *FLO11* (Javadekar *et al.*, 2000), and the recessive and semi-dominant genes *flo3*, *flo6*, *flo7*, *fsu2* and *fsu3*. The genetics of flocculation are further complicated by the finding that flocculation properties are influenced by the absence or presence of mitochondrial DNA (Teunissen *et al.*, 1993). In addition, mutations in several genes, including the regulatory genes *TUP1* and *SSN6*, also have been found to cause flocculation or flaky growth in non-flocculating yeast strains (Teunissen and Steensma, 1995).

Several of the dominant flocculation genes encode for membrane-anchored cell wall proteins and are referred to as flocculins or yeast lectins. They include *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*. All these genes encode proteins with a high degree of structural similarity (Stratford and Carter, 1993). Flocculins are anchored to the plasma membrane via a GPI (Glycosyl Phosphatidylinositol) anchor that is attached to the C-terminal end of

the protein (Figure 6). They also contain a large, central domain that is rich in serine and threonine residues and is thought to adopt a rod-like structure. This domain may enable the protein to traverse the cell wall entirely and to expose the N-terminal domain to the external environment. This N-terminal domain is highly variable between different flocculins and therefore may be responsible for the specific characteristics of each of the proteins. In the native protein, the N-terminal domain is preceded by a signal peptide sequence that ensures proper localisation in the cell wall (Lo and Dranginis, 1996).

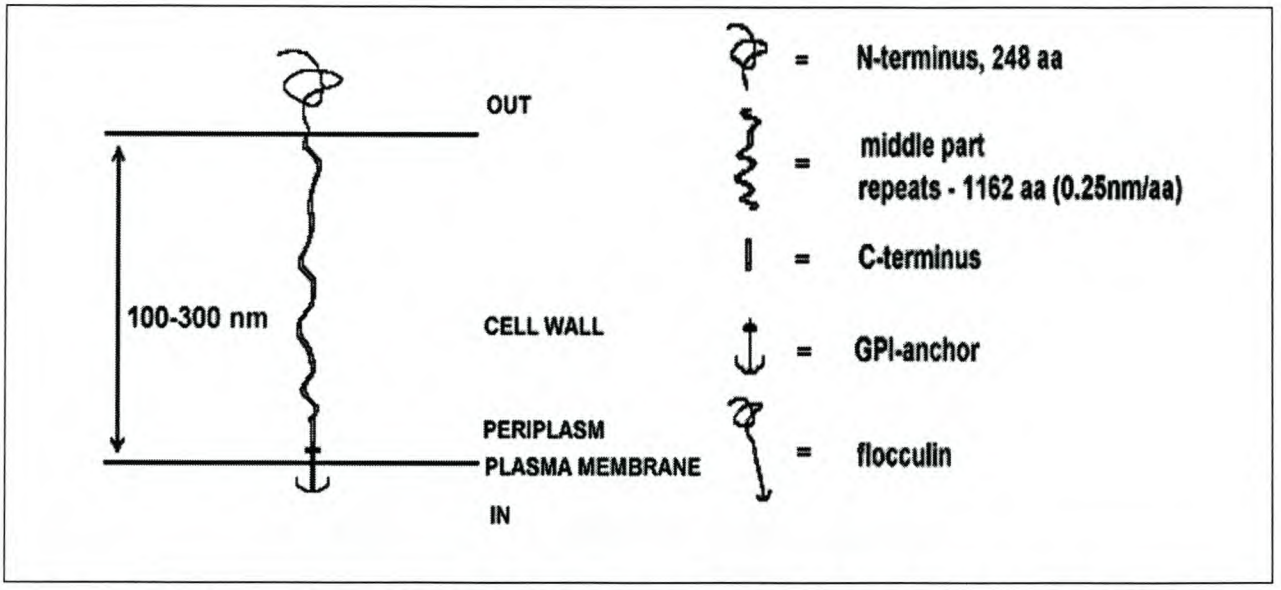

Figure 2.6. Schematic representation of a flocculin. The protein is involved in making contact with the neighbouring cells via binding to mannan residues on adjacent cells (Teunissen and Steensma, 1995).

Of all the genes that initially qualified as dominant flocculation genes, only *FLO8* does not encode for a flocculin-type protein, but rather for a transcriptional activator (Kobayashi *et al.*, 1996). Flo8p is required for the expression of several flocculins and binds to the upstream activating sequences of their promoters.

In this section, more information about these specific genes will be given, as well as an overview on the genetic regulation of flocculation.

2.3.1 LG*FLO1*

The NewFlo type of flocculation is found in bottom-fermenting yeast strains used in the beer-making industry. In these strains, flocculation is inhibited by both mannose and glucose and this differs from the normal Flo1 type of flocculation, which is only inhibited by mannose (see Figure 7). Since inhibition by glucose would correspond to one of the desired characteristics of industrial flocculation, as it would ensure that flocculation occurred only once glucose has been depleted, the gene that encodes for the specific sugar-binding protein (lectin) on the cell walls of these bottom-fermenting yeast strains was investigated.

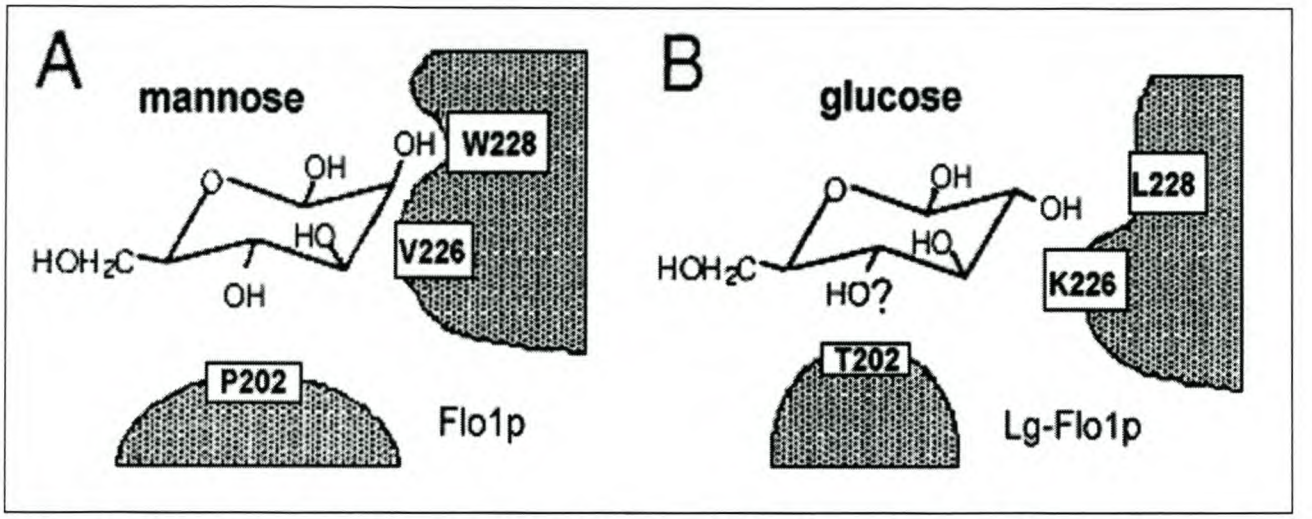

Figure 2.7. Model for sugar recognition by the Flo1 and Lg-Flo1 proteins (Kobayashi *et al.*, 1998).

In 1998, Kobayashi and co-workers identified the gene responsible for this phenomenon. The gene, designated Lg-*FLO1* (Lager-type *FLO1*), has an open reading frame of 5.8kb, which is longer than the coding region for the wild type *FLO1* gene (Watari *et al.,* 1994). The N- and C-termini of the gene show an identity of 61.7% and 55.1% respectively with that of the *FLO1* N- and C-termini, while the 5' and 3' flanking regions of the Lg-*FLO1* open reading frame show no significant homology with the corresponding regions of the *FLO1* gene (Kobayashi *et al.,* 1998).

It is supposed that the Lg-*FLO1* originated from a recombination between *FLO5* (*YHR211*) and *YAL065*, which is thought to be a pseudogene as the product of this gene has no effect on the cell when deleted or overexpressed. The region from amino acids 196 to 240 in both the Lg-*FLO1* and *FLO1* gene products is important for flocculation. Further analysis reported that Thr-202 in the Lg-*FLO1* protein and Trp-228 in the *FLO1* protein are involved in sugar recognition (Figure 7) (Kobayashi *et al.,* 1998).

2.3.2 DOMINANT GENES

2.3.2.1 *FLO1*

The *FLO1* gene is located on chromosome I and consists of a open reading frame of 4611 bp, which encodes for a protein of 1537 amino acids (Watari *et al.,* 1994). The protein is serine-threonine rich and has a molecular weight of 162 kDa (Bony *et al.,* 1997). Both the N- and C-terminal regions of Flo1p are hydrophobic and the C-terminal sequence contains a potential membrane-spanning region, also known as a GPI anchor. According to sequence analysis, the protein also contains four families of repeated Ser-Thr-rich sequences composed of 18, 2, 3 and 3 repeats (Watari *et al.,* 1994).

