Industrial yeast strains engineered for controlled flocculation

by

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

In many industrial fermentation processes, *Saccharomyces cerevisiae* yeast should ideally meet two partially conflicting demands. During fermentation a high suspended yeast count is of paramount importance to maintain a rapid fermentation rate, whilst efficient flocculation should ideally be initiated only on completion of the primary alcoholic fermentation, so as to enhance product clarification and recovery. Most commercial wine yeast strains are non-flocculent, probably because this trait was counter-selected to avoid fermentation problems. In this study, we assessed molecular strategies to optimise the flocculation behaviour of non-flocculent laboratory and wine yeast strains. For this purpose, the chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, of a non-flocculent *S. cerevisiae* laboratory strain (FY23) and two commercial wine yeast strains (BM45 and VIN13) were placed under the transcriptional control of the stationary phase-inducible promoters of the *S. cerevisiae ADH2* or *HSP30* genes.

Under standard laboratory media and culture conditions, all six promoter-gene combinations resulted in specific flocculation behaviours in terms of timing and intensity. The data show that the strategy resulted in the expected and stable expression patterns of these genes in both laboratory and industrial wine yeast strains. Most importantly, the data confirm that inducible expression of the native *FLO1* and *FLO5* open reading frames, albeit to varying degrees, are responsible for a quantifiable cell-cell adhesion phenotype that can be characterized as a Flo1 flocculation phenotype. On the other hand, we found that inducible expression of the native *FLO11* ORF under these conditions resulted in flor/biofilm formation and invasive growth phenotypes. However, the specific impact of the expression of individual dominant *FLO* genes with regard to characteristics such as flocculation efficiency, cell wall hydrophobicity, biofilm formation and substrate adhesion properties showed significant differences between the commercial strains as well as between commercial and laboratory strains. These adhesion phenotype differences may at least in part be attributed to wine yeast *FLO* gene open reading frames containing significantly smaller intragenic repeat regions than laboratory strains.

The data show that the *ADH2* regulatory sequences employed in this study were unsuitable for the purpose of driving *FLO* gene expression under wine-making conditions. However, *HSP30p*-based *FLO1* and *FLO5* wine yeast transformants displayed similar flocculent phenotypes under both synthetic and authentic red wine-making conditions, and the intensities of these phenotypes were closely aligned to those observed under nutrient-rich YEPD conditions. The fermentation activities of

HSP30p-based transgenic yeast strains were indistinguishable from that of their parental host wine yeast strains. The chemical composition of wines obtained using transgenic yeast strains were similar to those produced by parental strains. The BM45-derived *HSP30p-FLO5* transformant in particular was capable of generating compacted or 'caked' lees fractions, thereby providing a distinct separation of the fermented wine product and lees fractions. Furthermore, in this study we report a novel *FLO11* induced flocculation phenotype that seems to exclusively develop under authentic red wine-making conditions. This strong *FLO11* flocculation phenotype was not wine yeast strain dependant, possessed both Ca^{2+}-dependant and Ca^{2+}-independent flocculation characteristics and was insensitive to inhibition by both glucose and mannose. A distinct advantage of this unique *FLO11* phenotype was highlighted in its ability to dramatically promote faster lees settling rates. Moreover, wines produced by *HSP30p-FLO11* wine yeast transformants were significantly less turbid than those produced by their wild type parental strains. The benefit of this attractive property is it facilitates simpler and faster recovery of wines and also promotes greater volume recovery of the wine product.

OPSOMMING

In baie industriële gistingsprosesse moet die *Saccharomyces cerevisiae*-gis verkieslik aan twee gedeeltelik teenstellende eise voldoen. Tydens gisting is 'n hoë telling van gesuspendeerde gis van die uiterste belang om 'n vinnige gistingstempo te onderhou, terwyl doeltreffende flokkulasie ideaal gesproke eers ná die voltooiing van die primêre alkoholiese gisting geïnisieer moet word om produkverheldering en -herwinning te verhoog. Die meeste kommersiële wyngisrasse is nieflokkulerend, moontlik omdat daar teen hierdie kenmerk geselekteer is om gistingsprobleme uit te skakel. In hierdie ondersoek het ons molekulêre strategieë beoordeel om die flokkulasiegedrag van nieflokkulerende laboratorium- en wyngisrasse te optimaliseer. Vir hierdie doel is chromosomale kopieë van drie dominante flokkulasiegene, *FLO1*, *FLO5* en *FLO11*, van 'n nieflokkulerende *S. cerevisiae* laboratoriumras (FY23) en twee kommersiële wyngisrasse (BM45 en VIN13) onder die transkripsionele beheer geplaas van die promotors van die *ADH2*- of *HSP30*-gene wat deur die stasionêre fase geïnduseer kan word.

Onder standaard laboratoriummedia- en -kultuurtoestande het al ses promotor-geen kombinasies spesifieke flokkulasiegedrag in terme van tydsberekening en intensiteit veroorsaak. Die data toon dat hierdie strategie gelei het tot die verwagte en stabiele uitdrukkingspatrone van hierdie gene in beide die laboratorium- en industriële wyngisrasse. Van groter belang is dat die data bevestig dat induseerbare uitdrukking van die oop leesrame van die inheemse *FLO1* en *FLO5* verantwoordelik is vir 'n kwantifiseerbare sel-sel adhesie fenotipe wat as 'n Flo1 flokkulasie fenotipe gekenmerk kan word, hoewel tot wisselende grade. Aan die ander kant het ons gevind dat die induseerbare uitdrukking van oop leesrame van inheemse *FLO11* onder hierdie toestande gelei het tot flor/biofilmvorming en penetrasie-groei fenotipe. Die spesifieke impak van die uitdrukking van individuele dominante *FLO*-gene met betrekking tot kenmerke soos flokkulasie doeltreffendheid, selwand hidrofobisiteit, biofilmvorming en substraatadhesiekenmerke het egter opmerklike verskille getoon tussen die kommersiële rasse en tussen dié rasse en die laboratoriumrasse. Hierdie verskille in adhesie fenotipe kan ten minste gedeeltelik daaraan toegeskryf word dat die oop leesrame van die wyngis *FLO*-geen opmerklik kleiner intrageniese herhaalstreke bevat.

Dit data toon dat die *ADH2* regulatoriese sekwense wat in hierdie studie gebruik is nie geskik was om *FLO*-geenuitdrukking onder wynbereidingstoestande te dryf nie. *HSP30p*-gebaseerde *FLO1* en *FLO5* wyngistransformante het egter vlokkige fenotipe onder beide wynbereidingstoestande vertoon, en die intensiteit van hierdie fenotipe was soortgelyk aan dié wat onder voedingstofryke YEPD-toestande waargeneem is. Die

gistingsaktiwiteite van *HSP30p*-gebaseerde transgeniese gisrasse was ononderskeibaar van dié van hulle stamgasheer wyngisrasse. Die chemiese samestelling van wyn wat deur middel van transgeniese gisrasse verkry is, is soortgelyk aan dié wat deur die stamrasse verkry is. Die BM45-afgeleide *HSP30p-FLO5* transformant het veral die vermoë gehad om gekompakteerde of 'gekoekte' moerfraksies te genereer en sodoende 'n duidelike skeiding van die gegiste wynproduk en moerfraksies veroorsaak. In hierdie studie rapporteer ons ook oor 'n nuwe *FLO11*-geïnduseerde flokkulasie fenotipe wat blyk om uitsluitlik onder egte rooiwynbereidingstoestande te ontwikkel. Hierdie sterk *FLO11*-flokkulasie fenotipe was nie afhanklik van die wyngisras nie, het beide Ca^{2+}-afhanklike en Ca^{2+}-onafhanklike flokkulasiekenmerke getoon en was onsensitief vir inhibisie deur beide glukose en mannose. 'n Duidelike voordeel van hierdie unieke *FLO11*-fenotipe is na vore gebring deur sy vermoë om dramaties 'n vinniger moerafsaktempo te bevorder. Wyne wat met *HSP30p-FLO11* wyngistransformante geproduseer is, was ook noemenswaardig minder troebel as wyne wat deur die wilde tipe stamrasse geproduseer is. Die voordeel van hierdie aantreklike eienskap is dat dit eenvoudiger en vinniger herwinning van wyn fasiliteer en ook die herwinning van groter volumes van die wynproduk bevorder.

This dissertation is dedicated to my family and in fond remembrance of my dear friend
Sven Kroppenstedt

BIOGRAPHICAL SKETCH

Patrick Govender was born in Durban on 18 February 1966. Following Matriculation, he pursued a Bachelor of Science degree at the then University of Durban-Westville, graduating with majors in Biochemistry and Microbiology. His interest in Biochemistry led him to register for a Bachelor of Science (Honours) degree. Upon completing the Honours degree, he was offered a contract junior-lecturing position in the Department of Biochemistry at the University of Durban-Westville. During this period, Patrick enrolled for a MSc research study that involved *in vitro* receptor-mediated gene delivery to mammalian cells, for which he was awarded the degree *cum laude*.

In seeking variation and broader exposure within the academic research environment, Patrick embarked on a part-time Doctor of Philosophy (PhD) study under the supervision of Professor Florian Bauer and Professor Sakkie Pretorius at the Institute for Wine Biotechnology, University of Stellenbosch. Exposure to this environment and skills garnered during his Ph.D. studies has enabled Patrick to supervise both Honours (BSc) and MSc research students. Patrick is now pursuing a more independent research career investigating a range of different areas. One of those includes being part of a group that is based at the University of KwaZulu-Natal's School of Chemistry. The team has interests in microbial biotyping and novel drug development.

Patrick is married with two daughters and a son. In his spare time, he enjoys being with his family and is also a competitive angler.

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PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal Yeast.

Chapter 1   **General Introduction and Project Aims**

Chapter 2   **Literature Review**
Flocculation in *Saccharomyces cerevisiae*

Chapter 3   **Research Results**
Controlled expression of the dominant flocculation genes *FLO1, FLO5* and *FLO11* in *Saccharomyces cerevisiae*

Chapter 4   **Research Results**
Controlled expression of the dominant flocculation genes *FLO1, FLO5* and *FLO11* in industrial wine yeast strains

Chapter 5   **Research Results**
Wine yeast engineered for controlled flocculation

Chapter 6   **General Discussion and Conclusions**

CONTENTS

Chapter 1

INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Saccharomyces cerevisiae, also well-known as baker's, brewer's and wine yeast, has been employed by mankind over millennia, most prominently in the production of leavened bread dough and alcoholic beverages (Walker, 1998). As such, it has become world-wide the most relevant microorganism in food production. The desirable characteristics of this yeast, such as the ability to grow quickly and easily on different hexose carbon sources (glucose, fructose and maltose), its non-pathogenicity, and the absence of pyrogenic or allergenic substances such as those present in many prokaryotic cell walls, and, more recently, the relative ease of genetic manipulation, have contributed to making *S. cerevisiae* the preferred organism for the production of heterologous proteins and other substances of biotechnological interest (Guerra *et al.*, 2006). This is illustrated by the use of *S. cerevisiae* as cell factories for the production of insulin (Kjeldsen, 2000), L-lactic acid (Saitoh *et al.*, 2005) and polyketides (Kealey *et al.*, 1998; Maury *et al.*, 2005). Beyond this industrial interest, *S. cerevisiae* has played a significant role in advancing our understanding of biological systems. Indeed, this species is one of the preferred model systems for the study of fundamental cellular and molecular processes. As a consequence, the complete genome sequence of *S. cerevisiae* was the first of any eukaryotic organisms to be published (Goffeau *et al.*, 1996). The genome sequence is readily available and today represents probably the best annotated of all published genomes [*Saccharomyces* Genome Database, www.yeastgenome.org] (Cherry *et al.*, 1998).

On completion of many of the industrial processes mentioned in the preceding paragraph, the suspended yeast cells must be removed prior to further processing of the product. In the case of wine fermentation, such removal processes may involve filtration or other clarification strategies. These processes can be costly and can result in reduced quality of the final product. Considering the global trend in food production towards less interventionist, less energy-consuming and "greener" processes, wine makers would prefer to reduce or, if possible, entirely eliminate the need for such interventions (Bauer and Pretorius, 2002).

A strategy to achieve a satisfying level of clarification could be the optimization of a particular phenotypic trait of yeast strains that is referred to as flocculation. Yeast flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the

bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989). Flocculation could therefore allow the convenient separation of cells from the fermentation product. However, flocculation must not occur before fermentation has been completed since flocculent strains do not ferment efficiently and early flocculation may thus result in sluggish or stuck fermentation. An early arrest of fermentation yields products containing high residual sugars that are easily susceptible to spoilage and may result in substantial financial losses (Verstrepen *et al.*, 2001, 2003). Ideally, flocculation should therefore occur as close as possible after the end of the alcoholic fermentation process.

Flocculation is one of several phenotypes that are linked to the adhesion properties of yeast cells. Adhesion properties can refer to cell-cell and/or cell-substrate adhesion, and such properties directly impact on many phenotypes other than flocculation, such as invasive growth and pseudohyphal differentiation. All of these phenotypes have been shown to be mainly dependent of the expression of a limited number of genes that encode structurally related GPI-anchored cell wall proteins. The genes are referred to as *FLO* genes, and the expression of specific *FLO* genes, in particular *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*, has been shown to favour specific cell wall properties (Verstrepen and Klis, 2006). Some of these genes and proteins are at the centre of this dissertation and will be discussed in some depth in the literature review.

Two independent research studies have shown that most commercial wine yeast strains are either non-flocculating or flocculate inefficiently (Carstens *et al.*, 1998; Suzzi *et al.*, 1984). The structural *FLO* genes, however, appear to be present in most, if not all of these strains. In previous research studies, conventional hybridization technologies have been employed to create genetically improved wine yeasts that displayed increased flocculent abilities (Lahtchev and Pesheva, 1991; Romano *et al.*, 1985). However a significant drawback is associated with wine yeast selection using genetic techniques such as hybridization (mating, spore cell-mating, rare mating, cytoduction and spheroplast fusion), clonal selection of variants and mutagenesis in that these techniques lack the specificity to modify the flocculent ability of wine yeasts in a well-controlled manner and may result in the loss of some desirable traits (Pretorius and Bauer, 2002). However, the use of recombinant DNA technology and genetic engineering offers a reliable method for modifying the genetic framework of flocculation that is inherent to *S. cerevisiae* wine yeasts, thereby creating flocculent wine yeast strains without jeopardizing other desirable oenological properties of the parental host wine yeast strain.

In this study, we investigated the possibilities to induce desired adhesion phenotypes in laboratory and industrial wine yeast strains using recombinant DNA technology. The strategy is based on chromosomal promoter replacement, with the native promoter of specific *FLO* genes being replaced by promoters that would display the desired characteristics. The process was designed to result in self-cloned strains that only contain DNA sequences that were initially derived from *S. cerevisiae*. It should be noted that due to a general negative public perception, the implementation of genetically modified organisms is severely restricted in the food and alcoholic beverage industries. However, on a more promising note, the transgenic malolactic wine yeast strain ML01 that has been awarded GRAS status by the U.S. FDA was recently introduced to the North American wine making industry (Husnik *et al.*, 2006).

To induce genes in a manner that would restrict the possibility of flocculation during active metabolic sugar conversion, but lead to strong expression once sugars have been depleted, published data were screened for promoters that would provide such an expression pattern. For this purpose, the native promoters of the dominant flocculation genes *FLO1, FLO5,* and *FLO11* in non-flocculent laboratory and wine yeast strains were replaced with inducible promoters of the *ADH2* and *HSP30* genes that were generated using a PCR-based cloning strategy. The *ADH2* promoter is subjected to carbon catabolite repression and has been shown to be repressed several hundred-fold during growth on glucose (Gancedo, 1998; Price *et al.*, 1990). Derepression of the *ADH2* promoter only takes place in the absence or depletion of glucose (Ciriacy, 1997) and this event generally coincides with the transition to growth on ethanol (Noronha *et al.*, 1998). The *HSP30* promoter, on the other hand, has been shown to be induced during entry into the stationary phase of growth and coincides with the depletion of glucose from the medium, including in low-stress nutrient-rich wort and wine fermentation conditions (Donalies and Stahl, 2001; Regnacq and Boucherie, 1993; Riou *et al.*, 1997). Another advantage of the cloning strategy employed in this study is that no sub-cloning of the *FLO* gene open reading frames is required. Furthermore, expression levels are independent of plasmid-related artifacts such as variable copy-numbers and the increased risk of intragenic recombinations. Indeed, *FLO* genes contain intragenic tandem repetitive sequences that have been previously reported as difficult to clone or even as "unclonable" sequences (Teunissen *et al.*, 1993).

1.2   SCOPE OF THIS DISSERTATION AND AIMS OF THIS STUDY

The PhD project described within this dissertation is part of a larger research initiative at the Institute for Wine Biotechnology at the University of Stellenbosch to improve the fermentation performance of wine yeast. The main aim of this study was to investigate strategies to adjust the flocculation behaviour of laboratory and industrial yeast strains according to desired specifications. The thesis is divided into six chapters, including this introduction (**Chapter 1**).

In **Chapter 2**, a comprehensive literature review encompassing the various flocculation genes, their regulation and physiological factors affecting flocculation in *S. cerevisiae* is presented.

In **Chapter 3**, the haploid non-flocculent, non-invasive and non-flor forming *S. cerevisiae* FY23 laboratory yeast strain, which is isogenic to the strain S288C whose genome has been characterized, was employed as a model organism. The chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11* of the FY23 strain were placed under the transcriptional control of the promoters of the *ADH2* or *HSP30* genes. All transformed strains were evaluated for their growth, flocculating ability, hydrophobicity and flor forming capability in standard laboratory media. The novelty aspect of this study is that we could effectively compare specific roles of *FLO1*, *FLO5* and *FLO11* in cellular adhesion processes in an identical genetic background. The data generated clearly indicated the suitability of the promoter-*FLO*-gene constructs to induce industrially desirable phenotypes. Furthermore, the transgenic laboratory yeast strains created in this aspect of the study were employed to generate promoter replacement cassettes with extended homologous tail regions that would facilitate genetic manipulations of industrial yeast strains that are otherwise notoriously difficult to transform. This chapter has been published in Applied and Environmental Microbiology (Govender *et al.*, 2008).

With the promising results of promoter replacement strategy in a laboratory yeast strain, it was decided to study the effect of a similar approach using industrial wine yeast strains. Given that industrial strains of *S. cerevisiae* differ from most laboratory-bred strains, data obtained for laboratory strains may not always be the same for industrial yeast strains. Hence, the native promoters of dominant flocculating genes *FLO1*, *FLO5* and *FLO11* in two non-flocculating wine yeast strains BM45 and VIN13 were replaced with stationary phase-inducing promoters *ADH2* and *HSP30*. This exercise has provided us with a unique opportunity to study phenotypes that are associated with native *FLO1*, *FLO5* and *FLO11* wine yeast genes that are otherwise silent under standard laboratory media conditions and to compare such data with those obtained for transgenic laboratory strain. This comparison forms the basis of **Chapter 4**.

Standard culture conditions are usually very different from wine-making conditions, where multiple stresses occur simultaneously and sequentially. Thus, control of flocculation as mediated by *ADH2p* or *HSP30p* under laboratory conditions using rich media may not be the same during wine fermentation. Hence, in order to provide comprehensive proof for the controlled flocculating properties of the transformed wine yeast strains, they were subjected to fermentation in Merlot and synthetic MS 300 musts. Thorough evaluations of fermentation performance of the transgenic wine yeast strains, the effectiveness of inducible promoters and adhesion phenotypes under synthetic and authentic wine-making conditions are presented in **Chapter 5**.

Finally, **Chapter 6** presents a general conclusion and ideas on future work.

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Chapter 2

LITERATURE REVIEW

Flocculation in *Saccharomyces cerevisiae*

2.   FLOCCULATION IN *SACCHAROMYCES CEREVISIAE*

2.1   INTRODUCTION

Efficient fermentative conversion of carbohydrates in fruits, grains and other biomass to ethanol by *Saccharomyces cerevisiae* is the critical process for a wide range of products that include bioethanol, wine, beer and other alcoholic beverages (Bothast *et al.*, 1999; Pretorius, 2000). Strains of *S. cerevisiae* are also widely utilized in industrial production of small-molecule metabolites such as insulin (Kjeldsen, 2000), L-lactic acid (Saitoh *et al.*, 2005) and polyketides (Kealey *et al.*, 1998; Maury *et al.*, 2005).

Such wide-ranging industrial use has led to the isolation and, more recently, the scientific development of many highly specialized strains that are able to fulfill specific functions. In the wine industry alone, it is estimated that more than 300 different yeast strains are sold commercially, and that each of those strains displays some specific phenotypic traits that differentiate it from other strains (Pretorius and Bauer, 2002). In wine, such selected traits may relate to the ability of strains to produce specific aroma profiles, to ferment with various speeds and efficiencies, to produce reduced amounts of unwanted compounds such as H_2S and many more. All of these traits will directly or indirectly impact on the quality and style of the final product.

The cell wall properties of yeast strains can also significantly impact on their industrial suitability. Indeed, many cell wall-related phenotypes lead to specific characteristics that may positively or negatively impact on the production process as well as on the quality of the final product. Many of these cell wall phenotypes directly relate to the adhesion properties of industrial strains.

A phenotype of particular interest in this regard is referred to as flocculation. For example, the self-clearing of beers at the end of the fermentation by the flocculation and settling of ale yeast is a highly desirable characteristic of brewing yeast strains, and specific flocculation or adhesion phenotypes could theoretically be beneficial for many other industrial processes.

Yeast flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989a). This innate property of brewing yeast strains has been utilized in the latter phase of primary beer fermentation as a cost-effective method of separating biomass from the fermented broth. However, other industrial fermentation processes that primarily employ non-flocculent strains of *S. cerevisiae* have resorted to the use of

expensive separation procedures such as centrifugation and/or filtration to remove cells. Consequently there exists a major research impetus to genetically modify the flocculation profile of other industrial *S. cerevisiae* strains so that they could be of similar benefit to related industries.

This literature review attempts to provide an up to date understanding of both the physiological and genetics aspects of flocculation, both of which are deemed critical for the implementation of a successful genetic engineering approach to control flocculation.

2.2　CELL WALL ARCHITECTURE AND FUNCTION

To fully harness the flocculent ability and metabolic activities of *S. cerevisiae*, a thorough understanding of the relationship between architectural organisation and function of the envelop layers is of vital importance. Flocculation is indeed an intrinsic property of the cell wall (Stratford, 1992a), since isolated cell walls retain their original capacity to flocculate whereas isolated cell walls from non-flocculent cells will not flocculate (Eddy, 1955). Furthermore, heat-killed cells will flocculate if they were originally flocculent (Mill, 1964) and it is generally agreed that the yeast cell wall is an important indicator of the rate and extent of cell wall flocculation (Calleja, 1987).

The wall of yeast cells is a bilayered supramolecular structure that surrounds the entire cell, spans 100-200nm and may account for up to 30% of the dry weight of the cell (Klis *et al.*, 2002; Walker, 1998). The outermost layer is composed of mannoproteins, whilst the inner layer is composed largely of load-bearing polysaccharides (*β*-glucan), which are complexed to a smaller proportion of chitin (Fig. 2.1 and Table 2.1). However, it should be noted that the exact composition and proportions has been found to vary with the genetic background and with culture conditions (Aguilar-Uscanga and Francois, 2003)

Table 2.1 Cell wall composition of *S. cerevisiae* (Aguilar-Uscanga and Francois, 2003; Klis *et al.*, 2002; 2006)

Macromolecule	% of cell wall (dry weight)	Degree of polymerization	Average M_r (kDa)	Level of branching
Mannoproteins	30-50	highly variable	highly variable	high
β(1,6)-Glucan	5-10	150	24	moderate
β(1,3)-Glucan	30-45	1500	240	high
Chitin	1.5-6	120	25	linear
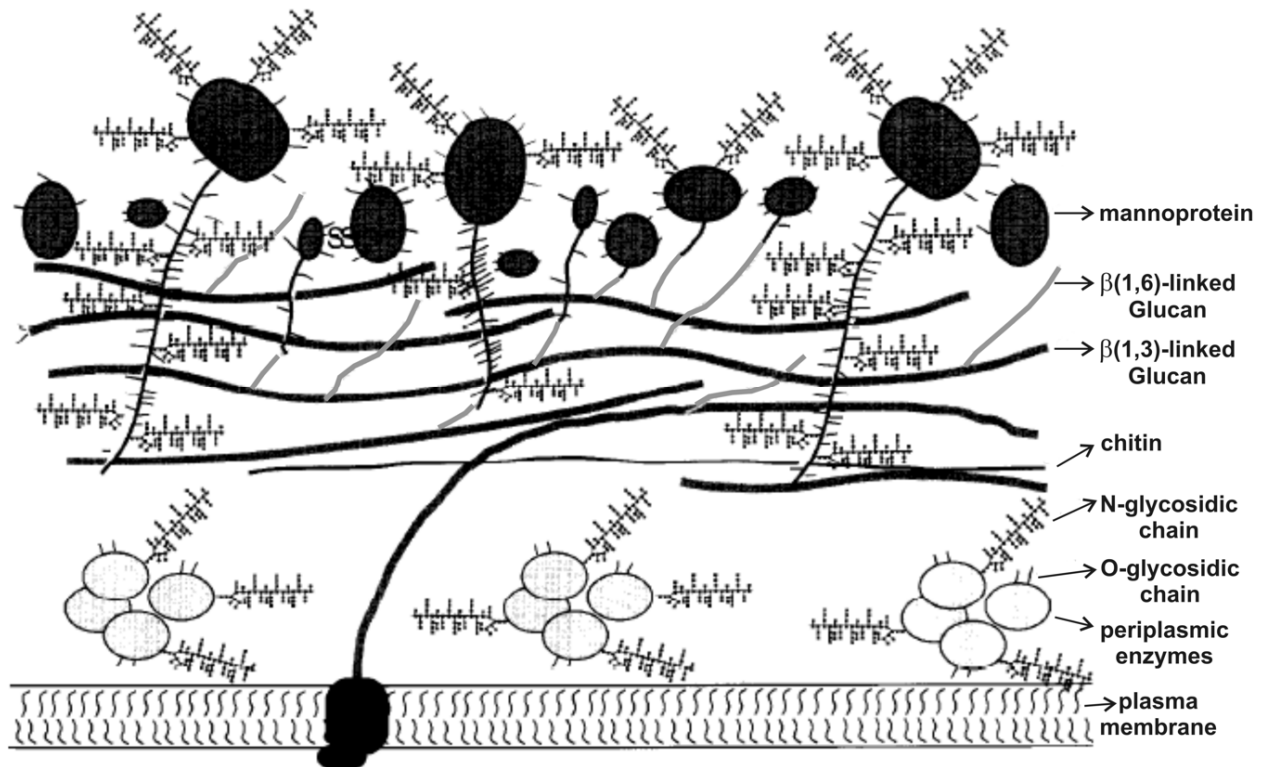
Figure 2.1 Composition and structure of the cell wall of *Saccharomyces cerevisiae*. The cell wall, which is located outside the plasma membrane, consists of two layers. The inner layer provides cell wall strength, and is made of β(1,3)- and β(1,6)-glucan that is complexed with chitin. The outer layer consists of mannoproteins, and determines most of the surface properties of the cell. The majority of mannoproteins are covalently linked to the inner glucan layer. Periplasmic enzymes are trapped between the plasma membrane and the inner skeletal layer (extracted from Schreuder *et al.*, 1996).

2.2.1. β-Glucan/Chitin network

The major load-bearing polysaccharide is a water-insoluble, moderately branched *β*(1,3)-glucan (Fleet, 1991). Branching of the polymer (about 3% branching points) ensures that *β*(1,3)-glucan molecules can only locally associate through hydrogen bonds, resulting in the formation of a continuous, three-dimensional network (Klis *et al.*, 2006; Manners *et al.*, 1973). The *β*(1,3)-glucan chains belong to the so-called hollow helix family and have a flexible shape that is comparable to a wire spring which can exist in various states of extension (Rees *et al.*, 1982). The helices of *β*(1,3)-glucan are composed of three hydrogen-bonded chains (a triple helix) or a single polysaccharide chain. The branch points are the 6-hydroxy groups, and substituents at this position do not interfere with formation of either single or triple helices (Stokke *et al.*, 1993; Williams *et al.,* 1991). The non-reducing ends of the *β*(1,3)-glucan molecules may function as attachment sites for covalent attachment of other polysaccharides (Kollar *et al.*, 1997). At the external face of the *β*(1,3)-glucan inner skeletal network (Fig. 2.1), highly

branched and consequently water-soluble β(1,6)-glucan chains are attached by a still uncharacterized link (Klis *et al.*, 2006; Lesage and Bussey, 2006). The β(1,6)-glucan polymer has a β(1,6)-linked glucose backbone that is branched with β(1,6)-linked side chains via 3,6- substituted glucose residues on 15% of the backbone residues (Magnelli *et al.*, 2002). The β(1,3)-glucan molecules have an estimated size of 1500 glucose units (or degree of polymerization or DP), whereas β(1,6)-glucans have about 150 DP (Table 2.1). This glucan network is highly elastic and is considerably extended under normal osmotic conditions and functions in the maintenance of osmotic homeostasis. Generally the osmolarity of the cytoplasm of *S. cerevisiae* is higher than outside the cell. Thus yeast constructs robust and elastic walls to limit the resulting water influx, which would otherwise disturb internal reaction conditions and cause excessive swelling of the cell eventually leading to rupture of the plasma membrane. Expansion of the wall produces a counteracting pressure by the wall, which efficiently prevents the influx of water (Klis *et al.*, 2006; Martinez de Maranon *et al.*, 1996; Morris *et al.*, 1986). The combination of substantial mechanical strength and high elasticity allows the wall to transmit and redistribute physical stresses, thus offering efficient protection against physical stress or mechanical damage (Morris *et al.*, 1986; Smith *et al.*, 2000).

Chitin is a linear polymer of β(1,4)-linked *N*-acetylglucosamine molecules that consists of approximately 120 residues. The reducing ends of chitin polymers may become covalently cross-linked to non-reducing ends of β(1,3)-glucan chains through a β(1,4)- glycosidic link . Chitin is deposited as a ring at the site of bud emergence, then as a disk (the primary septum), and finally in the lateral cell wall of the mother cell after septation (Kollar *et al.*, 1995; 1997). This seemingly demonstrates that chitin is not essential for the mechanical strength of the lateral walls. However, despite its small quantity (Table 2.1), chitin is essential for yeast survival, probably because of its central role in septation (Cabib *et al.*, 2001; Shaw *et al.*, 1991).

2.2.2 Cell wall mannoproteins

Yeast cell wall proteins (CWPs) are highly mannosylated polypeptides, often 50 to 95% carbohydrate by weight, and thus may be thought of as yeast proteoglycans or mannoproteins (Lesage and Bussey, 2006). The stress-bearing polysaccharides of the cell wall of *S. cerevisiae* function as a scaffold for the insertion of a fibrillar outer layer of glycoproteins that collectively form yeast mannan, so-called because of their high mannose content (Fig. 2.1). Mannoproteins are covalently linked to the β(1,3)-glucan-chitin network either indirectly through a β(1,6)-glucan moiety (reviewed in Klis *et al.*, 2002). In addition, some mannoproteins are linked to each other by hydrophobic

interaction or by disulphide bonds (Moukadiri *et al.*, 1999; Moukadiri and Zueco, 2001; Orlean *et al.*, 1986; Walker, 1998). Two types of cell wall protein (CWP) glycosylation modifications have been identified in *S. cerevisiae*, namely *O*-linked mannosylation and *N*-linked glycosylation (Fig. 2.2). In addition, the attachment of a glycosyl phosphatidylinositol (GPI) anchor to cell wall proteins has also been reported (Fig. 2.3). Whilst concise outlines of these processes are presented herein, they have been extensively reviewed by others (Ballou, 1990; Dean, 1999; Herscovics and Orlean, 1993; Lipke and Ovalle, 1998; Strahl-Bolsinger *et al.*, 1999).

2.2.2.1 *O*-linked mannosylation

The *O*-linked oligosaccharides (Fig. 2.2) are attached to the hydroxyl group of serine or threonine residues and consist of short linear chains consisting of only one to five mannose residues, with the first two residues being α(1,2)-linked and subsequent ones α(1,3)-linked (Ballou, 1990; Herscovics and Orlean, 1993). These oligosaccharide moieties resemble short rigid rod-like stalks that elevate protein domains from membranes or wall surfaces (Jentoft, 1990), presumably thereby lending them greater access to the extracellular environment. Although *O*-linked chains are relatively short oligosaccharides, predictive genome-wide identification studies have revealed that many cell wall proteins possess serine/threonine-rich domains (Caro *et al.*, 1997; De Groot *et al.*, 2003; Hamada *et al.*, 1998a), which are sites of mannose attachment. This suggests that *O*-mannosylation is a common feature of CWPs. Therefore the cumulative number of *O*-chains per CWP can be substantial and the amount of *O*-linked mannose in the cell wall significant.

2.2.2.2 *N*-linked glycosylation

In *S. cerevisiae*, *N*-glycosylated proteins bear an oligosaccharide that is glycosidically linked to the amide group of an asparagine residue. All *N*-modified glycoproteins acquire the same initial oligosaccharide in the Endoplasmic Reticulum (ER). This initial glycan moiety may undergo maturation to yield oligosaccharides chains, containing 9 to 13 mannose residues that are referred to as "core"-type oligosaccharides (Dean, 1999; Herscovics and Orlean, 1993). However, in some but not all cell wall mannoproteins the core structure may be extensively mannosylated in the Golgi. This results in an α(1,6)-linked mannose backbone chain of up to 50 mannose residues extending from the   *N*-glycan core and to which are attached shorter chains of α(1,2)-linked mannose residues that terminate in α(1,3)-linked mannose residues, thus forming a highly branched structure containing as many as 200 mannose residues (Fig. 2.2). In addition, phosphodiester-linked mannosyl side chains are present which gives yeast its overall

anionic surface charge (Ballou, 1990; Dean, 1999; Herscovics and Orlean, 1993). It is noteworthy at this juncture to mention that flocculation lectins were shown to specifically bind to the non-reducing termini of α(1,3)-linked mannan side branches that are two or three mannose residues in length (Ballou, 1990; Stratford, 1992b). For more details on the ability of cell-wall mannan to act as carbohydrate receptors to the flocculins, refer to the mechanism of flocculation that is presented in this review.
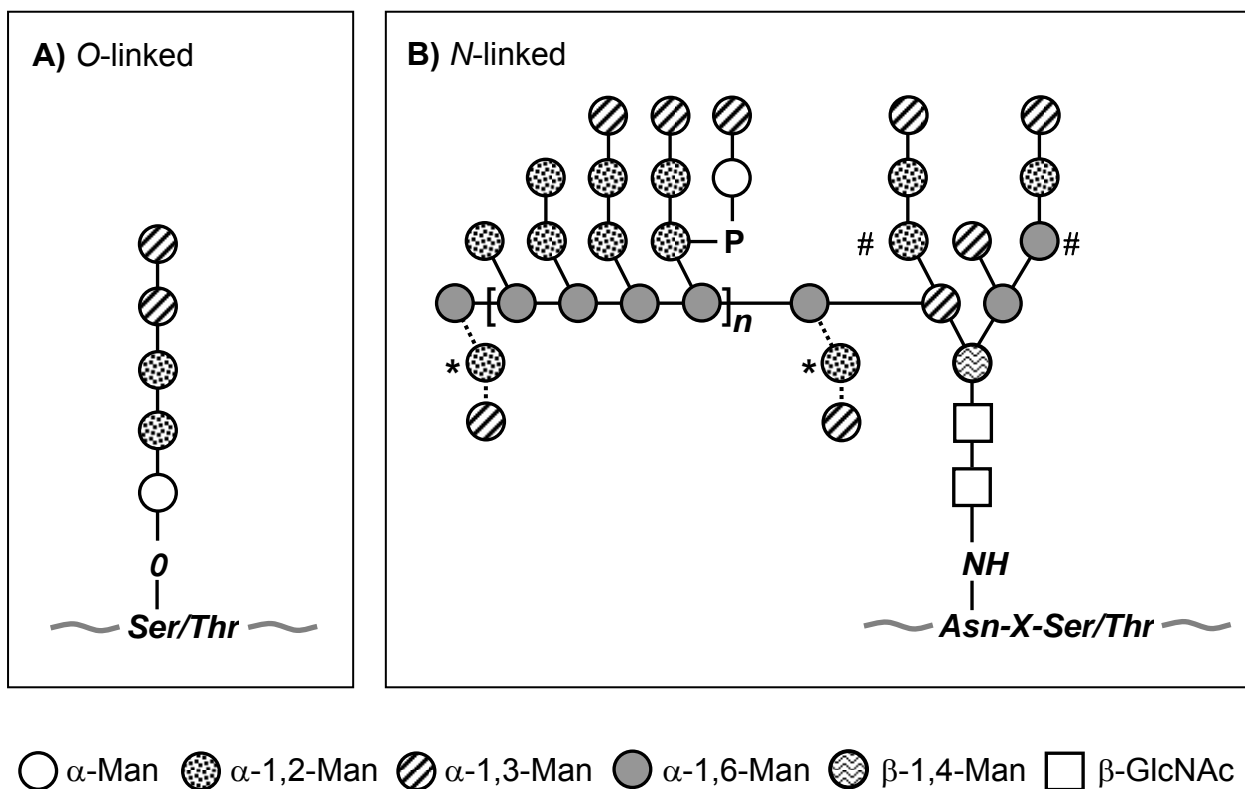
Figure 2.2   Glycosylation of cell wall proteins [CWP (—)] in *Saccharomyces cerevisiae.* A) *0*-linked mannosylation, short mannosyl side-chains linked to hydroxyl group of serine or threonine residues. The *0*-chains can vary in length from one to five mannose residues. B) *N*-linked glycosylation, carbohydrate side chains linked to amide group of asparagine residues. The number of repeating units (*n*) in *N*-chains varies and can be as high as 15 (Ballou, 1990). Asterisk denotes alternative positions of the α-1,2-linked mannose. This addition is proposed to prevent elongation and is not found in the cores to which outer chains are added. # Denotes additional sites of phosphorylation. Man, Mannose; GlcNAc, *N*-acetylglucosamine; P, phosphate (adapted from Herscovics and Orlean, 1993).

2.2.2.3 Cell wall anchorage of mannoproteins

On the basis of their covalent interactions with cell wall polysaccharides, two main classes of cell wall proteins can be discerned (Fig. 2.3 and 2.4), namely the glycosyl phosphatidylinositol (GPI)-modified cell wall proteins (GPI-CWPs) and the proteins with internal repeats cell wall proteins (Pir-CWPs) (reviewed by Klis *et al.*, 2006; Lesage and Bussey, 2006).

The GPI-CWPs are the most abundant class of cell wall proteins. Based on amino acid sequence similarity, some GPI-CWPs can be grouped into subclasses, including the flocculins, the agglutinins, and the *CRH1-UTR2*, the *TIR*, and the *SED1-SPI1* subclasses. A GPI anchorage signal can be located in approximately 70 secretory proteins of the *S. cerevisiae* genome (Caro *et al.*, 1997; De Groot *et al.*, 2003). Roughly 60% of these are known or putative plasma membrane proteins that possess an intact GPI-anchor that facilitates insertion into and attachment to the plasma membrane. In contrast, the remainder of these have been identified as intrinsic CWPs or were shown to contain a signal sufficient to direct a fusion protein (including a Flo1p fusion) to the cell wall (Hamada *et al.*, 1998b). All precursor GPI-CWPs are extensively processed in the ER, the N-terminal signal peptide is removed and a C-terminal signal peptide is replaced by an intact GPI anchor; in addition, *N*-glycosylation and *O*-glycosylation (as described above) are initiated. Further processing of the GPI anchor and the carbohydrate side-chains takes place in the Golgi. Finally, GPI proteins destined for the cell wall have their GPI-anchor trimmed at the plasma-membrane, prior to incorporation into the cell wall (Caro *et al.*, 1997; De Groot *et al.*, 2003; Frieman and Cormack, 2004; Lu *et al.*, 1995). Mature GPI-CWPs only have a remnant of the original GPI anchor, which is involved in a glycosidic linkage via its mannosyl reducing end with the non-reducing terminus of a β(1,6)-glucan chain from the inner polysaccharide skeletal network (Fig. 2.3). The core structure of this remnant is formed by an ethanolamine-PO_4-oligomannoside composed of four or five mannose residues, three of which are probably substituted with additional ethanolamine phosphate groups (Imhof *et al.*, 2004; Lipke and Ovalle, 1998).

The GPI-modified CWPs may have various functions. In many cases their exact function is still unresolved and they generally appear to contribute to cell wall stability. Of significance and direct relevance to the present study, various GPI-modified CWPs (Flo1p, Flo5p, Flo9p, Flo10p and Flo11p) have been shown to be involved in adhesion events like biofilm formation and flocculation (Fidalgo *et al.*, 2006; Govender *et al.*, 2008; Guo *et al.*, 2000; Verstrepen and Klis, 2006).
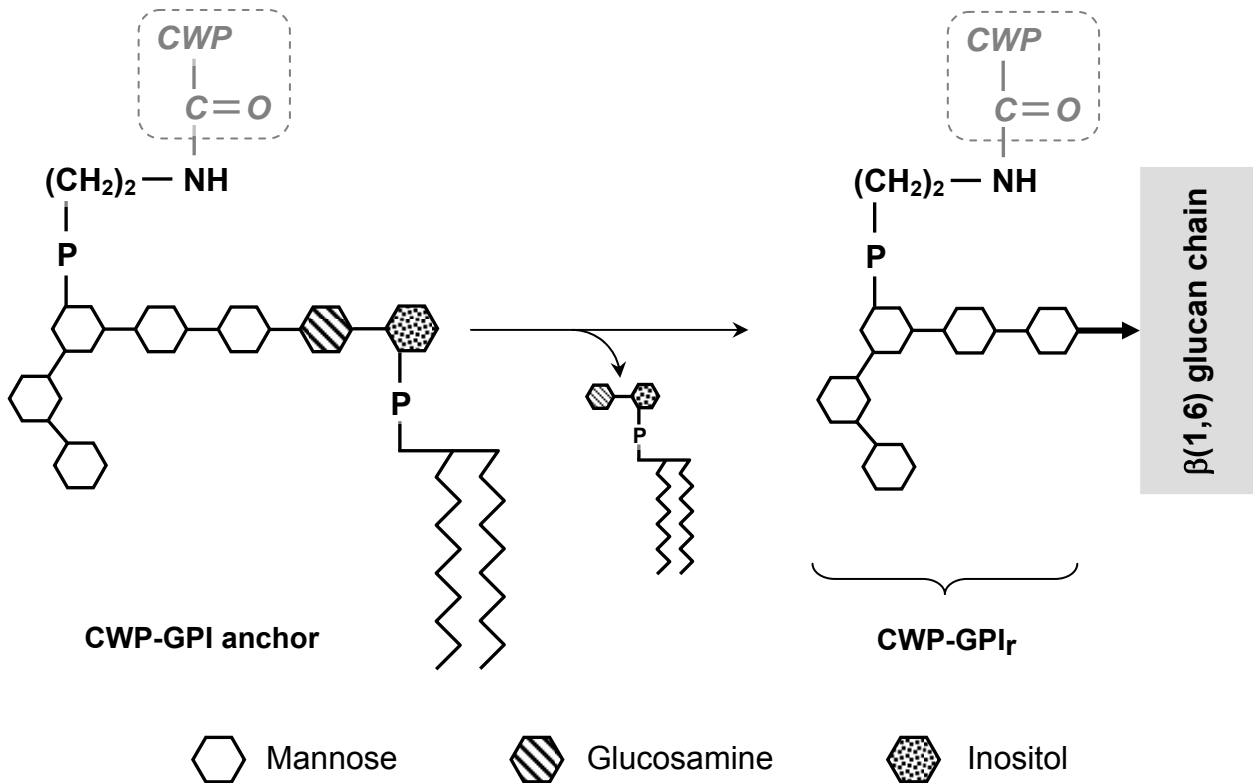
Figure 2.3   Structure of a yeast glycosyl phosphatidylinositol (GPI) anchor and a proposed model for the attachment of cell wall proteins to the β-glucan inner skeletal network. Certain classes of outer-layer mannoproteins are post-translationally modified by addition of a GPI anchor. After secretion of the GPI-anchored mannoprotein to the outer layer of the plasma membrane, the anchor is internally cleaved and the lipidless remnant forms a glycosidic linkage via its reducing end of its mannosyl moiety (indicated by arrow in bold) with the non-reducing terminus of a β(1,6)-glucan chain that is intrinsic to the outer fibrillar layer of the cell wall. This complex typifies the interaction of Flo proteins with the cell wall. CWP, cell wall protein; GPI$_r$, lipidless remnant of a GPI-anchor; P, phosphate; (adapted from Lipke and Ovalle, 1998).

In *S. cerevisiae*, a smaller group of CWPs includes the following four subclasses; Pir1/Ccw6, Hsp150/ Pir2/Ccw7, Pir3/Ccw8, and Cis3/Pir4/Ccw5. These proteins are collectively referred to as the Pir-proteins. The Pir proteins have comparable primary structures that include serine/threonine-rich domains that are potentially extensively *O*-mannosylated as described above (Mrsa *et al.*, 1997; Mrsa and Tanner, 1999). Due to the alkali-sensitive nature of the interaction between Pir proteins and β(1,3)-glucan, Klis and coworkers (2002) postulated that a glycosidic bond of yet unresolved nature links the reducing end of β(1,3)-glucan chains and a terminal mannose residue from the *O*-linked chains of the Pir proteins (Fig. 2.4). In contrast to GPI-CWPs, which are exclusively inserted into the outer fibrillar layer [β(1,6)-glucan] of the cell wall, the Pir-CWPs seem to be uniformly distributed throughout the inner skeletal layer, which is

consistent with their being directly connected to 1,3-*β*-glucan macromolecules (Kapteyn *et al.,* 1999). It has also been demonstrated that certain Pir proteins such as Hsp150 and Cis3 are linked to other CWPs through disulfide bonds (Moukadiri and Zueco, 2001). When interrogating the functions of Pir proteins, it becomes evident that they may play a protective role by regulating permeability of the cell wall.

alkali-sensitive

$$\beta\text{-(1,3)-glucan} \longrightarrow (Man)_{1-5} - O - Ser/Thr$$
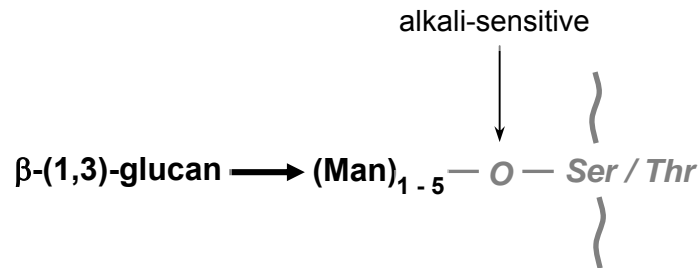
Figure 2.4 Hypothetical linkage between a Pir (proteins with internal repeats) protein and a β(1,3)-glucan polymer. According to this scheme, a β(1,3)-glucan molecule is directly linked via its reducing end to an oligomannoside moiety that is *O*-linked to a serine or threonine residue of a Pir-CWP. The arrow represents glycosidic linkages and points from the reducing end of a polysaccharide to a non-reducing end of the acceptor polysaccharide. This glycopeptide bond is an alkali-sensitive linkage (ASL) of as yet unresolved nature (adapted from Klis *et al.*, 2002).

