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Yeast Flocculation: Understanding cell surface structure-function relationships in industrial yeast strains

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ABSTRACT

Adhesion properties of microorganisms are crucial for many essential biological processes such as sexual reproduction, tissue or substrate invasion, biofilm formation and cell-cell aggregation. One of such controlled forms of cellular adhesion in yeast that occurs preferentially in the liquid environments is a process of asexual aggregation of cells which is also referred to as flocculation. The timing during growth and the causes of onset of yeast flocculation are of commercial interest to the brewing industry, as flocculation can determine the degree of attenuation of the wort. Early or premature flocculation is one common causes of 'hung' or 'stuck' fermentations giving rise to sweeter beer whereas a lack or delay in flocculation can cause filtration difficulties and some problems in obtaining a bright sparkling beer; in addition, the presence of excess yeast in beer during ageing can cause off flavours due to yeast autolysis. Despite this commercial interest, limited information is available about the onset of flocculation and the various factors that may be responsible in the process. In particular, what are the signals that trigger flocculation? Adhesion properties applicable in improving yeast biotechnology are dependent directly or indirectly on characteristics of cellular surfaces, usually the outer layer of the cell wall. Change in the structure and or composition of the cell wall leads to changes in the microbial adhesion properties. Exploring more into the cell wall and studying the nanoscale structure of the yeast cell wall would thus be beneficial to augment our understanding of flocculation.

In fond memory of my Supervisor

Late Prof (Dr.) Ashok K Adya and Late Dr. Forbes Wardrop

whose guidance and support shall always be missed.

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AUTHOR'S DECLARATION

I, **ASHIMA NAYYAR** declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the Abertay University, Dundee, United Kingdom. It has not been submitted before for any degree or examination in any other University.

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(Signature of candidate)

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CERTIFICATE

I certify that **ASHIMA NAYYAR**, a Ph.D. candidate has undertaken all the work described herein and is based on the original work done at the Abertay University, Dundee in partial fulfilment for the requirements for the award of Doctor of Philosophy in Industrial Biotechnology. This has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any similar title and represents an independent work on the part of the candidate.

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ACRONYMS/ABBREVIATIONS

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
cDNA	Complementary Deoxyribonucleic Acid
dia	Diameter
DNA	Deoxyribonucleac Acid
et al.	*et alia* (and others)
G	Relative Centrifugal Force
h	Hour(s)
Kb	Kilo Base
Min	Minute(s)
mRNA	Messenger RNA
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPM	Revolution Per Minute
s	Second(s)
TAE	Tris Acetate EDTA Buffer
Tm	Primer Melting Temperature

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Chapter One

General Introduction

1.1 Introduction

1.1.1 Sociobiology of the microorganism with the environment and with mankind

The adhesion properties of microorganisms, which may involve adhering of the microbe to other cells, tissues or solid substrates, have been the focus of wide ranging scientific and biotechnological interest (Verran and Whitehead, 2005; Verstrepen and Klis, 2006; Zhao and Bai, 2009). Adhesion properties are known to play an important role in governing many essential aspects of the life cycles of microorganisms like sexual reproduction (Chen *et al.,* 1995), cellular aggregation during processes such as flocculation and bio-film formation (Reynolds and Fink, 2001; Ramage *et al.*, 2009), invasion and/or pathogenic behaviour and many others. Many of these microbial adhesion phenotypes are controlled by factors such as nutrient availability or the presence of pheromones.

Adhesion properties are also dependent on the characteristics of the cellular surface, usually the outer layer of the cell wall. Microorganisms can adjust their adhesion properties by changing the structure of their external cell surface. Since these are adaptive responses to environmental parameters, they thus impart environment-specific adhesion phenotypes. Previous research has shown that the genes involved in conferring such adaptability involve a set of highly complex and diverse regulatory pathways. In *Saccharomyces cerevisiae*, adhesion mechanisms are controlled by combinational transcriptional factor networks as well as allele specific and epigenetic regulation (Verstrepen *et al.,* 2004 and Verstrepen *et al.,* 2005).