Bony and co-workers (1997) showed a direct correlation between the number of repeated sequences and the intensity of flocculation. The results of this study revealed

that a reduction in the copy number of these repeated sequences can be associated with a decrease in flocculation intensity.

Deletion studies have shown that a disruption of *FLO1* results in a loss of the flocculation phenotype. Transformation of *FLO1* into a non-flocculent yeast strain, however, is also capable of inducing flocculation in such a strain. Therefore, it is believed that *FLO1* is the most important lectin-type flocculation gene in *S. cerevisiae*.

2.3.2.2 *FLO5*

The *FLO5* gene is situated on chromosome VIII and has an open reading frame of 3 227 kb. The protein is 96% homologous to Flo1p (Teunissen and Steensma, 1995). The major difference between *FLO1* and *FLO5* is the number of repeats (Teunissen *et al.*, 1995). Upstream regions of *FLO1* and *FLO5* also differ. A MATα2-MAT**a**1 binding site (-724 relative to the ATG), which is absent from the *FLO5* promoter is found in the *FLO1* promoter. This might explain the mating typedependent regulation reported for *FLO1* and not for *FLO5* (Yamashita and Fukui, 1983).

In both Flo1p and Flo5p, the secretion signal is 24 amino acids long and is 80% identical. There also are no differences between the N-termini of the two proteins regarding charge and hydrophobicity. Flo5p, however, has an additional glycosylation site (Teunissen and Steensma, 1995).

The difference between Flo1-type and Flo5-type flocculation is that the latter is chymotrypsin-resistant, as well as sensitive to incubation at 70°C (Hodgson *et al.*, 1985).

2.3.2.3 *FLO8*

Liu and co-workers (1996) reported that Flo8p is a transcriptional activator of *FLO1*, since Northern blot analyses showed an enhancement of *FLO1* transcription in the presence of multiple copies of *FLO8*. In 1998, Kobayashi and co-workers showed that a *flo8* deletion strain loses its ability to grow invasively, to produce extracellular glucoamylases as to flocculate. This led them to believe that *FLO8* regulates these characteristics via the transcriptional regulation of the *FLO11* (invasive growth), *STA1* (glucoamylases) and *FLO1* genes (flocculation). These findings were later confirmed by Gagiano *et al.* (1999).

2.3.2.4 *FLO9* and *FLO10*

Both *FLO9* and *FLO10* were always considered to be pseudogenes (they have no effect on the cell when deleted or overexpressed), but were shown later to be lectin-encoding genes. The sequence of *FLO9* is not found in the genome of all strains and corresponds to a polymorphic locus.

Protein products of *FLO9* and *FLO10* show 94% and 58% similarity respectively with that of Flo1p. A comparison of the Flo10p N-terminus to those of Flo1p, Flo5p and Flo9p termini shows a 81.9% similarity among all proteins (Teunissen and Steensma, 1995). The specific role of *FLO10* is unclear (Teunissen and Steensma, 1995). Guo and co-workers (2000) showed that, when *FLO10* is placed under the control of a galactose-

inducible promoter, the levels of flocculation are stronger than that conferred by *FLO11,* while resulting in both invasive growth and adherence to agar medium. Overexpressed Flo10p could indeed overcome a *flo11* mutation and could restore the invasive growth phenotype to a Δ*flo11* deletion strain.

2.3.2.5 *FLO11* (*MUC1*)

FLO11 encodes for a protein that is 1367 amino acids long and the gene is located on chromosome IX. The promoter has been shown to be highly homologous to that of the glucoamylase-encoding *STA1-3* genes (Lambrechts *et al.,* 1996). The predicted protein is 37% similar to the product of *FLO1* and has a 36% sequence similarity (24% identity) to human intestinal mucin, *Muc2*.

Like all flocculins, the N-terminal domain contains a signal peptide sequence, whilst the C-terminal domain is homologous to GPI anchor-containing proteins. In between the two termini, there is also a domain containing highly repeated threonine-rich and serine-rich sequences (Lambrechts *et al.,* 1996; Lo and Dranginis, 1996).

FLO11 differs from all the other flocculin genes in that it is located near a centromere rather than a telomere. Immunofluorescence studies have localized Flo11p to the cell wall (Guo *et al.,* 2000)

Flo11p is required for invasive growth and pseudohyphae formation (Lambrechts *et al.*, 1996), suggesting that it may be involved more specifically in the adhesion to external substrates.

2.3.3 *FLO2* - A SEMI-DOMINANT FLOCCULATION GENE

The gene *FLO2* is situated on chromosome XII. It is 3.1 kb in size and shows no homology to *FLO1*. It is classified as a semi-dominant gene, since no phenotype can be associated with the gene when a wild type copy of the *FLO1* is present. However, if *FLO1* is deleted, *FLO2* is expressed and can overcome the *flo1* phenotype. The *FLO2* gene is suppressed by the suppressor *fsu3*. This suppressor only supresses the *Flo2* gene and not any other flocculation gene (Sieiro *et al.,* 1997).

Other semi-dominant flocculation genes were identified through genetic analysis before 1985. Most of these, however, were later reclassified either as alleles of *FLO1* (*FLO6* and *FLO7*) or as suppressors of specific flocculation genes (*FSU1*) and therefore are not included here.

2.3.4 MITOCHONDRIAL MUTATIONS

In some strains, flocculation is possible even in the absence of the *FLO* genes and mitochondrial DNA appears to control some of the genes that are involved in flocculation. Indeed, mitochondrial petite mutations may cause changes in the cell surface characteristics, which may lead to defects in the secretory mechanisms necessary for

flocculation. Mitochondrial mutations may cause a specific decrease in or a total loss of flocculation, but may also increase the flocculation ability of the cells (Stratford, 1992).

2.3.5 THE TRANSCRIPTIONAL REGULATION OF FLOCCULATION

Flocculation is regulated largely at the transcriptional level, and the transcriptional regulation of several *FLO* genes has been studied in some detail. Most efforts have focused on the transcriptional regulation of the *FLO11* gene and, to a lesser extent, of the *FLO1* gene. However, the studies on the transcriptional regulation of *FLO11* did not, for the most, assess its role in flocculation, but rather investigated the regulatory pathways involved in the control of invasive growth and pseudohyphal differentiation. Considering the data that are available regarding the role of *FLO11*, as well as the results of the study presented in Chapter 3 of this document, it appears questionable whether the data generated for *FLO11* are relevant for the regulation of flocculation.

Nevertheless, a short summary of the complex mechanisms regulating *FLO11* is given below, since at least some of the aspects of this regulation appear to be relevant for *FLO1* as well. *FLO11* is regulated in response to nutrient availability in the direct environment of the cell (Bauer and Pretorius, 2001). The best characterised signalling pathways that transmit the nutrient signal to the promoter of *FLO11* are the invasive growth MAP kinase cascade (Madhani *et al.*, 1997) and the Gpa2p-cAMP-PKA pathway (Pan and Heitman, 1999; Rupp *et al.*, 1999). These pathways regulate *FLO11* via a set of transcriptional activators and repressors, which include Flo8p (Kobayashi *et al.*, 1998), Ste12p and Tec1p (Mösch *et al.*, 1996), Phd1p (Gimeno and Fink, 1994), Sok2p (Pan and Heitman, 1999), as well as Msn1p and Mss11p (Gagiano *et al.*, 1999).

The upstream regulatory region of *FLO11* is one of the largest yeast promoters identified to date and spans 2.4 kb upstream from the transcription start site which was shown to be required for the regulation of *FLO11* expression (Gagiano *et al.*, 1999). The data suggest that the previously mentioned signalling pathways and regulatory proteins converge on this promoter to regulate invasive growth and pseudohyphal differentiation.

At least some of the transcriptional activators, mentioned above, have also been identified as regulators of *FLO1*, in particular Flo8p. Indeed, the Ras-cAMP pathway appears to regulate both *FLO1* and *FLO11,* mainly via this protein, and Flo8p has been shown to bind to the promoters of both genes (Kobayashi *et al.*, 1998).

A large set of evidence also suggests that *FLO1* and *FLO11* are both regulated by chromatin-dependent processes, in particular the general repressor complex, Tup1p/Ssn6p, and the chromatin-remodelling Swi-Snf complex (Fleming and Pennings, 2001). In the presence of a high concentration of glucose, many genes are repressed by the general repression complex formed by the Tup1p-Ssn6 co-repressors (Smith and Johnson, 2000). Tup1p and Ssn6p bind to the promoters of *FLO1* and *FLO11* and thus prevent the expression of the lectin proteins (Teunissen *et al.*, 1995). As a consequence,

deletion of either of the two regulatory genes results in strains with a strong flocculation phenotype.