2.2.2.4 Functions of cell wall mannoproteins

The limited permeability of the external protein layer regulates to some extent whether compounds in the environment gain access to the metabolic machinery that is housed within the cell, and whether compounds are excreted or secreted (De Nobel *et al.*, 1990; Klis *et al.*, 2006; Zlotnik *et al.*, 1984). The carbohydrate side chains of the cell surface proteins are responsible for the hydrophilic properties of the wall, and may be involved in water retention and drought protection. The yeast cell wall may contain up to twenty different glycoproteins at any given time. The glycoprotein composition of the cell wall is dependant on specific environmental conditions and is also reflective of specific phases of the cell cycle (De Groot *et al.*, 2005; Kitagaki *et al.*, 1997; Shimoi *et al.*, 1998). This creates the opportunity for the cell to introduce a wide array of new phenotypes such as flocculation, biofilm formation, recognition of mating of partners, adherence to abiotic surfaces invasive and pseudohyphal growth (Cappellaro *et al.*, 1994; Fidalgo *et al.*, 2006; Govender *et al.*, 2008; Guo *et al.*, 2000; Ishigami *et al.*, 2006; Lambrechts *et al.*, 1996; Reynolds and Fink, 2001).

2.3   DEFINITION OF FLOCCULATION

The first scientifically recorded observation of flocculation was made by Louis Pasteur in 1876 (referenced by Stratford, 1992a). However, early literature is often controversial and reports included sexual agglutination and chain formation (or pseudohyphal formation) in yeast flocculation. To enable an unambiguous understanding of flocculation, it is necessary to clearly distinguish these three processes that can give rise to clumps of yeast cells. In yeast mating or sexual agglutination, complementary haploid strains corresponding to **a** and α sexes of *S. cerevisiae* exchange small peptide pheromones (**a**- and α-factors) that promote a series of physiological changes. These changes result in cell-to-cell aggregation before cell- and nucleus-fusion to form diploids. Adhesion between cells is due to protein-protein interactions between α- and **a**-agglutinins anchored in the complementary cell walls (Calleja, 1987). Chain formation in *S. cerevisiae* occurs when the bud cell fails to separate from the mother cell during cell division, resulting in chain formation (up to 100 cells) as the attached mother and daughter cells continue to form new buds. In this case, the cells are physically joined at their cell walls, and all bud formation occurs in a unipolar fashion (Calleja, 1987; Gimeno *et al.*, 1992; Lambrechts *et al.*, 1996).

Authentic flocculation on the other hand is easily distinguishable from mating and chain-formed cellular aggregates due to the unique ability of flocs to be dispersed by ethylenediaminetetraacetic acid (EDTA) whilst chains and sexual aggregates are insensitive to treatment with EDTA. Moreover, chain forming cells that are dispersed by mechanical shear are incapable of spontaneous reaggregation as do flocculent cells (Stratford, 1992a). Strains of *S. cerevisiae* do differ in their ability to flocculate (Fig. 2.5), and yeast strains can be differentiated on the basis of their ability to form flocs as suggested by Stewart and Russel (1981). Stewart and collaborators (1976) previously defined yeast flocculation as *"the phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended"*. This definition, although useful, is reflective of early literature as it also encompassed chain formation and therefore warranted further refinement. Presently, flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing thousands of cells that rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989a).
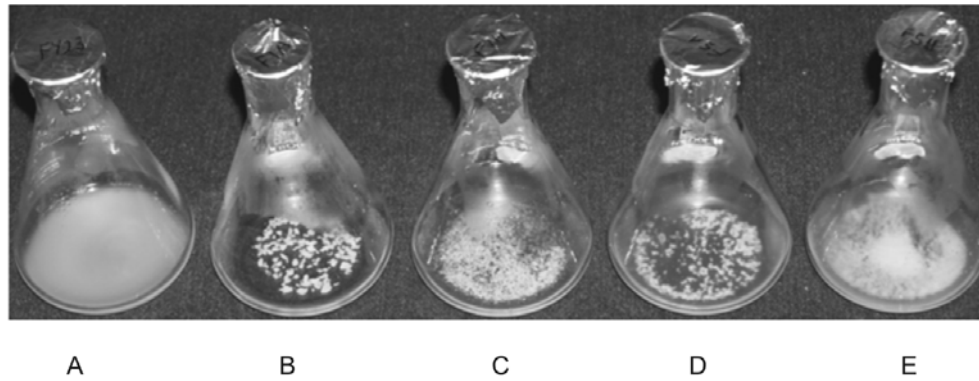
Figure 2.5   Laboratory cultures of non-flocculent (A) and flocculent (B, C, D and E) *S. cerevisiae* strains with varying flocculation intensities (Govender *et al.*, 2008).

2.4   THE MECHANISM OF FLOCCULATION

There are two main hypotheses that may explain the molecular mechanism of yeast flocculation.

2.4.1 The calcium-bridging hypothesis

Mill formally proposed the calcium-bridging hypothesis in 1964 and up until the early 1980s, it was widely accepted by the research community as a suitable explanation for the mechanism of yeast floc formation (Fig. 2.6). Essentially the theory suggested that flocculated cells are held together by salt bridges that involve Ca^{2+} ions linking negative charges of two carboxyl, phosphate and/or sulphate groups on the cell surface of interacting cells. Furthermore it was also proposed that these salt bridges would be stabilized by hydrogen bonds between complementary carbohydrate hydroxyl groups at the cell wall surface (Mill, 1964). The hypothesis was supported by the following findings:

- The thermal dissociation of flocs at 50-60°C implicated the involvement of hydrogen bonding interactions in maintenance of the bridging structure (Mill, 1964).

- The observed effect of pH on flocculation and irreversible inhibition of floc formation by 1,2-epoxypropane, which esterifies carboxyl functional groups, strongly points towards this group as the most likely combine sites (Jayatissa and Rose, 1976; Mill, 1964). This view was further corroborated when a correlation between the capacity for floc formation and the density of carboxyl groups on the cell surface was observed (Beavan *et al.*, 1979). The loss of flocculation in response to treatment with protein-denaturing agents and proteases suggested that carboxyl groups emanated from cell wall mannoproteins (Nishihara *et al.*, 1977; 1982; Stewart *et al.*, 1973).

- Evidence pertaining to the possible involvement of phosphodiester groups of phosphomannan which could serve as alternative binding sites for calcium ions was presented by Lyons and Hough (1970; 1971). However this could be viewed as contentious, as Jayatissa and Rose (1976) showed that removal of phosphodiester groups did not correlate with decreased flocculation.

Although there is little evidence that refutes the calcium-bridge hypothesis, its demise stemmed from the model's inability to accommodate the observed inhibition of flocculation by mannose and other sugars (Rose, 1993; Stratford, 1992a; Taylor and Orton, 1978). Furthermore, criticism against this theory has been levelled on the basis that it fails to explain the specificity of cell-cell interactions. This stems from the fact that it ignored the phenomena of mutual flocculation and co-flocculation and the presence of mannoprotein carboxyl groups on the non-flocculent cell surfaces (Calleja, 1987; Jin and Speers, 1998; Speers *et al.*, 1992).
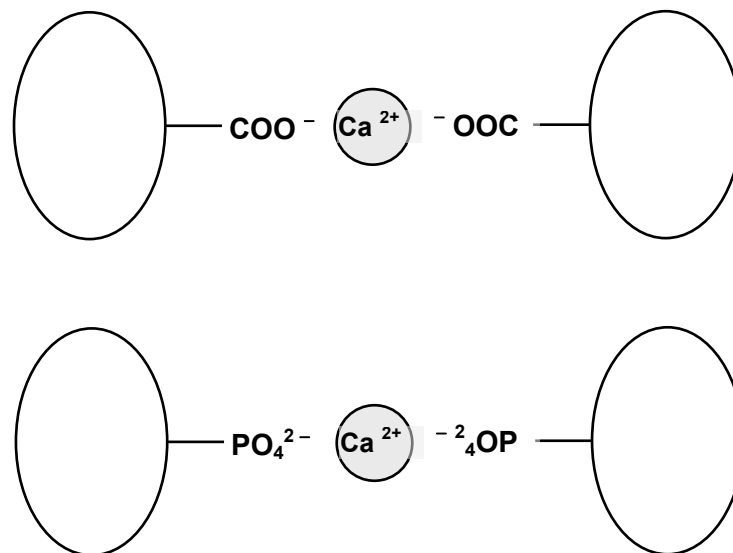
Figure 2.6 Schematic representations illustrating the calcium-bridging hypothesis. Flocculated cells are linked by salt bridges with Ca^{2+} ions joining either two carboxyl or two phosphate groups at the surface of aggregated yeast cells. The structures thus formed are stabilized by hydrogen bonds between complementary carbohydrate hydroxyl groups at the cell wall surface (adapted from Stratford, 1992a).

2.4.2 The lectin hypothesis

The Miki and coworkers (1982a) presented evidence for a new flocculation model that is now generally referred to as the lectin hypothesis (Fig. 2.7). This model proposed that specific surface proteins on flocculent cells recognize and bind to α-mannan carbohydrates on adherent yeast cells. The naming of this model is apt, especially since a lectin is currently defined as "carbohydrate binding proteins other than enzymes or antibodies" (Barondes, 1988). The model also suggests that Ca^{2+} ions may act as cofactors in maintaining the active conformation of surface proteins, thereby enhancing the capacity of lectins to bind α-mannan carbohydrates.

According to the lectin hypothesis, flocculation is mediated by the interaction between two distinct cell surface components (Fig. 2.7).The flocculation receptors, found both on flocculent and non-flocculent cells, are α-branched mannans, as suggested by the following findings;

- Mannose-specific inhibition of flocculation in *S. cerevisiae* (Taylor and Orton, 1978)

- Lack of coflocculation with yeast known to lack mannan in its cell wall, such as *Schizosaccharomyces pombe* (Miki *et al.*, 1982a)

- Mannan blocking with concanavalin A and chemical modification experiments (Miki *et al.*, 1982a; Nishihara and Toraya, 1987)

The carbohydrate nature of receptors for flocculation has been confirmed using known *mnn* mutants that varied in wall mannan structure. Results showed that flocculation receptors were the outer chain *N*-linked mannan side branches (Fig. 2.2) that are two or three mannose residues in length and that only a small proportion of the available receptors is sufficient to illicit flocculation (Stratford, 1992b). Stratford (1992a) using coflocculation studies concluded that whilst the general structure of mannan receptors across *S. cerevisiae* strains is similar, there are slight differences in the fine architecture of mannans between strains. Furthermore, Stratford (1993) concluded that receptor availability is not involved in the process of flocculation onset, which is the switch from single-cell yeast growth to multicellular aggregation. It should also be emphasized that flocculation can also be reversibly inhibited by the presence of other sugars (Stratford and Assinder, 1991).

The involvement of specific cell surface proteins in the lectin mechanism is supported by the fact that protein denaturation causes irreversible loss of flocculation capacity but does not affect receptor sites (Sieiro *et al.*, 1995). In the model presented by Miki and collaborators (1982a), the lectin component is represented as a dimeric protein complex in which disulphide bridges connect a smaller protein to a core protein

that is anchored in the cell wall. However, Teunissen and Steensma (1995) observed that flocculation is insensitive to protein reduction using dithiothreitiol and proposed a slightly modified lectin model. These authors proposed that cell wall-anchored flocculins might function as lectins. The major difference with Miki's model is that flocculins were touted as being cell wall-anchored glycoproteins that directly bind the mannan receptors of neighbouring cells. Therefore the flocculin fulfils the same role as the dimeric protein complex in the Miki model (1982a), combining anchoring and binding properties.

▶ Mannan   ● Ca $^{2+}$ >── Flocculin
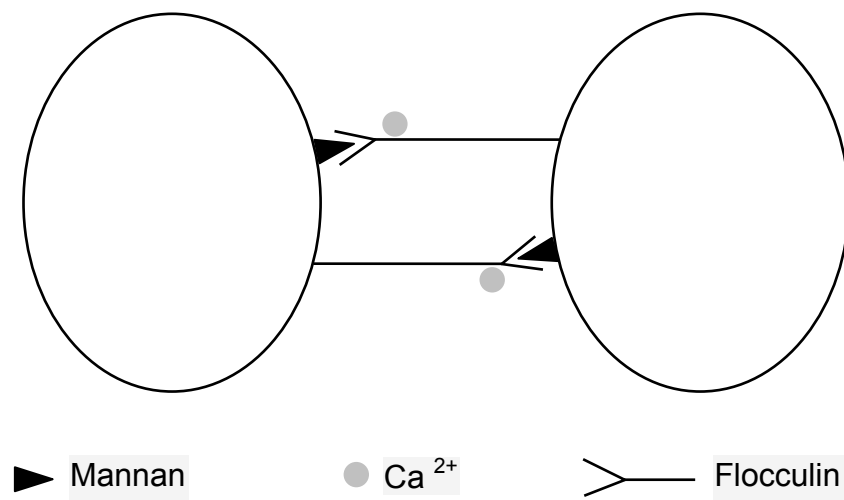
Figure 2.7   Flocculation model illustrating the lectin hypothesis. According to this model a Ca^{2+}-activated flocculin (*FLO* gene encoded lectin-like glycoprotein adhesin) interacts with neighbouring cells by binding to mannose residues belonging to cell wall associated mannoproteins (mannan receptor). Thus flocs are progressively built up to contain thousands of cells by simultaneous inter-binding of many cells (Teunissen and Steensma, 1995).

The revised lectin hypothesis as presented by Teunissen and Steensma (1995) has acquired considerable credibility since then. Predictive genome-wide identification studies have revealed that Flo proteins possess a carboxyl terminal GPI anchorage signal (Fig. 2.12) that facilitates insertion into and attachment to the cell wall (Caro *et al.*, 1997; De Groot *et al.*, 2003; Hamada *et al.*, 1998a; 1998b). As mentioned previously, the abundant serine and threonine residues that are located in the highly repetitive central domain of flocculins (Fig. 2.12 and Table 2.2) may undergo extensive *O*-glycosylation. It is believed that the short *O*-linked oligosaccharide side-chains enable the flocculins to obtain a long, semi-rigid rod-like structure that is stabilized by Ca^{2+} ions (Jentoft, 1990). Moreover, Kobayashi *et al.* (1998) found that modification of the extracellularly exposed N-terminal region was required to change the mannose-specific sugar recognition pattern of the Flo1 protein to the glucose/mannose-specific pattern. Further evidence has shown that Flo1p is a true cell wall mannoprotein which plays a

direct role in cell-cell interaction (Bony *et al.*, 1997; 1998; Javadekar *et al.*, 2000). Finally, the central role of Flo proteins in the flocculation process is further illustrated in that expression of a recombinant *FLO* gene can transform non-flocculent *S. cerevisiae* strains into flocculent ones (Chambers *et al.*, 2004; Cunha *et al.*, 2006; Govender *et al.*, 2008; Guo *et al.*, 2000; Verstrepen *et al.*, 2001; Wang *et al.*, 2008; Watari *et al.*, 1991; 1994). Although these lectins have been shown to be involved in flocculation, the precise nature of the lectin and the ligand involved in this interaction is still not very clear.

2.5   FACTORS AFFECTING FLOCCULATION

Until now, flocculation has been most studied with brewer's strains of yeasts because of its relevance to the brewing industry and consequently much of our current knowledge of factors that may affect flocculation is garnered from such studies. A schematic representation of the various factors that could affect flocculation is presented in Fig. 2.8. However, different studies often lead to contradictions, indicating that the flocculation behaviour is highly strain-specific and depends on multiple factors. Although it is difficult to compare reports on flocculation due to the numerous techniques employed and the variations therein, most *S. cerevisiae* strains flocculate under specific conditions (Jin and Speers, 2000; Stratford, 1992a; Verstrepen *et al.*, 2003).
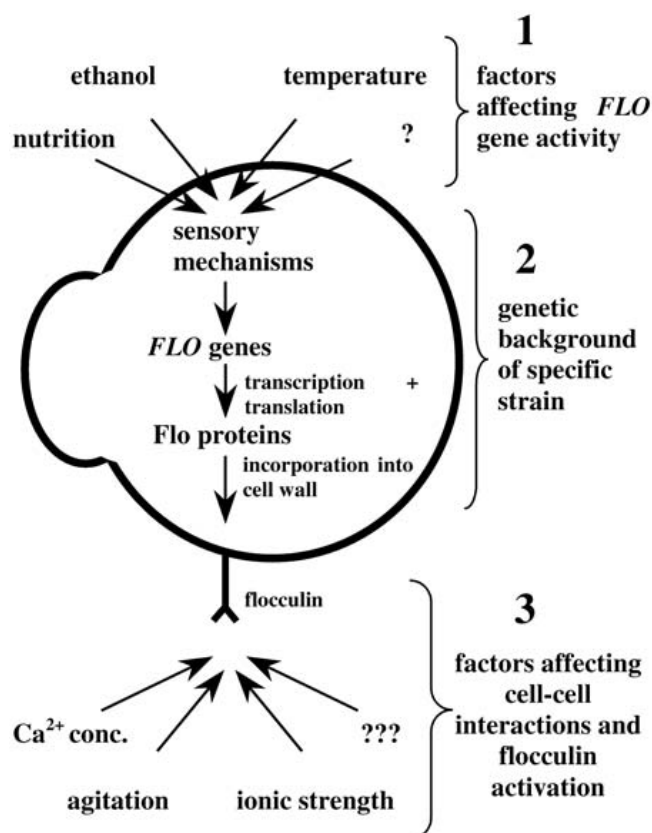
Figure 2.8   Factors affecting flocculation in *S. cerevisiae*. Three categories (1, 2 and 3) can be distinguished according to their mode of action (extracted from Verstrepen *et al.*, 2003).

2.5.1 Sugar inhibition

Mainly based on sugar inhibition, Stratford and Assinder (1991) presented evidence for two distinct categories of yeast strains suggesting two different lectin mechanisms: the Flo1 phenotype, which was only sensitive to mannose, and the NewFlo-type which was inhibited by mannose, glucose, sucrose and maltose. The Flo1 strains were generally laboratory strains that contained the *FLO1* gene and also other genes known to be involved in flocculation. In contrast, strains with the NewFlo phenotype were generally brewing strains of unknown genotype. In a related study, Masy and collaborators (1992) referred to the Flo1 phenotype as mannose sensitive (MS) and the NewFlo phenotype as glucose-mannose sensitive (GMS). Interestingly, they also reported a third group of strains in which flocculation is mannose-insensitive (MI) and independent of Ca^{2+} ions. They speculated that flocculation in these strains could be the result of hydrophobic interactions or other specific interactions not involving mannans. Stratford (1992a) suggested that mannose-insensitivity probably results from very low specificity to monosaccharides since lectins may have much greater affinity for tri- or polysaccharides than for simple sugars. Thus, it is highly probable that the flocculation mechanism in these strains would differ from the modified-lectin mechanism of Flo1 and NewFlo strains.

In growing conditions, almost all Flo1 phenotype strains seem to be insensitive to the presence of nutrients and constitutively expressed flocculence throughout all growth phases (Patelakis *et al.*, 1998; Soares and Mota, 1996; Stratford and Assinder, 1991). In contrast, strains with the NewFlo phenotype were generally brewing strains that flocculated exclusively in the stationary phase of yeast growth. It was shown that flocculation receptors were found at all stages of growth (Soares and Mota, 1996; Stratford, 1993), which signifies that flocculation onset is not likely to depend on receptor availability, but rather on the appearance of active lectins (Soares and Mota, 1996; Stratford and Carter, 1993). In agreement with this point of view, it was shown that Flo proteins are not permanently present on the yeast surface, but that their amount increases during growth. In the case of NewFlo strains, the absence of glucose, sucrose and maltose in the growth medium is an absolute prerequisite for flocculation, as these sugars block the NewFlo flocculin binding sites and thus inhibit flocculation (Stratford, 1992c). In support of this finding, Sampermans and coworkers (2005) reported that the triggering of flocculation was closely aligned with the attainment of the minimum level of the fermentable carbon source (glucose, maltose or fructose) in both minimal and rich media. A correlation between the availability of Flo proteins at yeast surface and the flocculation intensity was observed (Bony *et al.*, 1997; 1998; Javadekar *et al.*, 2000). Kobayashi and collaborators (1998) isolated a novel *FLO1* homologue, Lg-*FLO1* which encodes for a flocculin that binds both mannose and glucose and is believed to be

responsible for the NewFlo phenotype of most lager yeasts. In recent studies (Liu *et al.*, 2007a; 2007b), it was shown that the difference between the NewFlo and Flo1 flocculation phenotypes may at least be partially due to the deletion of two repeated regions that are located within the central domain of the Flo1 flocculin (Fig. 2.12).

Recently, it was described that fermentable sugars had a central role in the induction of flocculation loss both in growing conditions (Soares *et al.*, 2004) and in starved cells (Soares and Vroman, 2003; Soares, 2002) of ale brewing yeasts belonging to the NewFlo phenotype. Clearly nutrient starvation, such as shortage of fermentable carbon sources may act as a signal that induces the onset of flocculation that is unique to NewFlo type strains. This aspect will be discussed under genetic regulation of flocculation in this review. The switch from non-flocculence to flocculence probably allows the yeast cells to adapt to stress conditions. It is believed that flocculation may be a means to protect the cells present in the center of floc from the environmental stress or serve as a means of passive transport away from the stress (Verstrepen and Klis, 2006).

2.5.2 Inorganic ions

Early reports on the involvement of cations in flocculation were often conflicting. However, as mentioned previously there is a general consensus that calcium ions are integral to maintaining the active conformation of flocculins. According to Taylor and Orton (1978), an extremely low concentration (10^{-8} M) of calcium ions is required to induce flocculation. The controversial data involving Ca^{2+} and other cations can be associated with the use of differing cation concentrations, different flocculation assays and variable assay conditions (pH, temperature etc) and strains of different genetic backgrounds. For example, the *FLO5* and *FLO1* strains showed different patterns for the competing effects of other cations with Ca^{2+} (Kuriyama *et al.*, 1991). For low salt concentrations (cations other than Ca^{2+}), there is an observed flocculation enhancement, while inhibition is observed at high salt concentrations. It should be noted that magnesium can indirectly induce flocculation at low salt concentrations by stimulating release of intracellular calcium ions (Stratford, 1989b). In fact, Nishihara and coworkers (1976) reported that Mg^{2+} ions are required at a minimal concentration of 20 µ*M* for flocculation to occur. Although flocculation intensities were lowered, Stewart and Goring (1976) observed that Mg^{2+} and Mn^{2+} could mimic the activity Ca^{2+} ions. Sodium and potassium at concentrations below 10 mgL^{-1} are the only monovalent ions that are capable of influencing flocculation (Smit *et al.*, 1992), but they antagonise flocculation at concentrations above 50 mgL^{-1} (Nishihara *et al.*, 1982).

The increased flocculence observed at low salt concentrations may also be attributed to the lowering of yeast cell surface charge and the modifying effect on surface proteins, in a manner similar to that described as "salting in and salting out" for protein solubility (Stratford, 1992a). Cationic inhibition at high salt concentrations, 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). Strontium and barium acting in a concentration-dependant manner as calcium analogues have been implicated to competitively inhibit flocculation (Kuriyama *et al.*, 1991; Stratford, 1989b; Taylor and Orton, 1973).

2.5.3 pH

Low pH was initially considered not to be a dominant factor in flocculation. Rather, it was thought that high acidity resulted in a lowering of the overall negative cell-surface charge so that the electrostatic repulsion between cells disappeared which promotes cell-cell contact and flocculation (Stratford, 1992a). However, the flocculent Flo1 and NewFlo phenotypes were shown to differ with respect to pH tolerance. The Flo1 phenotype mediated in either a *FLO1 or FLO5* genetic background shows a very broad tolerance, exhibiting flocculation between pH 1.5 and 10 (Govender *et al.*, 2008; Stratford, 1996). In contrast, some but not all NewFlo strains display flocculation over a distinctly reduced pH range [pH 4 to 5] (Smit *et al.,* 1992; Stratford, 1996). A study by Dengis and collaborators (1995) of the combined effect of ethanol and pH on the flocculation of a NewFlo strain revealed that the addition of ethanol broadens the pH range in which flocculation can occur. It was suggested that adsorbed ethanol may induce flocculation by reducing the electrostatic repulsion between cells, by decreasing steric stabilization, and/or by allowing the protrusion of polymer chains into the liquid phase. Furthermore, many NewFlo strains do not flocculate in laboratory culture media, because the initial pH and buffering capacity of these media do not correspond to the pH range within which these yeasts flocculate. Once the pH was corrected, the brewing yeast strains were able to flocculate in laboratory culture media. With these strains, a simple change of pH at any desired time during fermentation allows for cell separation from the medium (Stratford, 1996). It has been suggested that the flocculins may be inactive at certain pH values due to conformational changes that occur when the electrostatic charge of surface proteins changes (Jin *et al.,* 2001; Jin and Speers, 2000). Another possible explanation for the induction of flocculation by changes in the pH is that the pH of the medium might directly influence *FLO* gene activity (Verstrepen *et al.,* 2003). Thus it seems that the pH optimum for flocculation is highly strain-dependent.

2.5.4 Temperature

Although it bears no industrial significance, Taylor and Orton (1975) reported that thermal deflocculation occurs at approximately 54 °C. Stewart and coworkers (1975) reported that there is minimal or no influence of temperature on flocculation phenotypes on condition that temperatures remain in the physiological range of 15-32 °C. However, the effects of temperature on the flocculation potential of different NewFlo industrial strains have been shown to be highly variable. Gonzales *et al.* (1996) found that flocculation of NewFlo larger strains was optimal above 10 °C and drastically decreased at temperatures below 5 °C. This finding was confirmed by other studies when a NewFlo lager strain was observed to flocculate between 24% at 5 °C and 67% at 25 °C (Jin *et al.*, 2001; Jin and Speers, 2000). In other instances, this trend is reversed in that flocculation is repressed at 25 °C, and cells sediment optimally at lower (5 °C) temperatures (Stratford, 1992a). Recently it was reported that a continuous mild heat shock at 37 °C has a negative impact on the phenotypic expression of flocculation in a *S. cerevisiae* brewing strain (Claro *et al.*, 2007). These contradictory results further emphasize the strain-specificity of flocculation.

2.5.5 Ethanol

There exists more than sufficient evidence to suggest that ethanol promotes flocculation (Amory *et al.*, 1988; Claro *et al.*, 2007; Dengis *et al.*, 1995; Eddy, 1955; Jin *et al.*, 2001; Jin and Speers, 2000; Mill, 1964; Sampermans *et al.*, 2005). However, Kamada and Murata (1984) observed ethanol to inhibit flocculation. Dengis *et al.* (1995) studied the effect of ethanol on flocculation in top- and bottom-fermenting yeast strains. Cell populations of top- and bottom-fermenting yeast strains that were harvested from the stationary phase of yeast growth responded differently in terms of flocculation to the addition of ethanol. Under these conditions, ethanol only induced flocculation in the top-fermenting strain. Thus it seems that the influence of ethanol is strain dependant (D'Hautcourt and Smart, 1999).

The mechanisms through which ethanol exerts its influence on flocculation are still unclear, although it has been suggested that adsorbed ethanol may induce flocculation by reducing the electrostatic repulsion between cells, by decreasing steric stabilization, and/or by allowing the protrusion of polymer chains (mannoproteins) into the liquid phase (Dengis *et al.*, 1995). In addition, increasing ethanol concentrations were correlated with a slight increase of cell-surface hydrophobicity and it was suggested that ethanol may act upon cell wall conformation and surface charge (Jin *et al.*, 2001; Jin and Speers, 2000). Sampermans and coworkers (2005) suggested that a shortage of nutrients (nitrogen source or fermentable carbon sources), combined with the presence of ethanol may be the signal that induces the onset of flocculation. However, the positive effect of ethanol on flocculation is concentration-dependant and 10% ethanol has been shown to significantly inhibit the extent of flocculation (Claro *et al.*, 2007).

2.5.6 Oxygen content

In aerobic cultures, flocculence was found to be constant (Miki *et al.*, 1982b). Straver *et al.* (1993) scrutinized whether flocculation onset could be altered by modifying the initial oxygen content of wort. It was found that poor aeration resulted in early and incomplete flocculation, while normal saturation with oxygen both delayed and intensified flocculation. It was also concluded that the flocculating ability of yeast cells during fermentation was triggered after growth limitation due to oxygen shortage and coincided with a sharp increase in cell surface hydrophobicity (CSH) of the cells. Remarkably, the poor growth and flocculation characteristics of yeast grown in de-aerated medium could be restored by addition of ergosterol and oleic acid to the medium. This seems to suggest that oxygen probably does not act directly on flocculation, but rather indirectly through its importance for the synthesis of unsaturated fatty acids and sterols (Straver *et al.*, 1993).

2.6   GENETICS OF FLOCCULATION

Primarily driven by the importance of flocculation to the brewing industry, a concerted attempt was made to understand the genetics of flocculation. Furthermore, an insight into the genetic control of flocculation has potential important practical applications and benefits in the biotechnology sector. To date, numerous genes have been shown to be either directly or indirectly involved in flocculation and other adhesion phenomena. This review will focus on aspects pertaining to the dominant flocculation genes that are members of a family of genes commonly known as the *FLO* genes. In addition, we will mainly focus on recent progress in our understanding of flocculation genetics and on new insights coming from genomic approaches. For earlier work, an extensive review by Teunissen and Steensma (1995) and studies by Lambrechts *et al.* (1996) and Lo and Dranginis (1996) is recommended.

2.6.1 Dominant flocculation genes

Structural and functional analysis of the genomic sequence of *S. cerevisiae* reveals that there are five unlinked dominant *FLO* genes (Verstrepen *et al.*, 2004). Four of these genes namely *FLO1*, *FLO5*, *FLO9* and *FLO10*, are located adjacent to their telomeres (Fig. 2.9). Consequently, this subset of dominant *FLO* genes is viewed as a subtelomeric gene family (Teunissen *et al.*, 1995). In addition, a fifth gene namely *MUC1* (Lambrechts *et al.*, 1996) or *FLO11* (Lo and Dranginis, 1996), which is neither centromeric nor telomeric, is also considered a dominant flocculation gene (Fig. 2.9).
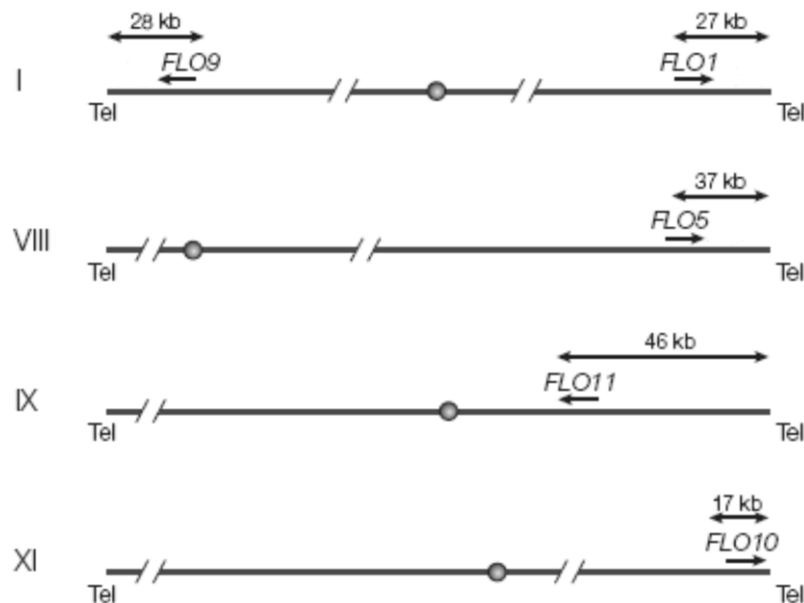
Figure 2.9 The chromosomal location of *FLO* genes in *S. cerevisiae* laboratory strain S288C. The numerals on the left indicate the relevant yeast chromosomes on which the *FLO* genes reside; the dots represent the centromeres. The four subtelomeric *FLO* genes are all located within 40 kb of the telomeres (Tel). *FLO11* is defined as neither centromeric nor telomeric (adapted from Verstrepen *et al.*, 2004).

Sequence analysis reveals that there are several DNA motifs that are conserved among different *S. cerevisiae FLO* genes. An outstanding feature is a highly conserved sequence motif of about 100 nucleotides that is present as a tandem repeat in the regions that encode the central domain in *FLO1* (18 copies), *FLO9* (13 copies) and *FLO5* (8 copies) [Fig. 2.10]. Interestingly, in a comparative study of six different *S. cerevisiae* strains, these tandem repeats were observed to vary in size (Verstrepen *et al.*, 2005). Both *FLO10* and *FLO11* also have tandem nucleotide repeats in the regions that encode domain B, but these are all distinct from that described for *FLO1*, *FLO5* and *FLO9*. It is also worth mentioning that there are other large motifs that are located at the 5′ and 3′ ends of each gene that are shared among several *FLO* genes of *S. cerevisiae* laboratory strain S288C (Verstrepen *et al.*, 2004).
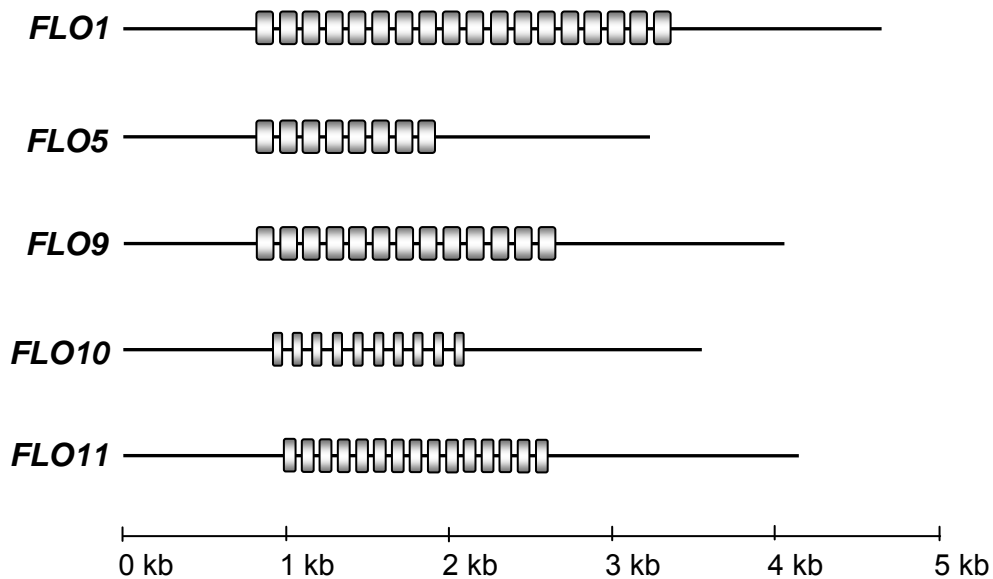
Figure 2.10   The *S. cerevisiae FLO* genes contain conserved intragenic tandem repeats (≥ 40 nucleotides). These repeats were shown to vary in size in six different *S. cerevisiae* strains. It should be noted that *FLO1*, *FLO5* and *FLO9* share the same repeat unit (adapted from Verstrepen *et al.*, 2005).

Verstrepen and coworkers (2004) suggested that the conservation of DNA motifs within individual *FLO* genes and among the *FLO* family is of functional significance and that these conserved sequences may provide sites for homologous recombination. As described in Fig. 2.11, the motifs within each adhesin gene, their presence in multiple copies and the chromosomal arrangement of *FLO* genes provide the basis for a constantly changing range of *FLO* genes encoding cell-surface molecules. The recombination between the tandem repeats of a motif within a single adhesin gene (Fig. 2.11a) can provide an inexhaustible source of diversity. It is important to note that the subtelomeric positioning of many of the adhesin genes is no coincidence. The relatively isolated position in the genome allows frequent recombination events without the risk of affecting other genes (Verstrepen and Klis, 2006). The proposal of Verstrepen and coworkers (2004) is supported by the following findings:

- The Lg-*FLO1* gene, which is a *FLO1* homologue that was isolated from a NewFlo bottom-fermenting brewery strain, was reported to have originated from a recombination event between YHR211 (*FLO5*) and YAL065 (*FLO* pseudogene) generated by chromosomal duplication and translocation in brewing yeast (Kobayashi *et al.*, 1998).

- The loss of intragenic repeats in the *FLO1* gene was associated with a loss of flocculation in strains of *S. cerevisiae* that that are used in brewing.

- Verstrepen *et al.* (2005) in an eloquently designed study, generated new *FLO1* alleles ranging from 2.9-5.4 kb and demonstrated that a decrease in the size of the intragenic repeat region of the *FLO1* ORF results in a quantitative decrease in *FLO1*-mediated flocculation and *vice versa*.

- Fidalgo *et al.* (2006) concluded that the difference between a flor-forming *Saccharomyces* yeast strain and a non-flor forming laboratory strain was due to an increase in the number of intragenic repeat sequences in the central domain of *FLO11*.

- In recent studies (Liu *et al.*, 2007a; 2007b), it was suggested that deletion of tandem repeats causes flocculation phenotype conversion from Flo1 to NewFlo.

These data support the hypothesis that the *FLO* gene family provides phenotypic plasticity to cell wall-related characteristics of yeast. This plasticity is hypothesised to provide *S. cerevisiae* with the ability to frequently adjust and switch cell wall related traits, thereby providing populations of cells with the necessary phenotypic diversity to adapt to particular changes in the environment that would necessitate adhesion-related phenotypic changes.
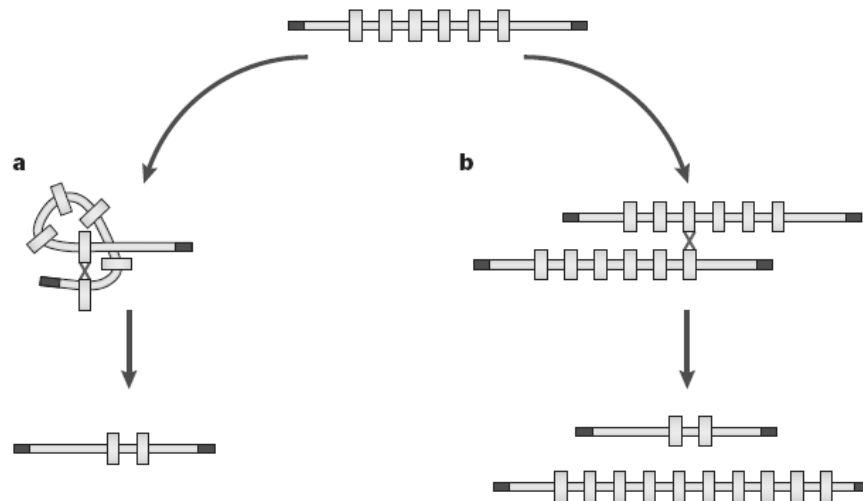
Figure 2.11   Recombination between repeated DNA motifs in flocculin genes generates new alleles. Boxes indicate nucleotide motifs; the regions shown in black at the ends of the chromosomes represent telomeres. (a) Intrachromosomal pairing coupled with a recombination event can generate a short gene with a reduced number of repeats. (b) An unequal crossover between two identical *FLO* genes on homologous chromosomes that have not aligned perfectly is shown. Similarly, *FLO* genes with significant homology that are located on different chromosomes could recombine to produce new chimeric genes. This event would generate both a long and a short form of a *FLO* gene (extracted from Verstrepen *et al.*, 2004).

2.6.2 *FLO* gene encoded adhesins

The *FLO* genes encode cell wall proteins that are collectively referred to as adhesins and they are characterized by a common modular organization (Fig. 2.12) that consists of three domains. Firstly, an amino-terminal domain (A) that is proposed to harbour the binding site to carbohydrate receptors (mannan) which confers adhesion (Kobayashi *et al.*, 1998). Initially attached to this domain is a secretory sequence that is removed as the protein migrates through the secretory pathway on its way to the cell wall. This is followed by a central domain (B) that is extremely rich in serine and threonine residues (Caro *et al.*, 1997), and thirdly, a carboxyl-terminal region (C) that contains a site for covalent attachment of a glycosyl phosphatidylinositol (GPI) anchors (Caro *et al.*, 1997; De Groot *et al.*, 2003; Hamada *et al.*, 1998a). A comparison of putative adhesins in terms of their number of amino acid residues and glycosylation sites is presented Table 2.2. The phylogenetic relatedness of Flo proteins is illustrated in Fig. 2.13 and it also concurs their grouping into a single family (Caro *et al.*, 1997).
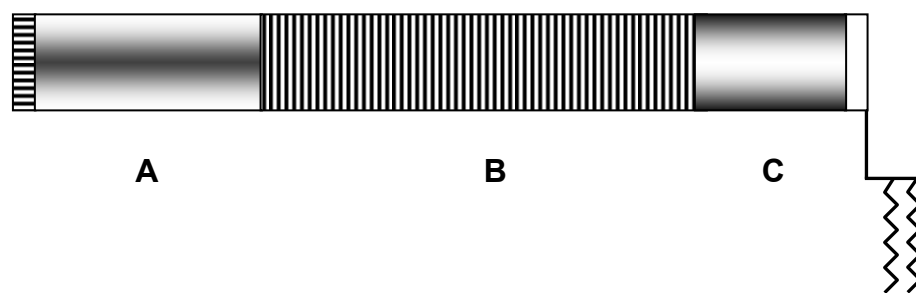
Figure 2.12   Organization of domains in flocculins (adhesins). Flocculins comprise three domains A, B and C, which are preceded by an amino-terminal signal sequence. The N-terminal domain (A) is considered to contain the carbohydrate receptor binding site that confers adhesion. The central domain (B) contains a serine/threonine-rich region. The carboxy-terminal domain (C) contains a site (no fill) for the covalent attachment of a glycosyl phosphatidylinositol (GPI) anchor (adapted from Verstrepen *et al.*, 2004).

Table 2.2 Comparison of putative *FLO* gene encoded adhesins in *S. cerevisiae* (Caro *et al.*, 1997).

Adhesin	number of amino acids	Number of *N*-sites	Ser/Thr (%)
Flo1p	1537	14	41
Flo5p	1075	6	40
Flo9p	1322	17	41
Flo10p	1169	12	41
Flo11p	1367	2	50
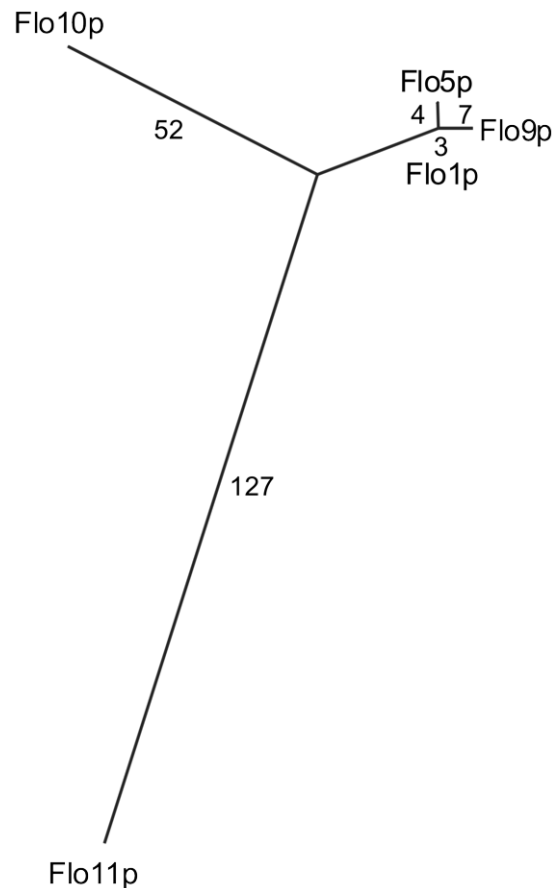
Figure 2.13   Phylogenetic tree of *FLO* gene encoded cell wall proteins. Each number corresponds to the phylogenetic distance *D* multiplied by 100 (extracted from Caro *et al.*, 1997).

In most laboratory strains of *S. cerevisiae*, the subtelomeric *FLO* genes (*FLO1*, *FLO5*, *FLO9* and *FLO10*) are transcriptionally silent and only *FLO11* is expressed. This simplifies the functional analysis of this family in that replacement of the native promoter of each these genes with a strong inducible promoter will allow an exclusive analysis of the function of a particular *FLO* gene. These studies have indicated that overexpression of these genes generates albeit to differing intensities, the following phenotypes:

- Flocculation (cell-cell adhesion); *FLO1*, *FLO5*, *FLO9* (unpublished results) and *FLO10* (Govender *et al.*, 2008; Guo *et al.*, 2000).

- Adherence to agar and plastic (cell-substrate adhesion); *FLO10* and *FLO11* (Govender *et al.*, 2008; Guo *et al.*, 2000).

- Flor formation or biofilm formation (cell-cell interaction in combination with the local environment); *FLO11* (Govender *et al.*, 2008).

The exact biological relevance of flocculation remains poorly understood. It has recently been suggested that flocculation may be a means to protect the cells that are present in the center of a floc from environmental stress. The flocculated cells settle to the bottom of the medium or float to the surface and thus may provide a means of passive transport in the medium away from the stress. Adherence on the other hand prevents cells from being dislodged when they find themselves in a nourishing environment (Verstrepen and Klis, 2006). It has also been suggested that Flo11p may empower cells under nutrient-limiting conditions to forage for new nutrients (Gagiano *et al.*, 2002). Fidalgo and coworkers (2006) recently demonstrated that increased cell surface hydrophobicity mediated by *FLO11* expression enables yeast cells to float by surface tension (flor or biofilm formation), thereby allowing cells direct access to oxygen within oxygen-deficient liquid environments.

2.6.3 Genetic regulation of flocculation

Intensive studies on the transcriptional regulation of the *FLO11* gene and to a far lesser extent of the *FLO1* gene, reveals that *S. cerevisiae* has evolved sophisticated mechanisms to sense and respond to environmental signals by activating developmental switches that result in coordinated changes in cell physiology, morphology and cell adherence.

FLO11 expression is tightly regulated in response to nutrient availability and other stress factors in the direct environment of the cell (Bauer and Pretorius, 2000; Verstrepen and Klis, 2006). The three best characterized signaling pathways that are implicated in transmitting the nutritional status of the environment to the promoter of *FLO11* include the Ras-cAMP-PKA (Gagiano *et al.*, 2002), the invasive growth MAP-kinase (Madhani and Fink, 1997; Madhani *et al.*, 1997) and the main glucose repression pathway (Gancedo, 1998; Vyas *et al.*, 2003). These pathways regulate *FLO11* via a set of transcriptional activators and repressors, which include Flo8p (Kobayashi *et al.*, 1999), Ste12p and Tec1p (Morillon *et al.*, 2000) and Mss11p (Gagiano *et al.*, 2002; van Dyk *et al.*, 2005). For an extensive review on *FLO11* regulation by these pathways see reviews by Palecek *et al.* (2002) and Verstrepen and Klis (2006). The data suggest that the above mentioned signalling pathways and regulatory proteins converge on this promoter to regulate the primary *FLO11* phenotypes of invasive growth and pseudohyphal differentiation. Moreover, these investigations have shown that *FLO11* transcriptional regulation is particularly dependent on the nutritional status and the specific composition of the growth environment.

Far less information is available regarding the regulation of other *FLO* genes. However, Verstrepen and Klis (2006) has suggested that other *S. cerevisiae FLO* genes may be controlled by similar (but not identical) pathways to those that control *FLO11*. This could well be the case as Kobayashi and co-researchers (1999) reported that Flo8p acts as a transcriptional activator of both *FLO1* and *FLO11* genes. The same researchers cautioned that the mechanism of the transcriptional regulation of *FLO1* and *FLO11* by Flo8p may be different from each other. However, in a more recent study, Bester and coworkers (2006) suggested that both Flo8p and Mss11p regulate transcription of *FLO1* and *FLO11* through similar mechanisms. Although the full extent of the *FLO1* promoter is yet to be determined, Fleming and Pennings (2001) reported that the Swi-Snf co-activator and Tup1-Ssn6 co-repressor control an extensive domain (> 5 kb) in which regulation of the *FLO1* gene takes place. The promoter region of *FLO1* was observed to contain a putative GCN4-box at position 268 and numerous stress responsive heat-shock elements (Teunissen *et al.*, 1993). It was suggested by Verstrepen (2003) that GCN4-box motif may repress *FLO1* expression under high nitrogen conditions whilst high ethanol concentrations may induce the *FLO1* expression through the stress responsive heat-shock elements. Indeed, it has been shown that *FLO1* expression is also controlled by nutritional status signals such as carbon and/or nitrogen starvation (Sampermans *et al.*, 2005) and other environmental indicators such as pH (Soares and Seynaeve, 2000) and ionic strength (Jin and Speers, 2000).