Adhesion properties also play an important role in controlling some phenotypes that have become relevant targets for the improvement of microorganisms during industrial process. In particular, some of these phenomena like flocculation, wherein the microorganisms display controlled asexual aggregation that leads to the formation of compact groups of cells or flocs, has been exploited to improve various processes, such as wine making, brewing, bioethanol production and waste water treatment.

Cell adhesion is promoted by the proteins that are expressed on the adherent cells known as the adhesins (see Fig 1.1). These cell adhesion proteins are critical to fungal cell interactions in cellular development, symbiosis and in pathogenesis, thus they mediate the interaction of the fungus with the outside world. They play an important role in mating, colony morphology changes, biofilm formation, fruiting body development and interaction with mammalian and plant hosts. Thus, depending on the kind of role they play, their interaction is either termed as “social” or “antisocial”.

Social interactions are the intraspecific interactions involved in mating and differentiation, the ones that we normally encounter in *Saccharomyces cerevisiae* and those involved in formation of colonies and biofilms that adhere and invade the substrate. In contrast to social interactions that are not of much harm to humans, these adhesins also promote antisocial interactions that basically constitute the pathogen binding to the host organisms. For example, eicosapentaenoic acid (EPA) galectins in *Candida glabrata* and the hydrophobic cell surface proteins and peptide binding Als proteins from *Candida albicans* promote such interactions. These agglutinin- like sequence (ALS) proteins also mediate colony and biofilm formation, but they also have equally antisocial

effects on human beings. Thus, proteins that facilitate cell interactions in fungal development may also play interactions with host organisms in commensal and pathogenic situations (Guo *et al.*, 2000; Kumamoto & Vinces, 2005; Nobile and Mitchell, 2006; Zara *et al.,* 2005).

Species	Adhesin (molecules per haploid cell)	Ligand (affinity[a])	Known physiological function(s)
S. cerevisiae	a-Agglutinin (10^4–10^5)	α-Agglutinin (10^{-9} M [r, p, c])	Mating
	α-Agglutinin (10^4–10^5)	a-Agglutinin (10^{-9} M [r, p, c])	Mating
	Flo1p	α-Mannosides	Flocculation
	Flo5p	α-Mannosides	Flocculation
	Flo9p	α-Mannosides	Flocculation
	Flo10p	α-Mannosides	Flocculation
	Flo11p (10^6)		Cell-cell adhesion in flocs, pseudohyphae, and biofilms; agar invasion; adhesion to plastic
		Unknown	Mating
	Lg-Flo1p		Flocculation

Figure 1.1 Major adhesins present on *Saccharomyces* cerevisiae cell wall, which play an important role in mating and adhesion properties. Affinity estimates were derived by radioligand binding (r), plasmon resonance (p) and/or 50% inhibitory concentration for competitive ligand (c).

Modified from Dranginis *et al.,* 2007

1.1.2 Social role of adhesins in *Saccharomyces cerevisiae*

1.1.2.1 The adhesins of Saccharomyces in mating

Among the social roles of adhesins, the best example to be quoted here is the *Saccharomyces cerevisiae* mating interactions. The mating types of this species secrete the a and α agglutinins that are specialized for mating. When these two mating agglutinins bind they eventually promote the fusion of the haploid cells.

1.1.2.1.1 S. cerevisiae a-agglutinin

a-agglutinin consists of two subunits, AGA1 and AGA2. a agglutinin shows less similarity to the other adhesins. While Aga1p is a 725- residue polypeptide with an N-terminal secretion signal sequence and a C- terminal GPI additional signal, which is expressed in both mating types and plays a role in agar invasion. But the binding specificity to Aga1p is provided by Aga2p which is expressed only in cells that express the a-agglutinin that is the a-cells. In the normal state the cells exhibit low concentrations of the a-agglutinin, while the concentration increases to about 5×10^4 per cell, once the cell detects the presence of sex pheromone α-factor in the surroundings. The Aga2p subunit then binds with either low intensity or high intensity with the α-agglutinin, which is expressed on the cells with opposite mating type.

1.1.2.1.2 S. cerevisiae α –agglutinin

α **–**agglutinin consists of a globular head and a highly glycosylated extended stalk (Cappellaro *et al.,* 1994). Studies suggest that the globular head of α**–** agglutinin is basically three tandem immunoglobulin (Ig)-like folds (Chen *et al.,* 1995).