Fleming and Penning (2001) showed that the repressive activity of these co-repressors is lifted through the antagonistic effects of the Swi-Snf chromatin remodelling complex, whose activity organises an extensive chromatin domain around the *FLO1* promoter. *TUP1* and *SSN6* are also repressed at the transcriptional level by the depletion of glucose, explaining, at least in part, the derepression observed for both *FLO1* and *FLO11* in glucose-limited growth media.

2.4 CONCLUSION

Although many questions regarding flocculation and its regulation remain, the past decade has witnessed major advances in this field of study. This review has highlighted some of these advances.

Physical and chemical studies revealed the importance of cell wall composition and the physical characteristics of the cellular surface in the process. The studies suggest that flocculation is the result of non-specific (hydrophobic, electrostatic) and specific (lectin-like proteins) interactions, and that it requires specific growth conditions, in particular regarding media composition, agitation and aeration.

Genetic studies of flocculation revealed several genes that are essential for the process to occur. These genes include *FLO1*, *FLO5*, *FLO10* and *FLO11*, which all encode lectin-like proteins. Furthermore, proteins that control the expression of these lectin-like proteins play a major role in the process, in which complex signalling pathways and numerous trancriptional activators are involved. A central transcriptional regulator appears to be Flo8p, which activates the expression of both *FLO1* and *FLO11* and acts downstream of the RAS-cAMP pathway (Kobayashi *et al.*, 1998).

Nevertheless, numerous question regarding the flocculation process remain unanswered. For example, no gene specific gene has as yet been associated with flocculation in top-fermenting yeast strains, and the specific roles of each of the lectin-encoding dominant flocculation genes has not been investigated fully. Furthermore, the complex regulatory processes that regulate the expression of the various lectin-like proteins are not yet fully understood. Considering that the expression of these cell wall proteins is associated with major changes in cellular physiology, the study of these processes is bound to reveal new insights into fundamental aspects of cellular biology.

2.5 REFERENCES

Amory, D. E., Rouxhet, P. G. and Dufour, J. P. (1988). Flocculence of brewery yeasts and their surface properties, chemical composition, electrostatic charge and hydrophobicity. *J Inst Brew* **94,** 79-84.

Ballou, C. E. (1990). Isolation, characterization, and properties of *Saccharomyces cerevisiae* mnn mutants with nonconditional protein glycosylation defects. *Methods Enzymol* **185,** 440-470.

Bauer, F. F. and Pretorius, I. S. (2001). Pseudohyphal and invasive growth in *Saccharomyces cerevisiae*. *Focus on Biotechnology – Applied Microbiology* **2,** 109-133.

Beavan, M. J., Belk, D. M., Stewart, G. G. and Rose, A. H. (1979). Changes in electrophoretic mobility and lytic enzyme activity associated with development of flocculating ability in *Saccharomyces cerevisiae* Yeast. *Can J Microbiol* **25,** 888-895.

Bony, M., Thines-Sempoux, D., Barre, P. and Blondin, B. (1997). Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *J Bacteriol* **179,** 4929-4936.

Carstens, E., Lambrechts, M. G. and Pretorius, I. S. (1998). Flocculation, pseudohyphal development and invasive growth in commercial wine yeast strains. *S Afr J Enol Vitic* **19,** 52-61.

Dengis, P. B., Néllisen, L. R. and Rouxhet, P. G. (1995). Mechanisms of yeast flocculation : Comparison of top- and bottom-fermenting strains. *Appl Environ Microbial* **61,** 718-728.

Fleet, G. H. (1991). "The yeasts". Rose, A.H. and Harrison, J.S. eds. 2nd edn, Academic Press, London. **4,** 199.

Fleming, A. B. and Pennings, S. (2001). Antagonistic remodelling by Swi-Snf and Tup1-Ssn6 of an extensive chromatin region forms the background for FLO1 regulation. *EMBO J* **20,** 5219-5231.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999). Divergent regulation of the evolutionarily closely related promoters of the *Saccharomyces cerevisiae STA2* and *MUC1* genes. *J Bacteriol* **181,** 6497-6508.

Gimeno, C. J. and Fink, G. R. (1994). Induction of pseudohyphal growth by overexpression of *PHD1*, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Mol Cell Biol* **14,** 2100-2112.

Guo, B., Styles, C. A., Feng, Q. and Fink, G. R. (2000). A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *PNAS* **97,** 12158-12163.

Hodgson, J. A., Berry, D. R. and Johnston, J. R. (1985). Discrimination by heat and proteinase treatments between flocculent phenotypes conferred on *Saccharomyces cerevisiae* by the genes *FLO1* and *FLO5*. *J Gen Microbiol* **131,** 3219-3227.

Javadekar, V. S., Sivaraman, H., Sainkar, S. R. and Khan, M. I. (2000). A mannose-binding protein from the cell surface of flocculent Saccharomyces cerevisiae (NCIM 3528): its role in flocculation. *Yeast* **16**, 99-110.

Jayatissa, P. M. and Rose, A. H. (1976). Role of wall phosphomannan in flocculation of *Saccharomyces cerevisiae. J Gen Microbiol* 96,165-174.

Klis, F. M. (1994) Review: cell wall assembly in yeast. *Yeast* **10,** 851-869.

Kobayashi, O., Hayashi, N., Kuroki, R. and Sone, H. (1998). Region of Flo1 proteins responsible for sugar recognition. *J Bacteriol* **180,** 6503-6510.

Kobayashi, O., Suda, H., Ohtani, T. and Sone, H. (1996). Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Mol Gen Genet* **251,** 707-715.

Kopecka, M. (1985). Electron microscopic study of purified polysaccharide components glucans and mannan of the cell walls in the yeast *Saccharomyces cerevisiae*. *J Basic Microbiol* 25, 161-174.

Lambrechts, M. G., Bauer, F. F., Marmur, J. and Pretorius, I. S. (1996). Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. *Proc Natl Acad Sci USA* **6,** 8419-8424.

Liu, H., Styles, C. A. and Fink, G. R. (1996). *Saccharomyces cerevisiae* S288C has a mutation in FLO8, a gene required for filamentous growth. *Genetics* **144,** 967-978.

Lo, W. S. and Dranginis, A. M. (1996). *FLO11*, a yeast gene related to the *STA* genes, encodes a novel cell surface flocculin. *J Bacteriol* **178,** 7144-7151.

Madhani, H. D., Styles, C. A. and Fink, G. R. (1997). MAP kinases with distinct inhibitory functions impart signalling specificity during yeast differentiation. *Cell* **91,** 673-684.

Masy, C. L., Kockerols, M. and Mestdagh, M. M. (1991). Calcium activity versus "calcium threshold" as the key factor in the induction of yeast flocculation in simulated industrial fermentations. *Can J Microbiol* **37,** 295-303.

Miki, B. L. A., Poon, N. H., James, A. P. and Seligy, V. L. (1982a). Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. *J Bacteriol* **150,** 878-889.

Miki, B. L. A., Poon, N. H. and Seligy, V. L. (1982b). Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*. *J Bacteriol* **150,** 890-899.

Mösch, H. -U., Roberts, R. L. and Fink, G. R. (1996). Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **93,** 5352-5356.

Mota, M. and Soares, E. V. (1994) Population dynamics of flocculating yeasts. *FEMS Microbiol Rev* **14,** 45-51.

Nishihara, H., Toraya, T. and Fukui, S. (1982). Flocculation of cell walls of brewer's yeast and effects of metal ions, protein-denaturants and enzyme treatments. *Arch Microbiol* **131,** 112-115.

Pan, X. and Heitman, J. (1999) Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19,** 4874-4887.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millenium: novel approaches to the ancient art of winemaking. *Yeast* **16,** 675-729.

Rupp, S., Summers, E., Lo, H. J., Madhani, H. and Fink, G. R. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. *EMBO J* **18,** 1257-1269.

Shankar, C. S. and Umesh-Kumar, S. (1994). A surface lectin associated with flocculation in brewing strains of *Saccharomyces cerevisiae*. *Microbiol* **140,** 1097-1101.

Sharon, N. and Lis, H. (1990). Legume lectins - a large family of homologous proteins. *Fed Am Soc Exp Biol* **4,** 3198-3208.

Sieiro, C., Reboredo, N. M., Blanco, P. and Villa, T. G. (1997). Cloning of a new *FLO* gene from the flocculating *Saccharomyces cerevisiae* IM1-8b strain. *FEMS Micro Biol Lett* **146,** 109-115.

Smit, G., Straver, M. H., Lugtenberg, B. J. J. and Kijne, J. W. (1992). Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Appl Environ Microbiol* **58,** 3709-3714.

Smith R. L. and Johnson A. D. (2000) Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem Sci* **25,** 325-330.

Speers, R. A., Tung, M. A., Durance, T. D. and Stewart, G. G. (1992). Colloidal aspects of yeast flocculation: a review. *J Inst Brew* **98,** 525-531.