Besides *FLO* gene activity being regulated at the transcriptional level, it has also been shown to be modulated by other regulatory systems. In particular, data suggest that these genes are often under promoter-specific epigenetic control allowing *S. cerevisiae* cells in a homogenous population to reversibly switch between active *FLO* gene expression and silent modes (Halme *et al.*, 2004). It is thus highly likely that cells can modulate differential expression of the *FLO* genes thereby optimally tailoring the adhesion properties of the cell wall in response to specific environmental signals.

2.7   CONCLUSION

Whilst certain facets of the flocculation phenomenon are reasonably well-understood, the precise structure of adhesins and ligand involvement in this interaction are not well understood. The transcriptional control of the subtelomeric *FLO* genes (*FLO1*, *FLO5*, *FLO9* and *FLO10*) is also rather poorly defined. The data clearly suggest that flocculation by *S. cerevisiae* is a highly variable and extraordinarily complex phenotype. Considering the complexity of *FLO* gene regulation and the contributing factors, it may be concluded that flocculation is a 'difficult' process to control. This scenario does not augur well for the needs of industries employing batch fermentation processes that are dependent on constant and predictable yeast performance. Furthermore, manipulation of both physiological and environmental factors offers winemakers and brewers limited avenues to control or alter flocculation during fermentations.

It has been proposed that recombinant DNA techniques can be engaged to genetically modify industrial yeast strains with respect to controlled expression of their flocculation genes, thereby generating more stable strains with an appropriate flocculation profile (Pretorius and Bauer, 2002). To this end, *S. cerevisiae* laboratory strains carrying specific *FLO* genes under transcriptional control of late fermentation promoters have shown potential of meeting the requirements of industry when studied under standard laboratory media and growth conditions (Govender *et al.*, 2008; Verstrepen *et al.*, 2001). It may be argued that improvements in this regard may be short-lived due to the *FLO* genes undergoing frequent recombination events involving internal tandem repeats which may result in irreversible decrease in flocculence. However, as suggested by Verstreppen (2003), such problems can be overcome by storage of the original genetically-modified production strain at temperatures that prevent genetic alterations. Thus the original, flocculent strain can be readily propagated whenever the current production strain displays undesirable flocculation characteristics.

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Chapter 3

RESEARCH RESULTS I

Controlled expression of the dominant flocculation genes *FLO1*, *FLO5* and *FLO11* in *Saccharomyces cerevisiae*

Controlled expression of the dominant flocculation genes *FLO1*, *FLO5* and *FLO11* in *Saccharomyces cerevisiae*

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3.1   ABSTRACT

In many industrial fermentation processes, *Saccharomyces cerevisiae* yeast should ideally meet two partially conflicting demands. During fermentation, a high suspended yeast count is required to maintain a satisfactory rate of fermentation, while at completion efficient settling is desired to enhance product clarification and recovery. In most fermentation industries, currently used starter cultures do not satisfy this ideal, probably because non-flocculent yeast strains were selected to avoid fermentation problems. In this paper, we assessed molecular strategies to optimise the flocculation behaviour of *S. cerevisiae*. For this purpose, the chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, of the haploid non-flocculent, non-invasive and non-flor forming *S. cerevisiae* FY23 strain were placed under the transcriptional control of the promoters of the *ADH2* or *HSP30* genes. All six promoter-gene combinations resulted in specific flocculation behaviours in terms of timing and intensity. The strategy resulted in stable expression patterns providing a platform for the direct comparison and assessment of the specific impact of the expression of individual dominant *FLO* genes with regard to cell-wall characteristics such as hydrophobicity, biofilm formation and substrate adhesion properties. The data also clearly demonstrate that flocculation behaviour of yeast strains can be tightly controlled and fine-tuned to satisfy specific industrial requirements.

3.2   INTRODUCTION

Industrial fermentations for the production of bioethanol, wine, beer and other alcoholic beverages are performed in batch processes. At the end of fermentation, the suspended yeast cells must be removed prior to further processing of the fermentation product. The separation of suspended yeast cells may have to be achieved by centrifugation or filtration, which are time consuming and expensive procedures.

Alternatively, clarification can be achieved by natural settling of the yeast. While single yeast cells tend to settle over time, natural settling only becomes a viable option in industrial processes when cells aggregate, a process also referred to as flocculation. Flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989). Although flocculation could provide a seemingly ideal solution to the removal of yeast cells after primary fermentation, it should not occur before the fermentation is completed. Early flocculation may indeed result in sluggish or stuck fermentation, and final products with high residual sugars and unsatisfactory aromatic characteristics (Verstrepen *et al.*, 2001).

Flocculation in *S. cerevisiae* is mediated by specific cell surface lectins (or flocculins) that are capable of binding directly to mannose residues of mannan molecules on adjacent cells (Miki *et al.*, 1982; Stratford, 1989). This interaction leads to cellular aggregation and finally settling. In some specific cases, cellular aggregation does not lead to settling, but to yeast cells rising to the surface of the substrate and forming an air-liquid interfacial biofilm. This behavior is also referred to as flotation or flor formation (Pretorius, 2000; Zara *et al.*, 2005).

In *S. cerevisiae,* two distinct flocculation phenotypes have been characterized on the basis of their sensitivity to sugar inhibition, namely Flo1 (mannose sensitive) and NewFlo (mannose and glucose sensitive) (Stratford and Assinder, 1991). Most brewer's yeast strains are of the NewFlo phenotype and flocculation in these strains is initiated after the end of exponential respiro-fermentative phase of growth (Sampermans *et al.*, 2005). The late onset of flocculation in the NewFlo phenotype makes them ideally suited to their task by aiding separation of biomass from the brew.

The genetic basis of flocculation has been the object of several investigations. These studies suggest that a family of subtelomeric genes, *FLO1, FLO5, FLO9* and *FLO10*, encode specific lectins that are responsible for flocculation (Teunissen and Steensma, 1995). A non-subtelomeric gene, *FLO11/MUC1* (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996), on the other hand, encodes a protein that has been associated

with flocculation, flor formation, invasive growth and substrate adhesion (Bayly *et al.*, 2005; Guo *et al.*, 2000; Ishigami *et al.*, 2004; Lo and Dranginis, 1996; Zara *et al.*, 2005). All Flo proteins are glycosyl-phosphatidylinositol (GPI)-linked glycoproteins that share a common three domain structure consisting of an N-terminal lectin domain, a central domain of highly repeated sequences rich in serine and threonine residues, and a carboxyl terminal domain containing a GPI anchoring sequence (reviewed in Verstrepen and Klis, 2006). In recent studies (Liu *et al.*, 2007a; 2007b), evidence was presented that the difference between the NewFlo and Flo1 flocculation phenotypes may at least be partially due to variations in the number of repeat sequences within the *FLO1* coding sequence.

The regulation of *FLO* gene expression is complex and in particular the promoter of *FLO11* has been intensively studied. *FLO11* expression is tightly controlled by environmental factors, and several signalling cascades, including the Ras-cAMP-dependent kinase complex, the filamentous growth controlling MAP-kinase (MAPK) and the main glucose repression pathways have been directly linked to *FLO11* regulation (reviewed in Verstrepen and Klis, 2006). Two transcriptional regulators, Mss11p and Flo8p, have been shown to play a central role in the control of flocculation and flotation phenotypes (Bester *et al.*, 2006; Liu *et al.*, 1996; van Dyk *et al.*, 2005). These investigations have shown that *FLO11* transcriptional regulation is particularly dependent on the nutritional status and the specific composition of the growth environment. Less information is available regarding the regulation of other *FLO* genes, although it has been shown that *FLO1* expression is also controlled by nutritional status signals such as carbon and/or nitrogen starvation (Sampermans *et al.*, 2005) and other environmental indicators such as pH (Soares and Seynaeve, 2000) and ionic strength (Jin and Speers, 2000).

In addition to this transcriptional regulation, *FLO* gene activity has been shown to be modulated by other regulatory systems. In particular, data suggest that these genes are often under promoter-specific epigenetic control allowing *S. cerevisiae* cells in a homogenous population to reversibly switch between active *FLO* gene expression and silent modes (Halme *et al.*, 2004). Furthermore, sequence analysis reveals that several DNA motifs in the central domain are conserved amongst different *FLO* genes, promoting diversity of adhesins by frequent intragenic recombination events (Verstrepen *et al.*, 2005).

Considering the complexity of *FLO* gene regulation, it is evident that manipulation of both physiological and environmental factors offers winemakers and brewers limited avenues to control or alter flocculation during fermentations. It is therefore not surprising that industrial yeast strains generally possess a less than optimal flocculation profile

(Carstens *et al.*, 1998; Verstrepen *et al.*, 2003). For this reason, the replacement of the native promoter of these genes with less complex promoters conferring expression patterns that would be better adapted to industrial needs may result in yeast strains that display improved flocculation behaviour for specific industrial purposes.

In previous attempts to modify flocculation behaviour, the flocculation genes *FLO1* and *FLO5* were introduced into non-flocculent *S. cerevisiae* brewing yeast strains (Barney *et al.*, 1990; Ishida-Fujii *et al.*, 1998; Watari *et al.*, 1990; 1991; 1994). However, the resultant modified yeast strains flocculated constitutively and displayed reduced fermentation performance or increased fermentation times. In an approach similar to the one described here, Verstrepen *et al.* (2001) brought the chromosomal *FLO1* gene of the haploid non-flocculent *S. cerevisiae* FY23 laboratory strain under the transcriptional control of the *HSP30* stationary phase promoter. The resulting strain showed strong flocculation towards the end of fermentation, resulting in a distinctly clearer beer than the beer obtained with wild-type cells (Verstrepen *et al.*, 2001).

In this study, we assess the suitability of six genome-integrated promoter-gene combinations to control stationary-phase specific flocculation. For this purpose, the native promoters of the dominant flocculation genes *FLO1, FLO5,* and *FLO11* in the haploid *S. cerevisiae* FY23 strain were replaced with the inducible promoters *ADH2* and *HSP30*. The *ADH2* promoter is subjected to carbon catabolite repression and has been shown to be repressed several hundred-fold during growth on glucose (Gancedo, 1998; Price *et al.*, 1990). Derepression of the *ADH2* promoter generally coincides with transition to growth on ethanol (Noronha *et al.*, 1998). The *HSP30* promoter, on the other hand, has been shown to be induced during entry into the stationary phase of growth and coincides with the depletion of glucose from the medium, including in low-stress nutrient-rich wort and wine fermentation conditions (Donalies and Stahl, 2001; Regnacq and Boucherie, 1993; Riou *et al.*, 1997). In addition, the *HSP30* promoter is activated by several stress factors including heat shock and sudden exposure to either ethanol or sorbate (Piper *et al.*, 1994; Regnacq and Boucherie, 1993; Seymour and Piper, 1999). Unlike *ADH2* regulation, which is reasonably well understood (Di Mauro *et al.*, 2000; Verdone *et al.*, 2002; Young *et al.*, 1998), the mechanism by which *HSP30* is induced in response to stress remains unclear.

Since the dominant *FLO* genes are transcriptionally silent in the *S. cerevisiae* FY23 strain due to a nonsense mutation in the *FLO8* gene (Liu *et al.*, 1996; Verstrepen *et al.*, 2005; Winston *et al.*, 1995), expression regulated by either the *ADH2* or *HSP30* promoter constructs allows both assessments of the phenotypic consequences of the expression of a particular Flo protein and of the transcriptional character of a promoter in the same genetic background. Indeed, it is difficult to compare reports on flocculation in

the literature due to the numerous techniques employed and the variations therein, coupled with the different yeast strain genetic backgrounds (Jin and Speers, 2000). Therefore, the inducible expression of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11* in the haploid laboratory strain *S. cerevisiae* FY23 strain reported in this study, presents a unique opportunity to compare the adhesion characteristics (flocculation, invasive growth and flor formation) of the aforementioned flocculation genes.

Our data show that each promoter-ORF combination leads to specific flocculation and adhesion behaviour and results in additional important changes in cell-surface properties, including hydrophobicity. The data indicate that highly specific flocculation behaviour can be stably conferred to individual yeast strains.

3.3   MATERIALS AND METHODS

3.3.1 Strains. The yeast strains employed in this study are listed in Table 3.1. All strains were derived from *Saccharomyces cerevisiae* strain FY23 (Liu *et al.*, 1996; Winston *et al.*, 1995). *Escherichia coli* DH5α (Gibco BRL/Life Technologies, Rockville, MD) was used as a host for all plasmid amplifications.

TABLE 3.1 *S. cerevisiae* employed in this study

Strain	Genotype	Reference
FY23	*MATa leu2 trp1 ura3 flo8-1*	(Winston *et al.*, 1995)
FY23-F1A	*MATa leu2 trp1 ura3 flo8-1 FLO1p::SMR1-ADH2p*	This study
FY23-F1H	*MATa leu2 trp1 ura3 flo8-1 FLO1p::SMR1-HSP30p*	This study
FY23-F5A	*MATa leu2 trp1 ura3 flo8-1 FLO5p::SMR1-ADH2p*	This study
FY23-F5H	*MATa leu2 trp1 ura3 flo8-1 FLO5p::SMR1-HSP30p*	This study
FY23-F11A	*MATa leu2 trp1 ura3 flo8-1 FLO11p::SMR1-ADH2p*	This study
FY23-F11H	*MATa leu2 trp1 ura3 flo8-1 FLO11p::SMR1-HSP30p*	This study

3.3.2 Media and cultivation conditions. Yeast strains were routinely cultivated at 30°C in rich YEPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose. Single yeast colonies from 3-day old YEPD plates were used to inoculate starter cultures in 40 mL YEPD broth contained in 250 mL Erlenmeyer flasks, which were incubated at 30°C with shaking (160 rpm) for 18 h. These were used to inoculate precultures at an initial cell density of 5×10^5 cells mL^{-1}, which were incubated at 30°C with shaking (160 rpm) for 18 h. Thereafter, yeast cells for inoculation of experimental cultures were routinely prepared as follows using ice cold reagents. Yeast from precultures were harvested by centrifugation (4000 rpm, 5 min), washed

once with 100 mM EDTA, pH 7 to ensure deflocculation; once with 30 mM EDTA, pH 7 and finally resuspended in 30 mM EDTA, pH 7. To determine the onset of flocculation, flocculent ability, glucose utilisation and growth rate of yeast in nutrient rich medium, experimental cultures were seeded at an initial cell density of 5×10^5 cells mL^{-1} into 40 mL YEPD, and incubated at 30°C with shaking (160 rpm). At 2 h intervals, for a period of 24 h and a 48 h time point, cell populations were harvested and deflocculated as described previously. The flocculation ability of FY23-F11A and FY23-F11H strains was also assessed in media that possessed a similar composition to YEPD, but with an alternative carbon source, namely YEPE [3% (vol/vol) ethanol] and YEPGE [3% (vol/vol) ethanol together with 3% (vol/vol) glycerol]. In addition, flocculation and invasive growth tendencies were also assessed in chemically defined synthetic complete (SC) media containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI, USA), supplemented with all nutrients (Sherman *et al.*, 1991) and 2% (wt/vol) glucose as sole carbon source (SCD); 0.2% (wt/vol) glucose (SCLD); 3% (vol/vol) ethanol (SCE); and 3% (vol/vol) ethanol with 3% (vol/vol) glycerol (SCGE). In addition to the above, medium containing only the auxotrophic requirements (Sherman *et al.*, 1991) of strain FY23 (leucine, uracil and tryptophan) was also used (SCD$_{LUT}$ and SCLD$_{LUT}$). Flor medium containing 0.67% YNB and 3% (vol/vol) ethanol adjusted to pH 3.5 (Ishigami *et al.*, 2004) containing all nutritional requirements was used to assess flor formation. For selection of sulphometuron methyl (SM) resistant yeast transformants, SC medium containing 0.67% YNB and 2% (wt/vol) glucose was supplemented with amino acids for strain FY23 and 80-100 μg/mL SM (DuPont Agricultural Products, France). *E. coli* was grown at 37°C in Luria-Bertani (LB) medium [1% (wt/vol) Bacto tryptone, 0.5% (wt/vol) yeast extract and 1% (wt/vol) NaCl] and bacterial transformants were selected using LB medium containing 100 mg/l ampicillin. In this study, 2% (wt/vol) agar (Difco) was used for all solid media. Bacterial and yeast strains were stored in LB containing 40% (vol/vol) glycerol and YPD supplemented with 15% (vol/vol) glycerol respectively (Ausubel *et al.*, 1995).

3.3.3 DNA manipulation, construction of promoter-replacement cassettes and yeast transformations. Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Expand™ High Fidelity PCR system (Roche Diagnostics GmbH) was employed for polymerase chain reaction (PCR) amplifications. The amplification products were purified from agarose gels and cloned into pGEM®-T Easy vector (Promega Corporation, Madison, USA) according to the specifications of the manufacturer. Standard procedures for bacterial transformations and plasmid isolation from *E. coli* were performed (Sambrook *et al.*, 1989). Standard procedures for isolation and manipulation of DNA were employed in all other aspects of this study (Ausubel *et al.*, 1995). The strategy for construction of promoter replacement cassettes was adapted from Verstrepen and Thevelein (2004). The *ADH2* promoter

region bearing a *FLO1*p homologous sequence was amplified from pDLG5 plasmid (la Grange *et al.*, 1996) by PCR with ADH2-F and ADH2::FLO1-R primers (Table 3.2). The SM-resistance yielding *SMR1-410* (*SMR1*) marker gene inclusive of promoter and terminator sequences was PCR amplified from plasmid pWX509 (Casey *et al.*, 1988) with the SMR1-R and FLO1::SMR1-F primer pair. The *ADH2-FLO1*p 834 base pair (bp) fragment in pGEM-T Easy was recovered by double restriction digest with *Bgl*II and *Spe*I, whilst the *FLO1*p-*SMR1* insert (2936 bp) was released by triple digestion with *Alw*441, *Bam*HI and *Sph*I. Both fragments were subsequently ligated. The *FLO1*p-*SMR1-ADH2-FLO1*p vector was PCR amplified by using shorter primers FLO1-F and FLO1-R and ligation reaction mixture as template. The integrating *FLO1* promoter replacement cassette (3762 bp) was extracted from agarose gels and purified. A similar strategy was employed for the construction and synthesis of *FLO1*p-*SMR1-HSP30-FLO1*p, *FLO5*p-*SMR1-ADH2-FLO5*p, *FLO5*p-*SMR1-HSP30-FLO5*p, *FLO11*p-*SMR1-ADH2-FLO11*p and *FLO11*p-*SMR1-HSP30-FLO11*p integrating promoter transplacement cassettes. The primer pairs for different open reading frames (ORF) are as follows: for *FLO5*, FLO5-F and FLO5-R; *and* for *FLO11*, FLO11-F and FLO11-R. Note that the *HSP30p* containing region was amplified using FY23 chromosomal DNA as template. Yeast transformation with 5 μg of DNA was performed according to the lithium acetate method described by Gietz and Schiestl (1995). Chromosomal integration was achieved by a double cross-over homologous recombination event, in which the *FLO1*, *FLO5* or *FLO11* gene was placed under transcriptional control of either the *ADH2* or *HSP30* promoter. The deletion of native promoters was confirmed by PCR using homologous primer sets. The primer pairs for transgenic strains were as follows: FY23-F1A and FY23-F1H (FLO1-F and FLO1-R); FY23-F5A and FY23-F5H (FLO5-F and FLO5-R); FY23-F11A and FY23-F11H (FLO11-F and FLO11-R). In addition, the integration of promoter replacement cassettes in transformed yeast was further confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants. The primer pairs for different transformants were as follows: for FY23-F1A, FLO1-F2 and ADH2-R; for FY23-F1H, FLO1-F2 and HSP30-R; for FY23-F5A, FLO5-F2 and ADH2-R; for FY23-F5H, FLO5-F2 and HSP30-R; for FY23-F11A, FLO11-F2 and ADH2-R; and for FY23-F11H, FLO11-F2 and HSP30-R. The wild type FY23 strain served as a control in these confirmation experiments.

3.3.4 Growth and enumeration of yeast populations. The cell density of suitably diluted yeast suspensions in 30 mM EDTA (pH 7) was determined either by direct cell counting with a hemocytometer or by measuring absorbance at 600 nm in a Cary 50 UV-Vis spectrophotometer (Varian Inc., CA, USA) using a standard curve as reference.

TABLE 3.2 Primers used in this study

Primer name	Primer sequence (5'→3')	Underlined sequence
FLO1::SMR1-F	<u>TGCGTCACTTTTCCTACGGTGCCTCGCACATGA ATGTTATCCGGCGCACG</u>GGTACCGGCTTGGCT TCAGTTGCTG	Homologous *FLO1*p region matching to nucleotides (nt) −813 to −764.
FLO5::SMR1-F	<u>GCAATAAACCACATGGCTACCGCACTTCTTGTC ACTATCC</u>GGTACCGGCTTGGCTTCAGTTGCTG	Homologous *FLO5*p region corresponding to nt −1995 to −1956.
FLO11::SMR1-F	<u>TCACTGCACTTCAACTATGCCTTATAGCAACCA AGAAGCTAGAAAATGCCAACTATTAAAAAGATA ACCTCT</u>CGGTACCGGCTTGGCTTCAGTTGCTG	Homologous *FLO11*p region corresponding to nt −2710 to −2639.
SMR1-R	CATG<u>GGATCC</u>AGCTTGCAATTTTTGACGGCCCC	*Bam*HI restriction site.
ADH2-F	TGAC<u>AGATCT</u>AACTCGTTCCAGTCAGGATTG	*Bgl*II restriction site.
ADH2-R	TGATAGTTGATTGTATGCTTTTTGTAGC	
ADH2::FLO1-R	<u>CTGCCAAAAACATATAGCGATGAGGCATTGTCA TTTTTGGATGTTCTGTTTACTGGTGAC</u>TGATAGT TGATTGTATGCTTTTTGTAGC	Homologous *FLO1*p region corresponding to nt −26 to +34.
ADH2::FLO5-R	<u>GCTAATCAATTTAAAGAAAATCAATTGCGGAATT TACTGCAGAGC</u>TGATAGTTGATTGTATGCTTTTT GTAGC	Homologous *FLO5*p region corresponding to nt −58 to −14.
ADH2::FLO11-R	<u>GGACCAAATAAGCGAGTAGAAATGGTCTTTGCA TAGTGTGCGTATATGGATTTTTGAGGC</u>TGATAG TTGATTGTATGCTTTTTGTAGC	Homologous *FLO11*p region corresponding to nt −26 to +34.
HSP30-F	CATG<u>AGATCT</u>GATGGCATTGCACTCAAG	*Bgl*II restriction site.
HSP30-R	TATTAAAGTCTCAAACTTGTTGTTTTG	
HSP30::FLO1-R	<u>GCGATGAGGCATTGTCATTTTTGGATGTTCTGT TTACTGGTGAC</u>AAAAGATATTAAAGTCTCAAACT TG	Homologous *FLO1*p region corresponding to nt −26 to +18.
HSP30::FLO5-R	<u>GCTAATCAATTTAAAGAAAATCAATTGCGGAATT TACTGCAGAGC</u>TATTAAAGTCTCAAACTTG	Homologous *FLO5*p region corresponding to nt −58 to −14.
HSP30::FLO11-R	<u>GGACCAAATAAGCGAGTAGAAATGGTCTTTGCA TAGTGTGCGTATATGGATTTTTGAGGC</u>AAAAGA TATTAAAGTCTCAAACTTGTTG	Homologous *FLO11*p region corresponding to nt −26 to +34.
FLO1-F	AAGTGTGCGTCACTTTTCCTACGGT	
FLO1-F2	ATGGCACTAGTCGATCGAGG	
FLO1-R	AGCGATGAGGCATTGTCATTT	
FLO5-F	GCAATAAACCACATGGCTACC	
FLO5-F2	GGTTGTGTTCTA GGACTTTCTGACG	
FLO5-R	AGTGGTGCTAATCAATTTAAAGAA	
FLO11-F	CCTCTCACTGCACTTCAACTATGC	
FLO11-F2	TTACGGCCTAATGTCGAGAC	
FLO11-R	GGACCAAATAAGCGAGTAGA	

Non-underlined sequences correspond to *ADH2*, *HSP30*, *and SMR1-410* or *FLO* gene sequences as denoted by the primer name. Nucleotide numbering has been done by assigning the A in ATG start codon of the open reading frame as base 1.

3.3.5 Glucose determination. Cells were pelleted from 1 mL samples of experimental cultures by microcentrifugation (10000 rpm, 1 min). The cell-free extracts were subsequently filtered through a 0.22 μm cellulose acetate filter and stored at $-20°C$. The concentration of glucose in the culture medium was determined using a GAGO-20 glucose assay kit (Sigma, Missouri, USA) according to the specifications of the manufacturer, using a BIOTEK 800ELX microplate reader (BIOTEK Instruments Inc., Winooski, VT, USA).

3.3.6 Flocculation assays. The flocculent ability of yeast strains was established using the modified Helm's assay as described by D'Hautcourt and Smart (1999). The percentage flocculation reported in this paper reflects the arithmetic mean of three independent determinations. To assess the influence of pH on flocculation, a composite suspension buffer with a very wide buffering range was adapted from Stratford (1996) to replace the buffer employed in the above protocol. This buffer contained 10 mM calcium chloride, 50 mM Tris base, 50 mM succinic acid, 100 mM potassium hydroxide and 4% (vol/vol) ethanol. The pH of the composite suspension buffer was adjusted with 5 M HCl and flocculation was assessed as described above. To investigate sugar inhibition of *FLO1* and *FLO5* flocculation phenotypes, either mannose or glucose was added at varying concentrations to both the washing and suspension buffers that are employed in the modified Helm's assay (1999).

3.3.7 RNA extraction and cDNA synthesis. FY23, FY23-F11A and FY23-F11A strains were precultured and treated as described earlier. Experimental batch cultures were inoculated in triplicate at an initial density of 5×10^5 cells mL^{-1} into 100 mL YEPD, and incubated at $30°C$ with shaking (160 rpm) for 12, 16 and 48 h. To investigate the transcription of *FLO* genes, samples from batch cultures were washed with ice-cold H_2O, pelleted and resuspended in ice-cold AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). Total RNA was isolated as previously described (Schmitt *et al.*, 1990). DNA contamination was eliminated by DNase I (Roche diagnostics) treatment. One μg total RNA was used as template for cDNA synthesis using the ImProm-II™ reverse transcription system according to the manufacturer instructions (Promega). cDNA samples were diluted 50-fold with H_2O before real-time PCR analysis.

3.3.8 Quantitative real-time PCR analysis (QRT-PCR). Primers and probes used for QRT-PCR analysis are listed in Table 3.3 and were designed using Primer Express software ver. 3 (Applied Biosystems, CA, USA). Reagents were purchased from Applied Biosystems and Kapa Biosystems (Cape Town, South Africa). QRT-PCR runs and collection of spectral data were performed with the 7500 cycler (Applied Biosystems). SYBR Green was used for the detection of *PDA1* and *FLO11* amplicons with final primer concentrations of 100 nM. Specific probes and primers were designed to

differentiate between the cDNA species corresponding to the extensively homologous *FLO1* and *FLO5* genes. Probes were modified by the addition of a 3' minor groove binder (MGB) and non-fluorescent quencher, as well as the 5' attachment of fluorescent dyes as indicated in Table 3.3 (Applied Biosystems). Probe and primer concentrations were 250 nM and 900 nM respectively in QRT-PCR reactions. Cycling conditions during QRT-PCR were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. When using SYBR Green, a dissociation curve analysis was included to verify amplicon authenticity. Preliminary data analyses were performed with Signal Detection Software (SDS) ver 1.3.1. (Applied Biosystems). Individual QRT-PCR reaction runs were performed at least in duplicate. The relative expression value for each sample was defined as $2^{-Ct}_{(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data was normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample thus giving normalized relative expression for a target gene as $2^{-Ct}_{(target)}/2^{-Ct}_{(PDA1)}$.

TABLE 3.3 Real-time PCR Primers and probes used in this study

Primer/probe name	Primer/probe sequence (5'→3')	Modifications
FLO1-F (TaqmanMGB)	ATGCCTCATCGCTATATGTTTTTG	none
FLO1-R (TaqmanMGB)	GCTCCTGAGGCCACACTAGTTAG	none
FLO5-F (TaqmanMGB)	AGCACCACTAAAAAAAATGACAATTG	none
FLO5-R (TaqmanMGB)	GCCAGAAAGGCCAAGATTACC	none
FLO1-probe	CAGTCTTTACACTTCTGGC	6-FAM 5' label , 3' Minor Groove Binder/ Non-Fluorescent Quencher
FLO5-probe	ACCACTGCATATTTT	VIC dye 5' label , 3' Minor Groove Binder/ Non-Fluorescent Quencher
FLO11-F-(QRT-PCR)	CCTCCGAAGGAACTAGCTGTAATT	none
FLO11-R-(QRT-PCR)	AGTCACATCCAAAGTATACTGCATGAT	none
PDA1-F-QRT-PCR	GGAATTTGCCCGTCGTGTT	none
PDA1-R-QRT-PCR	GCGGCGGTACCCATACC	none

3.3.9 Determination of hydrophobicity of yeast cell surfaces. The hydrophobicity of yeast cell surfaces was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent (Hinchcliffe *et al.*, 1985). Cultures in YEPD were incubated at 30°C for 48 h with shaking (160 rpm). The harvested cells from an experimental culture were deflocculated, washed and

diluted to a density of 5×10^6 cells mL^{-1} in 30 mM EDTA (pH 7). Yeast cells from a 20 mL aliquot of this suspension were washed twice and resuspended in 20 mL of phosphate-urea-magnesium (PUM) buffer pH 7.1 (Hinchcliffe *et al.*, 1985). The absorbance of this suspension (*I*) was determined at 660 nm. Aliquots of 2.4 mL (three replicates) were dispensed into borosilicate glass tubes (15 x 75 mm) and 200 µL xylene was layered over the yeast suspension. Tubes were rubber-capped; samples were vortexed at maximum speed for 30 s and allowed to stand undisturbed for 15 min. The absorbance of the residual buffer layer (*F*) at 660 nm was determined. The average modified hydrophobic index (MHI) for a sample was calculated using the equation: MHI = 1- (*F*/*I*)

3.3.10 Invasive growth plate assays. Yeast cultures, processed as previously described, were adjusted to an optical density (measured at a wavelength of 600 nm) of 1.0 and 10 µl aliquots were dropped onto SCLD and SCLD$_{LUT}$ plates without piercing the agar surface and incubated for 5 days at 30°C (van Dyk *et al.*, 2005). Using a gloved finger, superficial growth of yeast colonies was physically removed by washing plates under a steady stream of water. Plates were allowed to air dry and cells that invaded the agar were photographed.

3.3.11 Flor formation and buoyant cell density. Cells were pre-cultured in YEPD, deflocculated and washed as described previously. Subsequently 3×10^8 cells were recovered by microcentrifugation (10000 rpm, 1 min), washed once and resuspended in 1 mL flor medium (pH 3.5) and added to test tubes (16 x 165 mm) containing 4 mL flor medium. Biofilm formation was photographed in natural light after 5 days of static incubation at 30°C. Alternatively, the cultures were incubated statically at 30°C for 60 h, after which 1 mL samples were withdrawn from just below the meniscus. The optical density of samples was determined spectrophotometrically at 600 nm.

3.3.12 Analysis of stress-induced expression of *FLO1* and *FLO5* encoded flocculins. FY23, FY23-F5H and FY23-F1H strains were precultured and treated as described earlier. Experimental cultures were inoculated at an initial density of 5×10^5 cells mL^{-1} into 40 mL YEPD, and incubated at 30°C with shaking (160 rpm) for 10 h. The incubation of untreated cells was extended for another 45 min at 30°C, whereas other cultures were exposed to the following stress treatments: heat shock for 30 min at 42°C; heat shock for 45 min at 42°C; 3% (vol/vol) ethanol for 30 min at 30°C; 6% (vol/vol) ethanol for 30 min at 30°C and 6% (vol/vol) ethanol and heat shock for 30 min at 42°C. Ethanol (100%) was added directly to culture medium to yield a final concentration of 6% (vol/vol) and cultures were incubated at defined temperatures with shaking at 160 rpm. All cultures were placed on ice before flocculation was determined using the modified Helm's assay.

3.4   RESULTS

A

Promoter replacement cassette
with homologous ends

*Bam*HI and *Bgl*II
ligation site

B

C

← 6557
← 4361

2027 →

564 →
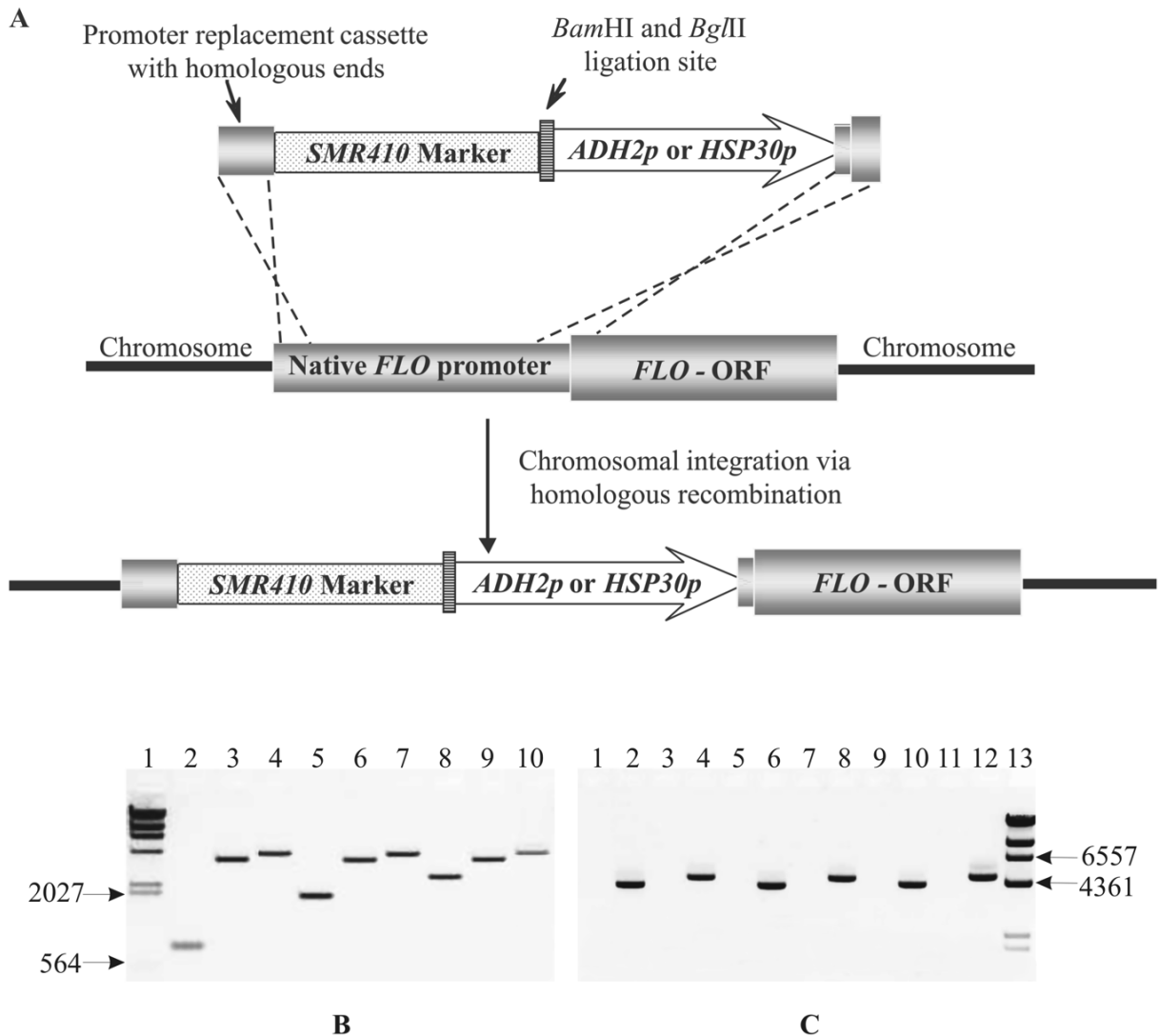
Figure 3.1   Chromosomal integration of either *ADH2* or *HSP30* promoter upstream of a dominant *FLO* gene in *S. cerevisiae* strain FY23. (A) Promoter transplacement strategy. (B) The deletion of native promoters was confirmed by PCR using homologous primer pairs described in materials and methods. The amplification of the native promoter sequence was only observed in the wild type FY23 strain; *FLO1p* (837 bp, lane 2), *FLO5p* (1988 bp, lane 5) and *FLO11p* (2748 bp, lane 8) whilst only the integration cassette was amplified in FY23-F1A (*FLO1*p-*SMR1-ADH2-FLO1*p, 3762 bp, lane 3); FY23-F1H (*FLO1*p-*SMR1-HSP30-FLO1*p, bp 4247, lane 4); FY23-F5A (*FLO5*p-*SMR1-ADH2-FLO5*p, 3753 bp, lane 6); FY23-F5H (*FLO5*p-*SMR1-HSP30-FLO5*p, 4232 bp, lane 7); FY23-F11A (*FLO11*p-*SMR1-ADH2-FLO11*p, 3798 bp, lane 9) and FY23-F11H (*FLO11*p-*SMR1-HSP30-FLO11*p, 4277 bp, lane 10). Lane 1 contained DNA molecular weight marker (phage lambda DNA restricted with *Hind*III). (C) The integration of promoter replacement cassettes were confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA as template, described in materials and methods. The amplification of *FLO1p-SMR1-ADH2p* (4189 bp, FY23-F1A, lane 2), *FLO1p-SMR1-HSP30p* (4668 bp, FY23-F1H, lane 4), *FLO5p-SMR1-ADH2p* (4099 bp, FY23-F5A, lane 6), *FLO5p-SMR1-HSP30p* (4578 bp, FY23-F5H, lane 8), *FLO11p-SMR1-ADH2p* (4334 bp, FY23-F11A, lane 10), *FLO11p-SMR1-HSP30p* (4813 bp, FY23-F11H, lane 12) is only evident in transformants, whilst lacking in the wild type FY23 strain with corresponding primer pairs (lanes 1, 3, 5, 7, 9 and 11). Lane 13 contained DNA molecular weight marker (phage lambda DNA restricted with *Hind*III).

3.4.1 Yeast transformation. Following initial selection on SC plates containing SM, putative transformants were inoculated individually into YEPD broth and cultivated for 48 h at 30°C with shaking (160 rpm). A majority of strains transformed with the combinations *FLO1*p-*SMR1*-*ADH2p*-*FLO1*p, *FLO1*p-*SMR1*-*HSP30p*-*FLO1*p, *FLO5*p-*SMR1*-*ADH2p*-*FLO5*p or *FLO5p*-*SMR1*-*HSP30p*-*FLO5*p visually displayed strong flocculent phenotypes suggesting that integration had occurred at the desired loci (Fig. 3.1A). Three independent transformants of each strain were selected for further analysis. No flocculent phenotype was detectable for putative transformants of FY23-F11A and FY23-F11H. A visual assessment of biofilm formation on flor medium was therefore used for the initial screen of putative *FLO11* transformants, and three independent flor-forming strains were retained for further analysis. For each of the selected strains, the deletion of native promoters was confirmed by PCR using homologous primer sets (Fig. 3.1B). In addition, integration at the correct gene locus was also confirmed by PCR (Fig. 3.1C) using primers in which the upstream primer was located outside the region of the inserted promoter replacement cassette.

3.4.2 Stability, growth rates, glucose consumption and flocculation. To assess the stability of the integrated promoter constructs, the selected transformed strains were cultivated in rich, non-selective medium in repeated batch cultures for more than 100 generations. For each strain, twenty individual colonies were then assessed for their flocculation (*FLO1* and *FLO5* constructs) and flor forming (*FLO11* constructs) behaviour. All tested colonies displayed the relevant phenotypes. The timing and intensity of the phenotypes were in all cases similar to those observed during the initial screen, indicating that the integration and the resulting expression patterns are stable.

The growth rate and sugar utilization capabilities of the wild type strain FY23 and its six transformants were assessed in YEPD containing 2% glucose at 2-hourly intervals (Fig. 3.2 and 3.3). No significant differences between the wild-type FY23 strain and the transformants regarding biomass growth, cell numbers and sugar utilization capabilities were observed. As seen during the initial screen, strains transformed with combinations involving *FLO1* and *FLO5* ORFs showed flocculent behaviour. Maximal flocculent ability of these strains was displayed 2-4 h after glucose depletion (Fig. 3.2B and 3.3B). In the *ADH2p*-*FLO1* and *ADH2p*-*FLO5* transformants, flocculation was observed approximately 2 h after glucose exhaustion, while maximum flocculation potential was evident after an additional 4 h (Fig. 3.2B).

After 48 h growth in YEPD containing 2% (wt/vol) glucose, *FLO1* transformants [FY23-F1A (98 ± 1%) and FY23-F1H (97 ± 1%)] were more flocculent than the corresponding *FLO5* transgenic yeast strains [FY23-F5A (84 ± 2%) and FY23-F5H (79 ± 3%)] (Fig. 3.2B and 3.3B). This also suggests that *ADH2p* controlled *FLO1* and

FLO5 phenotypes are slightly more flocculent than *HSP30p* regulated phenotypes. This difference was obvious with macroscopic evaluation, where it was evident that *ADH2p* induced *FLO1* and *FLO5* flocculent phenotypes are markedly stronger than *HSP30p* mediated *FLO1* and *FLO5* flocculation phenotypes. *ADH2p-FLO1* flocs also formed larger clumps that remained at the bottom of the flasks even when agitated at 200 rpm (Fig. 3.4).

Interestingly, *FLO1* and *FLO5* transformants displayed decreased flocculation capacities in minimal media (data not shown). In these conditions, the FY23-F1H and FY23-F5H strains, when cultivated in SCD medium containing all nutritional requirements or SCD$_{LUT}$ that contained only the auxotrophic requirements of the strains, displayed significantly higher flocculation abilities when compared to the FY23-F1A and FY23-F5A strains, with the latter strains not flocculating at all in SCD$_{LUT}$ medium. *FLO11* expression mediated by either the *ADH2* or *HSP30* promoter in nutrient rich YEPD [2% (wt/vol) glucose] (Fig. 3.2B and 3.3B), YEPE [3% (vol/vol) ethanol], or YEPGE [3% (vol/vol) ethanol and 3% (vol/vol) glycerol] medium and minimal media including SCD and SCD$_{LUT}$ did not yield a flocculent phenotype (results not shown).

The flocculent abilities of the wild type FY23 and six transformed yeast strains were studied over a broad pH range (Fig. 3.5). The FY23-F11A, FY23-F11H and FY23 wild type strain displayed no significant flocculation ability over the entire pH range. The FY23-F1A and FY23-F1H strains displayed relatively stable flocculation between pH 2 and 10, whereas flocculation was reduced by nearly 40% at pH 1. In contrast, flocculation exhibited by the FY23-F5A and FY23-F5H strains was stable between pH 3 and 10, whilst being reduced by approximately 20% at pH 2 and completely abolished at pH 1. This supports previous findings which reported that Flo1-type flocculation displays a broad tolerance to pH (Stratford, 1996) whilst a significantly reduced range (pH 4 to 5) was observed for NewFlo-type flocculation (Smit *et al*., 1992).