In summary, the a-agglutinin GPI-anchored subunit Aga1p has an adhesive role as well. It functions as a anchorage subunit of a-agglutinin and is expressed in both the mating types.

1.1.3 The *S. cerevisiae* flocculins: adhesins for social aggregation and foraging.

S. cerevisiae yeast cells undergo Ca^{2+} dependent, irreversible, asexual aggregation known as flocculation. Adhesins play a major role in this kind of

cell-cell interaction. Such interactions also take place between opposite mating types and are also responsible for biofilm formation.

Flocculation is associated with five flocculin genes that are expressed in *S. cerevisiae*, namely, FLO1, FLO5, FLO9 FLO10 and FLO11. Of these FLO11 is expressed in laboratory strains where it exhibits profusion of phenotypes. Flo11p shows a variety of roles in yeast that helps cells change and adapt during nutritional deficiencies by switching to pseudohyphal states (Lambrechts *et al.,* 1996 and Lo *et al.,* 1998). Such filamentous chains of pseudohyphae help the yeast to invade the substrate and develop in response of starvation of nitrogen and glucose. Pseudohyphal differentiation involves changes in gene transcription, in cell cycle progression as well as changes in the morphology. For the cell to become invasive the prior step of adhesion is required and that is partially contributed by the Flo11p. Apart from this, FLO11 plays a significant role in biofilm formation on agar, adhesion on plastic and for the formation of specialized floating biofilms called flors that are produced on sherry wines (Reynolds *et al.,* 2001).

In contrast to Fl011p, Flo1p and Flo5p, which are 95% similar, are the major proteins responsible for flocculation behaviour. These proteins are large and have a typical flocculin structure: a hydrophobic N terminus with signal sequence, a hydrophobic C terminus with GPI anchor consensus sequence and a central domain comprising of the Thr rich tandem repeats. These adhesins are responsible for cell-cell interaction. Flo1p, Flo5p and Flo11p are collectively called as the “Flo1-type flocculins” whose activity is inhibited by mannose but not by glucose (Bayly *et al.,* 2005). Whereas the “New-Flo1 type” has a variant of Flo1p called as the Lg-Flo1.

FLO9 and FLO10 also share sequence similarity with Flo1p and also play a role in flocculation. These flocculins proteins have varied roles when expressed at normal levels in the cell, once over-expressed they could potentially substitute for one another. For example, overexpression of Flo1p does not cause agar invasion unlike Flo11p but promotes flocculation, while expression of Flo10p can promote both flocculation and Flo11p like pseudohyphal development. Therefore, the roles of flocculins are overlapping but not identical and the flocculin members have partial functional redundancy.

1.1.4 Importance of microbial cell adhesion especially in *Saccharomyces cerevisiae*

To produce a high quality beer, the yeast should possess certain desirable features to ensure efficient fermentation. These are:

- Effective fermentation of the sugar, from the wort
- High ethanol tolerance levels
- Imparting the desired flavour to the beer
- Efficient yeast removal from the wort at the end of fermentation.

Thus, it is widely desired in the brewing industry that the yeast must also leave a clear beer and give a yeast crop suitable for repitching into subsequent brews (Stewart and Russell, 1981). It is therefore important for yeast biotechnologists to study the flocculation properties of particular yeast cultures while considering the selection of a yeast strain for brewing purposes.

The process of flocculation is a subject of significant scientific and biotechnological interest because of its relevance in industrial fermentation processes such as production of foods, fermented beverages, biofuels and pharmaceuticals (Bauer *et al.*, 2010).

1.1.5 Medical and industrial relevance of fungal adhesion

The cell wall, serves just not only to stabilize and shield cells from mechanical forces, but also serves as a tool for microbes to interact with their environment and vice versa.

One of the most critical functions of the cell surface is its ability to adhere to other cells and surfaces. Adhesion is very useful in preventing cells from being washed away, when they find themselves in a nourishing environment. This allows them to form biofilms that offer protection from hazardous conditions. For example, pathogenic yeasts exploit their capacity to adhere to abiotic surfaces such as plastic prostheses to gain access to the bloodstream and internal organs of patients (Kojic and Darouiche, 2004). Prostheses and catheters can serve as carriers for fungal biofilms and this may provide an internal reservoir of highly drug resistant infective cells (Kojic and Darouiche, 2004).