Stewart, G. G., Russell, I. and Garrison, I. F. (1975). Some considerations of the flocculation characteristics of ale and lager yeast strains [*Saccharomyces cerevisiae*]. *J. Inst. Brew.* 81, 248-257.

Stratford, M. (1992) Yeast flocculation: receptor definition by mnn mutants and concanavalin A. *Yeast* **8,** 635-645.

Stratford, M. and Assinder, S. (1991). Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. *Yeast* **7,** 559-574.

Stratford, M. and Brundish, H. M. (1990). Yeast flocculation: Cationic inhibition. *Yeast* **6**, 77-86.

Stratford, M. and Carter, A. T. (1993). Yeast flocculation: lectin synthesis and activation. *Yeast* **9,** 371-378.

Stratford, M., Coleman, H. P. and Keenan, M. H. (1988). Yeast flocculation: a dynamic equilibrium. *Yeast* **4**, 199-208.

Stratford, M. and Keenan, M. H. (1987) Yeast flocculation: kinetics and collision theory. *Yeast* **3,** 201-206.

Stratford, M. and Keenan, M. H. (1988). Yeast flocculation: quantification. *Yeast* **4**, 107-115.

Straver, M. H., Van der Aar, P. C., Smit, G. and Kijne, J. W. (1993a). Determinants of flocculence of brewer's yeast during fermentation in wort. *Yeast* **9,** 527-532.

Straver, M. H., Kijne, J. W. and Smit, G. (1993b). Cause and control of flocculation in yeast. *Trends Biotechnol* **11,** 228-232.

Teunissen, A. W., Van den Berg, J. A. and Steensma, H. Y. (1993). Physical localization of the flocculation gene *FLO1* on chromosome I of *Saccharomyces cerevisiae*. *Yeast* **9,** 1-10.

Teunissen, A. W., Van den Berg, J. A. and Steensma, H. Y. (1995). Localization of the dominant flocculation genes *FLO5* and *FLO8* of *Saccharomyces cerevisiae*. *Yeast* **11,** 735-745.

Teunissen, A. W. and Steensma, H. Y. (1995). Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast* **11,** 1001-1013.

Van Haecht, J. L., Defosse, C., Van den Bogaert, B. and Rouxhet, P. G. (1982). Surface properties of yeast cells: chemical composition by XPS and isoelectric point. *Colloids Surf* **4,** 343-358.

Van Hamersveld, E. H., Van Loosdrecht, M. C. M. and Luyben, K. C. A. M. (1994). How important is the physicochemical interaction in the flocculation of yeast cells? *Colloids Surf* **2,** 165-171.

Verstrepen, K. J., Derdelinckx, G., Delvaux, F., Winderickx, J., Thevelein, J. M., Bauer, F. F. and Pretorius, I. S. (2001). Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59,** 69-76.

Watari, J., Takata, Y., Ogawa, M., Sahara, H., Koshino, S., Onnela, M., Airaksinen, U., Jaatinen, R., Pentilla, M. and Keranen, S. (1994). Molecular cloning and analyisis of the yeast flocculation gene *FLO1*. *Yeast* **10,** 211-225.

Yamashita, I. and Fukui, S. (1983). Mating signals control expression of both starch fermentation genes and a novel flocculation gene *FL08* in the yeast *Saccharomyces*. *Agric Biol Chem* **47,** 2889-2896.

CHAPTER 3

RESEARCH RESULTS

The controlled expression of dominant flocculation genes in *Saccharomyces cerevisiae*

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The controlled expression of dominant flocculation genes in *Saccharomyces cerevisiae*

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ABSTRACT

The term flocculation refers to the non-sexual, calcium-dependent aggregation of yeast cells, which is usually followed by the sedimentation of the resulting flocs. The process is of importance in the brewing and wine-making industries as a potentially effective and cheap method to clarify the product after fermentation. However, flocculation by naturally-flocculating yeast strains is difficult to control and premature flocculation may result in stuck or sluggish fermentation and a loss of product. For this reason, most commercial yeast strains are either non-flocculating or flocculate inefficiently. In this study, the chromosomal promoters of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, were replaced by the stationary phase-inducible promoters of the *HSP30* and *ADH2* genes in a non-flocculating FY23 strain. The data show that some of the genetically engineered strains were able to flocculate efficiently, and specific promoter-gene combinations resulted in specific flocculation behaviours. Promoters fused to the *FLO1* ORF resulted in the most efficient flocculation, while strains with a high expression of the *FLO11* gene only flocculated in non-fermentable carbon sources. These strains also showed an ability to adhere to and invade the solid agar-containing growth media. In all cases, the intensity of the flocculation phenotype increased with a reduction in nitrogen sources.

3.1 INTRODUCTION

Flocculation can be defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs that sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997).

The flocculation process is influenced by physical, chemical and genetic factors. These factors can also be divided into those that depend on characteristics of the cell and those that dependent on the external environment. Cellular characteristics that influence flocculation efficiency include cell surface charge and hydrophobicity (Smit *et al.,* 1992), which play a complementary role to specific receptor-ligand-type interactions of lectins (sugar-binding proteins) with polysaccharides, in particular mannans, in the cell wall of adjacent cells (Stratford, 1992). Environmental factors include temperature, pH, as well as the concentration of ions, oxygen, sugar and inositol. Furthermore, flocculation is dependent on the growth phase and cell density (Pretorius, 2000).

Several chromosomal genes have been shown to play a role in flocculation. They include the so-called dominant flocculation genes, *FLO1*, *FLO5* and *FLO11,* and other recessive or semi-dominant genes (Stratford, 1992). Most of the identified dominant genes encode for so-called flocculins, which are lectin-like proteins that interact with sugar molecules in the cell wall of adjacent cells (Stratford, 1992). On the other hand, some dominant flocculation genes, for example *FLO8*, were characterised as transcriptional activators of *FLO1*, *STA1* and *FLO11* (Kobayashi *et al.*, 1996, Gagiano *et al.*, 1999).

Flocculation in industrial strains frequently leads to processing problems, since strains may flocculate too early during fermentation, i.e. before the fermentation process is finished, resulting in stuck fermentations. This can result in products with a low alcohol and excessive sugar content, as well as unsatisfactory aromatic characteristics, including severe off-flavours (Verstrepen *et al.*, 2001).

On the other hand, flocculation may be desirable once fermentation has been completed, since efficient settling is needed to minimise problems with wine or beer clarification. The process has also been linked to enhanced ester production (Pretorius, 2000).

The aim of this study was to assess the possibility of achieving controlled flocculation behaviour in *Saccharomyces cerevisiae* through genetic modification. For this purpose, the chromosomal copy of open reading frames (ORFs) of the dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, were linked to the promoters of the *HSP30* and *ADH2* genes. These promoters are known to result in high levels of expression during the stationary phase, while generally being repressed during fermentative growth (Riou *et al.*, 1997; Denis *et al.*, 1992).

For this purpose, various promoter-replacement cassettes were constructed, which consisted of a dominant selection marker (*SMR1*) that was combined with either the *HSP30* or *ADH2* promoters on a linear fragment of DNA through fusion PCR. The

fragments were flanked by sequences homologous to the 5' and 3' regions of the chromosomal promoters of the three dominant flocculation genes investigated, namely *FLO1*, *FLO5* and *FLO11*. After transformation, these linear DNA fragments were expected to integrate into the yeast genome through homologous recombination, replacing the native promoters of the three genes by the stationary phase-inducible heterologous promoters. The strategy is designed to generate new strains with changed flocculation behaviours without integrating any DNA from other organisms, since both the dominant marker gene *SMR1*, which is a point-mutated version of the *S. cerevisiae ILV2* gene, as well as the two promoters, originate from *S. cerevisiae*.

Here we assess the flocculation properties conferred by these constructs on the naturally non-flocculating yeast strain FY23. The data show that the three structural genes play partially overlapping, but not identical, roles in various cellular attachment processes. The data also show that the genetically engineered strains are able to flocculate and that specific promoter-gene combinations result in specific flocculation behaviours. Promoters fused to the *FLO1* ORF resulted in the most efficient flocculation, followed by strains containing the modified version of *FLO5*. Strains with a high expression level of *FLO11*, on the other hand, only flocculated in non-fermentable carbon sources. The *FLO11* strains also showed a strongly enhanced ability to adhere to the agar-containing solid media and to grow invasively into the growth media. In all cases, the intensity of the flocculation phenotype increased with a reduction in nitrogen sources, probably indicating the contribution of other genes to the process. These results clearly show that genetic modification can result in yeast strains with desired flocculation properties.