The relationship between sugar concentration and inhibition of flocculation in *FLO1* and *FLO5* transformants was also assessed (Fig. 3.6). Increasing concentrations of mannose was shown to have a progressively inhibitory effect on the flocculation of all these transformants and flocculation was completely inhibited at 900 mM mannose (Fig. 3.6A). On the contrary no inhibitory effect was evident in the presence of glucose (Fig. 3.6B). Although Kobayashi *et al*. (1998) reported residual flocculation of 22% at 10 mM mannose for a *FLO1* expressing *S. cerevisiae* strain displaying Flo1-type flocculation, the overall mannose inhibitory profile reported is similar to this finding. It can be suggested that the concentration of mannose required for complete inhibition of Flo1-type flocculation is variable and strain dependent. This may simply be a consequence of Flo1p concentrations within the cell wall, with higher concentrations of

Flo1p requiring a higher level of mannose to achieve inhibition. In addition, changes in *FLO1* sequences between different strains may be responsible for the difference. Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (Stratford and Assinder, 1991), this result clearly demonstrates that *FLO1* and *FLO5* encoded flocculins exhibit Flo1-type flocculation.
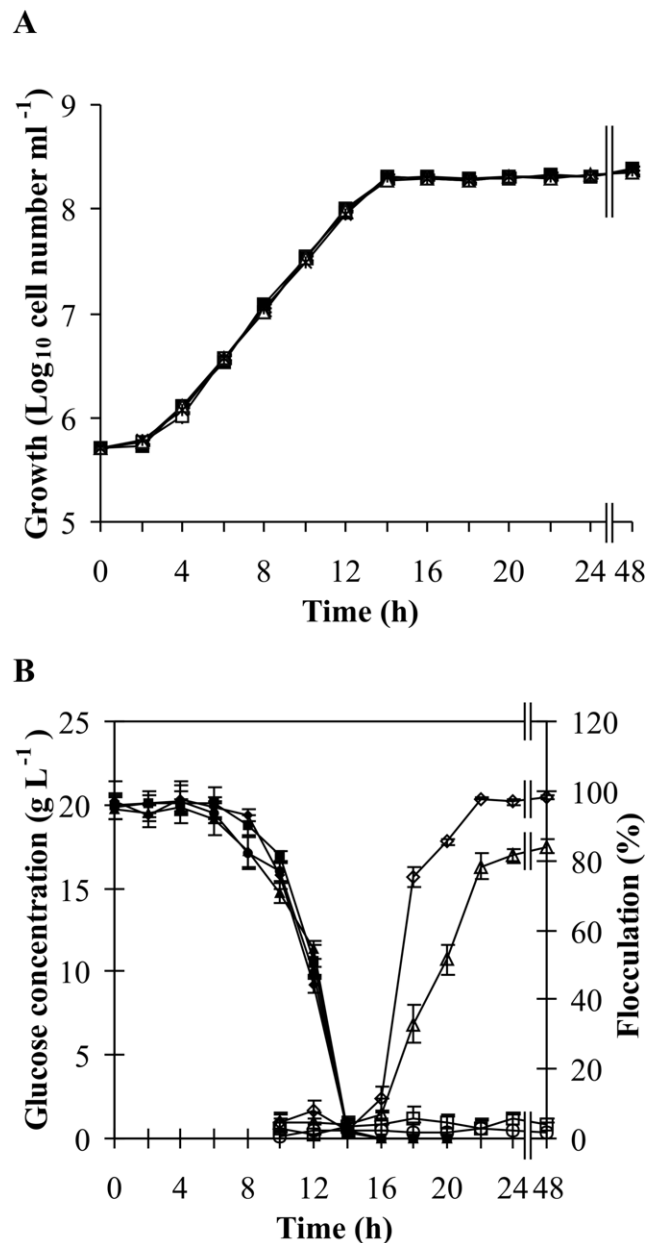
Figure 3.2   (A) Growth of FY23 wild type (□); FY23-F1A (■); FY23-F5A (△); and FY23-F11A (∗) strains. (B) Glucose utilization of FY23 wild type (■); FY23-F1A (◆); FY23-F5A (▲); and FY23-F11A (●) strains. Flocculation profile of FY23 wild type (□); FY23-F1A (◇); FY23-F5A (△); and FY23-F11A (○) strains. Yeast strains were cultivated in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.
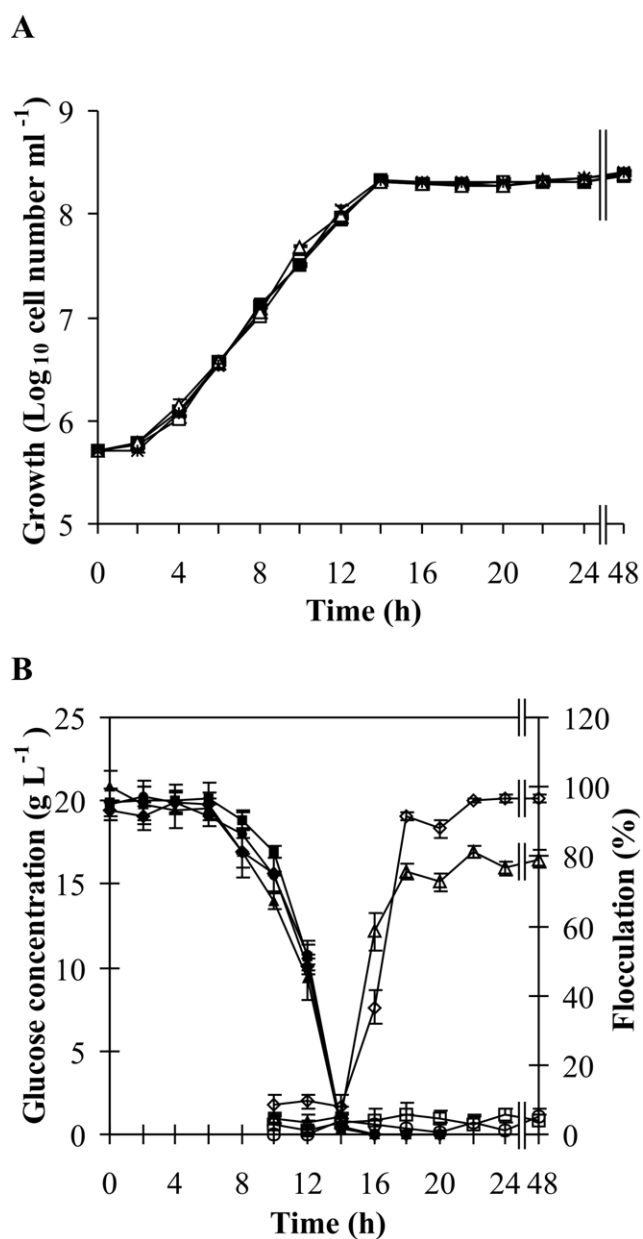
Figure 3.3   (A) Growth of FY23 wild type (□); FY23-F1H (■); FY23-F5H (△); and FY23-F11H (✱) strains. (B) Glucose utilization of FY23 wild type (■); FY23-F1H (◆); FY23-F5H (▲); and FY23-F11H (●) strains. Flocculation profile of FY23 wild type (□); FY23-F1H (◇); FY23-F5H (△); and FY23-F11H (○) strains. Yeast strains were cultivated in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Each point reflects the mean of experiments performed in triplicate and error bars represent standard deviations.
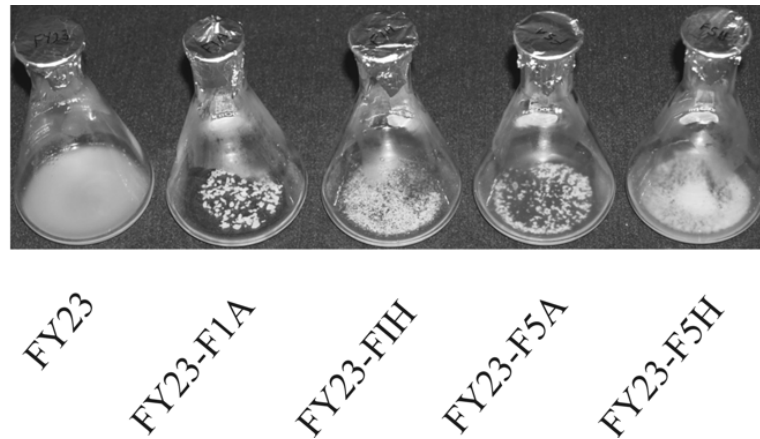
Figure 3.4 Floc formation by FY23 wild type; FY23-F1A; FY23-F1H; FY23-F5A; and FY23-F5H strains. Yeast strains were cultivated for 48 h in YEPD containing 2% glucose at 30°C with shaking (160 rpm) and photographed *in situ*.
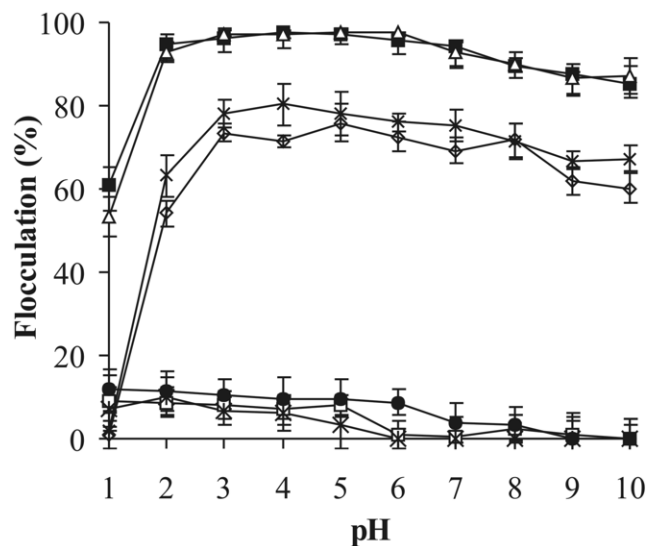
Figure 3.5 The effect of pH on flocculation of FY23 wild type (□); FY23-F1A (■); FY23-F1H (△); FY23-F5A (×); FY23-F5H (◇); FY23-F11A (●); FY23-F11H strains (✳). Yeast strains were grown for 48 h in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Flocculation was determined using a modified Helm's assay as described by D'Hautcourt and Smart (1999) that incorporated a composite suspension buffer with a very wide buffering range from Stratford (1996). Each point reflects the mean of experiments performed in triplicate and error bars represent standard deviations.

A

B
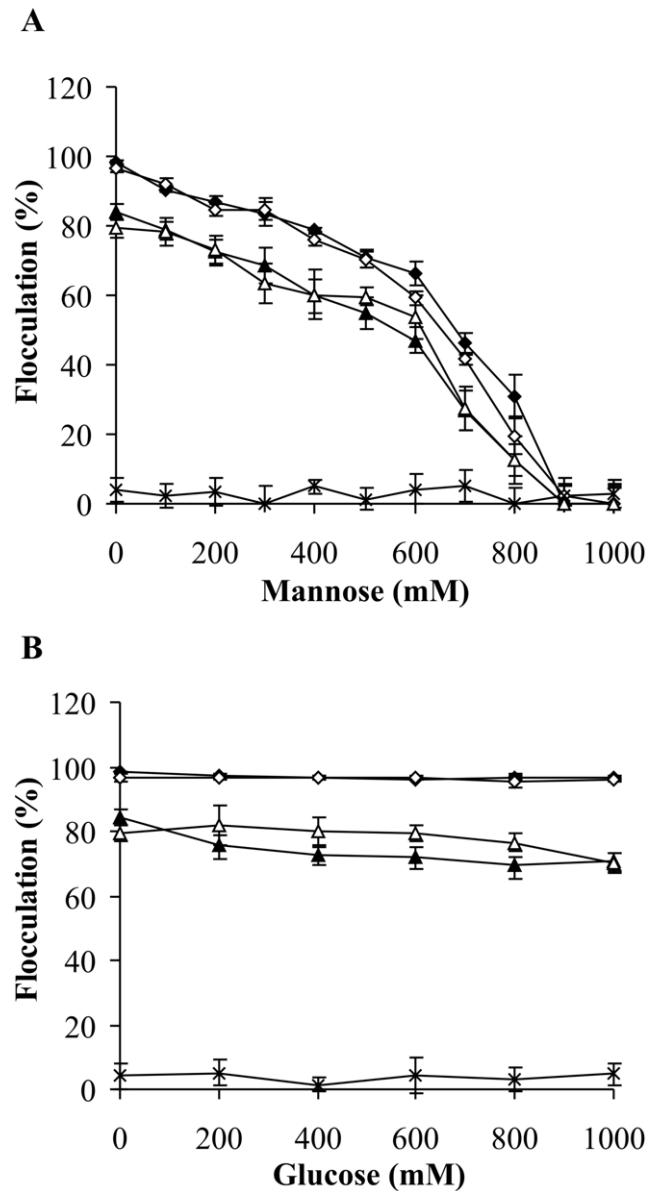
Figure 3.6   The effect of mannose (A) and glucose (B) on flocculation of FY23 wild type (×); FY23-F1A (◆); FY23-F1H (◇); FY23-F5A (▲) and FY23-F5H (△) strains. Yeast strains were grown for 48 h in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Flocculation was determined using a modified Helm's assay as described in the materials and methods. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

3.4.3 Quantitative real-time PCR analysis. In order to verify whether the *ADH2* or *HSP30* mediated *FLO* gene expression is similar to the reported expression patterns of these two promoters, total RNA of FY23, FY23-F11A and FY23-F11H cultures was processed from different growth phases after 12 (exponential), 16 (entry/early stationary) and 48 h (late stationary), and quantitative real-time PCR was performed. It is clearly evident (Fig. 3.7) that both *ADH2* and *HSP30* are tightly repressed in the presence of glucose at 12h. Entry into stationary phase shows strong induction. RNA levels, while slightly decreased in the late stationary phase, are maintained at high

levels. Similar data were observed for the *FLO1* and *FLO5* constructs (data not shown). These transcription levels are well aligned with the onset of flocculation and adhesion phenotypes in all strains (Fig. 3.2 and 3.3). Moreover, the data confirm that only the *FLO* gene carrying a modified promoter is activated, and that the two other genes that were monitored, do not contribute to the observed phenotypes.
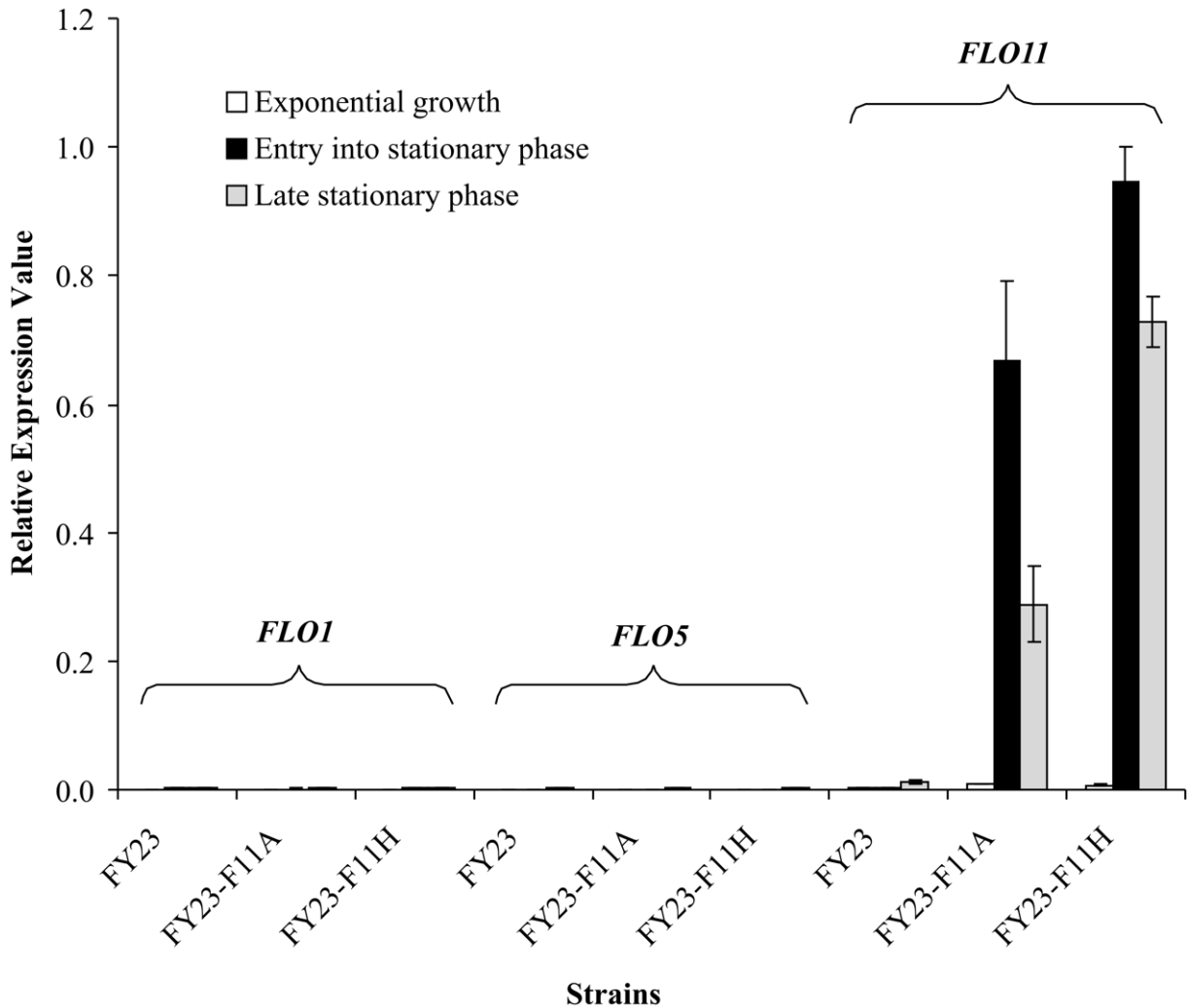
Figure 3.7   QRT-PCR relative expression of *FLO1*, *FLO5* and *FLO11* transcripts in FY23 wild type, FY23-F11A and FY23-F11H strains. Samples were taken from sampling points corresponding to exponential growth phase (white), entry into stationary growth phase (black) and upon completion of fermentation (grey). As indicated, a bracket denotes the expression of a particular *FLO* gene. The relative expression value for each sample was defined as $2^{-Ct}_{(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data was normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample thus giving normalized relative expression for a target gene as $2^{-Ct}_{(target)}/2^{-Ct}_{(PDA1)}$. The highest mRNA expression level was arbitrarily set to 1.0. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

**3.4.4 Heat shock and/or ethanol stress induction of flocculation in FY23-F1H and FY23-F5H strains.** Both heat shock treatment and exposure to ethanol were reported as suitable induction conditions for the *HSP30* promoter (Piper *et al.*, 1994; Seymour and Piper, 1999). Thus it was probable that flocculent phenotypes conferred on transformed strains FY23-F1H and FY23-F5H under transcriptional regulation of *HSP30p* could be triggered when desired in response to these stress conditions. To assess these possibilities only non-flocculent exponential growing cell populations of these strains (determined from Fig. 3.2 and 3.3) were subjected to heat shock treatments and/or exposure to differing ethanol concentrations. The results clearly indicate that heat shock treatment for 45 min at 42°C elicited the strongest induction of flocculation in both FY23-F1H (94%) and FY23-F5H (65%) strains (Fig. 3.8). On the other hand, exposure to 3% (vol/vol) ethanol induced flocculation to a lesser extent in both FY23-F1H (70%) and FY23-F5H (28%) transformants. Both strains displayed similar flocculent abilities (approximately 10%) when exposed to 6% (vol/vol) ethanol while no induction was evident for an ethanol/heat shock combination treatment.
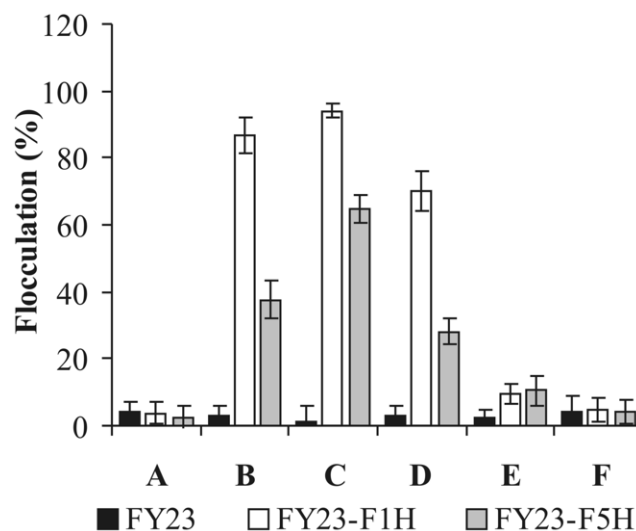
Figure 3.8   Stress-induced expression of *FLO1* and *FLO5* encoded flocculins in *HSP30* transformants. Yeast strains cultivated for 10 h in YEPD, were subjected to the following treatments: A – untreated (45 minutes at 30°C); B - heat shock for 30 minutes at 42°C; C - heat shock for 45 minutes at 42°C; D - 3% (vol/vol) ethanol for 30 minutes at 30°C; E - 6% (vol/vol) ethanol for 30 minutes at 30°C and F - 6% (vol/vol) ethanol and heat shock for 30 minutes at 42°C. The results are averages of three independent determinations and error bars represent standard deviations.

3.4.5 Flor formation and invasive growth. As shown in Fig. 3.9A, only transgenic yeast FY23-F11A and FY23-F11H strains formed a biofilm after 5 days in flor medium at 30°C under static conditions. The FY23-F11A strain produced a distinctly thicker biofilm (Fig. 3.9A) and displayed 3-fold higher suspended cell densities when evaluated 60 h post inoculation (Fig. 3.9B).
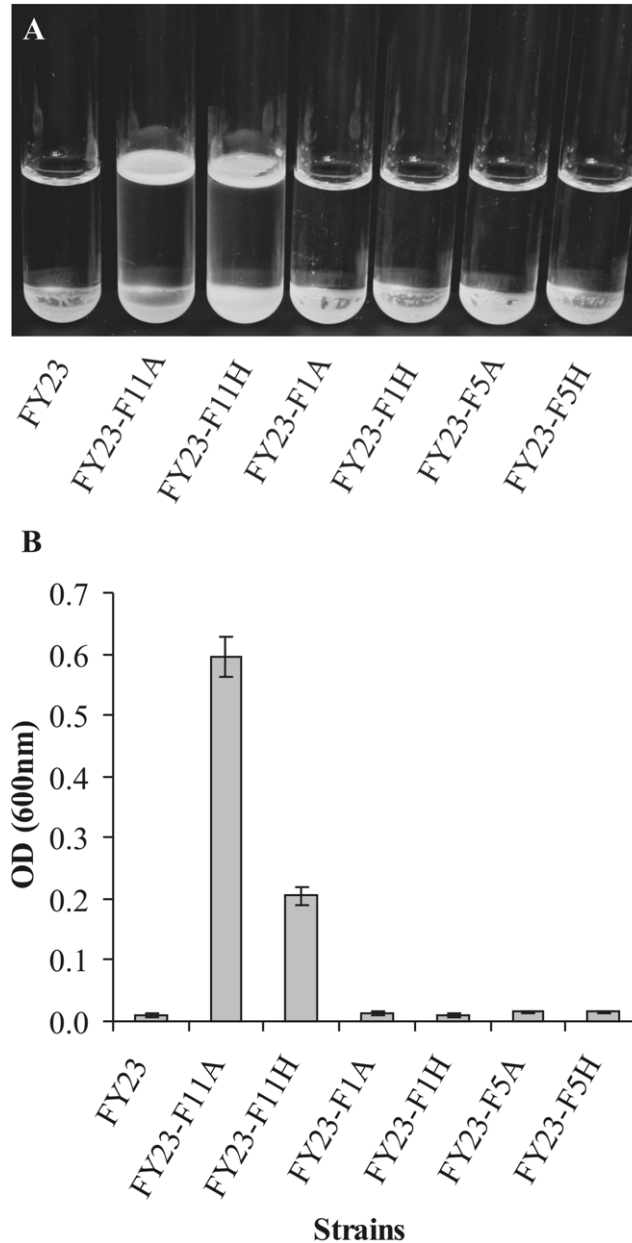
Figure 3.9   Biofilm formation (A). Cells were pre-cultured in YEPD broth and recovered by centrifugation, washed once with flor medium and resuspended at a density of 6×10^7 cells mL^{-1} in 5 mL flor medium contained in 16 x 165 mm glass test tubes. The tubes were photographed after 5 days of static incubation at 30°C. For buoyant cell density determinations (B), the cultures were incubated statically at 30°C for 60 h, after which 1 mL samples were withdrawn from just below the meniscus. The optical density of samples was determined spectrophotometrically at 600 nm. The results are averages of three independent determinations and error bars represent standard deviations.

The ability of the wild-type FY23 strains and its six transformants to invade agar is shown in Fig. 3.10. Only *ADH2* promoted *FLO11* expression resulted in an invasive growth phenotype in SCLD and SCLD$_{LUT}$ agar media. Moreover, the FY23-F11A strain grew as a larger sized colony on SCLD agar and it displayed more aggressive invasive growth behaviour when compared to growth and invasiveness on SCLD$_{LUT}$ plates.
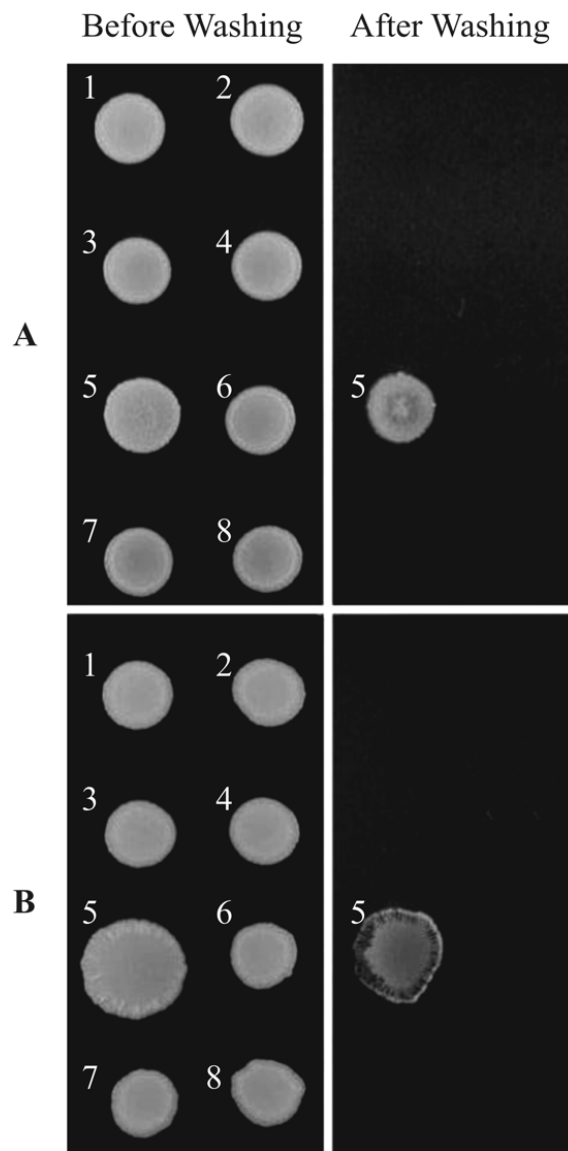
Figure 3.10   Haploid invasive growth of FY23-F5A (1); FY23-F5H (2); FY23-F1A (3); FY23-F1H (4); FY23-F11A (5); FY23-F11H (6) and FY23 wild type (7, 8) strains after 5 days growth at 30°C on SCLD$_{LUT}$ (A) and SCLD (B) media.

3.4.6 Effect of *FLO* gene expression on cell surface hydrophobicity. The hydrophobicity of yeast cell surfaces (Fig. 3.11) from yeast populations grown in YEPD for 48 h was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent. The order of enhancement in terms of the modified hydrophobic index (MHI) is FY23-F11A (0.83) > FY23-F11H (0.79) > FY23-F1A (0.64) > FY23-F1H (0.61) > FY23-F5A (0.29) > FY23-F5H (0.26) > FY23 wild type (0). Thus, it may be concluded that insertion of *FLO* gene encoded glycoproteins Flo1p, Flo5p and Flo11p into the yeast cell wall is responsible for increased cell surface hydrophobicity (CSH).
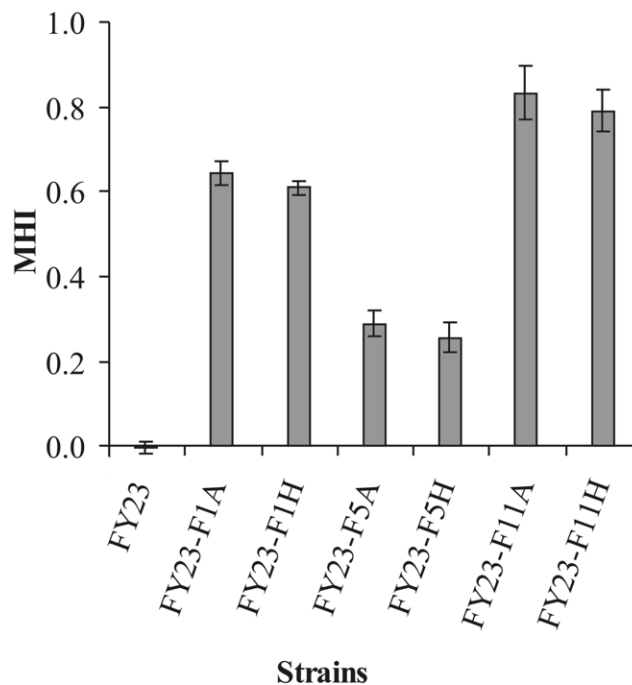
Figure 3.11   The impact of *ADH2* and *HSP30* expression of *FLO* genes on cell surface hydrophobicity (CSH). The wild type FY23 strain and transformants were cultivated for 48 h in YEPD containing 2% glucose at 30°C with shaking (160 rpm). The modified hydrophobic index (MHI) was determined according to the biphasic-solvent partition assay described by Hinchcliffe (1985). The results are averages of three independent determinations, and error bars represent standard deviations.

3.5   DISCUSSION

This is the first report that uses genome-integrated promoter-ORF combinations to compare the impact of various flocculation gene and promoter combinations on cell-surface properties and cell-surface dependent phenotypes. The data show that integration confers stable (both in timing and intensity) expression properties to the targeted genes, and demonstrate the possibility of adjusting flocculation and flor forming behaviour to specific industrial requirements. Importantly, all the engineered yeast strains displayed vegetative growth and fermentation properties that are comparable to that of the host strain, indicating that those industrially relevant characteristics were not compromised by modified *FLO*-gene expression.

In this study, the genomic *FLO1*, *FLO5* and *FLO11* open reading frames were brought under the transcriptional control of promoters of *ADH2* and *HSP30* genes by replacement of their native promoter sequences. The distinct advantage of the cloning strategy employed here over those used by other research groups (Chambers *et al.*, 2004; Cunha *et al.*, 2006) is that no sub-cloning of the *FLO* genes is required. Furthermore, expression levels are independent of plasmid-related artifacts such as variable copy-numbers and the increased risk of intragenic recombinations. Indeed, *FLO* genes contain intragenic tandem repetitive sequences that have been previously reported as difficult to clone or even as "unclonable" sequences (Teunissen *et al.*, 1993). Our data therefore provide reliable baseline information regarding the intrinsic ability of the three Flo genes to induce adhesion-related phenotypes.

The data show that *FLO1*-based constructs induce flocculation most efficiently, while still leading to significant flocculation; *FLO5*-based constructs are less efficient. *FLO11*-based constructs on the other hand induce flocculation only weakly. These constructs however strongly induced flor formation and cell adhesion, phenotypes that were not observed with *FLO1* or *FLO5*. Strains expressing *FLO11* also presented the highest cell surface hydrophobicity. Hydrophobicity was significantly lower in strains expressing *FLO5*, while *FLO1* expressing strains presented intermediate hydrophobicity levels. These data suggest that hydrophobicity *per se* is not a major determinant of adhesion-related phenotypes, but that the specific sequences of the Flo-genes are mainly responsible for phenotype specificity.

The observed flocculation patterns were in all cases consistent with the reported and the measured expression patterns conferred by the two promoters. In the case of *HSP30p-FLO1* and *HSP30p-FLO5* transformants, the onset of flocculation occurred towards the end of the respiro-fermentative exponential growth phase, and was concomitant with the depletion of glucose from the medium. This is consistent with a previous study which showed in particular that the levels of *HSP30* mRNA increased before glucose exhaustion and climaxes with glucose exhaustion (Regnacq and

Boucherie, 1993). The study also confirms the stress inducible nature of *HSP30p* controlled expression of *FLO1* and *FLO5* genes to yield flocculent phenotypes in response to specific stress conditions that include heat shock or exposure to ethanol. Although an ethanol concentration of 6% (vol/vol) is recommended for maximal induction of *HSP30p*, it is possible that this concentration brings about a toxic effect in the laboratory strains, which could be responsible for the absence of flocculation in these cells (Claro *et al.*, 2007; Piper *et al.*, 1994).

Other groups have previously engineered the expression of individual *FLO* genes. The *FLO1* gene was constitutively expressed, thereby creating transgenic yeast strains that exhibited a constitutive flocculation property irrespective of the growth phase (Barney *et al.*, 1990; Ishida-Fujii *et al.*, 1998; Watari *et al.*, 1991; 1994). The possibility of incomplete attenuation associated with constitutively flocculating yeast may lead to sluggish or stuck fermentations and as such these transgenic yeast strains are not ideally suited for industrial batch-wise fermentation processes. Cunha and coworkers (2006) reported controlled expression of the *FLO5* gene by employing a modified *ADH2* promoter. However, the native core promoter and ORF sequences of the *FLO5* gene used by Cunha *et al*. (2006) was sourced from the YEp-*FLO5* plasmid. This plasmid was originally created by Bidard and coworkers (1994) and was reported to contain the *FLO5* gene from the S. *cerevisiae* 17-13D strain. However, later studies by this research team retracted and confirmed that the *FLO5* gene used in the initial study was in fact identical to the *FLO1* gene sequence (Bidard *et al.*, 1995). We therefore assume that Cunha *et al.* (2006) used the *FLO1* gene in their studies. This implies that our research study is the first to report inducible promoter-controlled *FLO5* and *FLO11* gene expression.

Cunha *et al.* (2006) employed a multicopy plasmid-based strategy fusing the poly-T, *UAS1* and *UAS2* regions of the *ADH2* promoter upstream of the native core promoter and ORF of the *FLO1* gene for expression in the laboratory yeast strain W303-1a. The same modified promoter was also employed to control *FLO1* gene expression by cloning an integrative cassette to disrupt the *CAN1* gene in a commercial baking yeast strain (Fleischmann). Similarly to our study, the strains were reported to flocculate after glucose exhaustion in nutrient rich medium (Cunha *et al.*, 2006). However, when using the native *ADH2* promoter, the onset of flocculation observed for *ADH2p-FLO1* and *ADH2p-FLO5* transformants in our study is in line with data published by Lee and DaSilva (2005) who reported a similar native *ADH2* promoter-mediated expression pattern for β-galactosidase in *S. cerevisiae* transformed with a chromosomally integrated *ADH2p-lacZ* cassette. Moreover, the native *ADH2* promoter on multicopy plasmids was shown to drive β-xylanase production only after glucose exhaustion (Kealey *et al.*, 1998; Luttig *et al.*, 1997), clearly suggesting that modification of the native *ADH2* promoter as suggested by Cunha *et al.* (2006) is not necessary.

Chambers *et al.* (2004) employed the glucose-repressible *S. cerevisiae JEN1* promoter to regulate *FLO1* gene mediated flocculation. However, the FY23-F1A and FY23-F5A strains reported here display a much later onset of flocculation in comparison to their *JEN1-FLO1* transgenic *S. cerevisiae* strain W303. These observations are clearly significant as an early onset of flocculation might lead to a 'stuck' or 'hanging' fermentation because of insufficient contact between settled yeast cells and the medium. Some authors have reported non-detectable to significant decreases in ethanol production when converting non-flocculent yeast strains into flocculent strains (Cunha *et al.*, 2006; Ishida-Fujii *et al.*, 1998; Verstrepen *et al.*, 2001; Watari *et al.*, 1990; 1991; 1994). Although decreased ethanol production will not meet the requirements of the bioethanol production, it may be attractive to the alcoholic beverage industries that are currently faced with a growing consumer demand for lower alcohol beers and wines (Heux *et al.*, 2006; Nevoigt *et al.*, 2002).

The decreased flocculation abilities observed for all strains in chemically defined minimal media may be attributed to starvation for auxotrophically required nutrients as recent studies by Pronk (2002) recommend increased supplementation of auxotrophic nutrients in comparison to those used in this study as prescribed by Sherman (1991). Lee and DaSilva (2005) reported 10-fold lower β-galactosidase activities for transgenic *S. cerevisiae* strains expressing *lacZ* under transcriptional control of the *ADH2* promoter when grown in minimal media containing 2% glucose (wt/vol) which further supports these findings. Comparison of the relative promoter strengths of *ADH2p* and *HSP30p* for *FLO* gene expression in minimal media seems to suggest an increased nutritional demand for assimilable nitrogen by *ADH2p*. Although this study shows that *ADH2p* is responsible for later induction of flocculation and stronger flocculent phenotypes in nutrient rich medium than *HSP30p*, it is most probable that *ADH2p* controlled flocculation may not be suitable for certain industrial batch fermentation processes such as winemaking due to grape musts being sometimes deficient in assimilable nitrogen compounds (Henschke and Jiranek, 1993).

Although no observable adhesion phenotype was evident for the *FLO11* transformants used in this study, Bayly *et al.* (2005) presented evidence that *FLO11* encoded flocculin yielded a strongly flocculent Flo1 phenotype in untransformed *S. cerevisiae* strain YIY345. However, it was also reported that *FLO11* overexpression in *S. cerevisiae* strain Σ1278b promotes very weak calcium-independent flocculation, while overexpression in *S. cerevisiae* strain S288C does not promote cell to cell adhesion (Guo *et al.*, 2000; Verstrepen and Klis, 2006). It is possible that flocculent ability of FY23-F11A and FY23-F11H strains may be too weak to be assessed by the modified Helm's assay employed in this study.

As mentioned previously, a nonsense mutation in the *FLO8* gene ensures that the dominant *FLO* genes are transcriptionally silent in the *S. cerevisiae* FY23 strain employed in this study (Liu *et al.*, 1996; Verstrepen *et al.*, 2005; Winston *et al.*, 1995). Thus it is possible to eliminate contributions by other dominant *FLO* genes and exclusively assess the phenotypic consequences of *FLO11* expression. Therefore it may be concluded that *ADH2* and *HSP30* facilitated *FLO11* expression is sufficiently responsible for flor formation. This finding is further supported by earlier reports that identified *FLO11* as a primary factor for flor formation in other *S. cerevisiae* strains types (Ishigami *et al.*, 2004; 2006; Zara *et al.*, 2005).

It has been proposed that flor wine yeast only begin to form flor via a *FLO11* mediated mechanism when glucose repression of *FLO11* transcription is eliminated due to depletion of grape sugar after alcoholic fermentation (Ishigami *et al.*, 2004). Based on the findings of this study, it can be suggested that the *ADH2* or *HSP30* promoter can be utilised to induce flor formation in non-flor wine yeast in a manner that will mimic natural flor wine yeast. The FY23-11A strain displayed decreased invasive growth in minimal agar that contained only auxotrophic nutritional requirements in comparison to complete nutrient supplementation further supports the previously mentioned notion that the *ADH2* promoter displays an increased demand for assimilable nitrogen. Surprisingly, no invasive growth phenotype was associated with the FY23-F11H strain. This suggests that growth on solid media is not ideal for induction of the *HSP30* promoter.

This study highlights that specific adhesion properties appear primarily defined by the properties of specific flocculins, and not by general cell wall properties such as hydrophobicity. Each *FLO* gene leads to specific phenotypes and phenotype intensities, with *FLO1* and *FLO5* resulting in cell aggregation and flocculation, whereas *FLO11* expression leads to invasive growth and flor formation. Clearly the timing and intensities of the phenotypes are entirely dependent on the transcriptional regulation of each individual *FLO* gene.

The data clearly demonstrate that the flocculation behaviour of industrial yeast can be fine-tuned to optimise specific production processes. The modified yeast strains used in this study contain only yeast-derived DNA sequences and can be regarded as self-cloned strains. Such modified strains are generally recognized as safe (GRAS) and may be approved more readily for industrial exploitation (Verstrepen *et al.*, 2003). The bioengineering of *S. cerevisiae* strains capable of controlled flocculation reported in this study may also benefit downstream processing in the pharmaceutical and nutraceutical industries which employ *S. cerevisiae* in batch-wise fermentations for the biosynthesis of high-value natural products such as isoprenoids, flavanoids and long chain polyunsaturated fatty acids. We are currently investigating the impact of the same constructs in industrial wine yeast strains.

3.6   ACKNOWLEDGEMENTS

3.6   REFERENCES

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Chapter 4

RESEARCH RESULTS II

Controlled expression of the dominant flocculation genes *FLO1*, *FLO5* and *FLO11* in industrial wine yeast strains

Controlled expression of the dominant flocculation genes *FLO1*, *FLO5* and *FLO11* in industrial wine yeast strains

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4.1　ABSTRACT

Most commercial wine yeast strains are non-flocculent, probably because this trait was counter-selected to avoid fermentation problems. Controlled flocculation phenotypes could, however, provide significant benefits in many industrial processes. In laboratory strains, it has previously been demonstrated that it is possible to adjust flocculation and adhesion phenotypes to desired specifications by changing the expression of dominant flocculation (*FLO*) genes. However, the *FLO* gene family is characterized by high allele heterogeneity and is subjected to epigenetic regulation. Extrapolation of data obtained in laboratory strains to industrial strains is therefore not always feasible. In this paper, we assess the impact of the controlled expression of the chromosomal copies of the *FLO1*, *FLO5* and *FLO11* open reading frames on the flocculation and adhesion properties of two non-flocculent and popular commercial wine yeast strains, BM45 and VIN13. To this end, the chromosomal promoters of the dominant flocculation genes *FLO1*, *FLO5* and *FLO11* were replaced by the stationary phase-inducible promoters of the *HSP30* and *ADH2* genes. The data show that the strategy resulted in the expected and stable expression patterns of these genes in both strains. The native promoters of the *FLO1* gene in the wine yeast strains showed significant sequence differences from the native promoter in the laboratory strain, which may in part explain the diminished expression of this gene in the two commercial strains. The twelve transgenic wine yeast strains displayed specific adhesion behaviour in terms of timing and intensity. However, the specific impact of the expression of individual dominant *FLO* genes with regard to characteristics such as flocculation efficiency, cell wall hydrophobicity, biofilm formation and substrate adhesion properties showed significant differences between strains and when compared to the corresponding phenotypes observed in laboratory strains. In particular, *FLO5*-dependent flocculation was significantly stronger than *FLO1*-dependent flocculation in the wine yeast strains. The data suggest that the optimization of the flocculation pattern of individual commercial strains may have to be based on a strain-by-strain approach.

4.2   INTRODUCTION

Due to its ability to efficiently ferment the hexoses glucose, fructose and maltose from natural raw materials such as rice, wheat, barley, corn and grape juice, the yeast *Saccharomyces cerevisiae* has traditionally been employed in many food production processes, most prominently in the production of alcoholic beverages and in the baking industry. More recently, the relative ease and availability of genetic tools has resulted in *S. cerevisiae* increasingly being utilized as a cell factory for the production of various enzymes or metabolites such as insulin (Kjeldsen, 2000), L-lactic acid (Saitoh *et al.*, 2005) and others (Kealey *et al.*, 1998; Maury *et al.*, 2005).

In many of these industrial processes, the suspended yeast cells have to be removed prior to further processing of the product. In the case of wine fermentation, such removal processes may involve filtration or other clarification strategies. These processes can be costly and can result in reduced quality of the final product. For this reason, wine makers would prefer to reduce or, if possible, entirely eliminate the need for such interventions.

A strategy to achieve a satisfying level of clarification could be the optimization of a particular phenotypic trait of yeast strains that is referred to as flocculation. Yeast flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989). Flocculation could therefore allow the convenient separation of cells from the fermentation product. However, flocculation must not occur before alcoholic fermentation has been completed since flocculent strains do not ferment efficiently and early flocculation may thus result in sluggish or stuck fermentation. Ideally, flocculation should therefore occur as close as possible after the end of the alcoholic fermentation process.

Flocculation in *S. cerevisiae* is mediated by specific cell surface lectins (adhesins or flocculins) that are capable of binding directly to mannose residues of mannan molecules on adjacent cells (Miki *et al.*, 1982; Stratford, 1989). This interaction leads to cellular aggregation and finally settling. In some specific cases, cellular aggregation does not lead to settling, but to yeast cells rising to the surface of the substrate and forming an air-liquid interfacial biofilm. This behavior is also referred to as flotation or flor formation (Pretorius, 2000; Zara *et al.*, 2005). In *S. cerevisiae,* two distinct flocculation phenotypes have been characterized on the basis of their sensitivity to sugar inhibition, namely Flo1 (mannose sensitive) and NewFlo (mannose and glucose sensitive) (Stratford and Assinder, 1991). Most brewer's yeast strains are of the NewFlo

phenotype and flocculation in these strains is initiated after the end of exponential respiro-fermentative phase of growth (Sampermans *et al.*, 2005). The late onset of flocculation in the NewFlo phenotype makes them ideally suited to their task by aiding separation of biomass from the brew.

The genetic basis of flocculation has been the object of several investigations. These studies suggest that a family of subtelomeric genes, *FLO1, FLO5, FLO9* and *FLO10*, encode specific lectins that are responsible for flocculation (Teunissen and Steensma, 1995). A non-subtelomeric gene, *FLO11/MUC1* (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996), on the other hand, encodes a protein that has been associated with flor formation, invasive growth and substrate adhesion (Bayly *et al.*, 2005; Guo *et al.*, 2000; Ishigami *et al.*, 2004; Lo and Dranginis, 1996; Zara *et al.*, 2005). All Flo proteins are glycosyl-phosphatidylinositol (GPI)-linked glycoproteins that share a common three domain structure consisting of an N-terminal lectin-like domain, a central domain of highly repeated sequences rich in serine and threonine residues, frequently arrayed as tandem repeats, and a carboxyl terminal domain containing a GPI anchoring sequence (reviewed in Verstrepen and Klis, 2006). The intragenic repeats present in the central domain mediate frequent recombination events that lead to high allele heterogeneity of these genes and were shown to be responsible for quantitative alterations in their flocculation phenotypes (Liu *et al.*, 2007b; Verstrepen *et al.*, 2005). The evidence also suggests that the difference between the newFlo and Flo1 flocculation phenotypes may at least be partially due to variations in the number of repeat sequences within the *FLO1* coding sequence (Liu *et al.*, 2007a; 2007b).

In previous attempts to modify flocculation behaviour of laboratory yeast strains, the dominant *FLO1* gene from donor *S. cerevisiae* strains was employed to convert non-flocculent yeast strains into flocculent strains (Chambers *et al.*, 2004; Cunha *et al.*, 2006; Wang *et al.*, 2008; Watari *et al.*, 1991; 1994). On the other hand, Govender *et al.* (2008) and Verstrepen *et al.* (2001) used a promoter replacement strategy. In this strategy, the native chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, of the haploid non-flocculent, non-invasive and non-flor forming *S. cerevisiae* FY23 strain was brought under the transcriptional control of stably integrated inducible promoters (*ADH2p* or *HSP30p*) thereby generating strains with specific flocculation and adhesion behaviours. The data showed that this strategy allowed stable and controlled expression of these genes and imparted stationary phase-specific flocculation behaviour. A distinct advantage of the promoter replacement strategy over those used by other research groups (Chambers *et al.*, 2004; Cunha *et al.*, 2006; Wang *et al.*, 2008; Watari *et al.*, 1991; 1994) is that no sub-cloning of the *FLO*

genes is required. Indeed, the intragenic tandem repetitive sequences have previously been reported as difficult to clone or even as "unclonable" sequences (Teunissen *et al.*, 1993; Verstrepen and Thevelein, 2004). In addition, the modified yeast strains contain only yeast-derived DNA sequences and can be regarded as self-cloned strains, and such strains may be approved more readily for industrial exploitation (Verstrepen and Thevelein, 2004).

The data generated with the laboratory yeast S288C by Govender *et al.* (2008) therefore suggested the suitability of the promoter-*FLO*-gene constructs to induce desirable phenotypes. However, wine yeast strains are known to be significantly different from the standard laboratory strain. The aim of the present study was therefore to compare the phenotypes observed in the laboratory strain with those generated in recombinant wine yeast strains. To this end, the natural chromosomal promoters of the *FLO1*, *FLO5* and *FLO11* flocculation genes were replaced by the stationary phase-inducible promoters of the *HSP30* and *ADH2* genes. This exercise has provided us with a unique opportunity to study phenotypes that are associated with native *FLO1*, *FLO5* and *FLO11* genes in wine yeast strains that are otherwise silent, and to compare such data with those obtained for the laboratory strain that was previously published.

The data presented here confirm that inducible expression of the native *FLO1* and *FLO5* open reading frames, albeit to varying degrees, are responsible for a quantifiable cell-cell adhesion phenotype that can be characterized as a Flo1 flocculation phenotype. However, in contrast to our findings with transgenic laboratory yeast, the data clearly indicate that in the conditions used here, *ADH2p*-controlled *FLO1* and *FLO5* phenotypes of transgenic wine yeast strains are distinctly more flocculent than comparable *HSP30p* regulated phenotypes. Furthermore, it was also clearly evident that *FLO1* and *FLO5* transgenic laboratory yeast strains were markedly more flocculent than the corresponding wine yeast strains. Interestingly, in these strains, *FLO5*-based constructs induce flocculation more efficiently than *FLO1*-based constructs, irrespective of the promoter involved. On the other hand, we found that inducible expression of native *FLO11* ORF in wine yeasts under these conditions resulted in variable flor/biofilm formation and invasive growth phenotypes.

4.3   MATERIALS AND METHODS

4.3.1 Strains. All yeast strains used in this study are listed in Table 4.1. *Escherichia coli* DH5α (Gibco BRL/Life Technologies, Rockville, MD) was used as a host for all plasmid amplifications.