The industrial importance of fungal adhesion is of considerable economic importance for food processing companies, because adherent fungi can form highly resistant biofilms in industrial installations. However, a positive impact of cell-cell adhesion occurs in industrial brewing and winemaking when separating yeast biomass at the end of fermentation. This cell-cell adhesion between yeast cells is called flocculation.

1.1.6 Flocculating microorganisms of industrial relevance

1.1.6.1 Bacterial flocculation

Bacterial flocculation is controlled by extracellular interactions, but the precise mechanism for the formation of bacterial flocs is not well understood. Bacterial

flocculation plays a major role in the process of sludge treatment during sewage processing. The formation of bacterial flocs affects the physico-chemical properties of the sludge thus improving the aggregate structure settling and ensuring solid-effluent separation. Many mechanisms leading to bacterial floc formation have been suggested. For example, it may involve extracellular polymeric substances such as glycoproteins, together with the involvement of divalent ions (Park and Novak, 2009).

1.1.6.2 Yeast flocculation

Yeast flocculation has been defined as the asexual, reversible, calcium dependent aggregation of cells to form flocs that has many yeast cells. Such flocs are desired in some fermentation industries such as brewing, as they help to rapidly sediment the yeast cells to the bottom of the fermentor at the end of the fermentation process (Bony *et al.,* 1997 and Stratford, 1989). This process is an ideal, cost effective procedure that can happen in the brewing process only if the flocculation of yeast cells occurs at the desired time required for the product recovery. A variety of commodities are produced by yeast: beer, wine, champagne, fermented foods, bioethanol, insulin (Kjeldsen, 2000), L-lactic acid (Saitoh *et al.,* 2005) and polypetides (Maury *et al.,* 2005).

Flocculence refers to the ability of yeast cells to flocculate under optimal conditions, which is a cell wall property independent of its environment. Thus while one studies flocculation one needs to consider both the cell wall properties and the effects of the fermenter environment (Speers *et al.*, 2006).

In some cases, co-flocculation may occur between flocculent and non flocculent strains. In such a phenomenon the non flocculent cells adhere to the flocculent

cells (Miki *et al.,* 1982). So far co flocculation or mutual flocculation has not been reported.

Flocculation is reported to be a calcium dependent phenomenon. According to a lectin–like mechanism, yeast flocculation is caused by specific cell wall proteins known as lectins, present only in flocculent cells (Miki *et al.,* 1982). The N–terminal part of this lectin-like protein binds to mannose residues of neighbouring cells. In this adhesion process, calcium ions ensure the correct conformation of the lectins (Miki *et al.* 1982; Stratford, 1989).

1.1.7 The mechanism of flocculation in *S. cerevisiae*

The lectin hypothesis proposed that specific surface cell wall anchored glycoproteins or flocculins specifically recognise and bind to α- mannan carbohydrates of neighbouring yeast cell walls (Miki *et al.,* 1982; Hough *et al.*1982 ;Taylor and Orton 1978). Furthermore, such binding is supported by calcium ions that act as co-factors in maintaining the active conformation of the surface proteins, thereby enhancing the capacity of these lectin like proteins to interact with the α- mannan carbohydrates (Bauer *et al.,* 2010). FLO gene products, the Flo proteins, are responsible for carrying the lectin domains that enhance flocculation and can thus transform non flocculent *S. cerevisiae* strains into flocculent ones (Watari *et al.,* 1994).

Flocculation is believed to be reversibly inhibited by sugars that directly affect flocculation bonding by competing with the sugars (mainly mannose) of the yeast cell wall. Flo1 phenotype strains are inhibited by sugars like mannose and its derivatives. NewFlo phenotype strains are inhibited by mannose, maltose,

glucose and sucrose. Finally the MI strains are the mannose insensitive strains (Stratford & Assinder, 1991; Masy *et al.,* 1992).

There are two phenotypes in brewing yeast strains that are defined by the type of zymolectins they produce.

Flo 1 Phenotype

In the Flo1 phenotype, the zymolectins produced bind to only mannose residues and the zymolectins are inhibited only by mannose. In this yeast type, flocculation is not affected by the growth stage of the yeast. Many ale strains fall into this category.