3.2 MATERIALS AND METHODS

3.2.1 YEAST STRAINS AND CULTURE CONDITIONS

All strains used in this study are listed in Table 1 and are from the S288C (FY23) genetic background. The strains were grown at 30°C in standard yeast media, prepared according to Sherman *et al.*, (1991). Selective media contained 0.67% yeast nitrogen base (YNB) with ammonium sulphate (Difco) and specific amino acids that were added as required for each strain. Carbon sources included 4% glucose for SCD, 4% starch for SCS and 4% glycerol and 4% ethanol for SCGE. Agar was added to a final concentration of 2% for the solid media. For the selection of yeast transformants, minimal SD medium was supplemented with the required amino acids, as well as with 100 mg/l of SMM (Sulfomethyl-Meturon), which was used as the selective agent (Sherman *et al.*, 1991; Casey *et al.*, 1988).

Table 3.1. Yeast strains used in this study.

Strain	Relevant Genotype	Source of reference
S288C (FY23)	MATa *leu2 trp1 ura3 smr1*	Verstrepen *et al.*, 2001
JDFYS-1	MATa *leu2 trp1 ura3* P*FLO1*::SMR1-HSP30p	This study
JDFYS-2	MATa *leu2 trp1 ura3* P*FLO1*::SMR1-ADH2p	This study
JDFYS-3	MATa *leu2 trp1 ura3* P*FLO5*::SMR1-HSP30p	This study
JDFYS-4	MATa *leu2 trp1 ura3* P*FLO5*::SMR1-ADH2p	This study
JDFYS-5	MATa *leu2 trp1 ura3* P*FLO11*::SMR1-HSP30p	This study
JDFYS-6	MATa *leu2 trp1 ura3* P*FLO11*::SMR1-HSP30p	This study

3.2.2 PRIMERS AND CONSTRUCTION OF PROMOTER CONSTRUCTS

All primers used in this study are listed in Table 2. DNA fragments corresponding to either the *SMR1* gene or the promoters of *ADH2* and *HSP30*, each flanked by one short region of homology for the promoter area of the dominant flocculation genes, were generated through PCR using primer pairs 5'-MUC1-SMR1 and SMR1-3', 5'-HSP30 and HSP30-MUC1-3', 5'-ADH2 and ADH2-MUC1-3', 5'-FLO5-SMR1 and SMR1-3', 5'-HSP30 and HSP30-FLO5-3', 5'-ADH2 and ADH2-FLO5-3', 5'-FLO-SMR1 and SMR1-3', 5'-HSP30 and HSP30-FLO1-3', and 5'-ADH2 and ADH2-FLO1-3'. These PCRs resulted in fragments 5'-MUC1-SMR1-3', 5'-HSP30-MUC1-3', 5'-ADH2-MUC1-3', 5'-FLO5-SMR1-3', 5'-HSP30-FLO5-3', 5'-ADH2-FLO5-3', 5'-FLO1-SMR1-3', 5'-HSP30-FLO1-3' and 5'-ADH2-FLO1-3' respectively. All fragments were ligated into the pGEM-T-Easy plasmid. The *FLO1*, *FLO5* and *MUC1* homologous areas were integrated into the corresponding PCR primers and were from 20-25 bp in length.

The *SMR1* gene-containing fragment of the 2.9 kb fragment was cut from pGEM-T-Easy by digesting with the enzymes *Bam*HI, *Alw*44I and *Sph*I. Furthermore, 1.3kb and 800bp fragments were cut from pGEM-T-Easy plasmids containing the *HSP30* and *ADH2* promoters respectively through digestion with the enzymes *Bgl*II and *Spe*I.

The *SMR1* and promoter fragments were then ligated and were used as DNA templates for PCR amplification of the desired products. Six constructs were made, each having at its extremities short stretches of DNA homologous to the 5' and 3' regions of the promoters of the structural flocculation genes, *FLO1*, *FLO5* or *FLO11*, and consisting of the *SMR1* dominant selection marker and either the *HSP30* or *ADH2* promoter. The constructs were designated FLO1-SMR1-HSP30-FLO1 and FLO1-SMR1-ADH2-FLO1, FLO5-SMR1-HSP30-FLO5 and FLO5-SMR1-ADH2-FLO5, MUC1-SMR1-HSP30-MUC1 and MUC1-SMR1-ADH2-MUC1. The primers, FLO1-forward and FLO1-reverse, FLO5-forward and FLO5-reverse, and MUC1-forward and MUC1-reverse respectively were used for the amplification of each of the final constructs.

Table 3.2. Primers used in this study.

Name of primer	Primer sequence
FLO1-SMR1	5'-TGC GTC ACT TTT CCT ACG GTG CCT CGC ACA TGA ATG TTA TCC GGC GCA CGG GTA CCG GCT TGG CTT CAG TTG CTG-3'
FLO5-SMR1	5'-GCA ATA AAC CAC ATG GCT ACC GCA CTT CTT GTC ACT ATC CGG TAC CGG CTT GGC TTC AGT TGC TG-3'
MUC1-SMR1	5'-TCA CTG CAC TTC AAC TAT GCC TTA TAG CAA CCA AGA AGC TAG AAA ATG CCA ACT ATT AAA AAG ATA ACC TCT CGG TAC CGG CTT GGC TTC AGT TGC TG-3'
SMR1-3'	5'-CAT GGG ATC CAG CTT GCA ATT TTT GAC GGC CCC-3'
HSP30-5'	5'-CAT GAG ATC TGA TGG CAT TGC ACT CAA G-3'
HSP30-FLO1	5'-GCG ATG AGG CAT TGT CAT TTT TGG ATG TTC TGT TTA CTG GTG ACA AAA GAT ATT AAA GTC TCA AAC TTG-3'
HSP30-FLO5	5'-GCT AAT CAA TTT AAA GAA AAT CAA TTG CGG AAT TTA CTG CAG AGC TAT TAA AGT CTC AAA CTT G-3'
HSP30-MUC1	5'-GGA CCA AAT AAG CGA GTA GAA ATG GTC TTT GCA TAG TGT GCG TAT ATG GAT TTT TGA GGC AAA AGA TAT TAA AGT CTC AAA CTT GTT G-3'
ADH2-5'	5'- TGA CAG ATC TAA CTC GTT CCA GTC AGG AAT G-3'
ADH2-FLO1	5'-GCG ATG AGG CAT TGT CAT TTT TGG ATG TTC TGT TTA CTG GTG ACA AAA GAT GAT AGT TGA TTG TAT GCT TTT TGT AGC-3'
ADH2-FLO5	5'-GCT AAT CAA TTT AAA GAA AAT CAA TTG CGG AAT TTA CTG CAG AGC TGA TAG TTG ATT GTA TGC TTT TTG TAG C-3'
ADH2-MUC1	5'-GGA CCA AAT AAG CGA GTA GAA ATG GTC TTT GCA TAG TGT GCG TAT ATG GAT TTT TGA GGC AAA AGA TGA TAG TTG ATT GTA TGC TTT TTG TAG C-3'
FLO1-forward	5'-AAG TGT GCG TCA CTT TTC CTA CGG T-3'
FLO1-reverse	5'-AGC GAT GAG GCA TTG TCA TTT-3'
FLO5-forward	5'-GCA ATA AAC CAC ATG GCT ACC-3'
FLO5-reverse	5'-AGT GGT GCT AAT CAA TTT AAA GAA-3'
MUC1-forward	5'-CCT CTC ACT GCA CTT TCA TAT TCC-3'
MUC1-reverse	5'-GGA CCA AAT AAG CGA GTA GA-3'

3.2.3 CONSTRUCTION OF YEAST STRAINS WITH *HSP30* AND *ADH2* PROMOTERS IN FRONT OF FLOCCULATION GENES

Amplified PCR products were transformed into yeast strain FY23. The yeast transformations were performed using the lithium acetate method, as described by Ausubel *et al.* (1994). Positive SMM-resistant yeast colonies were recovered and the

integration of the fragment was verified by means of PCR. These colonies were then assessed for their flocculation, adhesion and invasive growth behaviour.

3.2.4 FLOCCULATION ASSAY

Yeast cultures (100 ml) were grown in 250 ml Erlenmeyer flasks to the desired phase of growth, and 1 ml each was transferred into two microcentrifuge tubes. After centrifugation, the pellets were resuspended in either 990 µl of water and 10 µl of EDTA (0.5 M, pH 8) or in 1 ml of a solution containing 0.63 g/l $CaCl_2$ and 6.8 g NaOAc and adjusted to a pH of 4.5. The mixtures were vortexed for 15 seconds and 100 µl of each suspension was resuspended in 900 µl of distilled water. The tubes were left to stand for 5 min and 200 µl were pipetted from just below the meniscus of each tube and resuspended in 800 ml of dH_2O.

Ten µl of 0.5M EDTA (pH 8.0) were added to both tubes, which were vortexed for 2 min. The OD at 600nm was measured for both tubes and the percentage of flocculation was determined by the equation $100 \text{ X } (OD_{600(Tube1)} - OD_{600(Tube2)}) / OD_{600(Tube1)}$.