Table 4.1 *S. cerevisiae* strains employed in this study

Strain	Genotype	Reference
BM45	Industrial wine yeast strain (unknown genotype)	Lallemand Inc., Montreal, Canada
BM45-F1A	*FLO1p::SMR1-ADH2p*	This study
BM45-F1H	*FLO1p::SMR1-HSP30p*	This study
BM45-F5A	*FLO5p::SMR1-ADH2p*	This study
BM45-F5H	*FLO5p::SMR1-HSP30p*	This study
BM45-F11A	*FLO11p::SMR1-ADH2p*	This study
BM45-F11H	*FLO11p::SMR1-HSP30p*	This study
EC1118	Industrial wine yeast strain (unknown genotype)	Lallemand Inc., Montreal, Canada
FY23	*MAT*a *leu2 trp1 ura3 flo8-1*	(Winston *et al.*, 1995)
FY23-F1A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO1p::SMR1-ADH2p*	(Govender *et al.*, 2008)
FY23-F1H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO1p::SMR1-HSP30p*	(Govender *et al.*, 2008)
FY23-F5A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO5p::SMR1-ADH2p*	(Govender *et al.*, 2008)
FY23-F5H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO5p::SMR1-HSP30p*	(Govender *et al.*, 2008)
FY23-F11A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO11p::SMR1-ADH2p*	(Govender *et al.*, 2008)
FY23-F11H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO11p::SMR1-HSP30p*	(Govender *et al.*, 2008)
NT50	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, Cape Town, South Africa
VIN13	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, Cape Town, South Africa
VIN13-F1A	*FLO1p::SMR1-ADH2p*	This study
VIN13-F1H	*FLO1p::SMR1-HSP30p*	This study
VIN13-F5A	*FLO5p::SMR1-ADH2p*	This study
VIN13-F5H	*FLO5p::SMR1-HSP30p*	This study
VIN13-F11A	*FLO11p::SMR1-ADH2p*	This study
VIN13-F11H	*FLO11p::SMR1-HSP30p*	This study
WE372	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, Cape Town, South Africa

4.3.2 Media and culture conditions. Yeast strains were routinely cultivated at 30°C in rich YEPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose. Single yeast colonies from 3 day old YEPD plates were used to inoculate starter cultures in 40 mL YEPD broth in 250 mL Erlenmeyer flasks, which were incubated at 30°C with shaking (160 rpm) for 16 h. These precultures were used to inoculate a second preculture at an initial cell density of 5×10^5 cells mL^{-1}, which were incubated at 30°C with shaking (160 rpm) for 16 h. Thereafter, yeast cells for inoculation of experimental cultures were routinely prepared as follows using ice-cold reagents: Yeast from precultures were harvested by centrifugation (4000 rpm, 5 min), washed once with 100 mM EDTA, pH 7 to ensure deflocculation; once with 30 mM EDTA, pH 7 and finally resuspended in 30 mM EDTA, pH 7. To determine the onset of flocculation, flocculent ability, glucose utilisation and growth rate of yeast in nutrient rich medium, experimental cultures were seeded at an initial cell density of 5×10^5 cells mL^{-1} into 40 mL YEPD contained in 250 mL Erlenmeyer flasks, and incubated at 30°C with shaking (160 rpm). At 2 h intervals, for a period of 24 h and at a 48 h time point, cell populations were harvested and deflocculated as described previously. In addition, the flocculation potential of *FLO11* wine yeast transformants was assessed in chemically defined synthetic complete (SC) media containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI, USA); 3% (vol/vol) ethanol (SCE) and 3% (vol/vol) ethanol with 3% (vol/vol) glycerol (SCGE) as non-fermentable carbon sources. Invasive growth was assessed in chemically defined synthetic complete (SC) media containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids and 0.2% (wt/vol) glucose (SCLD). Flor medium containing 0.67% YNB without amino acids and 3% (vol/vol) ethanol adjusted to pH 3.5 (Ishigami *et al.*, 2004) was used to assess flor formation. For selection of sulphometuron methyl (SM) resistant BM45 and VIN13 transformants, SC medium containing 0.67% YNB and 2% (wt/vol) glucose was supplemented with 280 and 300 μg mL^{-1} SM (DuPont Agricultural Products, France) respectively. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium [1% (wt/vol) Bacto tryptone, 0.5% (wt/vol) yeast extract and 1% (wt/vol) NaCl] and bacterial transformants were selected using LB medium containing 100 mg L^{-1} ampicillin (Sigma-Aldrich, Missouri, USA). In this study, 2% (wt/vol) agar (Difco) was used for all solid media. Bacterial and yeast strains were stored in LB containing 40% (vol/vol) glycerol and YEPD supplemented with 15% (vol/vol) glycerol respectively (Ausubel *et al.*, 1995).

4.3.3 DNA manipulation and construction of promoter-replacement cassettes. To ensure high fidelity amplification, *Pyrobest*™ DNA Polymerase PCR system (Takara Bio Inc., Otsu, Japan) was employed in all amplification reactions in which the amplicon was to be used as a DNA template in a subsequent PCR amplification or as a vector cassette for yeast transformation. All other routine PCR reactions, were performed

using *Takara Ex Taq*™ PCR system (Takara Bio Inc., Otsu, Japan). All primers employed in this study are listed in Table 4.2. Procedures for bacterial transformations and plasmid isolation from *E. coli* were performed as described by Sambrook *et al.* (1989). Standard procedures for isolation and manipulation of DNA were employed in all other aspects of this study (Ausubel *et al.*, 1995). The *FLO5* and *FLO11* promoter replacement cassettes containing either the *ADH2p* or *HSP30p* and bearing extensive 5' and 3' *FLOp* homologous tail regions (ranging from 437 to 672 bp), were amplified by PCR (Fig. 4.1) from the previously reported FY23 transgenic yeast strains (Govender *et al.*, 2008). The *FLO5p-SMR1-ADH2-FLO5p* (4690 bp), *FLO5p-SMR1-HSP30-FLO5p* (5169 bp), *FLO11p-SMR1-ADH2-FLO11p* (4940 bp) and *FLO11p-SMR1-HSP30-FLO11p* (5419 bp) cassettes were amplified using genomic DNA that was isolated from FY23-F5A, FY23-F5H, FY23-F11A and FY23-F11H yeast strains, respectively, as templates. The primer pair employed for the above *FLO5* cassettes was FLO5-F2 and FLO5-R2, whilst the FLO11-F2 and FLO11-R2 primer set was used in the preparation of the above *FLO11* cassettes. A similar procedure used for *FLO1* did not result in targeted integration.

The sequence of the *FLO1* promoter region (spanning -1290 to -818 nucleotides) in FY23 strains was compared to that of BM45 and VIN13 wine yeast strains by using any one of the following four forward primers: FLO1-F; FLO1-F2; FLO1-F3 or FLO1-F4 in combination with the FLO1-R reverse primer. The amplicons obtained with the FLO1-F and FLO1-R primer pair (Fig. 4.2) from BM45 and VIN wild type strains were isolated from 1% (w/v) agarose gels; column purified (Roche Diagnostics GmbH, Mannheim, Germany) cloned into pGEM®-T Easy vector (Promega Corporation, Madison, USA) and sequenced. A consensus sequence consisting of 287 nucleotides was located in the *FLO1p* region of BM45 and VIN13 wild type strains and deposited in GenBank (BM45, accession no. FJ238617 and VIN13, accession no. FJ238616). A 124 nucleotide sequence from this consensus sequence (Fig. 4.3) was used in the design of the BVFLO1::SMR1-F primer. The partial promoter replacement cassettes corresponding to *SMR1-ADH2-FLO1p* (3733 bp) and *SMR1-HSP30-FLO11p* (4218 bp) cassettes were amplified using genomic DNA that was isolated from the FY23-F1A and FY23-F1H yeast strains, respectively, as templates (Fig. 4.3). The SMR1-F and FLO1-R2 primer pair was used in the preparation of the aforesaid cassettes. The *FLO1p-SMR1-ADH2-FLO1p* (3857 bp) and *FLO1p-SMR1-HSP30-FLO1p* (4324 bp) promoter replacement vectors were amplified using the corresponding partial cassette as template DNA and the BVFLO1::SMR1-F and FLO1-R2 primer pair. Promoter replacement cassettes were isolated from 1% (w/v) agarose gels and column purified.

4.3.4 Wine yeast transformations and strain verification. Yeast transformation with freshly prepared electro-competent cells was performed with 10 μg of DNA according to the electroporation protocol described by Ausubel and coworkers (1995). The remaining freshly prepared electro-competent cells were cryopreserved according to the method described by Suga and co-authors (2000) and employed in subsequent transformations. Electroporation of yeast was performed with a Bio-Rad MicroPulser™ (Bio-Rad Laboratories, CA, USA) using the instrument's pre-programmed setting for *S. cerevisiae* (Sc2) and HiMax electroporation cuvettes (Cell Projects Ltd., Kent, UK) with a 0.2 cm electrode gap. To limit the carry-over of untransformed cells, single colonies of putative transformants following initial selection on SC plates containing SM, were inoculated individually onto fresh SC plates containing SM and cultivated at 30°C for 3 days. The deletion of native promoters was confirmed by PCR using homologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants as template.

The primer pairs for BM45 and VIN13 transgenic derivatives were: F1A and F1H (FLO1-F and FLO1-R2); F5A and F5H (FLO5-F3 and FLO5-R2); F11A and F11H (FLO11-F3 and FLO11-R2). In addition, the integration of promoter replacement cassettes in transformed yeast was further confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants as template. The primer pairs for BM45 and VIN13 transgenic strains were: F1A, FLO1-F and ADH2-R; F1H, FLO1-F and HSP30-R; F5A, FLO5-F3 and ADH2-R; F5H, FLO5-F3 and HSP30-R; F11A, FLO11-F3 and ADH2-R; and F11H, FLO11-F3 and HSP30-R. The wild type BM45 and VIN13 strains served as a control in the above confirmation experiments. To verify the integrity of the *ADH2p* and *HSP30p* elements driving *FLO* gene expression, the integrated promoter elements were amplified from transgenic yeast strains using heterologous primer sets: F1A and F1H (SMR1-F2 and FLO1-R2); F5A and F5H (SMR1-F2 and FLO5-R); F11A and F11H (SMR1-F2 and FLO11-R). Amplicons corresponding to the promoter elements were recovered from 1% (w/v) agarose gels, column purified, cloned into pGEM®-T Easy vector and sequenced.

The parental lineage of BM45 and VIN13 transgenic yeast strains was evaluated using primers (δ-F and δ-R) that are specific for delta (δ) sequences as described by Ness and coworkers (1993). The BM45, EC1118, NT50, VIN13 and WE372 industrial wine yeast wild type strains served as controls in these experiments. Intragenic repetitive domain polymorphism located within *FLO* genes of wine yeast was evaluated using primers sets designed by Verstrepen *et al.* (2005) i.e. *FLO1* (FLO1-reps-F and FLO1-reps-R); *FLO5* (FLO5-reps-F and FLO5-reps-R) and *FLO11* (FLO11-reps-F and FLO11-reps-R).

Table 4.2 Oligonucleotide primers used in this study

Primer name[a]	Primer sequence (5'→3')
δ-F	CAAAATTCACCTATWTCTCA
δ-R	GTGGATTTTTATTCCAACA
ADH2-R	TGATAGTTGATTGTATGCTTTTTGTAGC
*BVFLO1-F	GCTTCGCAGACGAATGTTTTCCG
*BVFLO1::SMR1-F	<u>GCTTCGCAGACGAATGTTTTCCGACACATGATACTTATCACCG</u>
	<u>AAAAACCTTATTTCACGGAAAAAACCTTATTTACATTAAAGTTTG</u>
	<u>AAAAATTTTCTTCTTTTCCGCAATATGGTGGGGCCT</u>GGCTTGG
	CTTCAGTTGCTGATCTCG
FLO1-F	AAGTGTGCGTCACTTTTCCTACGGT
FLO1-F2	ATGGCACTAGTCGATCGAGG
FLO1-F3	AGTGTTATGCTAGCCAGTTTCAGG
FLO1-F4	GCACATGCCAATTGTGTGCATAGC
FLO1-R	AGCGATGAGGCATTGTCATTT
FLO1-R2	GTGCCAGAAGTGTAAAGACTGC
FLO1-reps-F	CTAAGTCAATCTAACTGTACTGTCCCTGA
FLO1-reps-R	GATAGAGCTGGTGATTGTCCTGAA
FLO5-F2	GGTTGTGTTCTAGGACTTTCTGACG
FLO5-F3	ACTCAATTTGGACACTCGGTTCGG
FLO5-R	AGTGGTGCTAATCAATTTAAAGAA
FLO5-R2	TGTGCACAACATTCGAACGC
FLO5-reps-F	AAGGGTACGTTTACTCTTTTGACGATGACC
FLO5-reps-R	ACTGAAGAAGAAATTACTGAGGAGGAAATC
FLO11-F2	TTACGGCCTAATGTCGAGAC
FLO11-F3	GCTGCTTGTCTCACATCTAAACTTCG
FLO11-R	GGACCAAATAAGCGAGTAGA
FLO11-R2	CTGGGAAATCCGTTTGAGAG
FLO11-reps-F	TAGTGCCGCTCAATATGCAAGCTCCTGGCA
FLO11-reps-R	TGTTTGACTGCCAGGGTATTTGGATGATGA
HSP30-R	TATTAAAGTCTCAAACTTGTTGTTTTG
SMR1-F	GGCTTGGCTTCAGTTGCTGATCTCG
SMR1-F2	GTGTTTTAACTCGTCGGACACG

[a] F, forward primer and R, reverse primer. Non-underlined sequences correspond to *ADH2*, *HSP30, and SMR1-410* or *FLO* gene sequences as denoted by the primer name. *Corresponds to a homologous *FLO1* promoter region sequenced from BM45 and VIN13 industrial wine strains.

4.3.5 Enumeration of yeast populations. The cell density of suitably diluted yeast suspensions in 30 mM EDTA (pH 7) was determined either by direct cell counting with a hemocytometer or by measuring absorbance at 600 nm in a Cary 50 UV-Vis spectrophotometer (Varian Inc., CA, USA) using a standard curve as reference.

4.3.6 Stability of the integrated promoter replacement constructs. Single yeast colonies representing selected transformed strains from 3 day old YEPD plates were used to inoculate 20 mL YEPD broth contained in 100 mL Erlenmeyer flasks, which were incubated at 30°C with shaking (160 rpm) for 24 h. The cultures were then deflocculated with the addition of 50 µL sterile 400mM EDTA (pH 7). These deflocculated cultures were employed to inoculate a fresh batch of 20 mL YEPD broth contained in 100 mL Erlenmeyer flasks at an initial cell density of 5×10^5 cells mL^{-1}, which were incubated at 30°C with shaking (160 rpm) for 24 h. This batch culturing process was repeated for more than 100 generations. Final cultures were suitably diluted and spread on YEPD plates and incubated at 30°C for 48 h. Subsequently 50 colonies of each transformed strain were assessed for their resistance to SM, flocculation ability (*FLO1* and *FLO5* constructs), increased invasiveness (*ADH2p-FLO11* transformants) or lack of invasiveness (*HSP30p-FLO11* transformants) in SCLD plates. In this context, it is important to emphasize that both BM45 and VIN13 wild type strains displayed an innate capacity to invade agar when cultured on SCLD plates, whilst *HSP30p-FLO11* transformants lost this capacity. Biofilm formation by *FLO11* transformants was evaluated using flor medium.

4.3.7 Glucose determination. Cells were pelleted from 1 mL samples of YEPD cultures by microcentrifugation (10000 rpm, 1 min). The cell-free extracts were filtered through a 0.22 µm cellulose acetate filter and stored at −20°C. The concentration of glucose in the culture medium was determined using a GAGO-20 glucose assay kit (Sigma, Missouri, USA) according to the specifications of the manufacturer, using a BIOTEK 800ELX microplate reader (BIOTEK Instruments Inc., Winooski, VT, USA).

4.3.8 Flocculation assays. The ability of yeast strains to flocculate was established using the modified Helm's assay as described by D'Hautcourt and Smart (1999). Five replicates of the control and test reactions were performed for each sample. The percentage flocculation reported in this paper reflects the mean of three independent determinations. To investigate sugar inhibition of *FLO1* and *FLO5* flocculation phenotypes, either 1 M mannose or 1 M glucose was added at varying concentrations to both the washing and suspension buffers that are employed in the modified Helm's assay (1999).

4.3.9 Determination of hydrophobicity of yeast cell surfaces. The hydrophobicity of yeast cell surfaces was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent (Hinchcliffe *et al.*, 1985). Cultures in YEPD were incubated at 30°C for 48 h with shaking (160 rpm). The harvested cells from an experimental culture were deflocculated, washed and diluted to a density of 5×10^6 cells mL^{-1} in 30 mM EDTA (pH 7). Yeast cells from a 20 mL aliquot of this suspension were washed twice and resuspended in 20 mL of phosphate-urea-magnesium (PUM) buffer pH 7.1 (Hinchcliffe *et al.*, 1985). The absorbance of this suspension (*I*) was determined at 660 nm. Aliquots of 2.4 mL (three replicates) were dispensed into borosilicate glass tubes (15 × 75 mm) and 200 μL xylene was layered over the yeast suspension. Tubes were rubber-capped; samples were vortexed at maximum speed for 30 s and allowed to stand undisturbed for 15 min. The absorbance of the residual buffer layer (*F*) at 660 nm was determined. The average modified hydrophobic index (MHI) for a sample was calculated using the equation MHI = 1 – (*F*/*I*)

4.3.10 Analysis of stress-induced expression of flocculins. Strains were precultured and treated as described earlier. Experimental cultures were inoculated at an initial density of 5×10^5 cells mL^{-1} into 40 mL YEPD, and incubated at 30°C with shaking (160 rpm) for 10 h. The incubation of untreated cells was extended for another 45 min at 30°C whereas other cultures were exposed to the following stress treatments: heat shock for 30 min at 42°C; heat shock for 45 min at 42°C; 3% (vol/vol) ethanol for 30 min at 30°C; 6% (vol/vol) ethanol for 30 min at 30°C. Ethanol (100%) was added directly to culture medium to yield final concentrations of 3 or 6% (vol/vol) ethanol. All cultures were placed on ice before flocculation was determined using the modified Helm's assay.

4.3.11 Invasive growth plate assays. Yeast cultures, processed as previously described were adjusted to an optical density (measured at a wavelength of 600 nm) of 1.0 and 10 μL aliquots were dropped onto SCLD plates without piercing the agar surface and incubated for 5 days at 30°C (van Dyk *et al.*, 2005). Using a gloved finger, superficial growth of yeast colonies was physically removed by washing plates under a steady stream of water. Plates were allowed to air dry and cells that invaded the agar were photographed.

4.3.12 Flor formation. Cells were pre-cultured in YEPD, deflocculated and washed as described previously. Subsequently, 3×10^8 cells were recovered by microcentrifugation (10000 rpm, 1 min), washed once and resuspended in 1 mL flor medium (pH 3.5) and added to test tubes (16 x 165 mm) containing 4 mL flor medium. Biofilm formation was photographed in natural light after 5 days of static incubation at 30°C.

4.3.13 Statistical Analysis. In this study, paired t tests were employed to statistically compare data obtained for BM45 and VIN13 wild type strains to that of transgenic yeast strains. Paired t tests were performed using GraphPad InStat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego California)

4.4   RESULTS

4.4.1 Wine yeast transformation. Employing the transformation strategy presented in Fig. 4.1 using the homology regions derived from the S288C genetic background, SM resistant colonies were obtained for *FLO5* and *FLO11* transformations. However, no SM resistant colonies were obtained with the *FLO1* homologous DNA fragments. Putative SM-resistant transformants of *FLO5* and *FLO11* were inoculated individually into YEPD broth and cultivated for 48 h at 30°C with shaking (160 rpm). A majority (58 to 79%) of putative BM45 and VIN13 transgenic strains transformed with the combinations *FLO5p-SMR1-ADH2p-FLO5p* and *FLO5p-SMR1-HSP30p-FLO5p* visually displayed strong flocculent phenotypes in these conditions. Putative BM45-F11A, BM45-F11H, VIN13-F11A and VIN13-F11H transformants displayed no detectable flocculent phenotype. A visual assessment of both increased invasiveness (*ADH2p-FLO11* transformants) on SCLD plates and biofilm formation on flor medium was therefore used as an initial screen of putative *FLO11* transformants. This screening method identified putative *FLO11* transgenic BM45 and VIN13, with more than half of the sulfometuron methyl-resistant strains displaying flor-forming behaviours in all cases.

After 3 unsuccessful attempts at transforming BM45 and VIN13 wine yeast with the original *FLO1* promoter replacement constructs, the FY23-derived 527 bp *FLO1* promoter region (from nucleotide -1290 to -764) that had been targeted for homologous integration in the wine yeast strains was assessed through a series of nested PCRs. Only the first forward primer, which matched the 3' end of the target region, yielded a PCR fragment of expected size (aprox. 837 bp) from the FY23, BM45 and VIN13 wild type strains (Fig. 4.2). FY23 sequence derived primers situated further upstream did not yield PCR products in the wine yeast strains, suggesting significant sequence divergence between FY23 and the commercial strains. To overcome this obstacle, the 5' end of several independently obtained 837 bp amplicons from BM45 and VIN13 and corresponding to the region that appears preserved between FY23 and the commercial strains were sequenced (Fig. 4.2 and 4.3). BM45 and VIN13 derived sequences were identical for 287 bps, but significantly different (92% identity) from the sequence derived from FY23 (Fig. 4.3). These data are somewhat surprising when considering that,

although no or little information regarding the genetic background of BM45 and VIN13 exist, the two strains display very different phenotypical characteristics and were not expected to be closely related to each other. Furthermore, wine yeast strains are reportedly highly heterozygous. The molecular data presented here, however, suggest that both strains carry two very similar alleles of the *FLO1* gene.
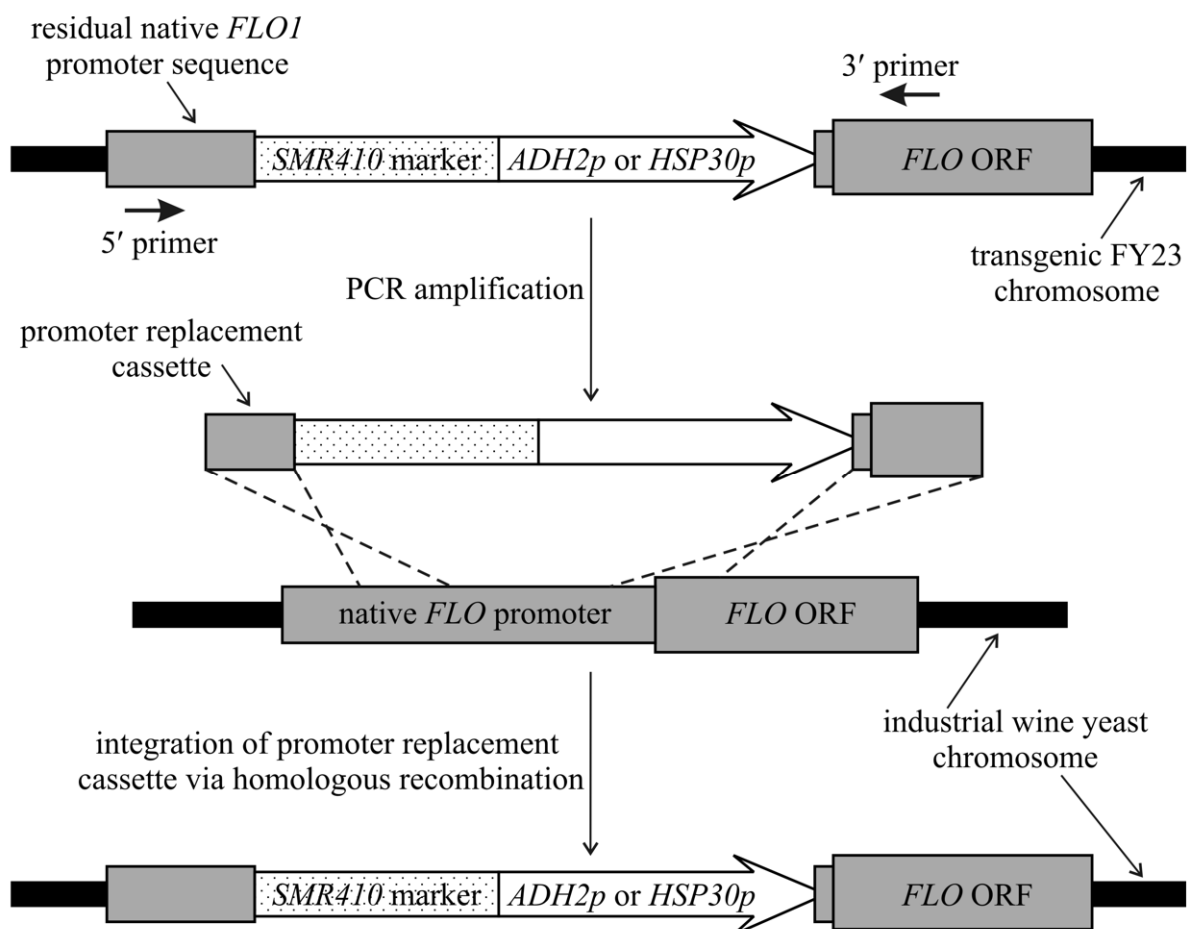
Figure 4.1   Promoter transplacement strategies demonstrating chromosomal integration of either *ADH2* or *HSP30* promoter upstream of the dominant *FLO5* or *FLO11* flocculation gene in industrial wine *S. cerevisiae* strains BM45 and VIN13. Promoter replacement cassettes bearing 5' and 3' tails (varying from 437 to 672 bp) that are homologous to the promoter regions of *FLO5* or *FLO11* were amplified using genomic DNA that was isolated from previously reported FY23-F5A; FY23-F5H; FY23-F11A or FY23-F11H transgenic yeast strains as template (Govender *et al.*, 2008).
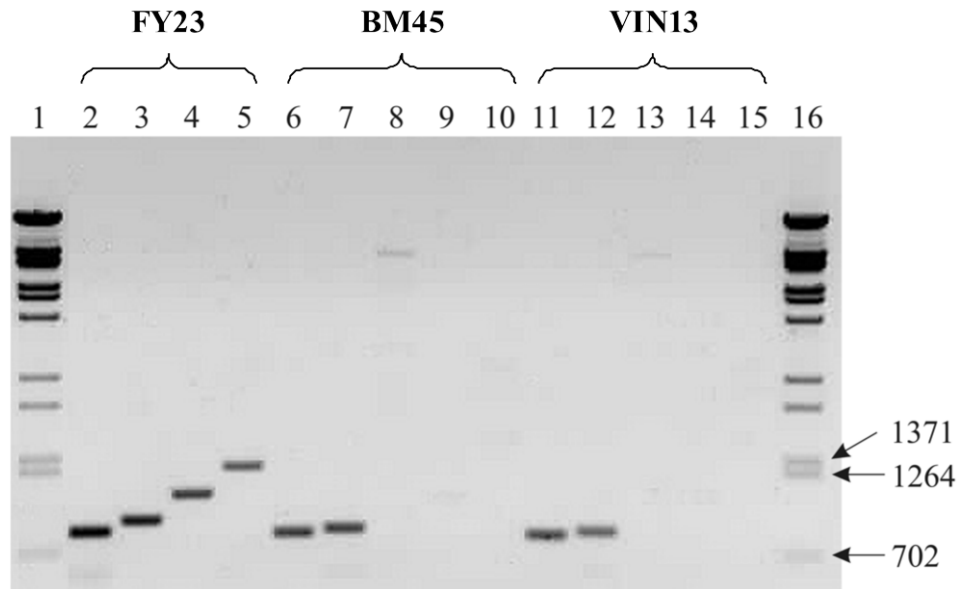
Figure 4.2   *FLO1* promoter comparison. PCR amplification of the *FLO1* promoter regions using different forward primers in combination with a single reverse primer and genomic DNA as template from FY23 [haploid laboratory strain isogenic to S288C (Winston *et al.*, 1995)], BM45 and VIN13 wine yeast strains. Lane 1 and 16, DNA molecular weight marker (bacteriophage λ DNA restricted with *BstE*II). Lane 2, FY23 837 bp amplicon (FLO1-F and FLO1-R primer pair); lane 3, FY23 904 bp amplicon (FLO1-F3 and FLO1-R); lane 4, FY23 1084 bp amplicon (FLO1-F4 and FLO1-R) and lane 5, FY23 1309 bp amplicon (FLO1-F2 and FLO1-R). BM45 amplicons in lanes 7, 8, 9, 10 and VIN13 amplicons in lanes 12, 13, 14, 15 with the preceding primer pairs respectively. Lane 6 and 11, BM45 and VIN13 amplicons (± 808 bp) obtained with the BVFLO1-F and FLO1-R primer pair.

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                      -800
                       ↓
    S288C  :  CTACGGTGCC.TCGCACATGAATGTTATCCGGCGCACGATACTTATCACC
    BM45   :  CTACGGTGGCTTCGCAGACGAATGTTTTCCGACACATGATACTTATCACC
    VIN13  :  CTACGGTGGCTTCGCAGACGAATGTTTTCCGACACATGATACTTATCACC

    S288C  :  GAAAAACCTTATTCTACGG..AAAACCTTATTTACATTAAAGTTGGAAAA
    BM45   :  GAAAAACCTTATTTCACGGAAAAAACCTTATTTACATTAAAGTTTGAAAA
    VIN13  :  GAAAAACCTTATTTCACGGAAAAAACCTTATTTACATTAAAGTTTGAAAA

    S288C  :  A.TTTCCTCTTTTTCCTAATAAGGTGGAGCTTTTGGCTTCCAGTATGCTT
    BM45   :  ATTTTCTTCTTTTTCCGCAATATGGTGGGGCCTTTGACTTCCAGTATGCTT
    VIN13  :  ATTTTCTTCTTTTTCCGCAATATGGTGGGGCCTTTGACTTCCAGTATGCTT

    S288C  :  TCACGGAATTATTTCTCATGTACATTTAGCTCCATTTCCAGTGCCTCCGA
    BM45   :  TCACGGAATTATTTCTGATGTACATTTAGCTCCATTTCCAGTGCCTCCGA
    VIN13  :  TCACGGAATTATTTCTGATGTACATTTAGCTCCATTTCCAGTGCCTCCGA

    S288C  :  TAGGGAGGCATCATGGTACTACCGTGACGGAGAATACGTAGGCTGACTTT
    BM45   :  TAGGAAGGCATCATGGTACTACCGTGACAGAGAATACGTAGGCTGACTTT
    VIN13  :  TAGGAAGGCATCATGGTACTACCGTGACAGAGAATACGTAGGCTGACTTT