NewFlo phenotype

In the NewFlo phenotype, the zymolectins produced bind to mannose and glucose residues and are inhibited by mannose, glucose, maltose and sucrose. Flocculation is typically expressed late in the exponential phase and into the early stationary phase. This group contains most lager strains and some ale strains.

1.1.8 Factors that lead to yeast flocculation – cell surface characteristics

The lectin hypothesis describes the mechanism that makes yeast cells stick together, but what factors promote this mechanism:

- Genetic background of the strain: for flocculation to occur the strain must carry the Flo genes responsible for encoding and regulating the production of Flo proteins. FLO genes are very unstable and have extremely high frequencies of mutation. This instability leads to deletion of FLO gene and loss of flocculation characteristics.

- Zymolectin concentration: The increase of zymolectin concentration in the cell wall may enhance flocculation. Depletion of nutrients, an increase in fermentation by products and temperature increases all causes an increase in zymolectin concentration in the cell wall.
- Mechanical factors that increase collision between the cells: Turbulence caused by CO_2 production, temperature gradients, or other factors will cause more collisions and increase cell aggregation. Thus, further agitation is also known to increase the flocculation rate.
- Factors that decrease repulsive electrostatic charge: Ethanol concentration, pH and changes in the cell wall composition.
- Factors that increase Cell Surface Hydrophobicity or CSH (CSH is the description of how much the cell surface repels water molecules): Increase in surface protein concentration, increase in zymolectin density due to hydrophobic regions in the protein, change in the ratio of phosphorus rich to nitrogen rich polypeptides in the cell wall and an increase in the production and accumulation of oxylipins, sterols and fatty acids in the cell wall.
- Reduction of zymolectin inhibiting sugars: Over the course of fermentation, sugars that competitively bind to the zymolectins will be consumed by yeast; this will make these sites available to cell wall mannans.
- Cell Age: Older cells tend to have rougher and more wrinkled cell walls than virgin cells which tend to increase the binding ability of the older cells. Older cells tend to have a more filamentous growth. It has

been seen that there is more zymolectin density on the cell wall of older cells.

Another important aspect that governs the degree of flocculation is cell surface hydrophobicity. Hydrophobic interactions play a major role in microbial adhesion phenomenon. It has been observed that an increase in flocculation is strongly correlated with an increase in cell wall surface hydrophobicity (Azeredo, *et al.,* 1997). Various methods have been applied to study cell surface hydrophobicity. Some are based on the specific binding properties of the microorganism, measuring the actual binding to the substances that carry hydrophobic groups such as hexadecane, octyl sepharose and polystyrene. Others give an estimate of the overall surface properties, such as salting out and contact angle properties.

Apart from cell surface hydrophobicity, other properties like electron donor/acceptor properties and zeta potential are also important in flocculation studies. Techniques like microbial adhesion to solvent techniques (MATS), based on cell surface affinities for a monopolar and non polar solvent, have been used to determine the electron donor or acceptor properties. While zeta potential and surface charge could be quantified by measurement of the electrophoretic mobility of cells on laser zeta compact equipment. Furthermore, using Smoluchowski's equation, zeta potential (mV) can be calculated (Vichi *et al.,* 2010).

Another important cell surface property, cell size is expected to influence the sedimentation of particles with a diameter >0.2 μm (Mortensen *et al.,* 2005). Therefore, possible modifications of yeast cell size during ageing could influence flocculation capacity, due to the effects of gravity (Vichi *et al.,* 2010).

1.1.9 Physiology of yeast flocculation

Even though flocculation is a cell wall related phenomenon, environmental conditions play a significant role in governing the extent and the onset of flocculation. Some factors that govern the flocculation include salt and sugar content, pH, temperature, aeration and agitation. Of the metal ions that promote flocculation, Ca^{2+} ions are widely accepted to "activate" the flocculation process (Taylor and Orton 1973). Calcium ions appear to be mandatory for flocculation to occur and as low as 10^{-8} M Ca^{2+} may flocculation. However, Nishihara *et al.* (1982) also reported the need of Mg^{2+}, at a concentration of 20μM, for flocculation to occur. Stewart and Goring (1976) also reported the significance of Mg^{2+} and Mn^{2+}, that could imitate the role of Ca^{2+}. Lower concentrations of sodium and potassium (1-10mg/L) may also induce flocculation to some extent. Conversely, some chemicals inhibit flocculation including the alkaline earth metal ions like Sr^{2+}, Ba^{2+}, Na^{+}, Mn^{2+},Cs salts, Al^{3+},La^{3+} and Li^{+} (Stratford, 1989; Kuriyama *et al.,* 1991).