3.2.5 DETERMINATION OF THE INFLUENCE OF HEAT SHOCK TREATMENT AND GLUCOSE DEPLETION ON FLOCCULATION

To assess the effect of heat stress on the flocculation efficiency, yeast cells were grown to the mid-exponential phase (OD_{600} of 1.0) at 30°C. Two times 5 ml were transferred into two 10 ml thin-walled glass tubes. One of the tubes was plunged into a water bath at 42°C for 30 min, whereas the other tube remained at 30°C. A flocculation assay (3.2.4) was then carried out for both cultures.

To assess the effect of glucose depletion, the strains were grown in 20 ml of rich YPD medium containing 4% glucose to an OD_{600} of 1. The cultures were then centrifuged at 3000 rpm for 2 min, and the culture supernatant was discarded. Strains were resuspended either in YPD or SCGE liquid medium. The cultures were then left to grow at 30°C for 45 min, followed by flocculation assays as described above (3.2.4).

3.2.6 INVASIVE GROWTH AND CELL ADHESION

For invasive growth study, cells were grown up to an OD of 1.0. Ten µl of each culture were then spotted on plates containing 2% glucose, 0.2% glucose and 2% glycerol / 3% ethanol respectively. Plates were left to grow for five days at 30°C. To assess invasive growth, cells were washed off the plates under running water and rubbed with a gloved finger.

To assess cell adhesion, cells were grown to stationary phase in a YPD medium. Ten µl of each culture was then taken and spotted on three different media containing either 2% glucose, 0.2% glucose or 2% glycerol/3% ethanol as carbon source. Plates were left in the incubator at 30°C for 1 h, and then washed under running water.

3.2.7 TEST FOR FLOCCULATION IN DIFFERENT CARBON SOURCES

Cells were grown in either rich (2% Yeast Extract) or minimal (6.7 g/l of YNB with ammonium sulphate) media containing the following carbon sources: 2% of either glucose, sucrose, fructose, galactose or 2% glycerol/3% ethanol. Cells were grown for two days in 10 ml of media in glass tubes at 200 rpm. To assess flocculation visually, tubes were briefly vortexed and then left to stand on the bench without agitation. Photos were taken after 30, 60 and 90 min.

3.3 RESULTS

3.3.1 FLOCCULATION ASSAYS

All the strains containing the six different promoter-ORF fusions, as well as the FY23 WT, were inoculated to an initial OD_{600nm} of 0.05 in YPD. No flocculation could be observed during the early stages of growth. Strains reached stationary phase after two days of growth. After three days, flocculation became visually detectable in the various cultures of strains in which P*ADH2* had been fused to the structural flocculation genes *FLO1* and *FLO5*. On the other hand, strains that had integrated the *HSP30* promoter only showed flocculating phenotypes after several more days, indicating that transcriptional activation by this promoter was probably less efficient.

Figure 3.1. Flocculation of strains transformed with P*ADH2* after 10 days of growth. The name of each strain is indicated in the figure. The tube on the left contains YPD without any yeast.

In Figure 1, the three strains containing the promoter of the *ADH2* gene fused to each of the three different structural flocculation genes are shown together with the wild type

strain FY23. The figure shows the flocculation phenotype after 10 days of growth, when flocculation appeared at its strongest. The results of the flocculation assays are presented in Table 3. The data clearly show that the expression of the *FLO1* gene results in the strongest flocculation, followed by that of *FLO5*. However, no flocculation can be observed visually in the strain P*ADH2-FLO11*. However, flocculation assays revealed a low percentage of flocculation, indicating some activity of Flo11p in these conditions.

Table 3.3. Comparison of the ability of three dominant flocculation genes to activate flocculation in stationary phase when regulated by P*ADH2* in YPD media. Tests were done in triplicate.

Yeast strains	Flocculation
JDFYS-2 (ADH2-FLO1)	93%
JDFYS-4 (ADH2-FLO5)	85%
JDFYS-6 (ADH2-FLO11)	5%
FY23	1%

The same strains were also evaluated when grown on other fermentable carbon sources, including sucrose, fructose and galactose, and results similar to those in YPD media were observed (Table 4). Indeed, all the strains flocculated with similar efficiency in each medium. Flocculation was always highest in strains carrying the *ADH2* promoter fused to *FLO1*, followed by the same promoter fused to *FLO5*. Flocculation induced by the promoter of *HSP30* was always approximately 50% lower than that induced by P*ADH2*. As described above, overexpression of *FLO11* never resulted in visual flocculation, although a small percentage of flocculating cells was always detected by the flocculation assay.

Table 3.4. Levels of flocculation when strains were grown on different fermentable carbon sources.

Yeast strains	Flocculation
FY23-wt	-
JDFYS-1	++
JDFYS-2	++++
JDFYS-3	++
JDFYS-4	+++
JDFYS-5	+
JDFYS-6	+

Flocculation scale: 75-100% = ++++, 50-74.9% = +++, 25-49.9% = ++, 5-24.9% = +, 0-5% = -.

3.3.2 CELL ADHESION AND INVASIVE GROWTH

FLO11 was initially identified for its ability to induce invasive growth when overexpressed (Lambrechts *et al.*, 1996). Invasion requires adhesion to the substrate, followed by directed budding to allow cells to penetrate below the surface of the agar. For this reason,

all the strains were compared for their ability to adhere to solid media (Fig. 2) and to invade the agar (Fig. 3).

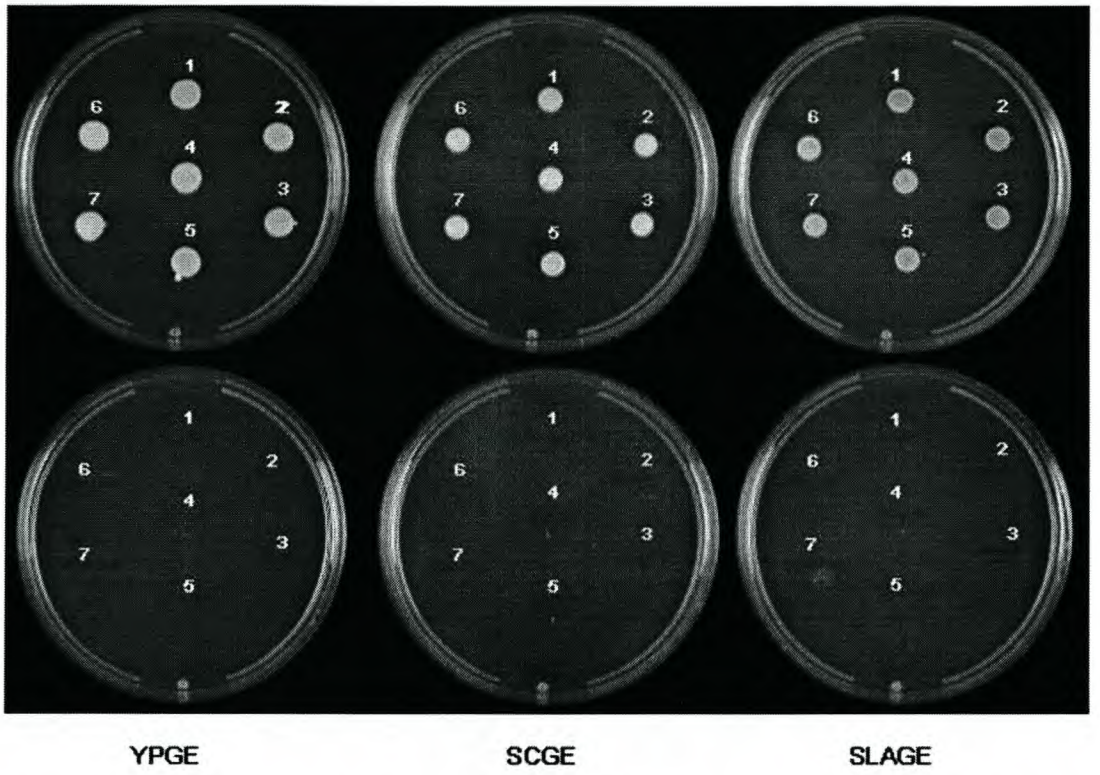

Figure 3.2. The six strains constructed in this study were compared to the wild type to investigate their ability to adhere to the agar. The strains 1-7 are as follows: 1-FY23-wt; 2-JDFYS 1(FLO1-HSP30); 3-JDFYS 2 (FLO1-ADH2); 4-JDFYS 3 (FLO5-HSP30); 5-JDFYS 4 (FLO5-ADH2); 6-JDFYS 5 (FLO11-HSP30); 7-JDFYS 6 (FLO11-ADH2). The top half of the picture represents the plates before washing, and the bottom half the plates after washing.