    S288C  :  TTCGTCAGTTTGTTGTCCGTTTACAAAATTGGTGAAT ← -518
    BM45   :  TTCGTCAGTTTGTTGTCCGTTTACAAAATTGGTGAAT
    VIN13  :  TTCGTCAGTTTGTTGTCCGTTTACAAAATTGGTGAAT
```

Figure 4.3   Partial sequence alignment of *FLO1* promoter region from *S. cerevisiae* strain S288C to that of BM45 and VIN13 wild type wine yeast strains using Vector NTI Advance 10 software and shading as performed with GeneDoc software (version 2.4.000). Nucleotide numbering was achieved by assigning the A in ATG start codon of the *FLO1* open reading frame of strain S288C as base 1. Nucleotide sequence variations are highlighted in grey. Underlined regions indicates the wine yeast consensus sequence (124 nucleotide) that was used in the design of the hybrid BVFLO1::SMR1-F primer.

The homologues sequence was used to construct the hybrid BVFLO1::SMR1 primer which contains a 124 nucleotide 5' tail that is homologous to the *FLO1p* region of the BM45 and VIN13 wine yeast strains. Employing the transformation strategy presented in Fig. 4.4, single SM-resistant colonies were obtained. These colonies were inoculated individually into YEPD broth and cultivated for 48 h at 30°C with shaking (160 rpm). Only a small proportion (3 to 5%) of these putative BM45 and VIN13 transgenic strains, transformed with the two combinations *FLO1p-SMR1-ADH2p-FLO1p* and *FLO1p-SMR1-HSP30p-FLO1p*, visually displayed flocculent phenotypes. This percentage is significantly lower than what had been observed in the case of *FLO5p* and *FLO11p* transformants, where more than 50% of SM-resistant colonies had shown the expected flocculation or adhesion phenotypes. The 5' (124 bp) and 3' (78 bp) flanking homologous regions of the *FLO1* promoter replacement cassettes is

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significantly shorter than those used in *FLO5* and *FLO11* promoter replacement cassettes (varying from 437 to 672 bp), which may have contributed to the decreased *FLO1p* transplacement efficiencies. This observation is in line with previously published data which suggested that, although strain dependant, an increase in the length of flanking homology sequences can drastically increase the efficiency of DNA fragment transplacement efficiencies (Manthey *et al.*, 2004).
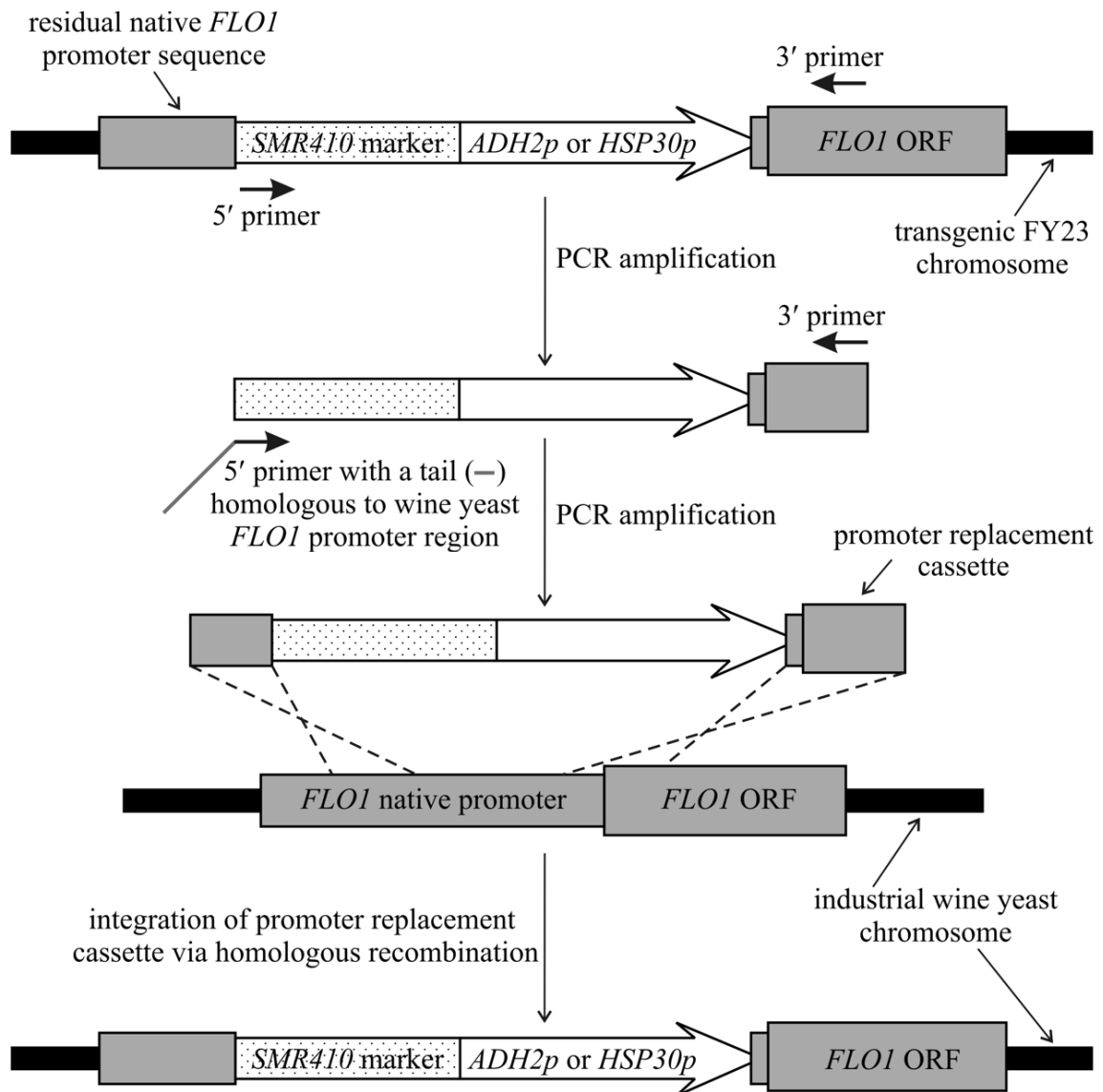
Figure 4.4   Promoter replacement strategy illustrating chromosomal integration of either *ADH2* or *HSP30* promoter upstream of the dominant *FLO1* flocculation gene in industrial wine *S. cerevisiae* strains BM45 and VIN13. Partial promoter replacement cassettes corresponding to *SMR1-ADH2p-FLO1p* and *SMR1-HSP30p-FLO1p* were amplified using genomic DNA obtained from FY23-F1A and FY23-F1H yeast strains that were cloned previously (Govender *et al.*, 2008). Thereafter, complete promoter replacement cassettes containing a 5' tail (124 bp) that is homologous to the *FLO1* promoter region of BM45 and VIN13 wine yeast were amplified by employing partial cassettes as template DNA.

With the aid of the screening systems described above, three independent transformants of each strain were selected for further analysis. For each of the selected strains, the integration of promoter replacement cassettes at specific loci were confirmed by PCR (Fig. 4.5A and 4.5B) using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA as template. Additionally, the deletion of native promoters for at least one allele was confirmed by PCR (Fig. 4.6A and 4.6B) using homologous primer pairs that contained a forward primer from outside the site of integration. As can be seen in Fig. 4.6, we also found a surprisingly significant number of strains were both copies of the *FLO*-gene promoters had been replaced by the new constructs. In order to eliminate any possible variability that may be associated with copy number, all results for transgenic wine yeast strains presented in this study were obtained with single copy integrants.
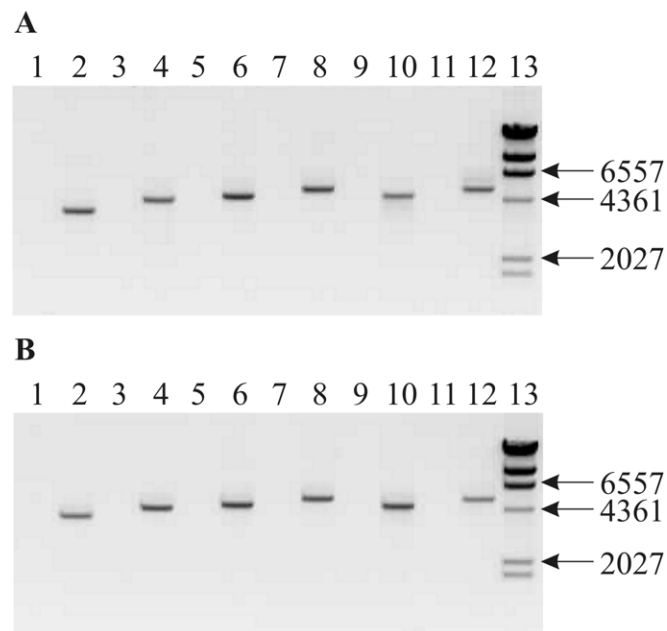
Figure 4.5   The integration of promoter replacement cassettes at specific loci were confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA as template. The primer pairs for BM45 and VIN13 transgenic strains were as follows: F1A, FLO1-F and ADH2-R; F1H, FLO1-F and HSP30-R; F5A, FLO5-F3 and ADH2-R; F5H, FLO5-F3 and HSP30-R; F11A, FLO11-F3 and ADH2-R; and F11H, FLO11-F3 and HSP30-R. (A) Wild type BM45, BM45-F1A, BM45-FIH, BM45-F5A, BM45-F5H, BM45-F11A, BM45-F11H strains and (B) wild type VIN13, VIN13-F1A, VIN13-FIH, VIN13-F5A, VIN13-F5H, VIN13-F11A, VIN13-F11H strains. The amplification of *FLO1p-SMR1-ADH2p* (3717 bp amplicon, F1A, lane 2), *FLO1p-SMR1-HSP30p* (4196 bp amplicon, F1H, lane 4), *FLO5p-SMR1-ADH2p* (4554 bp amplicon, F5A, lane 6), *FLO5p-SMR1-HSP30p* (5033 bp amplicon, F5H, lane 8), *FLO11p-SMR1-ADH2p* (4597 bp amplicon, F11A, lane 10), *FLO11p-SMR1-HSP30p* (5076 bp amplicon, F11H, lane 12) is only evident in transgenic yeast strains, whilst lacking in the BM45 and VIN13 wild type strains with corresponding primer pairs (lanes 1, 3, 5, 7, 9 and 11). Lane 13 contained DNA molecular weight marker (bacteriophage λ DNA restricted with *Hind*III).
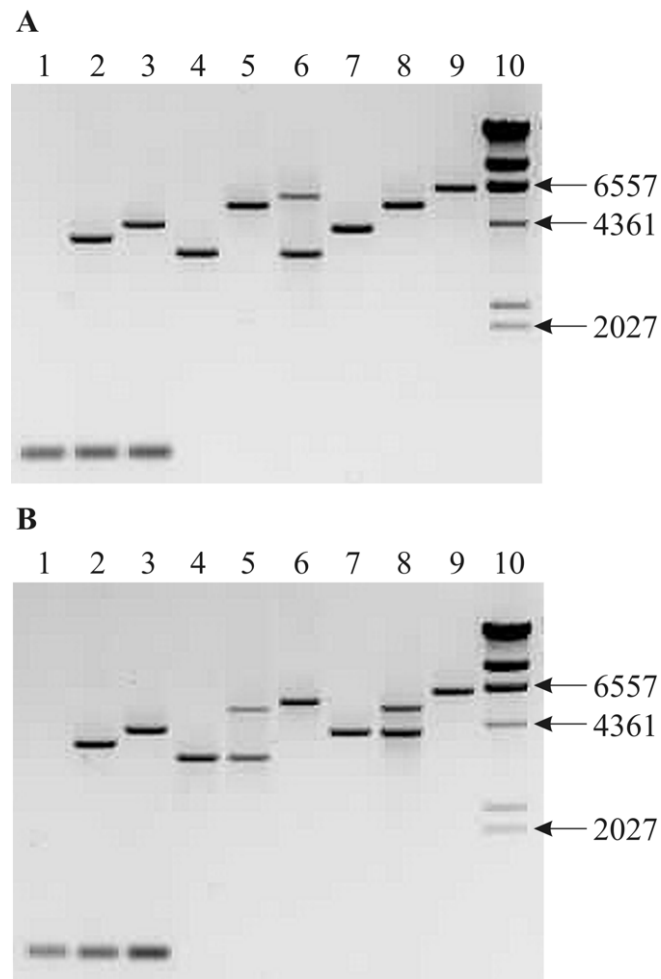
Figure 4.6   The deletion of native promoters in at least one locus was confirmed by PCR using homologous primer pairs that contained a forward primer from outside the region of integration as described in materials and methods. (A) BM45 and its transgenic strains (B) VIN13 and its transgenic strains. The native promoter amplicons of the wild type BM45 and VIN13 strains; *FLO1p* (870 bp, lane 1), *FLO5p* (3380 bp, lane 4) and *FLO11p* (4153 bp, lane 7). The integration cassette was amplified in F1A (*FLO1*p-*SMR1-ADH2-FLO1*p, 3795 bp, lane 2); F1H (*FLO1*p-*SMR1-HSP30-FLO1*p, bp 4280, lane 3); F5A (*FLO5*p-*SMR1-ADH2-FLO5*p, 5145 bp, lane 5); F5H (*FLO5*p-*SMR1-HSP30-FLO5*p, 5624 bp, lane 6); F11A (*FLO11*p-*SMR1-ADH2-FLO11*p, 5203 bp, lane 8) and F11H (*FLO11*p-*SMR1-HSP30-FLO11*p, 5682 bp, lane 9) transgenic yeast strains. Lane 10 contained DNA molecular weight marker (bacteriophage λ DNA restricted with *Hind*III). In order to eliminate any possible variability that may be associated with copy number, all results for transgenic wine yeast strains presented in this study were obtained with single copy integrants.

4.4.2 Transgenic yeast strain-typing and genetic stability. Since industrial strains of *S. cerevisiae* are not easy to differentiate from each other, the identity of all transformants was verified. For this purpose, primers that are specific for delta (δ) sequences as described by Ness *et al.* (1993) were used for all BM45 and VIN13 transgenic yeast strains. All strains were confirmed to be genetic descendants of BM45 and VIN13 wild type wine yeast strains. The stability of the integrated promoter constructs was assessed after repeated batch-culturing in nutrient-rich, non-selective medium for more than 100 generations. Thereafter, all tested colonies displayed resistance to SM, indicating stable integration of the *SMR1* marker gene. Flocculation phenotypes of these strains were also assessed, and 2% of BM45-F1A and 6% of VIN13-FIH colonies showed no visible flocculation. However, all other tested colonies of *FLO1* and *FLO5* transformants displayed the relevant phenotypes. In some instances, slight differences in flocculation intensities were observed. This is in contrast to the data reported for the laboratory strain, were the phenotype proved stable and reproducible over the same number of generations. Since the non-flocculent descendants still contained the modified promoter-ORF construct, the phenotypic differences observed may be attributed to genetic variations in the open reading frames of *FLO1* and *FLO5*.

4.4.3 Growth rates, glucose consumption and flocculation. The growth rate and sugar utilization capabilities BM45 and VIN13 strains and their transgenic descendants were evaluated in YEPD containing 2% glucose at 2-hourly intervals. No significant differences between the wild-type strains and the *ADH2p-FLO5* and *HSP30p-FLO5* transformants regarding biomass growth, cell numbers and sugar utilization capabilities were observed (Fig. 4.7 and 4.8). Similar trends in these parameters were observed for other transgenic strains reported in this study. In both BM45 and VIN13 *ADH2p-FLO5* transformants, an onset of flocculation was observed approximately 2 h after glucose exhaustion, while maximum flocculation potential was evident after an additional 6 h (Fig. 4.7B and 4.8B). Although there is parity with respect to *ADH2p* mediated *FLO5* expression, appreciably different *HSP30p* regulated *FLO5* expression profiles were observed in BM45-F5H and VIN13-F5H transformants. The onset of flocculation in BM45-F5H seemed to coincide with glucose depletion, and maximal flocculent ability was achieved after an additional 4 hours (Fig. 4.7B). However, the commencement of flocculation was considerably delayed in VIN13-F5H and full flocculation potential was only attained after 48 h (Fig. 4.8B).
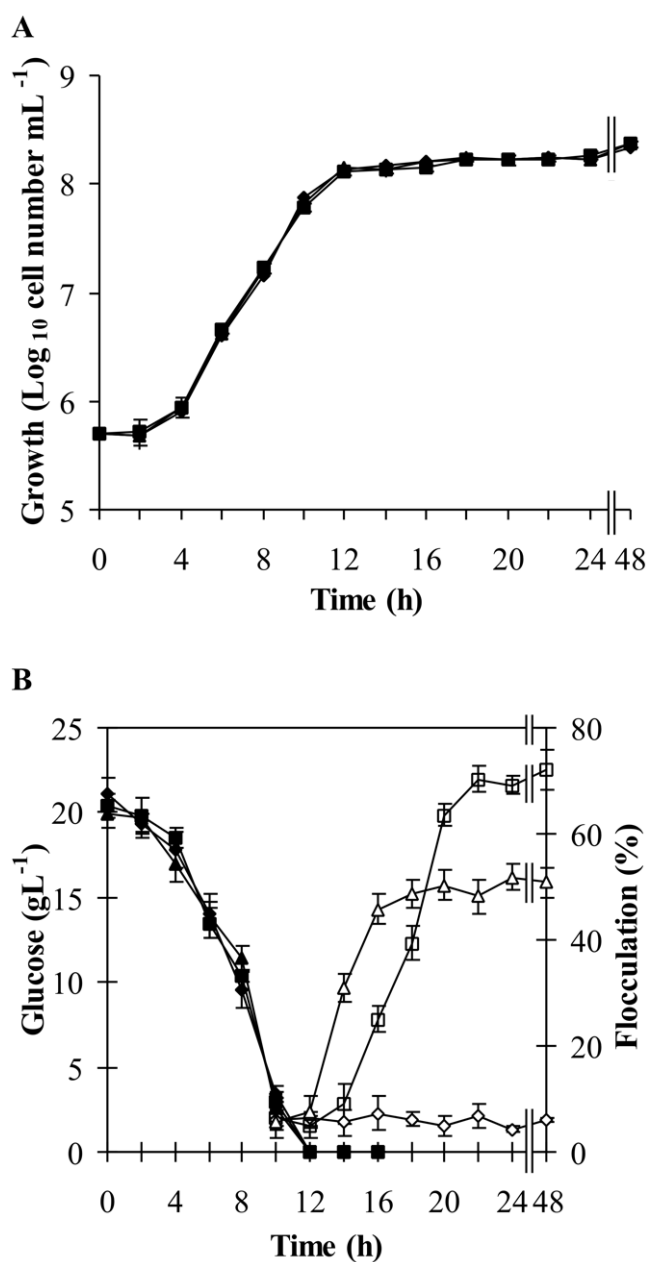
Figure 4.7   (A) Growth of BM45 wild type (◆); BM45-F5A (■) and BM45-F5H (▲) strains. (B) Glucose utilization of BM45 wild type (◆); BM45-F5A (■) and BM45-F5H (▲) strains. Flocculation profile of BM45 wild type (◇); BM45-F5A (□) and BM45-F5H (△) strains. Yeast strains were cultivated in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

A

B
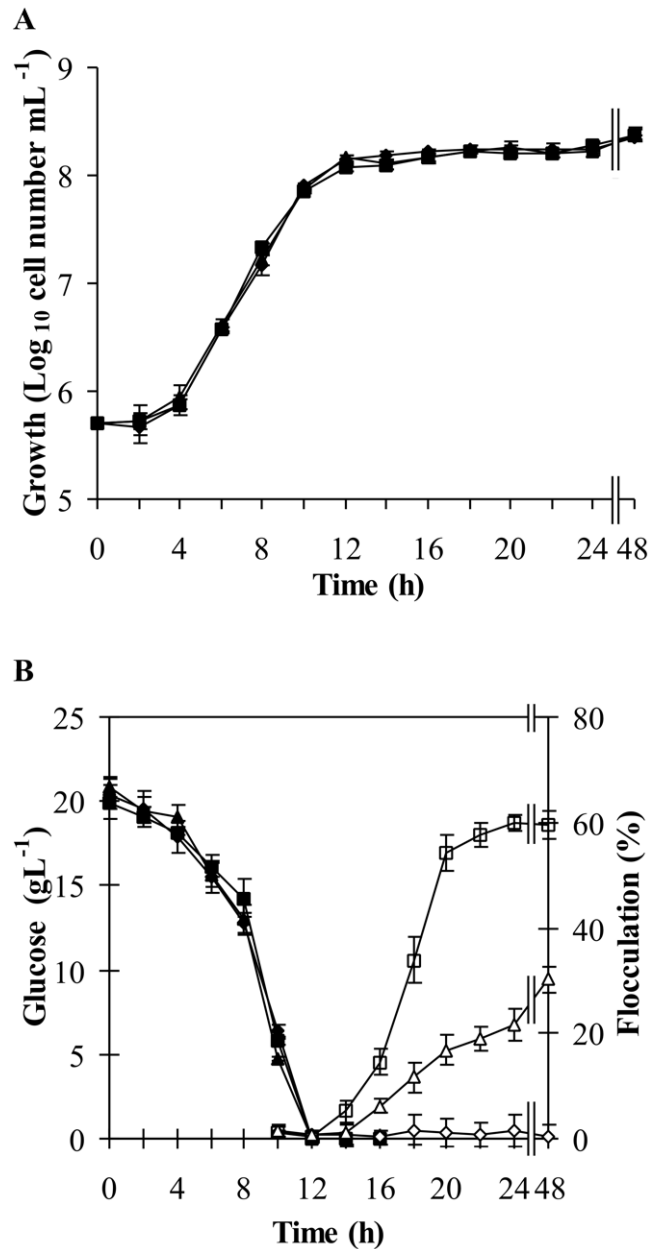
Figure 4.8   (A) Growth of VIN13 wild type (◆); VIN13-F5A (■) and VIN13-F5H (▲) strains. (B) Glucose utilization of VIN13 wild type (◆); VIN13-F5A (■) and VIN13-F5H (▲) strains. Flocculation profile of VIN13 wild type (◇); VIN13-F5A (□) and VIN13-F5H (△) strains. Yeast strains were cultivated in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

After 48 h growth in YEPD containing 2% (wt/vol) glucose, *ADH2p* controlled expression of *FLO5* in BM45 and VIN13 transformants [BM45-F5A (72.1 ± 3.9%), VIN13-F5A (59.4 ± 2.7%)] generated phenotypes that were significantly more flocculent than their *HSP30p* counterparts [BM45-F5H (50.8 ± 2.9%), VIN13-F5H (30.3 ± 2.5%)] (Fig. 4.9). A similar tendency was evident for the two promoters controlling *FLO1* expression in BM45 and VIN13 transformants (Fig. 4.9) that is BM45-F1A (49.4 ± 1.6%)

and VIN13-F1A (39.8 ± 2.8%) versus BM45-F1H (21.0 ± 2.5%) and VIN13-F1H (9.0 ± 1.3%). The above relationships also clearly illustrate that *FLO5* wine yeast transformants are more flocculent than their corresponding *FLO1* transgenic wine yeast strains. *FLO11* expression in both BM45 and VIN13 wine yeast strains, mediated by either the *ADH2* or *HSP30* promoter in nutrient rich YEPD medium (Fig. 4.9) and minimal media including SCE and SCGE with non-fermentable carbon sources (results not shown) did not yield a flocculent phenotype.
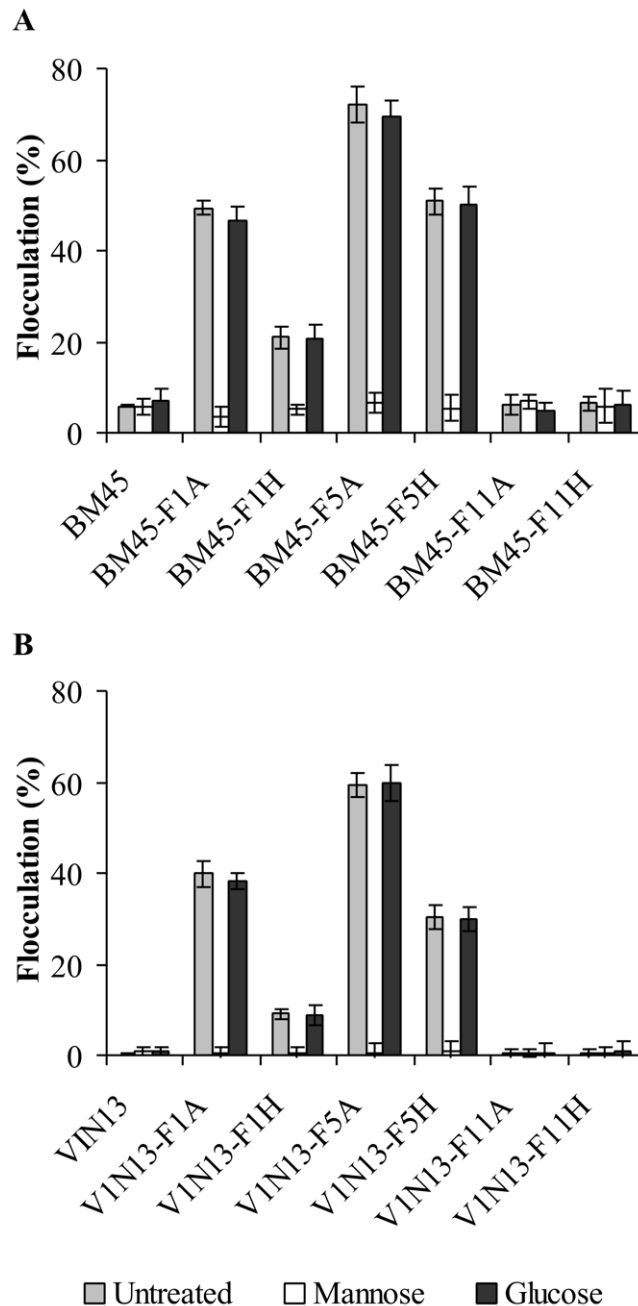
Figure 4.9   The effect of either 1 M mannose or 1 M glucose on flocculation of (A) BM45 and (B) VIN13 wild types and their transgenic strains. Yeast strains were cultivated in YEPD (2% glucose) for 48h at 30°C with shaking (160 rpm). The effect of either mannose or glucose on flocculation was determined using a modified Helm's assay as described in the materials and methods. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

The capacity of either 1 M glucose or 1 M mannose to inhibit flocculent phenotypes of BM45 and VIN13 transgenic yeast strains after 48 h growth in YEPD was evaluated (Fig. 4.9). The flocculent phenotypes displayed by both *FLO1* and *FLO5* wine yeast transformants were completely abolished on exposure to 1 M mannose. On the contrary, no inhibitory effect was evident in the presence of 1 M glucose. Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (Stratford and Assinder, 1991), this result clearly demonstrates that *FLO1* and *FLO5* transgenic wine yeast encoded flocculins exhibit Flo1-type flocculation.

4.4.4 Evaluation of the intragenic repetitive domains of *FLO1*, *FLO5* and *FLO11* from wine yeast. Each of the repetitive regions located within *FLO1*, *FLO5* and *FLO11* from BM45, VIN13, EC1118 and NT50 wild type wine yeast were amplified using PCR and compared to corresponding amplicons obtained from the haploid FY23 laboratory strain that is isogenic to S288C (Fig. 4.10). With the exception of the BM45 *FLO5* repeat region amplicon, all the other wine yeast intragenic repetitive domains displayed decreased lengths when compared to the corresponding amplicons obtained from FY23. No repeat region amplicon was evident for *FLO11* from the BM45 wild type strain. This result is not entirely surprising especially since Fidalgo and coworkers (2006) recently reported the presence of rearrangements within the central tandem repeat domain of the *FLO11* ORF from a flor-forming *S. cerevisiae* wine yeast strain. In addition, they found the *FLO11* coding region contained several point mutations and deletions (Fidalgo *et al.*, 2006). Given the innate flor-forming character of the BM45 wild type strain, it is possible that either one or both *FLO11* repetitive region primers employed in this study lacked specificity. It is also interesting to note in terms of the wild type wine yeast strains used in this study, that VIN13 consistently yielded smaller repeat region amplicons than BM45 for *FL01* and *FLO5*.
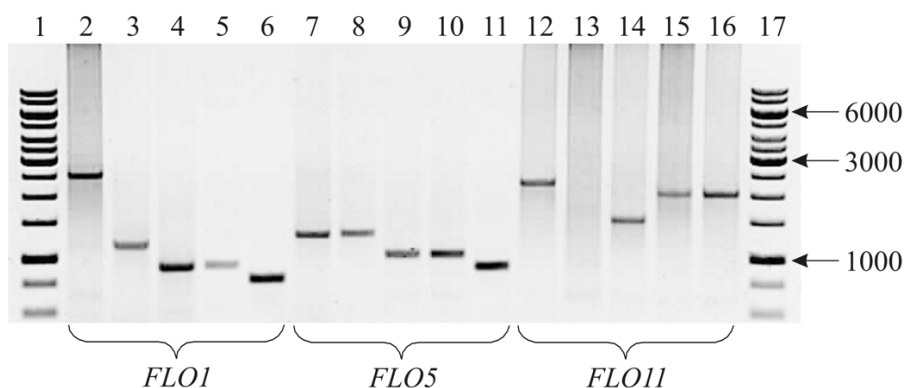
Figure 4.10   Evaluation of *FLO* intragenic repetitive domain polymorphisms using primers designed by Verstrepen and coworkers (2005) in *FLO1*, *FLO5* and *FLO11* ORFs in five wild type yeast strains. As indicated, a bracket denotes amplicons of a particular *FLO* gene. Lanes 2 (2529 bp), 7 (1288 bp) and 12 (2260 bp): FY23; lanes 3, 8 and 13: BM45; lanes 4, 9 and 14: VIN13; lanes 5, 10 and 15: EC1118; lanes 6, 11 and 16: NT50.

4.4.5 Heat shock and/or ethanol stress induction of flocculation in *FLO1* and *FLO5* transgenic wine yeast strains. Both heat shock treatment and exposure to ethanol were reported as suitable induction conditions for the *HSP30* promoter (Piper *et al.*, 1994; Seymour and Piper, 1999). It was thus expected that flocculent phenotypes conferred on transformed strains BM45-F1H, BM45-F5H, VIN13-F1H and VIN13-F5H under transcriptional regulation of *HSP30p* could be triggered when desired in response to these stress conditions. Non-flocculent exponential growing cell populations of these strains were subjected to heat shock treatments and/or exposure to differing ethanol concentrations. The results clearly indicate that an increased exposure to heat shock (45 min at 42°C) and a higher concentration of ethanol [6% (vol/vol)] elicited a strong induction of flocculation in both BM45-F1H and BM45-F5H strains (Fig. 4.11A). In contrast, exposure to both ethanol concentrations [3 and 6% (vol/vol)] induced flocculation to a greater extent than exposure to both heat shock regimes (30 or 45 min at 42°C) in VIN13-F5H transformants (Fig. 4.11B). In comparison to the wild type strain, statistically insignificant responses ($p > 0.05$) were recorded for VIN13-FIH under all stress conditions tested (results not shown).
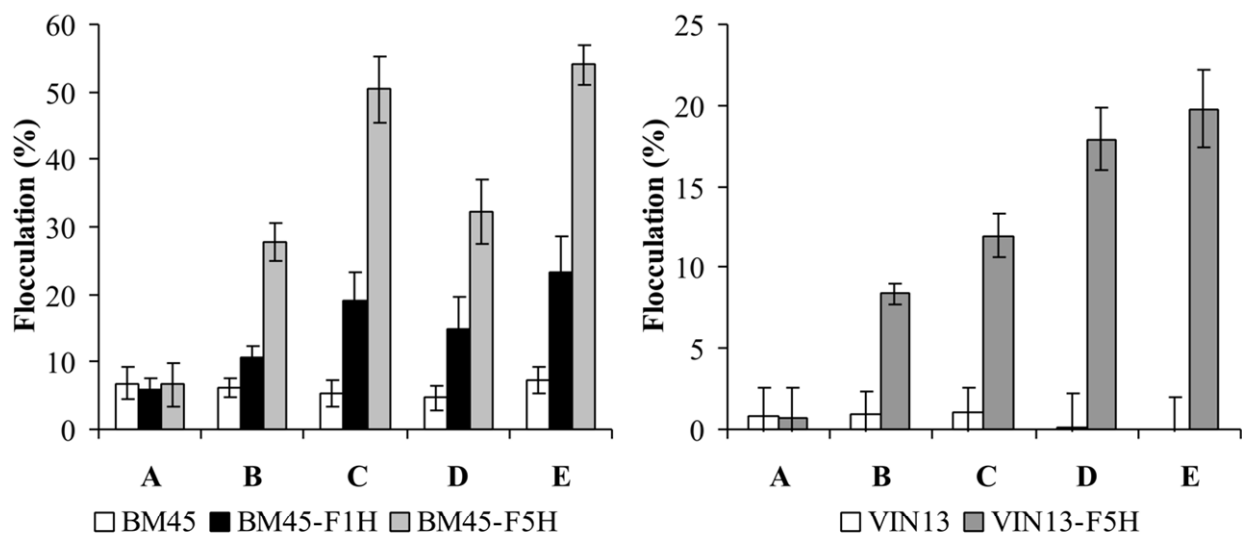
Figure 4.11   Stress-induced expression of *FLO1* and *FLO5* encoded flocculins in BM45 and VIN13 *HSP30p* transformants. Yeast strains cultivated for 10 h in YEPD, were subjected to the following treatments: A – untreated (45 minutes at 30°C); B - heat shock for 30 minutes at 42°C; C - heat shock for 45 minutes at 42°C; D - 3% (vol/vol) ethanol for 30 minutes at 30°C; E - 6% (vol/vol) ethanol for 30 minutes at 30°C. The results are averages of three independent determinations and error bars represent standard deviations.

4.4.6 Flor formation and invasive growth. The BM45 wild type wine yeast strain displays natural flor forming ability after 5 days in flor medium at 30°C under static conditions (Fig. 4.12A). It is visibly apparent that both BM45-F11A and BM45-F11A transgenic strains formed thicker biofilms with the latter strain producing the thickest biofilm. *ADH2p* or *HSP30p* driven expression of either *FLO1* or *FLO5* seemed to have no effect on the hereditary natural flor forming ability of BM45-F1A, BM45-F1H, BM45-F5A and BM45-F5H transformants. As shown in Fig. 4.12B, only transgenic yeast VIN13-F11A and VIN13-F11H strains formed a biofilm with the latter strain producing the thicker biofilm. The *FLO11* transformants (Fig. 4.12A and 4.12B) generally displayed higher buoyant cell densities in comparison to other strains; with *ADH2p* mediated *FLO11* expression clearly showing the highest suspended cell densities.
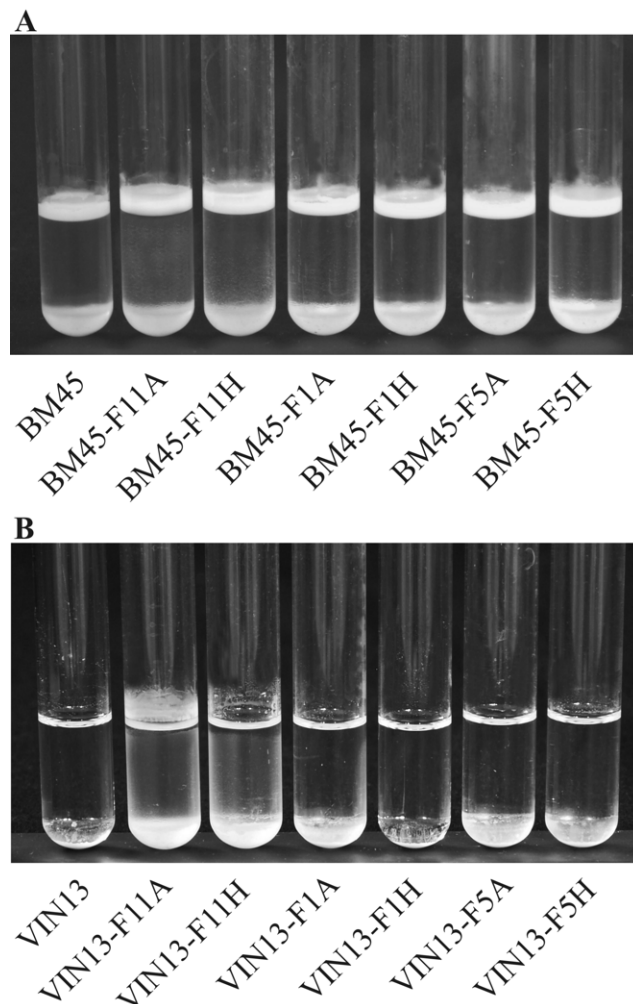
Figure 4.12   Biofilm formations by (A) BM45 and (B) VIN13 wild types and their transgenic derivatives. Cells were pre-cultured in YEPD broth and recovered by centrifugation, washed once with flor medium and resuspended at a density of 6 × 10^7 cells mL^{-1} in 5 mL flor medium contained in 16 x 165 mm glass test tubes. The tubes were photographed after 5 days of static incubation at 30°C.

The ability of the wild-type BM45 and VIN13 wild type wine yeast strains and their transgenic descendants to invade agar is shown in Fig. 4.13. BM45 and VIN13 wild type yeast strains exhibited natural invasiveness. *ADH2p* or *HSP30p* driven expression of either *FLO1* or *FLO5* seemed to have no effect on the inherited native invasive growth phenotype of BM45-F1A, BM45-F1H, BM45-F5A, BM45-F5H, VIN13-F1A, VIN13-F1H, VIN13-F5A, VIN13-F5H transformants. However, both BM45-F11A and VIN13-F11A grew as larger-sized colonies and presented different colony morphology on SCLD agar in comparison to their wild type parental strains. Moreover they displayed more aggressive invasive growth behaviour in comparison to their wild type parental strains. BM45-F11H and VIN13-F11H transformants were non-invasive.
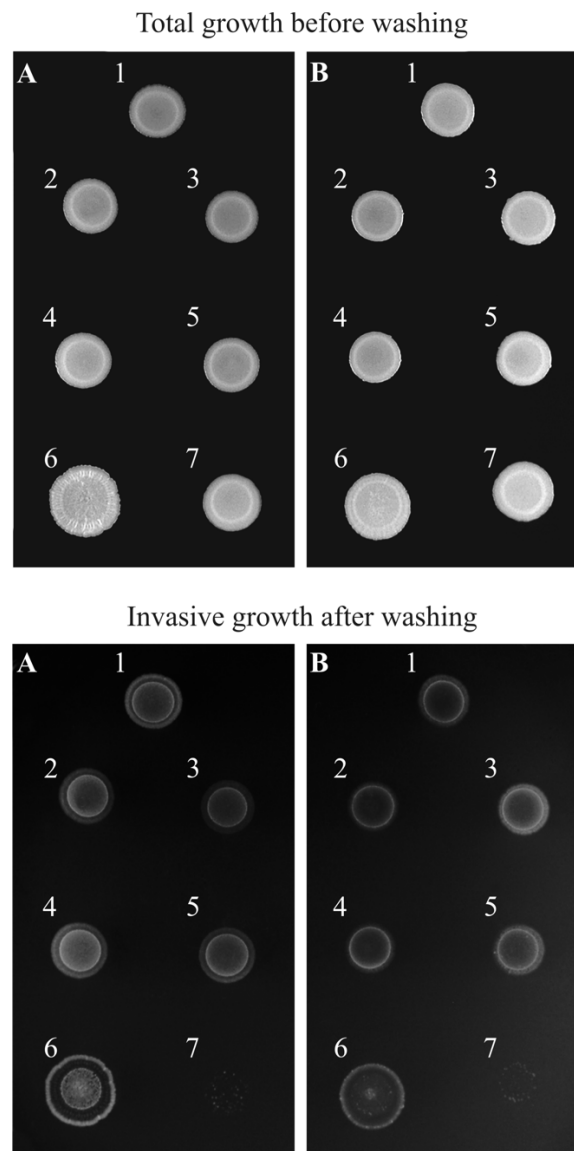
Figure 4.13   Invasive growth of (A) BM45 wild type (1); BM45-F1A (2); BM45-F1H (3); BM45-F5A (4); BM45-F5H (5); BM45-F11A (6) and BM45-F11H (8) and (B) VIN13 wild type (1); VIN13-F1A (2); VIN13-F1H (3); VIN13-F5A (4); VIN13-F5H (5); VIN13-F11A (6) and VIN13-F11H (8) strains after 5 days growth at 30°C on SCLD medium.

4.4.7 Effect of *FLO* gene expression on cell surface hydrophobicity. The hydrophobicity of yeast cell surfaces (Fig. 4.14) from yeast populations grown in YEPD for 48 h was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent. The VIN13 wild type yeast strain showed almost negligible natural hydrophobicity (Fig. 4.14B); whilst the BM45 wild type wine yeast strain displayed 50% more innate hydrophobicity (Fig. 4.14A). As a general trend, it was observed that *ADH2p* regulated expression of *FLO1*, *FLO5* or *FLO11* in transgenic wine yeast strains resulted in higher cell surface hydrophobicity (CSH) in comparison to *HSP30p* mediated expression of these genes. A statistically insignificant (*p* > 0.05) modified hydrophobic index (MHI) was only observed for VIN13-FIH, in comparison to the VIN13 wild type strain (Fig. 4.14B).
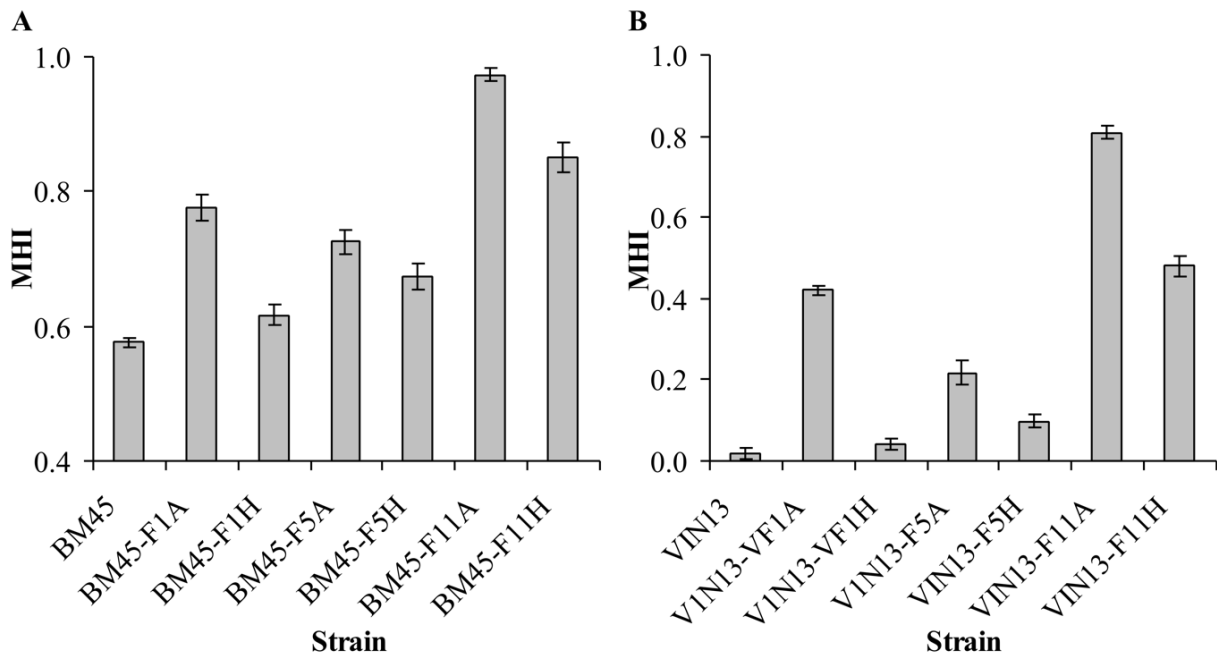
Figure 4.14   The impact of *ADH2* and *HSP30* expression of *FLO* genes on cell surface hydrophobicity (CSH). The wild type BM45 and VIN13 strains and their transgenic derivatives were cultivated for 48 h in YEPD containing 2% glucose at 30°C with shaking (160 rpm). The modified hydrophobic index (MHI) was determined according to the biphasic-solvent partition assay described by Hinchcliffe (1985). The results are averages of three independent determinations, and error bars represent standard deviations.

4.5   DISCUSSION

In a previous study, we reported on the stationary-phase specific expression of the flocculation genes *FLO1*, *FLO5* and *FLO11* in the haploid non-flocculent, non-invasive and non-flor forming *S. cerevisiae* FY23 laboratory strain (Govender *et al.*, 2008). By implementing a similar cloning strategy, we now assess the flocculation or adhesion potential of native *FLO1*, *FLO5* and *FLO11* open reading frames of non-flocculent BM45 and VIN13 wine yeast strains. Importantly, both sets of transgenic wine yeast strains displayed vegetative growth and fermentation properties that are comparable to that of their parental wild type strain, indicating that those characteristics were not compromised by modified expression of a specific Flo gene.

In BM45 and VIN13 wine yeast transformants, irrespective of the promoter involved, it was observed that *FLO5*-based constructs induce flocculation most efficiently, while *FLO1*-based constructs, although still leading to significant flocculation, are less efficient. This is in contrast to the observations made in the laboratory strain, where several reports suggest that *FLO1* imparts the strongest flocculation phenotype (Govender *et al.*, 2008; Guo *et al.*, 2000; Teunissen and Steensma, 1995). These data support the hypothesis that the *FLO* gene family provides phenotypic plasticity to cell wall-related characteristics of yeast. This plasticity is hypothesised to provide *S. cerevisiae* with the ability to frequently adjust and switch cell wall related traits, thereby providing populations of cells with the necessary phenotypic diversity to adapt to particular changes in the environment that would necessitate adhesion-related phenotypic changes. This phenomenon has also been linked to epigenetic regulation of such phenotypes (Verstrepen *et al.*, 2005).

On the other hand, phenotypic analysis of recombinant *FLO11* wine yeast strains confirmed the findings of our previous study in that *FLO11*-based constructs were incapable of promoting a flocculent phenotype. This remained the case in the BM45 host strain that possesses an innate flor-forming ability. Indeed, *FLO11* constructs strongly induced flor formation in both BM45 and VIN13. Transgenic strains expressing *FLO11* also presented the highest cell surface hydrophobicity. The same observations were made regarding the ability of strains to invade agarose. Although both BM45 and VIN13 host strains displayed native invasiveness, the *ADH2p-FLO11* derivatives displayed more aggressive invasive growth phenotypes. The *HSP30p-FLO11* descendants yielded non-invasive phenotypes, suggesting that growth on solid media may not provide good induction condition for the *HSP30* promoter. Previous studies reported that *FLO11* expression is driven by a promoter of approximately 3 kb, which is possibly the largest promoter found in the whole *Saccharomyces* genome (Rupp *et al.*, 1999; van Dyk *et al.*, 2005). It should be noted that insertion of our *FLO11* replacement

cassettes effectively deleted 3310 bp of the native *FLO11* promoter region. Thus, the non-invasiveness of *HSP30p-FLO11* wine yeast transformants seems to confirm the effectiveness of our expression strategy in that insertion of our inducible promoter replacement cassettes were deleterious to native BM45 and VIN13 host strain *FLO11* promoters that are responsible for their innate invasive behaviours.

Major variations were registered when comparing the size of intragenic repeat regions of *FLO* genes from industrial wine yeast strains to that of the FY23 laboratory strain employed in our previous study. With the exception of the BM45 *FLO5* repeat region amplicon, all other wine yeast intragenic repetitive domains displayed decreased lengths in contrast to corresponding amplicons obtained from FY23. Recently, Fidalgo *et al.* (2006) found that the number of repeated sequences in the central domain of *FLO11* from a flor yeast strain to be greatly increased and correlated this to the superior flor forming ability of the strain. In addition, Verstrepen and co-authors (2005) confirmed that an increase in the size of the intragenic repeat region of the *FLO1* gene results in a quantitative increase in *FLO1* mediated phenotypes (e.g. adhesion and flocculation) and *vice versa*. The decreased flocculation ability of BM45 and VIN13 *FLO1* and *FLO5* transgenic yeast strains in comparison to similar FY23 haploid transformants reported previously may therefore at least in part, be due to this reduced number of repeats (Govender *et al.*, 2008). These differences also provide an explanation for the greater flocculent abilities of BM45 *FLO1* and *FLO5* transformants when compared to VIN13 transformants, as well as the superior flocculation abilities of *FLO5* transgenic wine yeast to comparable *FLO1* wine yeast transformants. Although it is tempting to believe that smaller intragenic repeat regions are solely responsible for these fluctuations, it is also probable that the activities of especially the *FLO1* and to a lesser extent the *FLO5* promoter replacement cassettes may still be subject to upstream regulatory factors. This suggestion stems from a previous study that reported the Swi-Snf co-activator and Tup1-Ssn6 co-repressor control an extensive domain (> 5 kb) in which regulation of the *FLO1* gene takes place (Fleming and Pennings, 2001). Therefore, insertion of our *FLO1* and *FLO5* replacement cassettes, which knocked-out 845 bp and 2925 bp of the native *FLO1* and *FLO5* promoter regions respectively, may have been insufficient to eliminate all existing regulatory mechanisms of these genes.

The data here clearly indicate that *ADH2p* controlled *FLO1* and *FLO5* phenotypes of transgenic wine yeast strains are distinctly more flocculent than comparable *HSP30p* regulated phenotypes. This is in contrast to some of the findings in the laboratory yeast FY23 strain. Although both *ADH2p* and *HSP30p* are endogenous *S. cerevisiae* promoters, they may be subject to different regulation modalities in different strains (Nevoigt *et al.*, 2006). The study nevertheless confirms the

stress-inducible nature of *HSP30p* controlled expression of *FLO1* and *FLO5* genes to yield flocculent phenotypes in response to specific stress conditions that include heat shock and/or exposure to ethanol.

It has been proposed that flor wine yeast only begin to form flor via a *FLO11*-mediated mechanism when glucose repression of *FLO11* transcription is eliminated due to depletion of grape sugar after alcoholic fermentation (Ishigami *et al.*, 2004). Based on the findings of this study, it can be suggested that the *ADH2* or *HSP30* promoter can be utilised to induce flor formation in non-flor wine yeast in a manner that will mimic natural flor wine yeast. The data also clearly demonstrate that flocculation performance of industrial yeast strains can be tightly controlled and fine-tuned to satisfy specific industrial requirements, thus opening an avenue for the development of new commercial wine yeast strains that may be beneficial to the downstream processing of wine. We are currently investigating the fermentative potential of the 12 transgenic wine yeast strains under wine making conditions.

4.6   ACKNOWLEDGEMENTS

4.7   REFERENCES

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Chapter 5

RESEARCH RESULTS III

Wine yeast engineered for controlled flocculation

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Wine yeast engineered for controlled flocculation

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5.1   ABSTRACT

The fermentative and flocculation potential of twelve genetically engineered strains derived from BM45 and VIN13 commercial wine yeast strains in which the natural chromosomal promoters corresponding to *FLO1*, *FLO5* and *FLO11* flocculation genes were replaced by the stationary phase-inducible promoters of either the *HSP30* or *ADH2* genes, were evaluated under wine-making conditions using both synthetic MS300 and natural Merlot musts. The data show that the *ADH2* regulatory sequences employed in this study were unsuitable for the purpose of driving *FLO* gene expression under wine-making conditions. However, *HSP30p*-based *FLO1* and *FLO5* wine yeast transformants displayed flocculent phenotypes under both wine-making conditions, and the intensities of these phenotypes were closely aligned to those observed under nutrient-rich YEPD conditions. The BM45-derived *HSP30p-FLO5* transformant in particular was capable of generating compacted or 'caked' lees fractions, thereby providing a distinct separation of the fermented wine product and lees fractions. The BM45 and VIN13-derived *HSP30p-FLO11* transformants were exclusively and strongly flocculent under authentic red wine-making conditions, thus suggesting that this specific fermentation environment specifically contributes to the development of a flocculent phenotype which is insensitive to both glucose and mannose. Furthermore, this phenotype displayed both Ca^{2+}-dependant and Ca^{2+}-independent flocculation characteristics. A distinct advantage of this unique *FLO11* phenotype was highlighted in its ability to dramatically promote faster lees settling rates. Moreover, wines produced by BM45-F11H and VIN13-F11H transformants were significantly less turbid than those produced by their wild type parental strains. Furthermore, the fermentation activities of *HSP30p*-based transgenic yeast strains were indistinguishable from that of their parental host wine yeast strains. The benefit of this attractive property is that it facilitates simpler and faster recovery of wines and also promotes greater volume recovery of the wine product.

5.2   INTRODUCTION

Winemakers involved in high-volume wine production must ensure rapid and reliable wine fermentations to produce wines with consistent wine flavour and predictable quality. Thus it is common practice in modern winemaking to inoculate wine fermentations with pure yeast starter cultures that are supplied to wineries by several commercial manufacturers of active dried wine yeast (Pretorius, 2000). To meet the demands of a consumer-driven market, wine processing currently involves fining and clarification procedures to produce clear and physicochemical stable wines. Wine fining entails the purposeful addition of an adsorptive compound [bentonite, gelatin, albumin or industrially prepared yeast cell wall mannoproteins (Mannostab™)], followed by the settling or precipitation (cold stabilization) of partially soluble components from the wine. Further clarification is usually achieved by sedimentation, racking, centrifugation and filtration. Wine filtration involves the partial removal of large suspended solids by various grades of diatomaceous earth or filter sheets to the complete retention of microbes by perpendicular flow polymeric membranes (Boulton *et al.*, 1996; Pretorius and Bauer, 2002; Ribéreau-Gayon *et al.*, 2000). Moreover, studies have shown that filtration alters the aroma and colour of the wine and also removes molecules that would otherwise positively contribute to the impression of body and volume on the palate (Boulton *et al.*, 1996; Lubbers *et al.*, 1994; Moreno and Azpilicueta, 2004; Moreno *et al.*, 2007). Thus it may be concluded that the fining and clarification of wine are expensive and time-consuming procedures that ultimately negatively impact on the cost of the finished product.

Yeast flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989). Primarily driven by the importance of flocculation to the brewing industry, a concerted attempt was made to understand the genetics of flocculation. Structural and functional analysis of the genomic sequence of *Saccharomyces cerevisiae* reveals that there are five unlinked dominant *FLO* genes (Verstrepen *et al.*, 2004). Four of these genes namely *FLO1*, *FLO5*, *FLO9* and *FLO10*, are located adjacent to their telomeres. Consequently, this subset of dominant *FLO* genes is viewed as a subtelomeric gene family (Teunissen *et al.*, 1995). In addition, a fifth gene namely *MUC1* (Lambrechts *et al.*, 1996) or *FLO11* (Lo and Dranginis, 1996), which is neither centromeric nor telomeric, is also considered a dominant flocculation gene. The *FLO* genes encode cell wall proteins that are collectively referred to as adhesins and they are characterized by a common modular organization that consists of three domains. Firstly, an amino-terminal domain that is proposed to harbour the binding site to carbohydrate

receptors (mannan) which confers adhesion. This is followed by a central domain that is extremely rich in serine and threonine residues, and thirdly, a carboxyl terminal domain containing a GPI anchoring sequence that links the flocculin to the cell wall (reviewed in Verstrepen and Klis, 2006). The *FLO11*/*MUC1* gene encodes a protein that has been implicated in flocculation (Bayly *et al.*, 2005; Lo and Dranginis, 1996), flor formation, invasive growth and substrate adhesion (Bayly *et al.*, 2005; Guo *et al.*, 2000; Ishigami *et al.*, 2004; Lo and Dranginis, 1996; Zara *et al.*, 2005).

Efficient wine yeast flocculation after primary alcoholic fermentation leads to the formation of compacted sediments (Lahtchev and Pesheva, 1991) or 'caked' lees, thereby reducing the handling of wines and minimizing problems associated with wine clarification (Pretorius and Bauer, 2002). As such, this ultimately contributes to lower volume loss of the finished wine products. The fact that the natural flocculent ability of certain commercial wine yeast strains is advertised by retailers of active dry wine yeasts, further highlights the significance and attractiveness of this trait to the wine industry (http://www.maurivinyeast.com/media/51.pdf, 21 October 2008). To date two independent research studies have shown that most commercial wine yeast strains are either non-flocculating or flocculate inefficiently (Carstens *et al.*, 1998; Suzzi *et al.*, 1984). In previous research studies, conventional hybridization technologies have been employed to create genetically improved wine yeasts that displayed increased flocculent abilities (Lahtchev and Pesheva, 1991; Romano *et al.*, 1985). However, a significant drawback is associated with wine yeast selection using genetic techniques such as hybridization (mating, spore cell-mating, rare mating, cytoduction and spheroplast fusion), clonal selection of variants and mutagenesis in that these techniques lack the specificity to modify the flocculent ability of wine yeasts in a well-controlled manner and may result in the loss of some desirable traits (Pretorius and Bauer, 2002).

The use of recombinant DNA technology and genetic engineering offers a reliable method for modifying the genetic framework of flocculation that is inherent to *Saccharomyces cerevisiae* wine yeasts, thereby creating flocculent wine yeast strains without jeopardizing other desirable oenological properties of the parental host wine yeast strain. Being mindful of this, we showed in a recent study that by placing the native chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5* and *FLO11*, of the haploid non-flocculent, non-invasive and non-flor forming *S. cerevisiae* FY23 strain under the transcriptional control of stably integrated inducible promoters (*ADH2p* or *HSP30p*), that it is indeed possible to generate strains with specific flocculation and adhesion behaviours (Govender *et al.*, 2008). Using a similar genetic

engineering approach we also demonstrated in Chapter 4 that it is feasible to impart desirable late fermentation flocculation and adhesion behaviours to the non-flocculent BM45 and VIN13 commercial wine yeast strains.

It should also be noted that, due to their *N-* and *O*-linked carbohydrate moieties containing large amounts of mannose, Flo cell wall glycoproteins may also be termed mannoproteins (Bony *et al.*, 1997; Groes *et al.*, 2002; Straver *et al.*, 1994; Verstrepen and Klis, 2006). Mannoproteins from the yeast cell wall are enzymatically released into wine during alcoholic fermentation and/or during ageing on yeast lees by autolysis (Caridi, 2006). Research studies have attributed numerous valuable enological properties to parietal yeast mannoproteins, which include protection against protein haze formation (Brown *et al.*, 2007; Dupin *et al.*, 2000; Gonzalez-Ramos *et al.*, 2008; Waters *et al.*, 1994); chemical leeching of ochratoxin A (OTA) which is potent mycotoxin (Caridi, 2006; Ringot *et al.*, 2005); inhibition of potassium-bitartrate crystallization (Boulton *et al.*, 1996; Lubbers *et al.*, 1994; Ribéreau-Gayon *et al.*, 2000); retention of aroma compounds thus improving the color stability; and reducing the astringency of wines (Caridi, 2006; Chalier *et al.*, 2007; Lubbers *et al.*, 1994; Vidal *et al.*, 2004). Moreover, mannoproteins have been also shown to encourage lactic acid bacterial growth, thus contributing to malolactic fermentation. In addition to the desirable flocculent phenotypes displayed by transgenic wine yeast strains engineered in this study, these transgenic strains are also potentially attractive in that they are designed to over-express flocculin mannoproteins that could positively enhance the quality of wine fermented by these transformants in certain if not all enological properties described above.

In this study, the fermentative potential of twelve transgenic wine yeast strains expressing their native *FLO1*, *FLO5* or *FLO11* open reading frames that are under transcriptional control of stably integrated inducible promoters (*ADH2p* or *HSP30p*) is assessed under both natural and synthetic wine making conditions. We show that some of these strains display interesting oenological properties. In particular, we show that *HSP30-FLO11* transgenic yeast strains were able to produce significantly clearer wines with compact lees.

5.3   MATERIALS AND METHODS

5.3.1 Strains. All yeast strains used in this study are listed in Table 5.1

Table 5.1 *S. cerevisiae* strains employed in this study

Strain	Genotype	Reference
BM45	Industrial wine yeast strain (unknown genotype)	Lallemand Inc., Montreal, Canada
BM45-F1A	*FLO1p::SMR1-ADH2p*	This study
BM45-F1H	*FLO1p::SMR1-HSP30p*	This study
BM45-F5A	*FLO5p::SMR1-ADH2p*	This study
BM45-F5H	*FLO5p::SMR1-HSP30p*	This study
BM45-F11A	*FLO11p::SMR1-ADH2p*	This study
BM45-F11H	*FLO11p::SMR1-HSP30p*	This study
EC1118	Industrial wine yeast strain (unknown genotype)	Lallemand Inc., Montreal, Canada
NT50	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, Cape Town, South Africa
VIN13	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, Cape Town, South Africa
VIN13-F1A	*FLO1p::SMR1-ADH2p*	This study
VIN13-F1H	*FLO1p::SMR1-HSP30p*	This study
VIN13-F5A	*FLO5p::SMR1-ADH2p*	This study