Apart from the metal ions concentration in the medium, sugar concentration also governs the extent of flocculation. Initially Stratford and Assinder (1991) grouped flocculent yeast strains into two categories on the basis of their inhibition by mannose. For example, *S cerevisiae* strain MUCL28323, a top fermenting yeast strain, is inhibited by mannose and is thus classified as Flo1phenotype being mannose sensitive, while a bottom fermenting yeast like *S uvarum* MUCL28235 and M259, is inhibited just not by mannose but by maltose, glucose. The latter cells are thus classified as NewFlo phenotype, as such yeast strains are inhibited by several sugars. Later Masy *et al.* (1992) showed that some strains are not inhibited by mannose and glucose. Such

strains belong to the mannose-insensitive group. Thus sugar inhibition on flocculation is very much a strain dependent phenomenon.

The pH of the medium also plays a significant role in flocculation as it alters the cell surface charge. The Flo1 phenotype shows a very broad tolerance, exhibiting flocculation between pH 1.5 and 10 (Stratford, 1992). The NewFlo strains exhibit two distinct phenotypes: some flocculated over a broad range while some flocculated over a narrow range (Stratford, 1996). Thus for the latter strains, and in some brewing yeast strains, pH may act as a determinant to govern the timing of flocculation. With these strains, a simple change of pH at any desired time during fermentation, allows cell separation from the medium (Stratford, 1996).

Another important factor that determines flocculation is temperature which can influence flocculation development and expression. From studies, it has been concluded that temperature does not inhibit cell-cell interactions but it induces or represses the formation of cell wall components and may alter the availability of the Flo proteins that are involved in flocculation (Garsoux *et al.,* 1993 and Stratford, 1992).

Oxygen also seems to play an important role in flocculation and this is related to mitochondrial function (Nishihara *et al.,* 1977). The effect of oxygen/aeration in the medium leads to activation or repression of certain mitochondrial proteins that induce flocculation. Agitation serves two antagonistic effects as well, that is enhanced particle collision rate induces flocculation on one hand while on the other hand higher shear forces causes particle breakage (Stratford and Keenan, 1988). Thus the rate of agitation can govern the size of floc formation, as gentle

agitation intensity can lead to larger floc sizes, while vigorous agitation leads to smaller floc sizes that settle more slowly and give compact sediments (Burrel, 1996).

Next to these fermentation parameters, yeast management plays a crucial role in dictating the physiological state of yeast, and in its ability to flocculate. For instance, a highly flocculant yeast strain can take on the characteristics of a powdery yeast strain simply by being subjected to excessive aeration during the propagation phase the first time it is pitched. This is because the transcriptional activity of the flocculation genes depends on the physiological state of the yeast, the contents of minerals and trace elements in the yeast, and the stress to which the yeast is exposed. Likewise, manipulation of the yeast's cell surface by acidification, or coating the cell surface by trub particulate material can play a crucial role in the agglutination behaviour of yeast cells.

During fermentation, the drop in both pH and gravity depends on the largest possible contact area between the wort and yeast, which happens during maximum and uniform suspension of the yeast. The same dependence applies to the maturation phase when yeast activity reduces diacetyl and aldehydes. After completion of the maturation phase, however, continued yeast suspension poses both technological and quality problems for the brewer. Excessive yeast in suspension at that stage can cause filtration problems and pose a risk in autolysis with the related deterioration of foam, flavour, pH and stability.

If late sedimentation is one problem, early sedimentation is another. Such flocculation is also known as Premature Yeast Flocculation (PYF). In PYF, yeast starts to flocculate well before the beer has reached the final gravity. This

in turn, has a significant impact on the beer quality and stability. The cause of PYF is still not precisely understood but there are indications that there is so called PYF factor at work. The suspect is a polysaccharide that is introduced into the wort by the malt .Other factors may be anaerobic conditions during malting, as well as certain microorganisms.