Several media were used in these assays to assess the phenotypes. Since glucose represses both promoters that were investigated in this study, glycerol and ethanol were used as sole carbon sources. Furthermore, rich yeast extract medium was compared with minimal medium containing either high (SCGE) or low levels of nitrogen (SLAGE). These media are expected to induce the two promoters to different degrees, with SLAGE media resulting in the strongest induction, since nitrogen limitation is perceived as a stress signal by the cells. The data presented in Figs. 2 and 3 show that only strains overexpressing *FLO11* were able to adhere to and invade the growth media.

As expected, adhesion was strongest on SLAGE, but was also evident on SCGE. No adhesion could be observed on YPGE, probably reflecting lower levels of expression of the transgenes. As observed in the flocculation assays, the *ADH2* promoter appeared to confer higher levels of expression than the *HSP30* promoter, since the strain containing the P*ADH2*-*FLO11* fusion showed by far the highest adhesion levels. Strains containing either *FLO1* or *FLO5* overexpression constructs were unable to attach to the media under

the same conditions. The data also provide evidence that the inability of the FY23 strain to grow invasively is entirely due to the low expression levels of the native *FLO11* promoter, which probably are a result of the absence of the Flo8p transcriptional activator (Liu *et al.*, 1996). The ORF of the gene appears to be fully functional, since invasion levels conferred by the *ADH2* promoter compare well with those observed in invasive yeast strains. The increase in the intensity of invasive growth when nitrogen levels are low is also interesting to note. It is unlikely that this is due to the *FLO11* expression levels, since the *ADH2* promoter is activated by glucose depletion and is not known to respond to nitrogen-dependent signalling. It therefore is likely that other factors play a role and intensify the phenotype in response to nitrogen limitation.

A. Before washing B. After washing

Figure 3.3. The ability of the yeast strains to invade the agar plates. Cells were grown overnight in YPD and spotted onto SCGE plates. The *FLO11* strains are labelled "*MUC1*" on this plate.

3.3.3 ASSESSMENT OF THE INDUCTION OF FLOCCULATION BY HEAT AND NUTRIENT STRESSES

The data presented above assessed the ability of the different promoter-gene fusions to induce adhesion-related phenotypes during growth in specific conditions. To assess the effect of sudden induction of the three *FLO* genes, conditions that lead to the rapid induction of each of the two promoters were investigated. For the stress-responsive *HSP30* promoter, the effect of a heat shock on the strains that carried the *HSP30* promoter fused to the three flocculation genes was investigated (JDFYS 1, JDFYS 3 and JDFYS 5).

These test also allow an assessment of whether the *HSP30* promoter is responding as expected to specific stress signals when placed in a different chromosomal context.

Table 3.5. Percentage flocculation of yeast cells after heat shock treatment.

Yeast strain	% Flocculation
JDFYS 1 (FLO1-HSP30)	38%
JDFYS 3 (FLO5-HSP30)	5.8%
JDFYS 5 (FLO11-HSP30)	1.3%
FY23	1.5%

From the results in Table 5, it is clear that the HSP30 promoter is fully stress inducible. The data also confirm that, of the three genes investigated, the *FLO1* gene again confers the highest flocculation levels, while *FLO5* is significantly less efficient. *FLO11* appears unable to induce any flocculation in the conditions described above. Strains that had not been subjected to heat shock did not show any flocculation phenotype in the same growth medium.

To assess the rapid induction of the *ADH2* promoter constructs, the P*ADH2* strains were grown to an OD of 1.0 in glucose containing YPD and transferred to SCGE (4% glycerol/3% ethanol) medium. The strains were then left for 40 minutes at 30°C and subjected to the flocculation assay. As a control, cells were also transferred back to YPD. In the latter case, no flocculation could be observed.

Table 3.6. Percentage flocculation of yeast cells after nutrient limitation shock treatment.

Yeast strain	% Flocculation
JDFYS 2 (FLO1-ADH2)	17%
JDFYS 4 (FLO5-ADH2)	5.8%
JDFYS 6 (MUC1-ADH2)	3.5%

The data confirm the ability of P*ADH2* to rapidly induce the flocculation genes in response to specific signals. Of the three genes, *FLO1* is again the most efficient in inducing flocculation (Table 6). More importantly, these tests also prove that all the constructs were working correctly and that flocculation behaviour is presumably dependent on the level of gene transcription.

3.3.4 ASSESSMENT OF FLOCCULATION IN NON-FERMETABLE CARBON SOURCES

In the previous experiments, the strains had always been grown in fermentable carbon sources. To assess the effect of growth in a non-fermentable carbon source on

flocculation, all strains were grown in SCGE media. The results are presented in Figure 4.

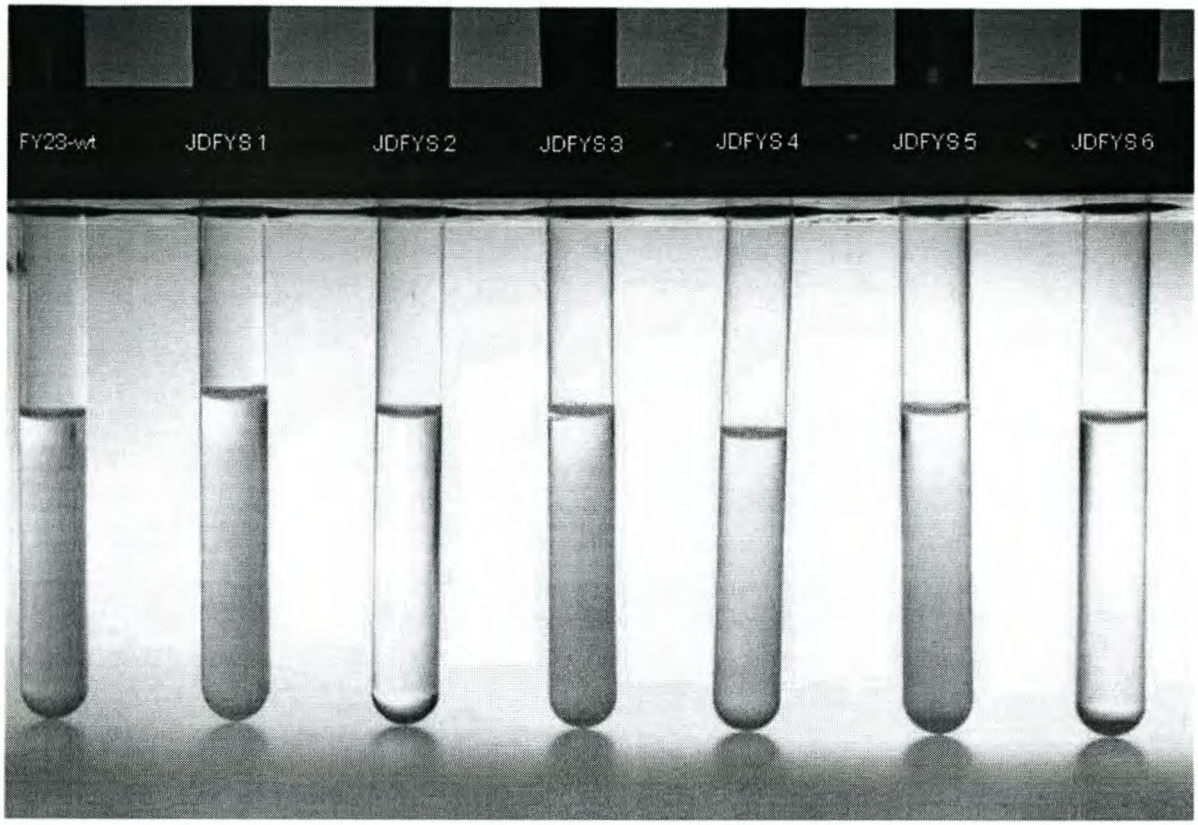

Figure 3.4. Ability of modified strains to flocculate in SCGE media.

In this medium, the *FLO11*-overexpressing strains (JDFYS5 and JDFYS6) were able to flocculate, contrarily to what had been observed in rich yeast extract-containing medium. This may indicate that flocculation, induced by *FLO11*, is inhibited by specific components within the rich growth substrate, or that other genes required for FLO11-dependent flocculation are down-regulated in these conditions.

3.4 DISCUSSION

For this study, strains with controlled flocculation ability were constructed by replacing the native promoters of three dominant flocculation genes with strong stress-inducible and stationary phase-inducible promoters. The data show that the inability of the laboratory yeast strain FY23 to flocculate is not related to mutations in any of the ORFs of the genes investigated, but is presumably due to the low expression levels of these genes.

The flocculation assays showed that the modified strains could flocculate efficiently in specific conditions, and very strong flocculation was obtained when *FLO1* was controlled by the *ADH2* promoter. Indeed, of the two promoters, the *ADH2* promoter always resulted in stronger phenotypes than the *HSP30* promoter. This does not, however, imply that this promoter is more suitable for industrial applications. Most of the industrial processes

stretch over long periods and use conditions that were not assessed in this work. Some of these conditions may require less intense flocculation.