VIN13-F5H	*FLO5p::SMR1-HSP30p*	This study
VIN13-F11A	*FLO11p::SMR1-ADH2p*	This study
VIN13-F11H	*FLO11p::SMR1-HSP30p*	This study
WE372	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, Cape Town, South Africa

5.3.2 Media and culture conditions. Yeast strains were routinely cultivated at 30°C in rich YEPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose. Single yeast colonies from 3 day old YEPD plates were used to inoculate starter cultures in 40 mL YEPD broth contained in 250 mL Erlenmeyer flasks, which were incubated at 30°C with shaking (160 rpm) for 16-18 h. These were used to inoculate precultures at an initial cell density of 5×10^5 cells mL^{-1} which were incubated at 30°C with shaking (160 rpm) for 16 h. Thereafter yeast cells for inoculation of experimental cultures were routinely prepared as follows using ice cold reagents. Yeast from precultures were harvested by centrifugation (4000 rpm, 5 min), washed once with 100 mM EDTA, pH 7 to ensure deflocculation; once with 30 mM EDTA, pH 7 and finally resuspended in 30 mM EDTA, pH 7. To determine the effect of initial glucose concentration on the flocculent ability of BM45-F5A and VIN13-F5A transgenic yeast strains in nutrient-rich medium, experimental cultures were seeded at an initial cell density of 1×10^6 cells mL^{-1} into 40 mL YEPD (containing 20, 30, 50, 100, 150 and 200 gL^{-1} glucose) and incubated at 30°C with shaking (160 rpm) until 24 h post-glucose exhaustion. Invasive growth was assessed in chemically defined synthetic complete (SC) media containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI, USA) and 0.2% (wt/vol) glucose (SCLD). For selection of sulphometuron methyl (SM) resistant BM45 and VIN13 transformants, SC medium containing 0.67% YNB without amino acids and 2% (wt/vol) glucose was supplemented with 280 and 300 μg mL^{-1} SM (DuPont Agricultural Products, France) respectively. Yeast strains were stored in YEPD supplemented with 15% (vol/vol) glycerol (Ausubel *et al.*, 1995). In this study, 2% (wt/vol) agar (Difco) was used for all solid media.

5.3.3 Fermentation media and conditions.

5.3.3.1 Defined synthetic grape must (MS300) fermentations. The defined medium (MS300) simulating standard grape juice containing 10% glucose (wt/vol) and 10% (wt/vol) fructose [20% (wt/vol)] total sugar with a total nitrogen concentration of 300 mg L^{-1} supplied as amino acids and ammonia, was prepared as previously described (Bely *et al.*, 1990). The fermentative potential of BM45 and VIN13 wild type strains and their 12 transgenic derivatives were assessed in triplicate. Yeast precultures in YEPD were prepared as described above and yeast was harvested by centrifugation (4000 rpm, 5 min) and resuspended in MS300 medium. Batch fermentations (200 mL) of MS300 medium contained in 250 mL Schott bottles equipped with fermentation airlocks were performed by the inoculation of precultured cells at a density of 2×10^6 cells mL^{-1} and were performed at room temperature. To determine the progress of fermentations, carbon dioxide release was monitored on a daily basis by measurement of fermentor weight loss. Samples were withdrawn for analysis under aseptic conditions as swiftly as possible to limit the fermentations exposure to oxygen. The flocculation potential of ADH2p-FLO1, *ADH2p-FLO5* BM45 and VIN13 transgenic yeast strains was assessed in aerobic shake-flask experiments using MS300 medium.

The flocculation potential of *HSP30-FLO11* BM45 and VIN13 transgenic yeast strains was assessed in fermentor vessels as described above and in aerobic shake-flask experiments using MS300 medium that contained either pectin (1 g L^{-1}) or diatomaceous earth (1 g L^{-1}).

5.3.3.2 Red wine fermentations. For red wine fermentations, grapes of *Vitis vinifera* Merlot (200 kg) were rinsed with sufited water, destemmed and crushed. As a precaution, damaged grape clusters (broken or with visual microbial alterations) were discarded in order to eliminate undesirable contamination. Red grape must [24.2% sugar (glucose and fructose), 5.8 g L^{-1} titratable acidity and pH 5.8] was sulfited to 40 mg L^{-1}. Thereafter red grape must was batch fermented in 20 L plastic buckets containing 3kg of red grape must that were adjusted to exactly 10 kg by the addition of grape skins and 4g diammonium phosphate (DAP). Yeast precultures in YEPD were prepared as described above and yeast was harvested by centrifugation (4000 rpm, 5 min). Thereafter, wild type and transgenic yeast populations were pre-acclimatized for wine fermentations by incubation at 30ºC for 4 h with shaking at 160 rpm in 50% (v/v) filter (0.22 µm cellulose acetate) sterilized Merlot must.

The fermentative potential of BM45 and VIN13 wild type strains and their 12 transgenic derivatives were assessed in triplicate. Assuming a ratio of 0.6 L kg^{-1} of grape must to grape pulp-seed-skin mixture, batch alcoholic fermentation of 6 L red grape must were performed by the inoculation of pre-acclimatized cell populations at a density of 2 x 10^6 cells mL^{-1} and was carried out at 27°C. Maceration during alcoholic fermentation was achieved by punching down fermentation caps three times per day. The residual glucose-fructose concentration was monitored on a daily basis with a balling meter. When residual glucose-fructose levels were approximately 10 g L^{-1} (6th day), the wines were hydraulically pressed (2 bar) from grape skins. The pressed wine (4.4 L) including lees was dispensed into 4.5 L glass jars equipped with fermentation airlocks and fermentation at was allowed to proceed to dryness (residual sugar ≤ 1.95 g L^{-1}). Racking entailed that wines from each fermentation were carefully siphoned-off (avoiding lees sediment carryover), sulfited to 40 ppm (free sulphur) and bottled (5 x 750 mL dark green glass bottles). Putative wild type and transgenic yeast populations from completed wine fermentations were established by plating out 100 µL of a dilution series onto YEPD plates containing 25 mg L^{-1} kanamycin sulphate (Roche, Germany) and 30 mg L^{-1} chloramphenicol (Sigma-Aldrich, Missouri, USA). After incubation at 30 °C for 2 to 3 days, colonies representing putative transgenic yeast strains were randomly selected from plates (25 colonies per replicate sample) and assessed for their resistance to SM, flocculation ability (*FLO1* and *FLO5* transformants), increased invasiveness (*ADH2p-FLO11* transformants) or lack of invasiveness (*HSP30p-FLO11* transformants).

Genomic DNA isolated from 25 colonies per replicate sample, putative wild type BM45 and VIN13 isolates were *S. cerevisiae* strain-typed using PCR with primers δ-F (5' CAAAATTCACCTATWTCTCA 3') and δ-R (5' GTGGATTTTTATTCCAACA 3') that are specific for delta sequences (Ness *et al.*, 1993). PCR reactions were performed using *Takara Ex Taq*™ PCR system (Takara Bio Inc., Otsu, Japan). Isolated genomic DNA from *S. cerevisiae* BM45, EC1118, NT50, VIN13 and WE372 industrial wine yeast wild type strains served as controls.

The lees component (5 mL aliquots) from individual batch fermentations was washed 3 times with an equal volume of sterile 0.9% saline and stored at -20°C for flocculation and sedimentation analysis. The lees from stored samples was recovered by centrifugation and resuspended in 50 mL 100 mM EDTA by vigorous vortexing. Thereafter the amorphous solid debris from the lees was allowed settle for 20-30 min and the yeast cellular fraction was recovered from just below the meniscus. Microscopic evaluation of cellular fractions determined whether extractions were to be repeated.

5.3.4 Analytical Methods

5.3.4.1 Fourier transform infrared (FT-IR) spectral measurements. FT-IR spectral scans were generated from samples using a WineScan FT 120 equipped with Michelson interferometer (FOSS Electric A/S, Hillerød, Denmark). Depending on the sample, either of three fixed instrument-preset programs (must, wine under fermentation or completed wine) was used. Samples were routinely centrifuged, vacuum-filtered (Whatman No. 1 cellulose filter paper, Kent, United Kingdom) and degassed before analysis. Samples (7-8 mL) were pumped through a heat exchanger (40 °C) and a CaF_2-lined cuvette (path-length 37 µm). Each sample was scanned in duplicate at 4 cm^{-1} intervals within the wavenumber range 5011 to 929 cm^{-1}, with the spectra being averaged for data processing. The Winescan instrument equipped with software version 2.2.1, constructed an interferogram (derived from 20 scans per sample) based on recorded frequencies of infrared radiation transmitted by the sample. Fourier transformation converted the interferogram into a single-beam transmittance spectrum. FOSS Zero liquid S-6060 was employed to generate a zero-beam spectrum. The single beam sample spectrum was divided by the zero-beam spectrum to yield a transmittance spectrum which was converted into a linear absorbance spectrum by a series of mathematical procedures (WineScan FT120 Type 77110 and 77310 reference manuals, FOSS Electric, Denmark, 2001).

5.3.4.2 High-Pressure Liquid Chromatography (HPLC). Wine and MS300 samples were centrifuged and filtered (0.22 µm cellulose acetate) before HPLC analysis. Chromatographic separations were performed on a 300 x 7.8 mm Aminex HPX-87H column (Bio-Rad Laboratories, California, US). The Agilent Technologies 1100 HPLC series system (Palo Alto, CA) consisted of a binary pump system (G1312A), automatic

injector furnished with a 50 µL loop, diode array detector (G1315A) set at 220 nm, refractive index detector (G1632A) maintained at 35 ºC, autosampler (G1313A), integrator and ChemStation chromatography software [Version Rev.A.10.01 (1635)]. The thermostatically controlled column chamber was set at 55 ºC and elution was performed with 5 mM H_2SO_4 at a flow rate of 0·5 mL min^{-1}. Quantification of glucose, fructose, glycerol, ethanol, acetic acid, citric acid, lactic acid, malic acid, succinic acid and tartaric acid was performed using external standards prepared from chemically pure compounds (Sigma-Aldrich, Missouri, USA).

5.3.4.3 Gas chromatography (GC) analysis. Wine and MS300 samples were centrifuged and filtered (0.22-µm cellulose acetate) prior to GC analysis. The analysis of the major volatile compounds in wine and MS300 samples was performed by direct injection (3 µL) on an Agilent Technologies (Palo Alto, CA) 6890 A Series gas chromatograph coupled to an HP 7673 auto-sampler-injector and a HP 3396A integrator. An Agilent Technologies DB-FFAP organic-coated, fused silica capillary column (60 m x 0.32 mm internal diameter) with a 0.5 µm coating thickness was employed. The initial oven temperature was 33 ºC (10 min hold) and thereafter ramped at 12 ºC min^{-1} to 240 ºC (5 min hold). The following parameters were employed: mode, split; split ratio, 15:1; split flow rate, 49.5 mL min^{-1}; injector temperature, 200 ºC; initial pressure, 84.5 kPa; flow mode, constant; column flow rate, 3.3 mL min^{-1}; detector temperature, 250 ºC, carrier gas, hydrogen; hydrogen flow rate, 30 mL min^{-1}; air flow rate, 350 mL min^{-1} and make up flow rate, nitrogen at 30 mL min^{-1}. A 100 µL aliquot of the internal standard 4-methyl-2-pentanol (0.5 mg L^{-1}) and 1 mL was diethyl ether added to each 5 mL sample of wine or MS300. Volatile components were extracted into the diethyl ether fraction of the aforementioned mixture by ultrasonication for 5 min, followed by centrifugation at 4000rpm for 3 min. The ether layer was removed and dried on $NaSO_4$ prior to analysis. Each sample extract was injected onto the column three times and reported values are an averaged amount of at least two injections. Peak identities and their concentrations were resolved by comparison to analytically pure standard reagents procured from Sigma (Missouri, USA) and Merck (Darmstadt, Germany). ChemStation software [version Rev.B.01.03 (204)] was used for data processing.

5.3.5 Biomass determination. Dry cell weight of MS300 batch fermentations was determined by filtering under vacuum 5 mL of culture through a pre-dried (350 W for 4 min in a microwave oven) and pre-weighed 0.45 µm Supor® membrane disc filter (Pall Corporation, NY, USA). The filter was reweighed after being washed with three volumes of distilled water and dried in a microwave oven at 350 W for 8 min. The dry weights of sample replicates were determined in duplicate.

5.3.6 Enumeration of yeast populations. The cell density of suitably diluted yeast suspensions in 30 mM EDTA (pH 7) was determined by direct cell counting with a hemocytometer.

5.3.7 Flocculation assays. The flocculent ability of yeast strains was established using the modified Helm's assay as described by D'Hautcourt and Smart (1999). Five replicates of the control and test reactions were performed for each sample. The percentage flocculation reported in this paper reflects the arithmetic mean of three independent determinations. To investigate sugar inhibition of *FLO1* and *FLO5* flocculation phenotypes, either 1 M mannose or 1 M glucose was added at varying concentrations to both the washing and suspension buffers that are employed in the modified Helm's assay (1999).

5.3.8 The sedimentation or Ca^{2+}-independent flocculation assay. The sedimentation or Ca^{2+-} independent flocculation ability of yeast cell populations that were harvested from the lees of red wines was assessed in 100 mM EDTA. Samples containing 1×10^8 cells were dispensed into 1.5 mL microcentrifuge tubes and the cells were recovered by centrifugation at 10000 rpm for 1 minute. For the control assay (in 5 replicates), cells were resuspended in 1 mL 100 mM EDTA (pH 7), properly agitated by high-speed vortexing for 30 seconds and inverted five times in a period of 15 seconds. Immediately thereafter 10 μL aliquots were withdrawn from just below the meniscus and added to 990 μL 100 mM EDTA, pH 7 contained in a cuvette. For the test assay (in 5 replicates), the cells were resuspended in 1 mL 100 mM EDTA (pH 7), samples were properly agitated by high-speed vortexing for 30 seconds and to promote aggregation, the microcentrifuge tubes were inverted five times in a period of 15 seconds. Test samples were left undisturbed for 15 minutes at room temperature. Thereafter 100 μL aliquots were carefully withdrawn from just below the meniscus and added to 900 μL 100 mM EDTA, pH 7 contained in a cuvette. The absorbance of both control and test samples was determined at 600 nm. The average of the 5 control observations was obtained (denoted A). Each of the five individual test replicate observations (denoted B) was used to determine the replicate percentage sedimentation as follows: replicate % sedimentation = (A-B)/A. The percentage sedimentation of a sample was determined from an average of 5 replicate percentages as calculated above. The percentage sedimentation reported reflects the arithmetic mean of three independent determinations.

5.3.9 RNA extraction and cDNA synthesis. Strain BM45 and its transgenic descendants were precultured in YEPD and treated as described earlier. Batch fermentations (200 mL) of MS300 medium contained in 250 mL Schott bottles equipped

with fermentation airlocks were performed by the inoculation of YEPD precultured cells in triplicate at a density of 2×10^6 cells mL^{-1} and were performed at room temperature. To determine the progress of fermentations, carbon dioxide release was monitored on a daily basis by measurement of fermentor weight loss. Samples from fermentations corresponding to the exponential yeast growth phase, entry into stationary yeast growth phase and late stationary yeast growth phase were withdrawn for analysis under aseptic conditions as swiftly as possible to limit the fermentations exposure to oxygen. Samples were washed with ice-cold H_2O, pelleted and resuspended in ice-cold AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). Total RNA was isolated as previously described (Schmitt *et al.*, 1990). DNA contamination was eliminated by DNase I (Roche diagnostics) treatment. One μg total RNA was used as template for cDNA synthesis using the ImProm-II™ reverse transcription system according to the manufacturer instructions (Promega). cDNA samples were diluted 50 times with H_2O before real-time PCR analysis.

5.3.10 Quantitative real-time PCR analysis (QRT-PCR). Primers and probes used for QRT-PCR analysis are listed in Table 5.2 and were designed using Primer Express software ver. 3 (Applied Biosystems, CA, USA). Reagents were purchased from Applied Biosystems and Kapa Biosystems (Cape Town, South Africa). QRT-PCR runs and collection of spectral data were performed with the 7500 cycler (Applied Biosystems). SYBR Green was used for the detection of *PDA1* and *FLO11* amplicons with final primer concentrations of 100 nM. Specific probes and primers were designed to differentiate between the cDNA species corresponding to the extensively homologous *FLO1* and *FLO5* genes. Probes were modified by the addition of a 3' minor groove binding (MGB) protein and non-fluorescent quencher, as well as the 5' attachment of fluorescent dyes as indicated in Table 5.2 (Applied Biosystems). Probe and primer concentrations in QRT-PCR reactions were 250 nM and 900 nM, respectively. Cycling conditions during QRT-PCR were as follows: 50˚C for 2 minutes, 95˚C for 10 minutes, 40 cycles of 95˚C for 15 seconds followed by 60˚C for 1 minute. When using SYBR Green, a dissociation curve analysis was included to verify amplicon authenticity. Preliminary data analyses were performed with Signal Detection Software (SDS) ver. 1.3.1. (Applied Biosystems). Individual QRT-PCR reaction runs were performed at least in duplicate. The relative expression value for each sample was defined as $2^{-Ct}_{(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data was normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample thus giving normalized relative expression for a target gene as $2^{-Ct}_{(target)}/2^{-Ct}_{(PDA1)}$. The highest mRNA expression level was arbitrarily set.

Table 5.2 Quantitative real-time PCR oligonucleotide primers and probes used in this study

Primer/probe name[a]	Primer/probe sequence (5'→3')	Modifications
FLO1-F (TaqmanMGB)	ATGCCTCATCGCTATATGTTTTTG	none
FLO1-R (TaqmanMGB)	GCTCCTGAGGCCACACTAGTTAG	none
FLO5-F (TaqmanMGB)	AGCACCACTAAAAAAAATGACAATTG	none
FLO5-R (TaqmanMGB)	GCCAGAAAGGCCAAGATTACC	none
FLO1-probe	CAGTCTTTACACTTCTGGC	6-FAM 5' label, 3' Minor Groove Binder/ Non-Fluorescent Quencher
FLO5-probe	ACCACTGCATATTTT	VIC dye 5' label, 3' Minor Groove Binder/ Non-Fluorescent Quencher
FLO11-F-(QRT-PCR)	CCTCCGAAGGAACTAGCTGTAATT	none
FLO11-R-(QRT-PCR)	AGTCACATCCAAAGTATACTGCATGAT	none
PDA1-F-QRT-PCR	GGAATTTGCCCGTCGTGTT	none
PDA1-R-QRT-PCR	GCGGCGGTACCCATACC	none

[a] F, forward primer and R, reverse primer.

5.3.11 Phase contrast microscopy. Yeast cell populations of BM45 wild type (A), BM45-F11H (B), VIN13 wild type (C) and VIN13-F11H (D) were harvested from lees on completion of Merlot red grape must fermentations. Cell populations at a density of 1×10^8 cells mL^{-1} in 100 mM EDTA contained in microcentrifuge tubes were resuspended by high-speed vortexing for 30 seconds. To promote aggregation, the microcentrifuge tubes were inverted five times. Cell suspensions were allowed to stand undisturbed for 30 minutes. Samples (10 µL) were transferred to glass slides with coverslips and viewed at a magnification of 60x, and photographed under phase contrast transillumination (green filter), at maximum intensity on an Olympus CKX41SF inverted microscope (Olympus Corporation, Tokyo, Japan).

5.3.12 Scanning Electron Microscopy (SEM). Samples of completely fermented red wines containing lees were homogenized, diluted and filtered through 0.22 µm Durapore® membrane filters (Millipore Corporation, MA, USA) and immediately frozen by plunging into sub-cooled nitrogen ('slush') prepared by placing liquid nitrogen under vacuum for several minutes. After about 10 seconds, each filter was removed from the cryogen and transferred without warming to a labeled well of a custom-made aluminium holder that was maintained at -196°C. Once all the samples were frozen, the holder was transferred to the stage of an Edwards freeze dryer (Edwards Vacuum Ltd, Sussex, UK)

pre-cooled to -60°C. A container with a thin layer of phosphorous pentoxide (Fluka, Steinheim, Germany) was placed in the chamber of the freeze dryer to assist in the drying process. The chamber was then evacuated below 10^{-2} Torr. The samples were kept frozen and under vacuum until all cellular water had sublimed (overnight) and gradually warmed to room temperature over a period of about 5 hours. Filters supporting the yeast samples were then removed from the freeze dryer and individually mounted on stubs for SEM observation using conductive carbon tape (Agar Scientific Ltd., Essex, UK). Stubs were rendered conductive by lightly sputter-coating with gold using a Polaron SC500 (Quorum Technologies Ltd., Sussex, UK). Samples were viewed with a LEO 1450 scanning electron microscope (Carl Zeiss, Jena, Germany) at 5 kV accelerating voltage and images were captured digitally.

5.3.13 Turbidimetric Analysis. Turbidity of the wines after racking was evaluated using a LP2000 turbidity meter (Hannah Instruments, Bedfordshire, UK). The turbidity meter was calibrated before use as detailed in the instruction manual. Bottled-wines (5 per replicate fermentation) were allowed to stand undisturbed for 5 days after racking. Thereafter, a 10 mL aliquot was removed from below the meniscus from each bottle and dispensed down the inside of a clean cuvette to avoid the formation of air bubbles. All measurements were taken with samples equilibrated to room temperature. The turbidity of wines is presented as Formazine Turbidity Units (FTU). Values reported in this study reflect the mean of experiments performed in triplicate (5 measurements per replicate) and error bars represent standard deviations.

5.3.14 Statistical Analysis. In this study, one-way analysis of variance (ANOVA) was employed to statistically compare data obtained for BM45 and VIN13 wild type strains to that of transgenic yeast strains. Analyses were performed using the statistical software package GraphPad InStat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego California).

5.4   RESULTS

5.4.1 Flocculation and fermentation profiles in MS300 medium. At the end of MS300 batch fermentations, the flocculent ability of BM45 and VIN13 wild type wine yeast strains and their transgenic derivatives were determined (Fig. 5.1). The results clearly illustrate that *HSP30p* driven expression of *FLO1* and *FLO5* in transgenic wine yeast strains yielded flocculent phenotypes. The flocculent phenotypes produced by BM45-F1H, BM45-F5H, VIN13-F1H and VIN13-F5H transformants in MS300 were similar to those described earlier in nutrient-rich YEPD medium (Chapter 4, Fig. 4.9). The above reinforces our earlier findings in this study that *FLO5* wine yeast transformants are more flocculent than their corresponding *FLO1* transgenic wine yeast strains (Section 4.4.3). Transgenic wine yeast strains containing *FLO1* or *FLO5* under the transcriptional control of *ADH2p* failed to generate flocculent phenotypes (Fig. 5.1). Since it was reported that Adh2 was found in aerobically grown yeast cells (Thomson *et*

al., 2005; Wills, 1976), the flocculent potential of BM45-F1A, BM45-F5A, VIN13-F1A and VIN13-F5A in MS300 medium was also assessed aerobically using shake-flask experiments. However, no flocculent phenotypes were displayed by BM45 and VIN13 transgenic yeast strains. This is quite surprising, especially since BM45-F1A, BM45-F5A, VIN13-F1A and VIN13-F5A yielded distinctly stronger flocculent phenotypes in nutrient-rich YEPD (Chapter 4, Fig. 4.9) than their comparable *HSP30p* wine yeast transformants. Congruent to earlier observations (Section 4.4.3), *ADH2p* or *HSP30p* regulated expression of *FLO11* in BM45 and VIN13 transgenic strains (Fig. 5.1) did not produce flocculent phenotypes under MS300 fermentation conditions. In addition, BM45 and VIN13 *FLO11* transgenic strains that were cultivated in MS300 medium containing either pectin or diatomaceous earth also showed no flocculation (data not shown).
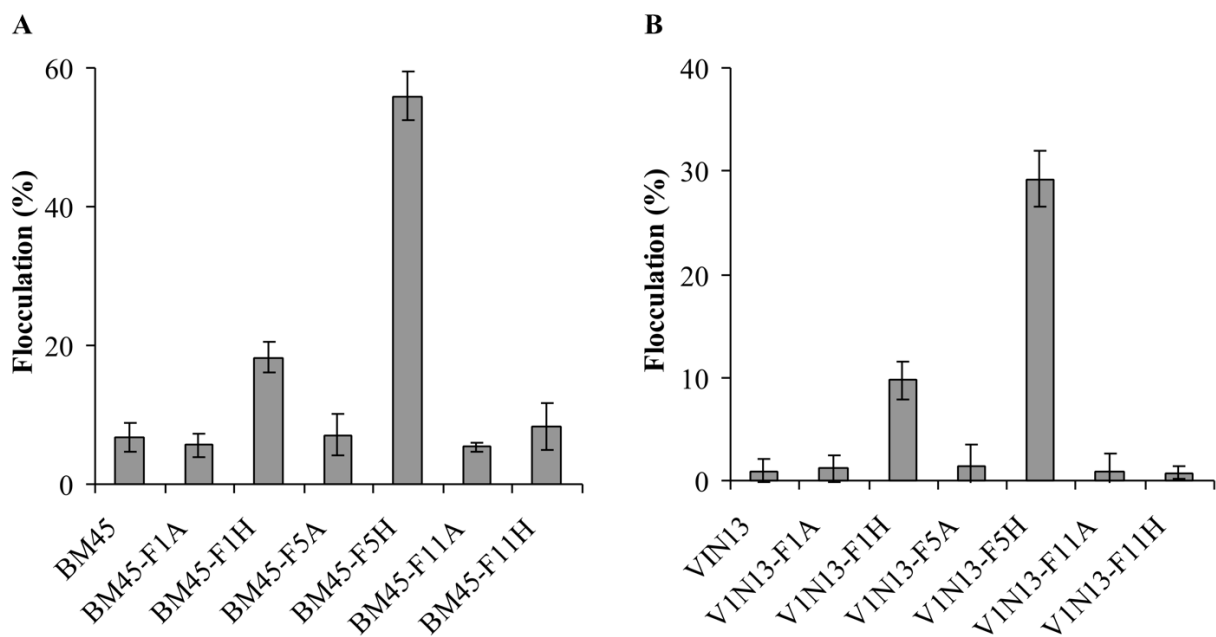
Figure 5.1   Flocculation of (A) BM45 and (B) VIN13 wild types and their transgenic strains on completion of fermentation in synthetic defined medium (MS300). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

Since *HSP30p* wine yeast transformants exclusively displayed flocculent phenotypes, only data pertaining to their MS300 fermentation profiles are presented. There were no significant differences ($p > 0.05$) observed in sugar utilization abilities of BM45 and VIN13 wild type wine yeast strains in contrast their *HSP30p* transgenic descendants (Fig. 5.2). However, glucose was consumed at a faster rate than fructose in all strains, thereby confirming the glucophilic status of the *S. cerevisiae* (Luyten *et al.*, 2002), which is attributable to differences in the affinities of hexose transporters for these sugars (Boles and Hollenberg, 1997; Reifenberger *et al.*, 1997). The fermentation profiles in terms of CO_2 released (fermentor weight loss) were similar for all strains that were evaluated (data not shown). Moreover, no significant differences ($p > 0.05$) were observed in the abilities of BM45 and VIN13 wild type wine yeast strains in comparison

to their *HSP30p* transgenic descendants to produce ethanol (Fig. 5.2), glycerol [BM45 and its transgenic strains (~7.57 gL^{-1}); VIN13 and its transgenic strains (~5.99 gL^{-1})] (data not shown) and biomass (Fig. 5.3).
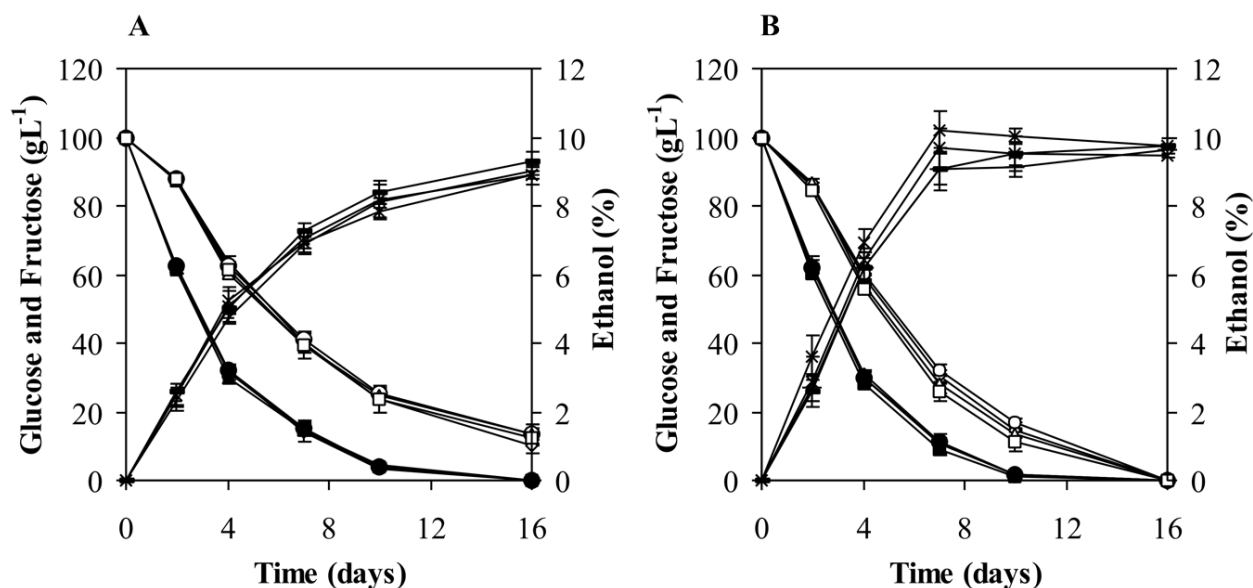
Figure 5.2   Fermentation profile of (A) BM45 and (B) VIN13 wild types and their transgenic *HSP30p* descendants in defined synthetic must (MS300). Glucose utilization by BM45 and VIN13 wild types (◆); BM45-F1H and VIN13-F1H (●); BM45-F5H and VIN13-F5H (▲); BM45-F11H and VIN13-F11H (■) strains. Fructose consumption by BM45 and VIN13 wild types (◇); BM45-F1H and VIN13-F1H (○); BM45-F5H and VIN13-F5H (△); BM45-F11H and VIN13-F11H (□) strains. Ethanol production by BM45 and VIN13 wild types (−); BM45-F1H and VIN13-F1H (ı); BM45-F5H and VIN13-F5H (✳), BM45-F11H and VIN13-F11H (×) strains.
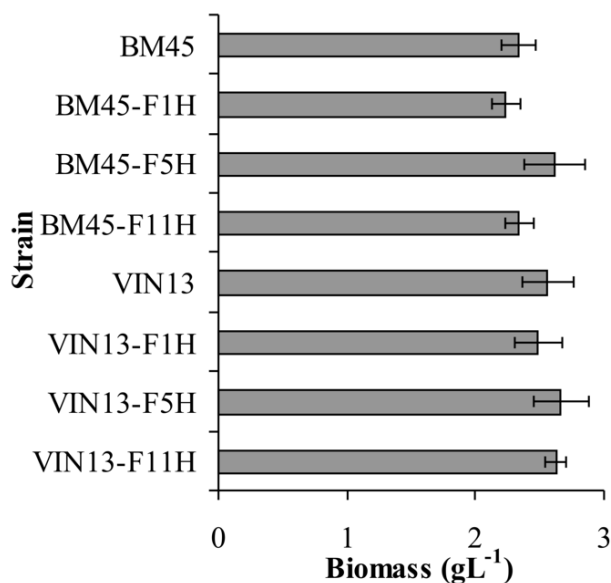
Figure 5.3   Biomass produced by BM45 and VIN13 wild types and their *HSP30p* transgenic strains on completion of fermentation using in defined synthetic must (MS300). The results are averages of three independent determinations, and error bars represent standard deviations

Furthermore, GC monitoring of volatile components at the end of MS300 batch fermentations also revealed no significant ($p > 0.05$) differences in all components analyzed for BM45 and VIN13 wild type wine yeast strains in comparison to their *HSP30p* transgenic derivatives (Tables 5.3 and 5.4).

Table 5.3 Volatile components in wines produced from chemically defined synthetic grape must (MS300) with BM45 wild type strain and its transgenic descendants

Volatile Component	Strain			
(mg L^{-1})	BM45	BM45-F1H	BM45-F5H	BM45-F11H
2-Phenyl Ethanol	12.10	11.28	11.57	11.27
2-Phenylethyl Acetate	0.35	0.28	0.36	0.30
Acetic Acid	1744.89	1752.31	1716.66	1666.59
Decanoic Acid	1.82	1.94	1.58	1.78
Diethyl Succinate	0.04	0.02	0.01	0.03
Ethyl Acetate	104.93	104.18	102.65	95.81
Ethyl Caprate	0.35	0.30	0.29	0.26
Ethyl Hexanoate	1.42	1.42	0.84	0.00
Ethyl Lactate	7.96	7.81	6.83	7.24
Hexanoic Acid	0.84	0.84	0.59	0.81
Isoamyl Acetate	0.39	0.38	0.33	0.33
Isoamyl alcohol	53.75	53.96	50.96	51.13
Isobutanol	36.52	48.26	43.65	44.22
Iso-Butyric Acid	0.73	0.77	0.76	0.63
Octanoic Acid	0.07	0.05	0.35	0.05
Propanol	22.82	18.45	19.58	20.09
Propionic Acid	3.56	3.08	3.04	3.54

No statistically significant differences were observed in comparison to the parental BM45 wild type strain.

Table 5.4 Volatile components in wines produced from chemically defined synthetic grape must (MS300) with VIN13 wild type strain and its transgenic descendants

Volatile Component	Strain			
(mg L^{-1})	VIN13	VIN13-F1H	VIN13-F5H	VIN13-F11H
2-Phenyl Ethanol	10.33	11.19	11.25	9.71
2-Phenylethyl Acetate	0.03	0.12	0.02	0.04
Acetic Acid	1094.84	992.08	1071.18	1096.94
Butyric Acid	0.26	0.29	0.25	0.27
Decanoic Acid	2.09	2.35	1.78	1.95
Diethyl Succinate	0.11	0.15	0.18	0.15
Ethyl Acetate	87.71	84.97	102.21	100.15
Ethyl Caprate	0.25	0.27	0.16	0.27
Ethyl Caprylate	0.02	0.03	0.02	0.03
Ethyl Hexanoate	1.45	1.45	1.34	1.47
Ethyl Lactate	8.36	7.83	9.86	9.17
Hexanoic Acid	1.04	1.06	0.96	1.10
Isoamyl Acetate	0.37	0.38	0.44	0.43
Isoamyl alcohol	48.13	54.08	60.03	64.08
Isobutanol	29.14	29.60	26.55	32.19
Iso-Butyric Acid	0.39	0.53	0.41	0.63
Octanoic Acid	0.53	0.62	0.44	0.55
Propanol	57.52	55.95	60.32	63.03
Propionic Acid	3.44	3.47	4.26	3.69

No statistically significant differences were observed in comparison to the parental VIN13 wild type strain.

5.4.2 *FLO* gene transcription in MS300 medium. The gene expression profiles of *FLO1*, *FLO5* and *FLO11* as mediated by either *ADH2p* or *HSP30p* in synthetic MS300 wine fermentations were evaluated. The total RNA of BM45, BM45-F1A, BM45-F1H, BM45-F5A, BM45-F5H, BM45-F11A and BM45-F5H cultures was processed from different growth phases corresponding to the exponential phase (Day 2), entry into stationary phase (Day 4) and late stationary phase (Day 10) and quantitative real-time PCR was performed. It is clearly evident (Fig. 5.4) that *HSP30p* is tightly repressed in the exponential phase of growth, whilst some expression is observed on entry into the stationary and strongly increased RNA levels in late stationary phases. Interestingly, an approximately 10-fold higher *HSP30p* induction of *FLO11* is observed in the late stationary phase of yeast growth in comparison to *FLO5*, whilst the intensity of *FLO5* transcripts was approximately 10-fold higher than *HSP30p* induced *FLO1* transcripts. Moreover, the data confirm that only the *FLO* gene carrying a modified promoter is activated, and that the other two genes that were monitored in parallel, do not contribute

to the observed phenotypes. In contrast, the activity of *ADH2p* seems to be tightly repressed at all phases of growth. These transcription levels are well aligned with the adhesion phenotypes observed in these strains (Fig. 5.1A).
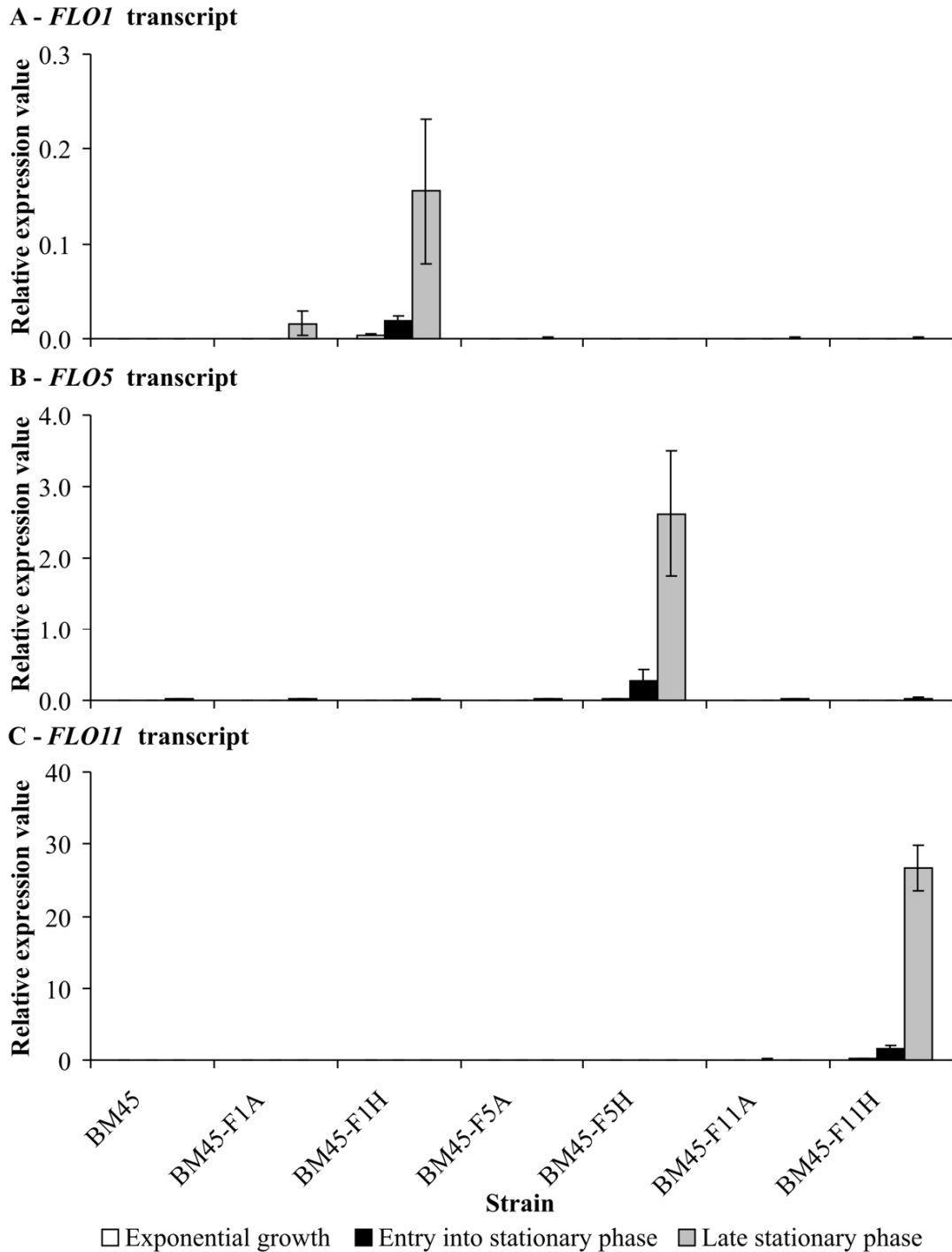
FIGURE 5.4   QRT-PCR relative expression of (A) *FLO1*, (B) *FLO5* and (C) *FLO11* genes in BM45 wild type and its *HSP30p* transformants at different stages of wine fermentation on defined synthetic must (MS300). Samples were taken from sampling points corresponding to exponential growth phase (white), entry into stationary growth phase (black) and late stationary phase (grey). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

5.4.3 The effect initial glucose substrate concentration on *ADH2p* controlled expression of *FLO5* encoded flocculins in BM45-F5A and VIN13-F5A transgenic strains. Cunha and coworkers (2006) showed that a commercial baking yeast strain (Fleischmann) transformed with an integrative cassette containing the *FLO1* ORF under transcriptional control of a modified *ADH2* promoter was capable of conditional flocculation that coincided with the depletion of glucose when cultivated in YEPD containing 200 gL^{-1} glucose. In addition, protein expression mediated by *ADH2p* in complex nutrient-rich medium was reported to be stronger than that observed in selective medium (Govender *et al.*, 2008; Lee and DaSilva, 2005). Considering the aforementioned research studies, the flocculent potential of BM45-F5A and VIN13-F5A transgenic strains was evaluated aerobically in shake-flask experiments using YEPD containing increasing glucose substrate concentrations. As shown in Fig. 5.5. The flocculent ability of the VIN13-F5A transformant was consistent until 30 gL^{-1} glucose whilst it was extended to 50 gL^{-1} glucose for the BM45-F5A transformant. Thereafter the results clearly demonstrate that the flocculent potential of both transformants decreases as the initial glucose substrate concentration increases. The VIN13-F5A transformant displays no flocculence at substrate concentrations from 150 gL^{-1} onwards whilst the BM45-F5A transgenic strain shows no flocculation at a glucose substrate concentration of 200 gL^{-1}. These observations are surprising and contradictory to that of Cunha *et al.* (2006) mentioned above and may suggest strain-specific transcriptional regulation of *ADH2p*. Although Cunha and coworkers (2006) modified the native *ADH2* promoter to eliminate significant basal expression in the presence of higher glucose concentrations, they may have inadvertently fine-tuned the *ADH2* promoter to operate efficiently under high glucose concentrations.
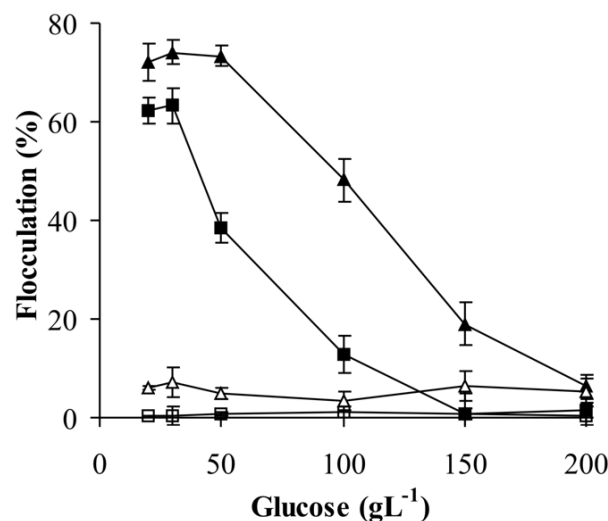
Figure 5.5   The effect of initial glucose substrate concentration on the flocculent ability of BM45-F5A and VIN13-F5A transgenic yeast strains. Flocculation of BM45 wild type (△); BM45-F5A (▲); VIN13 wild type (□) and VIN13-F5A (■) strains. Yeast strains were batch-cultured in YEPD containing increasing glucose concentrations (20, 30, 50, 100, 150 and 200 gL^{-1}) at 30°C with shaking (160 rpm) until 24 h post-glucose exhaustion. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

5.4.4 Yeast strain verification and fermentation profiles of red wine vinifications.
Using a microsatellite PCR strain typing method that targets delta (δ) sequences
confirmed that alcoholic red wine fermentations were performed by the inoculated BM45
and VIN13 wild type wine yeast strains. In addition, using a screening system that
incorporated sensitivity to SM; flocculation ability (*FLO1* and *FLO5* transformants);
increased invasiveness (*ADH2p-FLO11* transformants) or lack of invasiveness
(*HSP30p-FLO11* transformants) confirmed that alcoholic fermentations were performed
by the inoculated transgenic wine yeast strain. This is consistent with a previous study
that reported the addition of a pure wine yeast culture to red wine vinifications induced a
clear predominance of inoculated strain (Beltran *et al.*, 2002).

Since only *HSP30p* wine yeast transformants displayed flocculent phenotypes
(Fig. 5.7), only data pertaining to their red wine fermentation profiles are presented. No
significant differences (*p* > 0.05) were observed in the abilities of BM45 and VIN13 wild
type wine yeast strains in comparison to their *HSP30p* transgenic descendants to utilize
sugars; produce ethanol (Fig. 5.6) and succinic acid [BM45 and its transgenic strains
(~0.88 gL^{-1}); VIN13 and its transgenic strains (~1.01 gL^{-1})].
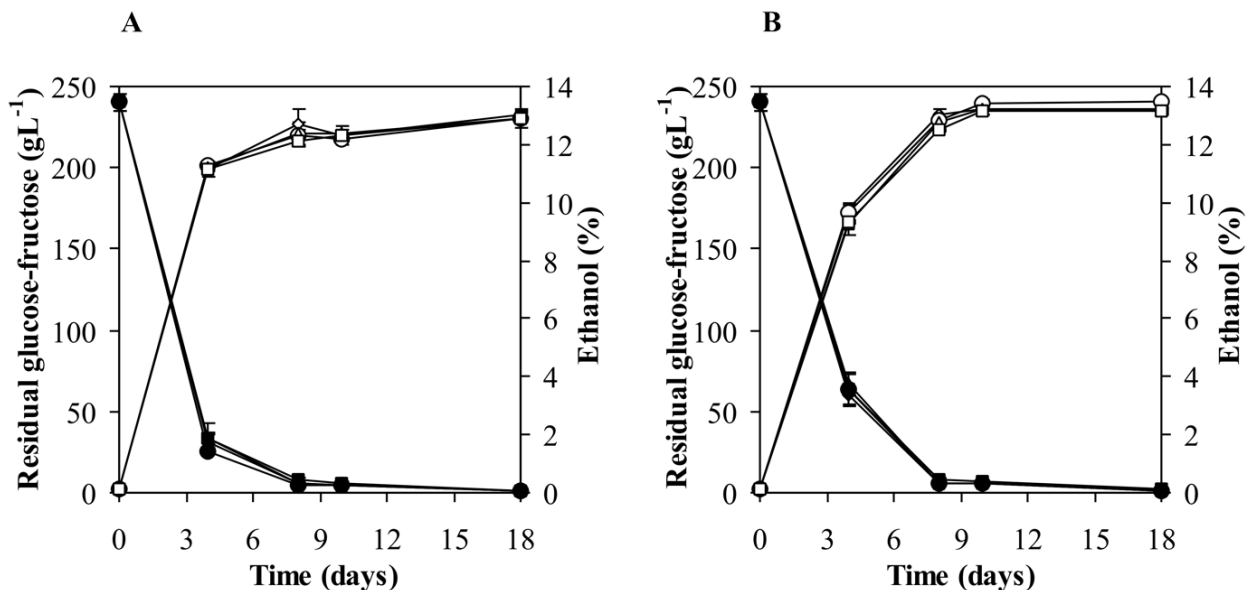
FIGURE 5.6 Fermentation profiles of (A) BM45 and (B) VIN13 wild types and their transgenic *HSP30p*
descendants in Merlot red grape must. Glucose-fructose utilization by BM45 and VIN13 wild types (◆);
BM45-F1H and VIN13-F1H (●); BM45-F5H and VIN13-F5H (▲); BM45-F11H and VIN13-F11H (■)
strains. Ethanol production by BM45 and VIN13 wild types (◇); BM45-F1H and VIN13-F1H (○);
BM45-F5H and VIN13-F5H (△); BM45-F11H and VIN13-F11H (□) strains.

Moreover, with the exception of decreased acetic acid production by BM45-F11H
and VIN13-F11H (~1.3 and ~1.5-fold reduction respectively), GC monitoring of volatile
components at the end of alcoholic fermentations revealed no significant (*p* > 0.05)
differences in all components analyzed for BM45 wild type wine yeast strains in
comparison to their *HSP30p* transgenic derivatives (Table 5.5 and 5.6). In addition, no

significant differences were observed in all components analyzed with FT-IR in red wines produced with BM45 and VIN13 transgenic yeast strains (Table 5.7 and 5.8). It may thus be suggested that *HSP30p* controlled expression of *FLO1*, *FLO5* or *FLO11* in BM45 and VIN13 transgenic yeast strains has seemingly no deleterious effect on the fermentative potential of these transgenic strains.

Table 5.5 Volatile components in wines produced from Merlot grape must with BM45 wild type strain and its transgenic strains

Volatile Component	Strain			
(mg L^{-1})	BM45	BM45-F1H	BM45-F5H	BM45-F11H
2-Phenyl Ethanol	68.77	68.92	67.77	63.93
2-Phenylethyl Acetate	0.02	0.02	0.02	0.01
Acetic Acid	405.14	376.87	367.61	***317.34**
Butanol	1.33	1.22	1.33	1.15
Butyric Acid	1.29	1.24	1.17	1.20
Decanoic Acid	0.27	0.27	0.28	0.28
Diethyl Succinate	2.49	2.67	2.29	2.52
Ethyl Acetate	56.60	52.75	52.26	44.51
Ethyl Butyrate	0.06	0.07	0.05	0.07
Ethyl Caprylate	0.00	0.00	0.01	0.06
Ethyl Hexanoate	0.17	0.21	0.19	0.24
Ethyl Lactate	7.53	7.36	6.97	7.26
Hexanoic Acid	1.16	1.19	1.12	1.14
Hexanol	1.20	1.29	1.08	1.21
Isoamyl Acetate	1.98	1.98	1.85	2.06
Isoamyl alcohol	392.61	409.27	384.47	404.93
Isobutanol	112.34	126.77	104.18	117.75
Iso-Butyric Acid	3.87	4.26	3.57	4.10
Iso-Valeric Acid	0.31	0.26	0.26	0.25
Methanol	128.50	128.34	121.70	111.77
Octanoic Acid	1.22	1.26	1.22	1.21
Propanol	37.82	35.72	33.79	30.62
Propionic Acid	1.86	1.81	1.67	1.78
Valeric Acid	0.53	0.44	0.35	0.37

*Statistically significant differences in comparison to the parental BM45 wild type strains.

Table 5.6   Volatile components in wines produced from Merlot grape must with VIN13 wild type strain and its transgenic strains.

Volatile Component	Strain			
(mg L^{-1})	VIN13	VIN13-F1H	VIN13-F5H	VIN13-F11H
2-Phenyl Ethanol	110.50	116.41	110.90	100.05
2-Phenylethyl Acetate	0.02	0.04	0.06	0.03
Acetic Acid	104.01	96.69	100.97	***70.26**
Butanol	1.96	2.07	1.74	1.43
Butyric Acid	1.07	1.14	1.11	0.94
Decanoic Acid	0.20	0.21	0.27	0.39
Diethyl Succinate	3.14	3.42	3.60	3.25
Ethyl Acetate	49.24	44.83	46.18	45.11
Ethyl Butyrate	0.02	0.04	0.04	0.08
Ethyl Caprylate	0.00	0.00	0.09	0.11
Ethyl Hexanoate	0.17	0.23	0.28	0.31
Ethyl Lactate	6.03	6.36	5.81	7.04
Hexanoic Acid	0.88	1.00	0.99	0.84
Hexanol	1.47	1.78	1.84	1.84
Isoamyl Acetate	1.04	1.08	1.15	1.13
Isoamyl alcohol	467.78	461.79	484.77	489.15
Isobutanol	62.14	55.65	59.39	64.01
Iso-Butyric Acid	2.40	2.29	2.29	2.75
Iso-Valeric Acid	0.29	0.31	0.61	0.34
Methanol	163.21	161.96	158.43	151.66
Octanoic Acid	0.91	1.01	1.11	1.02
Propanol	68.71	67.95	65.16	58.23
Propionic Acid	1.99	1.98	1.86	1.54
Valeric Acid	0.20	0.22	0.22	0.17

*Statistically significant differences in comparison to the parental VIN13 wild type strain.

Table 5.7 FT-IR analysis of oenological factors of Merlot red wines produced by BM45 wild type and its transgenic strains

Factor	Strain			
	BM45	BM45-F1H	BM45-F5H	BM45-F11H
pH	3.53	3.57	3.54	3.51