For these reasons, a well delineated rapid and timely onset of flocculation in the ferment and the resulting sedimentation of yeast are very important for the brewer. Thus flocculation behaviour is a critical criterion to consider in the choice of yeast strain.

1.1.10 Genetics of yeast flocculation

Gilliland and Thorne in the 1950's established the existence of flocculation genes by genetic crosses (Gilliland, 1951 and Thorne, 1951). Until the 1970's flocculation was thought to be a dominant characteristic and was presumed to be controlled by several genes. Lewis and Johnston (1974) suggested that FLO1 and FLO2 as the dominant genes and flo3 as the recessive gene. There are at least 35 genes that are involved in flocculation. The gene family consists of dominant genes like FLO1 and FLO5 (sharing 96% homology with FLO1 gene), and the two other dominant genes FLO9 (sharing 96% sequence homology with FLO1 gene) and FLO10 (sharing 58% homology with FLO1) that were later identified on the basis of sequence homology to FLO1 gene. The other dominant gene FLO11 shares a sequence homology of 37% with FLO1 gene thus sharing a distant homology with FLO1 gene while a high degree of homology with STA1 gene. Later another gene was described by Sieiro *et al.* (1997) sharing no homology with FLO1 gene but conferring a high degree of

flocculence. This FLO2 gene, is found on the right arm of chromosome XII. Gene FLO8 is somewhat controversial regarding its function in flocculation, but later results by Kobayashi and collaborators suggested that FLO8 gene mediates flocculation via transcriptional activation of FLO1 gene (Kobayashi *et al.,* 1996). Apart from the dominant genes there are a set of recessive or semi dominant genes like flo3, flo6 and flo7 and are likely to be allelic to their dominant counter parts FLO1, FLO5, FLO9 and FLO10. Expression of some of the human heterologous genes like *Ha-ras* and the viral *tax* gene has also been shown (Hinrichs *et al.,* 1988) to cause flocculation. Expression of the GTS1 has been reported (Bossier *et al.,* 1997) to lead to constitutive flocculation even in the yeast strains lacking FLO1 gene. It was later suggested that overexpression of GTS1 leads to thermo tolerance in strains thus leading to increased lethal shock resistance and changes in the cell wall long chain fatty acids profile. All these changes lead to lowering of the cell surface hydrophobicity (Domingues *et al.,* 2000).

Strains that exhibit the four dominant structural genes FLO1, FLO5, FLO9 and FLO10 belong to the FLO1 phenotype.It was later realised by Kobayashi and co-workers, that replacement of FLO1 gene that is Lg-FLO1 exists in cells in addition to the other genes in NewFlo phenotype strains (Kobayashi *et al.*, 1998). While mannose-insensitive strains that don't require Ca^{2+} ions, a distinct flocculation mechanism other than that involving FLO1 and NewFlo phenotype strains occurs. The most probable reasons for such flocculation are hydrophobic interactions.

1.1.10.1 Lectin like proteins that are responsible for flocculation:

The FLO1 gene product is localised on the cell surface by immunofluorescent microscopy (Bidard *et al.,* 1995). The flocculation level is related to the amount of Flo1p level on the cell surface and their concentration increases as the yeast cells progress from one phase of the growth to another. It's seen that the FLO proteins are incorporated polar into the cell wall at the bud tip and at the mother-daughter junction (Bony *et al.,* 1988). Fig 1.2 shows the molecular representative of Flo proteins. The deduced amino acid sequence from the FLO1gene revealed a serine and threonine rich protein with the N- and C-terminal regions that are hydrophobic and contain potential membrane spanning region. All this data suggests that Flo1p is an integral membrane protein, a true cell wall mannoprotein. The studies also indicated that the hydrophobic C-terminus, which is a putative GPI anchoring domain, is necessary for anchoring of the Flo1p in the cell wall as well as for the cell- cell interactions, while the N-terminal domain of the protein is responsible for the sugar recognition (Kobayashi *et al.*, 1998). The protein coded by Flo5 gene is Flo5p, which is a GPI-anchored protein attached to the yeast cell wall at the bud tip and the mother-daughter neck junction (Bony *et al.* 1997). It has been observed that there is irreversible loss of flocculation in FLO1 and FLO5 genes, if the cells are treated with pronase, proteinase K, trypsin or 2-mercaptoethanol. However, the FLO1 strain was sensitive to chymotrypsin and stable to 70^{o}C incubation whereas the FLO5 strain was thermolabile and chymotrypsin resistant (Hodgsin *et al.,* 1985)