The formation of a velum was also observed, i.e. the formation of a layer of cells on top of the medium, in the *FLO11* overexpressing strain JDFYS6 when grown in the thin glass tubes (data not shown). None of the other strains overexpressing flocculation genes showed a similar phenotype. The cells within the velum were tested for their hydrophobicity, and indeed, they appeared to be more hydrophobic. These data could, however, not be reproduced in an Erlenmeyer flask. The *FLO11* overexpressed cells also showed a tendency to stick to the surface of the test tube, a phenomenon that has previously been described by other scientists (Reynolds and Fink, 2001).

The cell adhesion and invasive growth studies clearly confirm the specific importance of *FLO11* for these processes. We also show that the *FLO1* and *FLO5* genes play a negligible role in invasive growth or cell adhesion. Interestingly, the *FLO11* overexpressing strains showed stronger phenotypes when the amount of nitrogen in the medium was reduced. This may indicate a requirement for other genes to establish an efficient invasive growth phenotype. Indeed, invasion is a complex phenomenon, which requires the coordination of numerous cellular processes that are at least in part regulated by nitrogen availability. It therefore appears that, while *FLO11* is indispensable for the process, other nitrogen-regulated genes contribute significantly.

The heat shock and glucose depletion tests revealed that both promoters confer rapid inducibility on the dominant flocculation genes. This may be of interest to some industrial processes, since it may allow tight control of the fermentation process and the possibility to stop fermentation at an earlier stage if this was desired.

Further research is required to optimise the timing and levels of expression of the dominant flocculation genes. The direct or indirect role of *FLO11* in velum formation also needs to be investigated further.

3.5 REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. and Smith, J. A. (1994). *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.

Bony, M., Thines-Sempoux, D., Barre, P. and Blondin, B. (1997). Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *J Bacteriol* **179,** 4929-4936.

Casey, G. P., Xiao, W. and Rank, G. H. (1988). A convenient dominant selection marker for gene transfer in industrial yeast strains of *Saccharomyces cerevisiae*: SMR1-encoded resistance to the herbicide sulfometron methyl. *J Inst Brew* **94,** 93-97.

Denis, C. L., Fontaine, S. C., Chase D., Kemp, B. E. and Bemis, L. T. (1992). $ADR1^{C}$ mutations enhance the ability of ADR1 to activate transcription by a mechanism that is independent of effects on cyclic-AMP dependent protein kinase phosphorylation of Ser-230. *Mol Cell Biol* **4,** 1507-1514.

Gagiano, M., Van Dyk, D., Bauer, F. F., Lambrechts, M. G. and Pretorius, I. S. (1999). Divergent regulation of the evolutionarily closely related promoters of the *Saccharomyces cerevisiae STA2* and *MUC1* genes. *J Bacteriol* **181,** 6497-6508.

Kobayashi, O., Suda, H., Ohtani, T. and Sone, H. (1996). Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Mol Gen Genet* **251,** 707-715.

Lambrechts, M. G., Bauer, F. F., Marmur, J. and Pretorius, I. S. (1996). Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. *Proc Natl Acad Sci USA* **6,** 8419-8424.

Liu, H., Styles, C. A. and Fink, G. R. (1996). *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. *Genetics* **144,** 967-978.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millenium: novel approaches to the ancient art of winemaking. *Yeast* **16,** 675-729.

Reynolds, T. B. and Fink, G. R. (2001). Baker's yeast, a model for fungal biofilm formation. *Science* **291,** 878-881.

Riou, C., Nicaud, J., Barre, P. and Gaillardin, C. (1997). Stationary phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. *Yeast* **13,** 903-915.

Sherman, F., Fink, G. R. and Hicks, J. (1991). *Methods in yeast genetics*. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, New York. 263.

Smit, G., Straver, M. H., Lugtenberg, B. J. J. and Kijne, J. W. (1992). Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Appl Environ Microbiol* **58,** 3709-3714.

Stratford, M. (1992) Yeast flocculation: receptor definition by mnn mutants and concanavalin A. *Yeast* **8,** 635-645.

Verstrepen, K. J., Derdelinckx, G., Delvaux, F., Winderickx, J., Thevelein, J. M., Bauer, F. F. and Pretorius, I. S. (2001). Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59,** 69-76.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

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4.1 GENERAL DISCUSSIONS AND OTHER PERSPECTIVES

The yeast *Saccharomyces cerevisiae* is able to undergo profound molecular and physiological changes in response to changing environmental conditions. Several phenotypical manifestations of these changes are, at least in part, associated with changes in cellular adhesion properties and include flocculation, pseudohyphal growth and invasive growth.

This work focused on one of these phenomena, flocculation. Flocculation can be defined as the non-sexual, calcium-dependent aggregation of yeast cells to form flocs that sediment from the media in which they are suspended (Stratford and Assinder, 1991). This process has a specific importance for the winemaking and brewing industries, since it potentially represents a cost-effective way to clarify wines or beers and may be of particular use in the production of bottle-fermented sparkling wines. The use of flocculating yeast strains, however, presents several problems, in particular due to the fact that flocculation may occur too early during the fermentation process. This usually results in products with high levels of residual sugar that are susceptible to microbial spoilage (Verstrepen *et al.*, 2001). For this reason, few industrial yeast strains appear to be able to flocculate efficiently (Carstens *et al.*, 1998).

The literature review focused on the physical, chemical and genetic factors that affect the flocculation process, while the research reported in chapter 3 assessed the possibility to adjust the genetic make-up of *S. cerevisiae* to allow controlled flocculation. The work also generated new information on and knowledge of the genes involved in the process and on their respective importance for various phenotypical manifestations of cellular attachment processes.

From a genetic perspective, little is known about flocculation in top-fermenting yeast strains. Our data strongly suggest that *FLO11* may be involved specifically in this process, since strains overexpressing this gene showed velum-forming behaviour in certain specific conditions.

More information is available regarding bottom-fermenting yeast strains, and several genes that play a role in the process have been characterised. Our data clearly highlight the important role of *FLO1* in this process and indicate that *FLO5*, while less efficient than *FLO1*, may also be an interesting target for further work on adjusting flocculation behaviour. It also appears clear that *FLO11* is not the ideal target gene to achieve efficient flocculation. However, the specific adhesion properties of the *FLO11*-overexpressing strains may make the gene an interesting target for other industrial processes, in particular in cases where attachment to specific substrates may be advantageous.

Of the two promoters investigated, the *ADH2* promoter appears better suited for the induction of a strong flocculating phenotype. However, none of the two promoters is likely

to find favour in industrial applications. Indeed, the *HSP30* promoter would present a risk in industrial fermentation processes, since these processes are inherently stressful and unwanted expression of the promoter cannot be excluded (Bauer and Pretorius, 2000). The *ADH2* promoter, on the other hand, while strongly expressed in our conditions, appears less strongly expressed in the stationary phase of wine fermentation. The solution may lie in creating new promoters by combining specific elements, i.e. upstream activating and repressing sequences, of current yeast promoters to achieve very specific expression patterns.

Further research is required to properly assess different promoter and gene combinations in industrial conditions. However, the work presented here clearly shows the feasibility of the approach and opens new possibilities for the future.

4.2 REFERENCES

Bauer, F.F. and Pretorius, I.S. (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine. *S Afr J Enol Vitic* **21,** 27-51.

Carstens, E., Lambrechts, M. G. and Pretorius, I. S. (1998). Flocculation, Pseudohyphal Development and Invasive Growth in Commercial Wine Yeast Strains. *S. Afr. J. Enol. Vitic.* **19,** 52-61.

Stratford, M. and Assinder, S. (1991). Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. *Yeast* **7,** 559-574.

Verstrepen, K. J., Derdelinckx, G., Delvaux, F., Winderickx, J., Thevelein, J.M., Bauer, F. F. and Pretorius, I. S. (2001). Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59,** 69-76.

teen laer ZR vlakke as die langdraers. Hierdie waarneming is soortgelyk aan vorige studies op xileemsap ZR vlakke van appel lote. Die hoë ZR vlakke gevind in die xileemsap van die apikale gedeeltes van lote ondersteun die hipotese van 'n kumulatiewe opbou van ZR soos die lengte van die loot toeneem. Die snoei van lote as kortdraers het gelei tot vroeër bot en 'n hoër finale bot persentasie as die snoei van langdraers. Die basale gedeeltes van lote het verhoogde ZR vlakke in al drie kultivars getoon, onafhanklik van die feit dat dit vanaf die kortdraer of die basale gedeelte van 'n langdraer was. Hierdie verskil in ZR vlakke in ogie weefsel van verskillende gedeeltes van die loot impliseer 'n verskil in ZR verbruik of omset.

Die resultate van hierdie studie het belangrike bestuursimplikasies vir die verbouing van wingerd in warmer gebiede, waar waterstofsiaanamied gebruik word om botprobleme te oorkom.

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