Volatile acids (gL^{-1})	0.39	0.38	0.38	0.34
Total acids (gL^{-1})	6.90	6.66	6.75	6.71
Malic acid (gL^{-1})	2.49	2.47	2.49	2.46
Lactic acid (gL^{-1})	0.30	0.33	0.28	0.29
Glucose (gL^{-1})	0.58	0.61	0.55	0.59
Fructose (gL^{-1})	0.84	0.83	0.89	0.87
Glycerol (gL^{-1})	9.67	9.37	9.38	9.41
Ethanol [% (v/v)]	12.79	12.65	12.71	12.69

No statistically significant differences were observed in comparison to the parental BM45 wild type strain.

Table 5.8 FT-IR analysis of oenological factors of Merlot red wines produced by VIN13 wild type and its transgenic derivatives

Factor	Strain			
	VIN13	VIN13-F1H	VIN13-F5H	VIN13-F11H
pH	3.46	3.49	3.48	3.42
Volatile acids (gL^{-1})	0.18	0.15	0.16	0.13
Total acids (gL^{-1})	7.02	7.07	7.11	6.97
Malic acid (gL^{-1})	2.61	2.80	2.89	2.67
Lactic acid (gL^{-1})	0.15	0.22	0.11	0.16
Glucose (gL^{-1})	0.72	0.79	0.73	0.84
Fructose (gL^{-1})	0.80	0.80	0.84	1.11
Glycerol (gL^{-1})	9.40	9.78	9.58	9.35
Ethanol [% (v/v)]	13.01	13.28	12.95	12.98

No statistically significant differences were observed in comparison to the parental BM45 wild type strain.

5.4.5 Flocculation in red wine fermentations. At the end of alcoholic red wine fermentations, the flocculent ability of BM45 and VIN13 wild type wine yeast strains and their transgenic derivatives were determined (Fig. 5.7). The transgenic wine yeast strains containing *FLO1*, *FLO5* or *FLO11* under the transcriptional control of *ADH2p* failed to generate flocculent phenotypes (Fig. 5.7) as was noted in MS300 fermentations (Fig. 5.1). The flocculent phenotypes produced by BM45-F1H, BM45-F5H, VIN13-F1H and VIN13-F5H transformants in red wine fermentations were similar to those described earlier in nutrient- rich YEPD medium (Section 4.4.3) and MS300 fermentations (Fig. 5.1). Astonishingly, *HSP30p* driven expression of *FLO11* in both BM45-F11H and VIN13-F11H strains yielded strong flocculent phenotypes that displayed both Ca^{2+}-dependent (Fig. 5.7) and Ca^{2+}-independent adhesion characteristics (Fig. 5.8). Moreover, the adhesion phenotypes displayed by both BM45-F11H and VIN13-F11H transgenic strains were not inhibited in the presence of either 1 M glucose or 1 M mannose (Fig. 5.9). Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (Stratford and Assinder, 1991), this result clearly demonstrates that *FLO11* transgenic wine yeast encoded flocculins exhibit neither Flo1-type nor NewFlo-type flocculation. This finding is supported by a previous study that reported that *FLO11* overexpression in *S. cerevisiae* strain Σ1278b promotes weak calcium-independent flocculation that is not inhibited by either glucose or mannose (Guo *et al.*, 2000). However, it should be noted that Bayly *et al.* (2005) presented evidence that *FLO11* encoded flocculin yielded a strongly flocculent Flo1 phenotype in untransformed *S. cerevisiae* strain YIY345. It was also reported that over-expression in haploid laboratory *S. cerevisiae* strains S288C and FY23 does not promote cell to cell adhesion (Govender *et al.*, 2008; Verstrepen and Klis, 2006). It therefore may be suggested that *FLO11* flocculation is variable and strain-dependent.

The ability of free-cell populations of BM45-F11H and VIN13-F11H, although suspended in 100 mM EDTA to re-aggregate spontaneously after mechanical agitation (Fig. 5.10), further confirms that the *FLO11* phenotype displayed under red wine-making conditions is indeed a *bona fide* flocculent phenotype. This clearly differentiates the flocculent phenotype from the formation of mating aggregates or chain formation that could also give clumps of yeast cells that cannot re-aggregate after mechanical agitation (Stratford, 1992). The phenotypes displayed by *HSP30* transgenic yeast derivatives of BM45 and VIN13 were also confirmed in small-scale (3 L) red wine fermentations (data not shown) using Cabernet Sauvignon and Petit Verdot grape varietals.
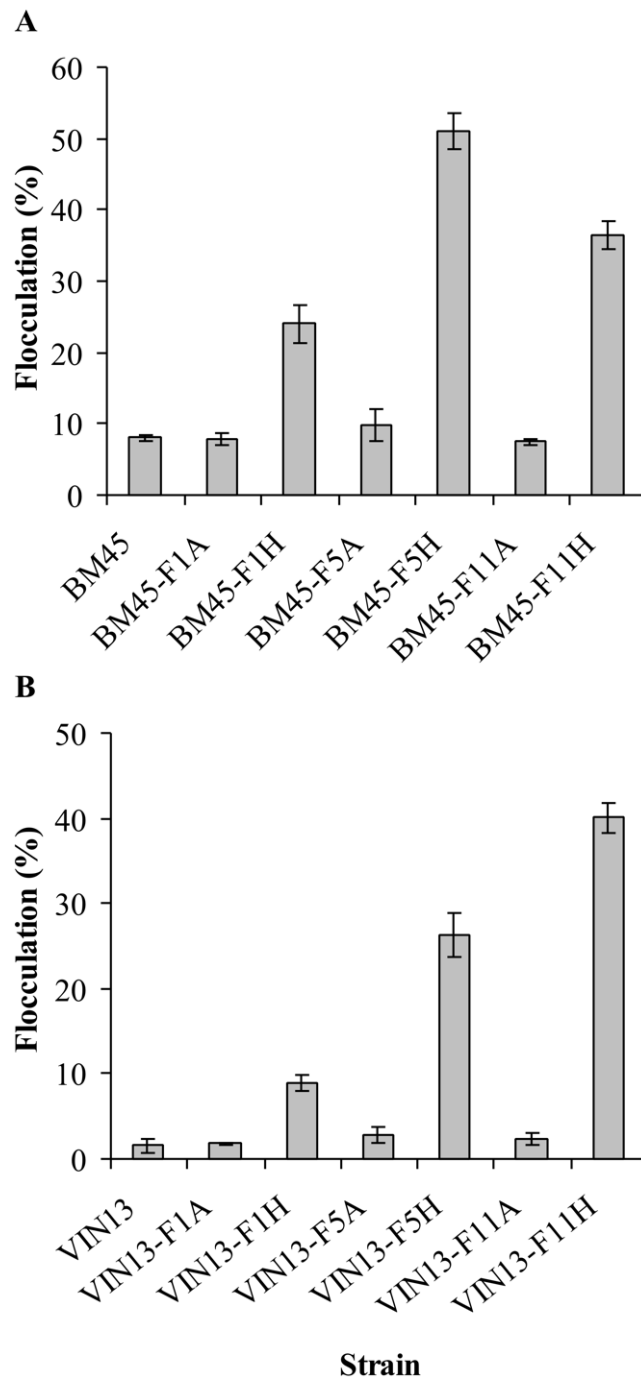
FIGURE 5.7   Flocculation of (A) BM45 and (B) VIN13 wild types and their transgenic strains on completion of fermentation in Merlot red grape must. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.
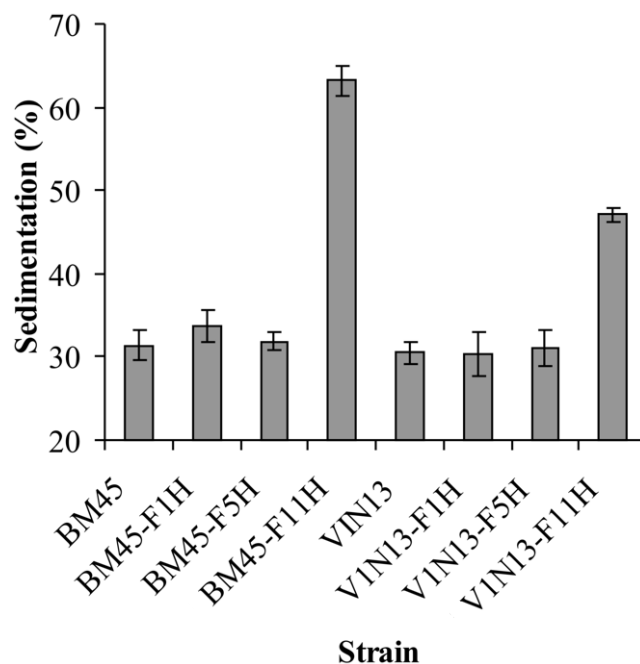
FIGURE 5.8 Ca^{2+}-independent sedimentation or flocculation of BM45 and VIN13 wild types and their transgenic *HSP30p* descendants on completion of fermentation on Merlot red grape must. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.
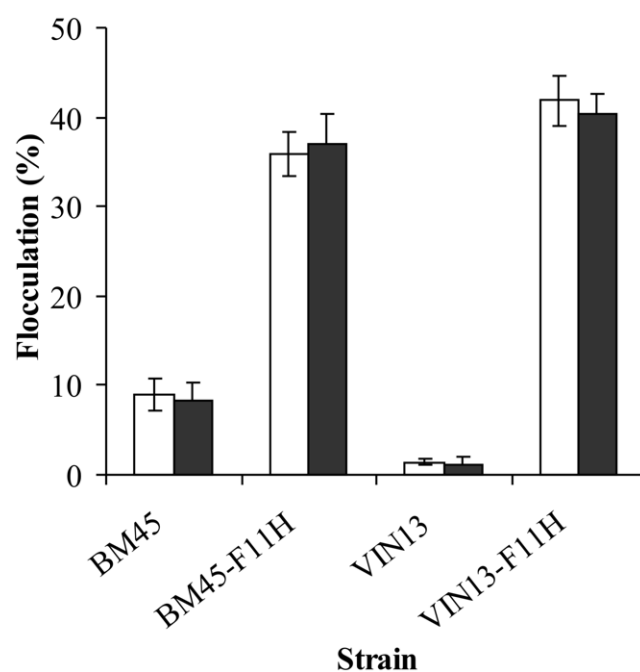
FIGURE 5.9 The effect of 1 M glucose (□) or 1 M mannose (■) on the flocculation of BM45-F11H and VIN13-F11H strains that were harvested from completed Merlot red grape must fermentation. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

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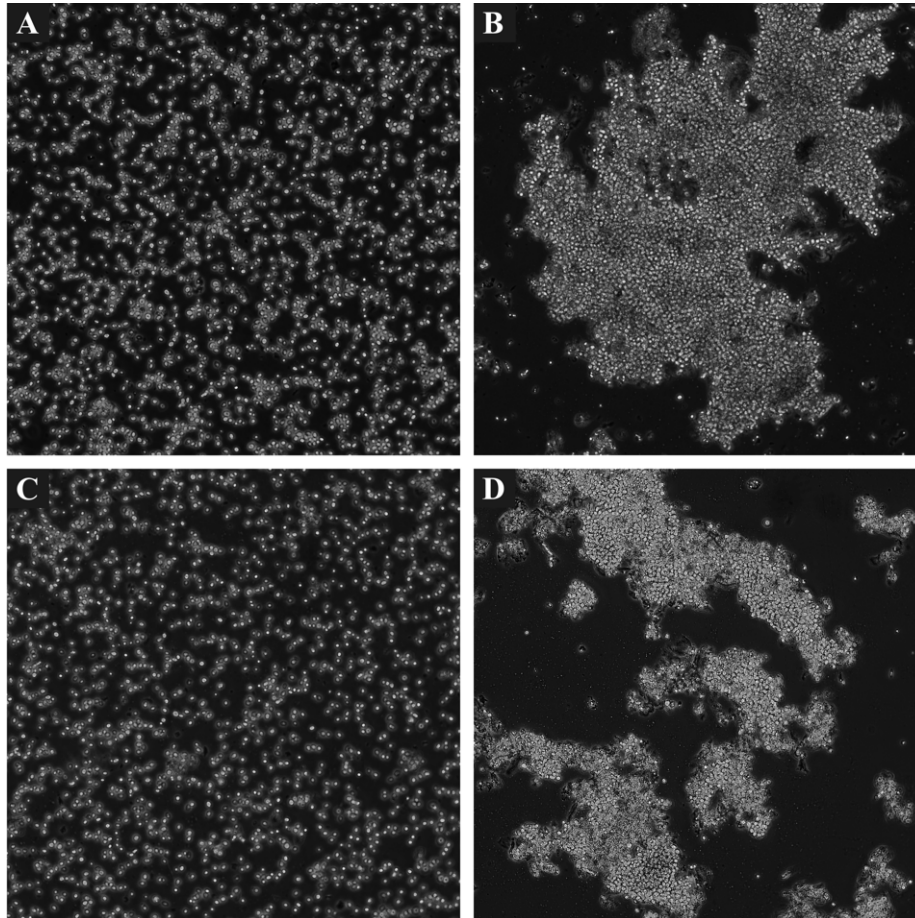
FIGURE 5.10 Microscopic demonstration that *HSP30p* controlled expression of *FLO11* encoded flocculins promotes yeast cellular interactions into 2-dimensional sheet aggregates. Yeast cell populations of BM45 wild type (A), BM45-F11H (B), VIN13 wild type (C) and VIN13-F11H (D) in 100 mM EDTA after aggregation, were photographed under phase contrast transillumination (green filter) at maximum intensity on an Olympus CKX41 inverted microscope (x 60 magnification).

5.4.6 Macroscopic and microscopic evaluation of lees. Red wines fermented with the BM45 and VIN13 wild type strains and BM45-F1H; BM45-F11H; VIN13-F1H; VIN13-F5H and VIN13-F11H transgenic strains generated lees fractions with slurry-like consistencies. In contrast, the BM45-F5H transgenic strain yielded very compacted lees fractions (lees was in the form of a slab), thereby promoting a greater volume recovery of fermented wine product. This improvement has financial cost-saving implications and can be directly attributed to the superior flocculent ability of the BM45-F5H transgenic strain. Although BM45-F5H displayed strongest calcium-dependent flocculation (Fig. 5.7B), its lees fraction together with those from BM45-F1H, VIN13-F1H and VIN13-F5H transgenic strains were observed to sediment at similar rates as those of their wild type parental strains. On the contrary, lees components from wines fermented with BM45-F11H and VIN13-F11H transgenic strains were observed to sediment at markedly faster rates that those of BM45 and VIN13 wild type strains (Fig. 5.11).
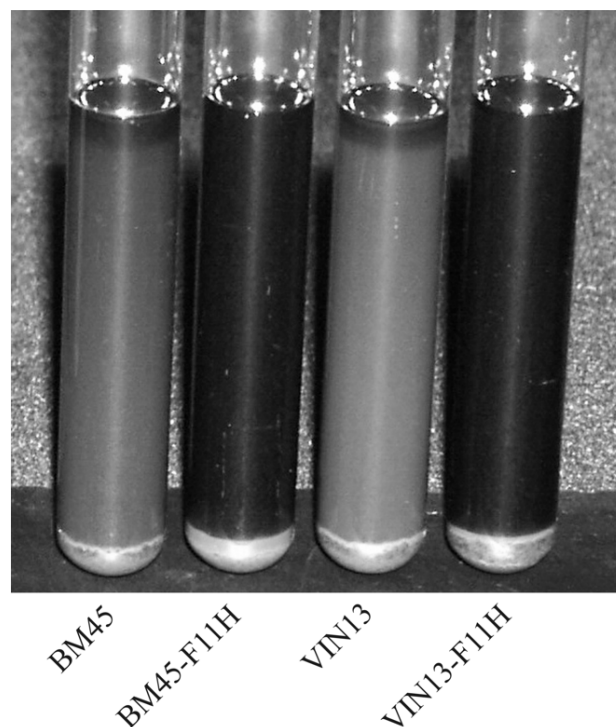
FIGURE 5.11 *HSP30p* controlled expression of *FLO11* encoded flocculins in BM45-F11H and VIN13-F11H effects rapid settling of Merlot red wine lees. Completely fermented red wines containing lees were homogenized before racking and 10 mL aliquots were dispensed into 16 x 165 mm glass test tubes. Samples were thoroughly agitated by vortexing for 30 seconds and allowed to stand undisturbed 30 min before *in situ* photography.

Scanning electron microscopy (Fig. 5.12) of lees clearly illustrates the presence of BM45-F11H and VIN13-F11H transformants co-aggregating with amorphous and crystalline solids. A similar interaction was not evident in images of BM45-F5H, VIN13-F5H and their wild type parental strains. The above mentioned co-aggregation phenomenon that is unique to *FLO11* transformants provides a possible reason for the faster rate of sedimentation of lees in wines fermented with *FLO11* transgenic yeast strains. The interaction with amorphous and crystalline solids would create bridges between cells, leading to aggregation and a dramatically increased weight of aggregates thereby promoting faster sedimentation. A qualitative cold stabilization procedure (filtered wine samples were stored at 4°C) showed that filtered red wines fermented with *FLO11* transgenic yeast strains contained significantly reduced to no red stained crystal deposits (potassium bitartrate crystals). Thus the crystalline solids associated with *FLO11* transformants (Fig. 12) may be cautiously identified as potassium bitartrate crystals. It may be speculated that the amorphous solids could be small insoluble pectic substances, and bitartrate crystals may also play a role. The above attributes (results not shown) of BM45-F11H and VIN13-F11H strains were also confirmed in small-scale (3 L) red wine fermentations using Cabernet Sauvignon and Petit Verdot grape varietals.
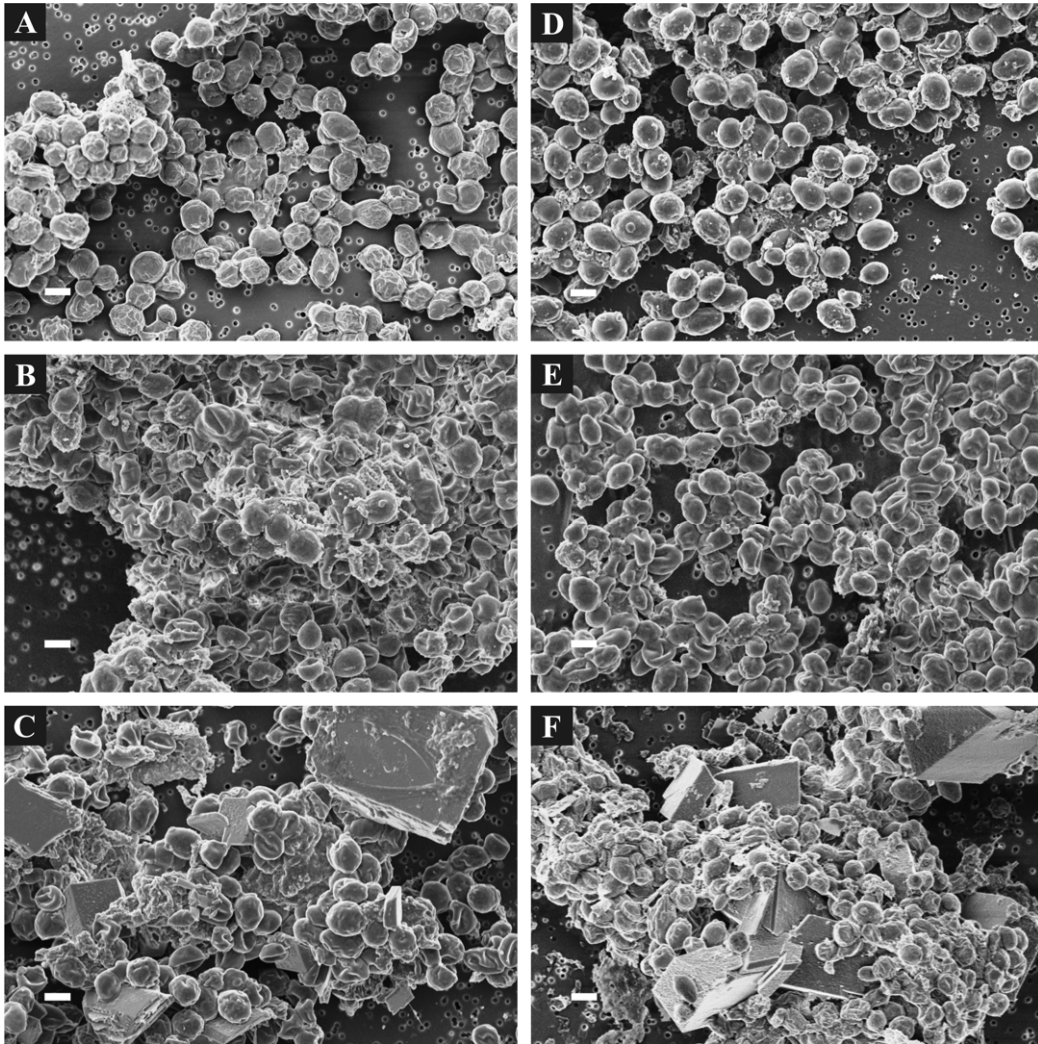
FIGURE 5.12 Scanning electron micrographs of lees from completed red wine fermentations (before racking) illustrating the exclusive co-aggregation of *HSP30p-FLO11* wine yeast transformants with other amorphous and crystalline lees components. (A) BM45 wild type; (B) BM45-F5H; (C) BM45-F11H; (D) VIN13 wild type; (E) VIN13-F5H and (F) VIN13-F11H strain. Scale bars represent 4 μm.

5.4.7 Turbidimetric analysis. Turbidimetric analysis indicated that red wines fermented with *FLO11* transgenic yeast strains are significantly (*p* < 0.05) less turbid than other wines produced in this study (Fig. 5.13). Comparatively, the BM45 wild type and its transgenic derivatives yielded substantially clearer wines than those fermented using VIN13 wild type and its transgenic strains. In comparison to their wild type parental strains wines produced with BM45-F11H and VIN13-F11H transformants displayed reductions in turbidity of 16% and 33% respectively. Small-scale (3 L) wine fermentations using Cabernet Sauvignon and Petit Verdot grape varietals (data not shown) confirmed the ability of BM45-F11H and VIN13-F11H to yield less turbid wines.
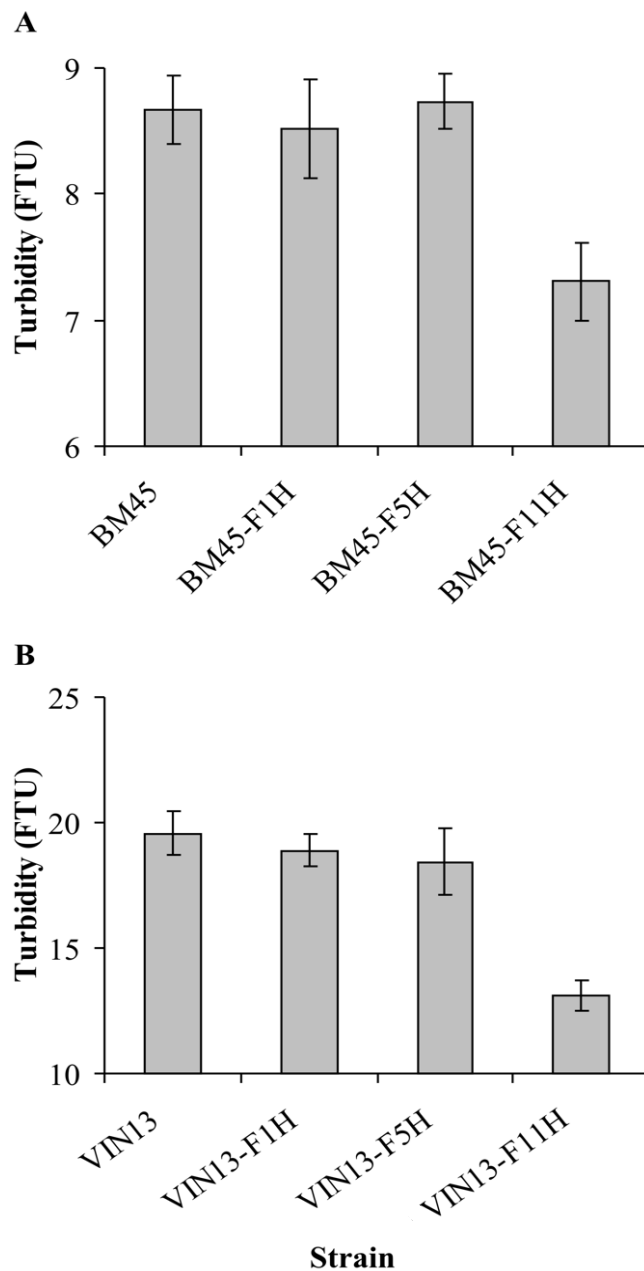
FIGURE 5.13 Turbidimetric analysis of red wines produced by (A) BM45 and (B) VIN13 wild types and their *HSP30p* transgenic strain derivatives. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

5.5   DISCUSSION

The suitability of *ADH2* and *HSP30* promoters to specifically drive stationary-phase expression of dominant *FLO* genes in both laboratory and industrial yeast genetic backgrounds cultivated in nutrient-rich standard laboratory medium (YEPD) has been previously demonstrated in (Section 4.4.3) and in other recent research studies (Cunha *et al.*, 2006; Govender *et al.*, 2008; Wang *et al.*, 2008). To date, a solitary study by Verstrepen *et al.* (2001) using laboratory yeast transformants demonstrated the effectiveness of *HSP30p* to mediate late fermentation expression of *FLO1* under industrial brewing conditions. The transformants displayed strong flocculation in the stationary phase of yeast growth, resulting in a distinctly clearer beer than the beer obtained with wild type cells. An extensive evaluation of phenotypes displayed in nutrient-rich (YEPD) and chemically defined laboratory media by transgenic BM45 and VIN13 wine yeast strains in which the genomic copy of *FLO1*, *FLO5* or *FLO11* open reading frames were brought under transcriptional regulation of stably integrated *ADH2p* or *HSP30p* was presented in Chapter 4. Under the conditions assayed, the data showed that some of our genetically engineered wine yeast strains were capable of stationary-phase specific flocculation that ranged from moderate to strong flocculation phenotypes.

In this study the fermentative and flocculation potential of 12 transgenic BM45 and VIN13 wine yeast strains (Chapter 4) were assessed under both natural and artificial wine-making conditions. Commercial wine yeast strains have been selected in the last century from natural spontaneous wine fermentations on the basis of their desirable oenological properties. These industrial yeasts are closely related to *S. cerevisiae* laboratory strains but have distinct physiological properties making them suitable for wine fermentation (Pretorius, 2000). Yeasts are subjected to multiple and changing stress conditions during alcoholic fermentation, which is a dynamic and complex process. Wine yeasts have evolved mechanisms to sense and respond to environmental changes and thus maintain metabolic activity and cellular integrity (Bauer and Pretorius, 2000). Unlike optimal laboratory growth media and conditions, grape must composition presents culture conditions that are far from optimal. Upon inoculation into grape must, the wine yeast must acclimatize to high sugar concentration (140-300 g L^{-1}), low pH (2.9-3.8) and high supplementary SO$_2$ levels (40-100 mg L^{-1}) before commencement of primary alcoholic fermentation. The metabolic activity of wine yeast during alcoholic fermentation permeates a cascade of stress conditions, which include rapid nutrient inadequacies and starvation (e.g. available nitrogen 50-600 mg L^{-1} at fermentation onset, is quickly depleted), temperature variation and ethanol toxicity [final ethanol concentration up to 15% (v/v)] (Fleet and Heard, 1993; Pizarro *et al.*, 2007).

In *S. cerevisiae* five alcohol dehydrogenase isoenzymes encoded by *ADH1* to *ADH5* are involved in ethanol metabolism. The isoenzymes corresponding to Adh1, Adh3, Adh4, and Adh5 reduce acetaldehyde to ethanol during alcoholic fermentation. In contrast, the isoenzyme Adh2 catalyzes the reverse reaction which is the oxidation of ethanol to acetaldehyde (Ciriacy, 1975; Lutstorf and Megnet, 1968). The *ADH2* promoter is subjected to carbon catabolite repression and has been shown to be repressed several hundred-fold during growth on glucose (Gancedo, 1998; Price *et al.*, 1990). Derepression of the *ADH2* promoter only takes place in the absence or depletion of glucose (Ciriacy, 1997) and this event generally coincides with the transition to growth on ethanol (Noronha *et al.*, 1998).

The *ADH2* promoter mediated distinctly stronger *FLO1* and *FLO5* flocculent phenotypes in BM45-F1A, BM45-F5A, VIN13-F1A and VIN13-F5A transgenic wine yeast strains in comparison to their respective *HSP30p* wine yeast transformants when cultivated in nutrient-rich YEPD containing 20 gL^{-1} glucose (Chapter 4, Fig. 4.9). In addition, we also reported a similar trend in a recent study that employed laboratory *S. cerevisiae* transgenic yeast strains (Govender *et al.*, 2008). However, in the present study, QRT-PCR clearly demonstrates that the *ADH2* promoter is tightly repressed in all phases of yeast growth under wine fermentation conditions using the synthetic grape must MS300. This was further supported by the inability of BM45-F1A, BM45-F5A, VIN13-F1A and VIN13-F5A transgenic wine yeast strains to flocculate under both synthetic MS300 and Merlot must fermentation conditions. This is surprising, especially since the derepression profile of the *ADH2* promoter strongly suggests that significant up-regulation of *FLO* gene expression, mediated by *ADH2p* should have at least been observed in the late stationary phase of yeast growth, which corresponds with glucose depletion and high ethanol levels.

Supportive of our research findings, a recent transcriptome study of the commercial wine yeast strain *S. cerevisiae* EC1118 under wine-making conditions using MS300 also demonstrated that there was no change in the expression profile of *ADH2* during all phases of yeast growth (Varela *et al.*, 2005). Furthermore gene expression analysis of a bottom-fermenting industrial lager *Saccharomyces* yeast strain under experimental brewing conditions revealed that transcript levels of alcohol dehydrogenases (Adh1, Adh2, Adh3 and Adh5) with the exception of Adh4 are reduced in the late stationary phase of yeast growth (James *et al.*, 2002).

In studies relating to *ADH2p* mediated expression of the heterologous xylanase (*XYN2*) in *S. cerevisiae*, Du Preez and coworkers (2001) made a startling observation in that the *ADH2* promoter was subject to transcriptional repression by extracellular ethanol. Furthermore, this phenomenon was shown not to be strain-specific and that

derepression of *ADH2p* occurred at ethanol concentrations below 50 mM. In essence it may be tentatively suggested that higher concentrations of extracellular ethanol could serve as a form of feedback inhibition that prevents overexpression of the *ADH2* gene during alcoholic fermentation. This assumption stems from a suggestion that *ADH2* overexpression during fermentation could create conditions of a futile metabolic cycle that leads to the accumulation of NADH and the toxic compound acetaldehyde, which could affect cellular demands for cofactors and also the redox status of yeast cells (Maestre *et al.*, 2008). Herein lies a possible reason that might explain firstly the inability of our engineered *ADH2p-FLO1* and *ADH2p-FLO5* transgenic wine yeast strains to flocculate under wine-making conditions, and secondly, the progressive loss of flocculent abilities in relation to higher glucose substrate concentrations when aerobically grown in nutrient-rich YEPD medium.

Therefore, it may be suggested that the native *ADH2* promoter utilized in this study is not an ideal candidate to drive *FLO* gene expression under wine-making conditions. However, *S. cerevisiae* is increasing being utilized as a cell factory as illustrated in the production of insulin (Kjeldsen, 2000), L-lactic acid (Saitoh *et al.*, 2005) and polyketides (Kealey *et al.*, 1998; Maury *et al.*, 2005). Batch fermentations with substantially lower initial glucose substrate concentrations than those employed in wine fermentations are employed for the production of these industrially important compounds. Thus it may be suggested that *ADH2p* based *FLO1* and *FLO5* transformants created in this study that possess the capacity for controlled flocculation may be of tremendous benefit to the downstream processing technologies employed in these industries.

In this study, QRT-PCR clearly shows that *HSP30p* based constructs were capable of driving *FLO* gene expression in transgenic wine yeast under defined synthetic grape must conditions. In all the *HSP30p-FLO* based constructs, *HSP30p* was tightly repressed in the exponential phase, induced on entry into stationary phase and strongly induced in the late stationary phase of yeast growth. The aforementioned profile is highly desirable and especially pertinent to expression of flocculin encoding *FLO* genes, in that flocculation should not occur before the fermentation is completed as an early onset of flocculation may result in sluggish or stuck fermentations which yield final products with high residual sugars and unsatisfactory aromatic characteristics (Verstrepen *et al.*, 2001). In addition, this is further supported by the flocculent ability of BM45-F1H, BM45-F5H, VIN13-F1H and VIN13-F5H transgenic wine yeast strains under both synthetic and Merlot must fermentation conditions. These findings are consistent with recent studies that reported almost identical *HSP30p* expression profiles in the commercial wine yeast strain EC1118 during fermentation of MS300 artificial must (Rossignol *et al.*, 2003; Varela *et al.*, 2005). Interestingly, these research groups also reported the phase specific up-regulation of *HSP30* that corresponded to the stationary phase of yeast growth.

As mentioned previously (Section 4.5), the integration of *HSP30p-FLO11* promoter replacement cassettes in this study effectively deleted a 3310 bp sequence of the native *FLO11* promoter, a region that is in excess of the reported approximately 3000 bp native *FLO11* promoter (Rupp *et al.,* 1999; van Dyk *et al.,* 2005). However, due to limited sequence identities between the *FLO1* promoter region of the laboratory *S. cerevisiae* SC288 strain and the wild type wine yeast strains employed in this study (Section 4.4.1), the deleted native *FLO1* promoter region by integration of *HSP30-FLO1* promoter replacement cassettes was limited to an approximately 845 bp sequence. Considering that Fleming and Pennings (2001) reported that the Swi-Snf co-activator and Tup1-Ssn6 co-repressor complexes control an extensive upstream *FLO1* domain (> 5000 bp) in which regulation of the *FLO1* gene takes place, it is most probable that the *HSP30* promoter of wine yeast integrated *FLO1*-based constructs are subject to some form of repression by the remnant of the *FLO1* native promoter sequence. In view of *FLO1* having an extensive native promoter sequence (5000 bp) and considering that the open reading frames of *FLO1* and *FLO5* share a 96% sequence identity (Jin and Speers, 1998), it may be guardedly suggested that the native promoter sequence of *FLO5* is similar to that reported for *FLO1*. Although a 2925 bp sequence of the native *FLO5* promoter was knocked-out on insertion of *HSP30p-FLO5* constructs, it is also probable that the newly inserted *HSP30* promoter in *FLO5* transgenic wine yeast strains is also governed albeit to a lesser extent than *HSP30p-FLO1* constructs, by the remaining native *FLO5* promoter sequences.

Thus it may be cautiously suggested that differential placement of the *HSP30* promoter in *FLO1*, *FLO5* and *FLO11* transgenic wine yeast strains may have contributed to the observed differences in mRNA levels associated with these open reading frames. Therefore, it may also be suggested that more appropriate positioning of *HSP30* promoter replacement cassettes upstream of native *FLO1* and *FLO5* ORFs may in fact yield flocculent phenotypes that are more equitable to those observed in *ADH2p* wine yeast transformants. For future studies, it becomes prudent to ensure that all necessary measures are undertaken to inactivate native promoter sequences when attempting to engineer transgenic yeast strains using the promoter replacement strategy.

In this study, transgenic yeast strains (BM45-F1H, BM45-F5H, BM45-F11H, VIN13-F1H, VIN13-F5H and VIN13-F11H) in which an ORF of a dominant chromosomal flocculation gene (*FLO1*, *FLO5* or *FLO11*) was placed under the transcriptional control of the stationary phase inducible *HSP30* promoter displayed metabolic fermentation profiles in both synthetic grape must and natural Merlot must that were almost indistinguishable from their parental host wine yeast strains, BM45 and VIN13. Considering that wines are

regarded as dry if their residual sugar content is less than 5 gL^{-1}, it is clearly evident that Merlot wines ($\leq$ 1.95 gL^{-1} residual sugars) produced by both parental host wine yeast strains and their *HSP30p* transgenic descendants were fermented almost equally well to dryness. Moreover, *HSP30p* transgenic wine yeast strains produced synthetic and Merlot wines that displayed almost identical volatile and aroma compound profiles. These observations were critical in validating one of the hypotheses of this study in that the introduction of genetically engineered promoter replacement cassettes designed for induction of late fermentation flocculation will not compromise desirable oenological properties of original non-flocculent host wine yeast strains.

Only BM45-F1H, BM45-F5H, VIN13-F1H and VIN13-F5H transformants displayed flocculent phenotypes in both synthetic MS300 and Merlot wine fermentations. Of these transformants, only the BM45-F5H strain was capable of generating compacted or 'caked' lees fractions thereby providing a distinct separation of the fermented wine product and lees fractions. The benefit of this attractive property is it facilitates simpler and faster recovery of wines and it also promotes a greater volume recovery of fermented wine product. This improvement has significant financial cost-saving implications and can be directly attributed to the superior flocculent ability of the BM45-F5H transgenic strain. As stated previously, under nutrient-rich YEPD conditions, *ADH2p* relative to *HSP30p* mediated markedly stronger *FLO1* and *FLO5* flocculent phenotypes in both BM45 and VIN13 derived wine yeast transformants. As such, the full potential of these phenotypes to yield compacted lees are yet to be realized. These phenotypes may be fully exploited in this regard by the use of recently identified stationary phase inducible promoters which are expressed more strongly than the *HSP30* promoter during wine fermentation conditions (Rossignol *et al.*, 2003; Varela *et al.*, 2005).

The *HSP30* promoter was shown to drive substantially higher *FLO11* expression under standard synthetic MS300 fermentations compared to the expression levels of other *FLO* genes employed in this study. However, both BM45-F11H and VIN13-F11H transformants displayed no detectable flocculent phenotypes under this condition, or in MS300 fermentations containing either pectin or diatomaceous earth. Surprisingly, *HSP30p* driven expression of *FLO11* in both BM45-F11H and VIN13-F11H strains under red wine-making conditions yielded strong flocculent phenotypes that displayed a combination of both Ca^{2+}-dependent and Ca^{2+}-independent flocculation characteristics. A distinct advantage of this unique *FLO11* phenotype was highlighted in its ability to dramatically promote faster lees settling rates. Moreover, wines produced by BM45-F11H and VIN13-F11H transformants were significantly less turbid (reduced by up to 33%) than those produced by their wild type parental strains. The present study

has provided sufficient evidence that seems to suggest that yeast cells expressing *FLO11* encoded mannoproteins are capable of interacting with suspended potassium-bitartrate complexes. It may be tentatively suggested that a combination of interactions that involve *FLO11*-based transformants and suspended insoluble components such as amorphous solids and potassium-bitartrate complexes may possibly promote faster lees settling rates that yields substantially clearer wines with enhanced stability. This improved stability is most probably due to inhibition of potassium-bitartrate crystallization (Boulton *et al.*, 1996; Lubbers *et al.*, 1994; Ribéreau-Gayon *et al.*, 2000). The development of commercial wine yeast strains in this regard will reduce the financial cost incurred in the downstream processing such as fining and filtration of red wines. The visual aspect of a red wine, described by its colour, brightness, turbidity or cloudiness, etc, is one of its most important attributes and it is the first characteristic seen by the consumer that has a direct influence on the acceptance of the wine (Revilla and González-San José, 2003). The ability of *FLO11* transformants to positively contribute to the aesthetic quality of red wines further highlights the importance of this finding and its potential contribution to the wine industry. The full impact of these mannoproteins to contribute to other valuable enological properties previously outlined in the discussion section warrants further investigation.

In our past and current studies, of the three media types (YEPD, MS300 and Merlot must) evaluated, both BM45-F11H and VIN13-F11H strains were exclusively flocculent under authentic red wine-making conditions, thus enunciating that this specific growth condition contributes to the development of a flocculent *FLO11* phenotype. This finding supports the suggestion of Gimeno and coworkers (1992) that the *FLO11* expression in *S. cerevisiae* results in a growth pattern that may be used in the natural environment to penetrate substrates such as grapes. This is indeed a remarkable finding and it adds to the plethora of phenotypic identities that can directly be attributed to the *FLO11* encoded glycoprotein which include Ca^{2+}-dependant flocculation (Bayly *et al.*, 2005; Lo and Dranginis, 1996), invasive growth and pseudohyphal formation (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996), flor formation (Fidalgo *et al.*, 2006; Ishigami *et al.*, 2004; Zara *et al.*, 2005) and adhesion to biotic and abiotic surfaces (Verstrepen and Klis, 2006; Verstrepen *et al.*, 2004). Moreover, the flocculent phenotype displayed by both BM45-F11H and VIN13-F11H transgenic strains was not inhibited in the presence of either glucose or mannose. Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (Stratford and Assinder, 1991), this result clearly demonstrates that *FLO11* transgenic wine yeast encoded flocculins exhibit neither Flo1-type nor NewFlo-type flocculation mechanisms. It can be suggested that the flocculent phenotype of BM45-F11H and VIN13-F11H transformants may at least in

part belong to a third group named mannose-insensitive (MI), which is insensitive to mannose (and glucose), and independent of Ca^{2+} ions (Masy *et al.*, 1992). Masy and coworkers postulated that flocculation in such strains could be produced by hydrophobic interactions or other specific interactions not involving mannans. However, Stratford (1992) suggested that mannose-insensitivity probably results from very low specificity to monosaccharides since lectins may have much greater affinity for tri- or polysaccharides than for simple sugars. Therefore, it is most likely that the flocculation mechanism of these *FLO11* transgenic wine yeast strains would deviate from the widely accepted lectin hypothesis that was proposed by Miki and coworkers (1982).

In summary, this study has clearly demonstrated that it is possible to harness the innate dominant *FLO* gene open reading frames of non-flocculent commercial wine yeast strains by use of self-cloning promoter replacement cassettes to yield conditionally flocculent wine yeast strains with oenological properties that are superior to their parental wild type strains.

5.6   ACKNOWLEDGEMENTS

5.7   REFERENCES

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

6.   GENERAL DISCUSSION AND CONCLUSION

6.1   GENERAL DISCUSSION AND CONCLUSION

While the phenotype "flocculation" is relatively easily and precisely defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Stratford, 1989), the exact biological relevance of this phenotype remains poorly understood. It has recently been suggested that flocculation may be a means to protect the cells that are present in the center of a floc from environmental stress or serve as a means of passive transport away from the stress (Verstrepen and Klis, 2006). Whatever the physiological role of the phenomenon, flocculation can be an industrially desirable trait. In wine, efficient and timely yeast flocculation leads to the formation of compacted sediments (Lahtchev and Pesheva, 1991) or 'caked' lees, thereby reducing the handling of wines and minimizing problems associated with wine clarification (Pretorius and Bauer, 2002). While centuries of unconscious or conscious selection of yeast strains in wine making environments has resulted in strains that are able to efficiently convert sugar to ethanol, few if any of these strains display desired flocculation phenotypes. Ideally, indeed, during fermentation, a high suspended yeast count is required to ensure a rapid fermentation rate, whilst upon completion, efficient flocculation should be initiated (Henschke, 1997). This evaluated several molecular strategies to transform non-flocculent wine yeast strains with desirable fermentation properties into strains that display inducible flocculation without impacting on desirable oenological traits. As our data show, such strains can be generated through the controlled expression of dominant flocculation genes. The data also clearly suggest that such strains have the potential to benefit wine production significantly.

The individual results in laboratory strains and industrial strains have been reported in the three research chapters of this dissertation. Most importantly, the data confirm that inducible expression of native *FLO1* and *FLO5* open reading frames, albeit to varying degrees, are responsible for a quantifiable cell-cell adhesion phenotype that can be characterized as a Flo1 flocculation phenotype. It was also clearly evident that *FLO1* and *FLO5* transgenic laboratory yeast strains were markedly more flocculent than the corresponding wine yeast strains. This difference could at least in part be attributed to wine yeast *FLO1* and *FLO5* open reading frames containing significantly smaller intragenic repeat regions. This is supported by Verstrepen and collaborators (2005) finding that a decrease in the size of the intragenic repeat region of the *FLO1* ORF results in a quantitative decrease in *FLO1*-mediated flocculation and vice versa. Furthermore, and of potential benefit to the winemaking industry, a *FLO5* wine yeast transformant was capable of generating compacted or 'caked' lees fractions, thereby providing a distinct separation of the fermented product and the lees fractions.

On the other hand, we found that inducible expression of native *FLO11* ORF under different laboratory growth conditions resulted in flor/biofilm formation and invasive growth phenotypes. Furthermore, in this study we report a novel *FLO11* induced flocculation phenotype that seems to exclusively develop under authentic red wine-making conditions. This strong *FLO11* flocculation phenotype was not yeast strain dependant, possessed both Ca^{2+}-dependant and Ca^{2+}-independent flocculation characteristics and was insensitive to inhibition by both glucose and mannose. These characteristics seem to indicate that the mechanism of this flocculation phenotype may deviate from currently known mechanisms. All of the *FLO11* phenotypic behaviours described in this study depend on cells that interact with one another and the local environment (Reynolds *et al.*, 2008). Of industrial significance, wines produced with *FLO11* transformants were markedly less turbid than those produced by their wild type parental strains. The benefit of this attractive property is it facilitates simpler and faster recovery of wines and also promotes greater volume recovery of the wine product.

The native promoters of the *FLO* genes under scrutiny in this study are tightly repressed and appear to be nonessential for routine growth under standard laboratory conditions. The transgenic yeast strains engineered in this study has thus provided us with a unique opportunity to study phenotypes that are associated with native *FLO1* and *FLO5* genes of laboratory and wine yeast that are otherwise silent. Although the *FLO11* gene is silent in the laboratory strain, it is important to emphasize that under certain growth conditions it is expressed in wine yeast strains as evidenced by their innate capacity to invade agar. This presents a fascinating conundrum as to why these *S. cerevisiae* strains maintain functional *FLO1*, *FLO5* and *FLO11* open reading frames. This seems to suggest that there possibly exists some form of natural selection pressure that maintains the integrity of these genes, and that these genes most probably continue to play a role in the life cycle of these yeasts. *S. cerevisiae* indeed requires a wide range of responses to enable survival in variable and rapidly changing deleterious environmental circumstances, and adjustable adhesion phenotypes, including filamentation, invasive growth, flocculation, and biofilm development (Fidalgo *et al.*, 2006; Guo *et al.*, 2000; Halme *et al.*, 2004; Lambrechts *et al.*, 1996; Lo and Dranginis, 1998; Reynolds and Fink, 2001) can provide a significant benefit. Furthermore, the maintenance of functional but silent subtelomeric *FLO* genes may be linked to the suggestion that subtelomeric sequences play a role in preventing nonfunctional meiotic crossovers near chromosome end (Barton *et al.*, 2003). Additionally, it has been recently demonstrated that the telomere-adjacent *IMD1* ORF is silenced by a natural telomere position effect (TPE) wherein a subtelomeric ORF is silenced by its own telomere (Barton and Kaback, 2006). Although the silent *FLO* genes provide a reservoir of tools to adjust adhesion phenotypes, the regulatory mechanisms that permit access to this silent information are yet to be explored.

The data show that our engineered promoter-replacement cassettes and the integration thereof confers stable (both in timing and intensity) expression properties to the targeted genes, and demonstrates the possibility of adjusting flocculation and flor forming behaviour to specific industrial requirements. Nevertheless due to chromosomal sequence differences between laboratory and industrial yeast strains, for future studies of this nature and as a precautionary measure, it would be extremely advisable to establish sequence identities between donor and acceptor strains for the targeted integration sites. Importantly, flocculent transgenic wine yeast strains under winemaking conditions displayed vegetative growth and fermentation properties that were comparable to that of their parental wild type strains, indicating that those characteristics were not compromised by modified expression of a specific *FLO* gene.

With regard to the specific promoters employed in this study there should be some room for further optimisation either through fine-tuning of these promoters or by substitution with other promoters. Nevertheless, this study gives some credence to the proposal that genetic engineering is an efficient tool to design flocculating wine yeast traits that would meet the winemaker's demands (Pretorius, 2000; Pretorius and Bauer, 2002). Furthermore, the GRAS status of genetically modified (GM) wine yeast strains created in this study was preserved by implementing a self-cloning strategy that was based on the use of host-derived material. This is of significance in that it has been suggested that the shortest path to commercial implementation and public acceptance of GM wine yeasts will probably lie in strains developed through self-cloning techniques (Pizarro *et al.*, 2007).

Future prospects arising from this study that warrant further investigation can be summarized as follows:

- The putative primary structure of Flo1p, Flo5p and Flo11p should be ascertained from sequencing of the native *FLO1*, *FLO5*, and *FLO11* open reading frames of the wine yeasts employed in this study. This could provide substantial insight into some of the phenotypic differences that were observed between laboratory and wine yeast strains. This exercise will contribute to a greater understanding of the relationship between structure and function in the flocculins.

- The *FLO11* phenotype that is unique to authentic red wine fermentations should be further interrogated so as to ascertain the mechanism of flocculation which could potentially deviate from those already described.

- The suitability of a similar strategy to convert other *S. cerevisiae* strains that are employed as cell factories in pharmaceutical and nutraceutical industries should also be explored as such strains may facilitate the downstream processing of such products.

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