FLO8 gene is also equally important for flocculation. Although it shares no significant homology with the FLO1 gene, it does mediate flocculation by

transcriptional activation of the FLO1 gene, since the level of FLO1 gene transcription is dependent on its transcription rate (Kobayashi *et al.*, 1996).

On the other hand, there are two dominant genes apart from FLO1 and FLO5, including the FLO9 gene. The N terminal region of Flo9p is similar to Flo1p. The same is true with Flo10p which is 58% similar to Flo1p (Teunissen *et al.*, 1995). The FLO11 gene produces “mucin like protein” and is required for invasive growth of haploid cells and for diploid cells to form pseudo hyphae in response of nitrogen starvation. The proteins share about 26% homology with the Flo1p.

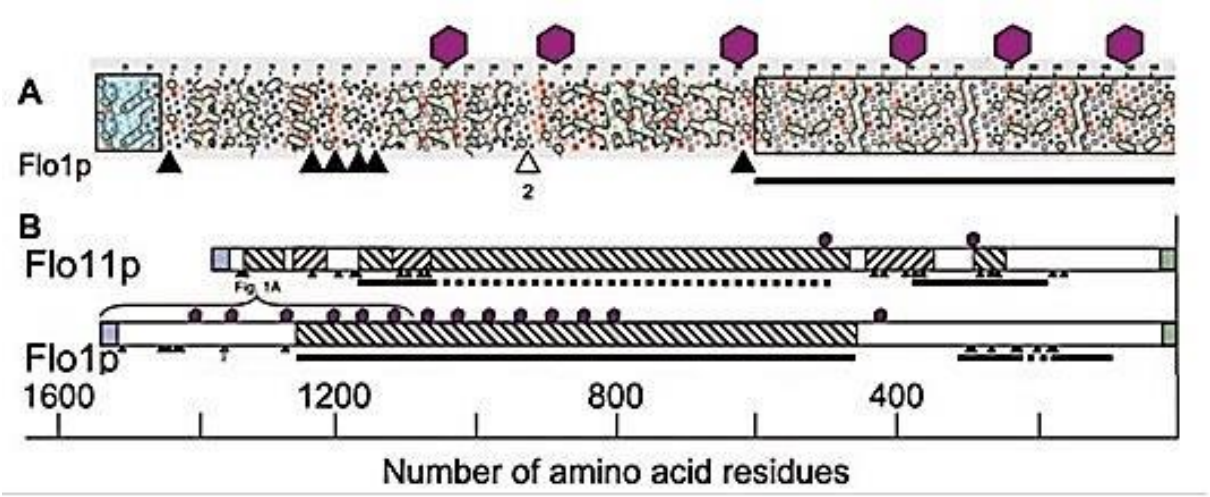


Figure 1.2 Molecular features of representative *Saccharomyces* adhesins. These features are based on HCA plots of the adhesins.

(A) HCA plot of the N-terminal 440 residues of Flo1p. HCA draws each open reading frame as a helical projection, which is vertically repeated. Individual amino acids are denorted as red for acidic, blue for basic and green for hyfrophobic, with hydrophic patched bounded by black lines. Thr residues are as hollow squares, Ser as dark centered squares, Gly as diamonds and Pro as red stars. Cys residues are marked with triangles below the HC plots and N-glycosylation sites are marked with maroon hexagons.Transparent boxes designate the N-terminal secretion signal (light blue) and the beginning of the tandem repeat region (open box).

(B) Summary of HCAs of representative yeast adhesins, aligned at the C termini, where they are linked to the cell wall polysaccharide through the

GPI anchor remnant. N- terminal secreation signals are blue while GPI addition signals are green in colour. Repeated sequences are boxed. Diagnol stripes in white boxes indicate the tandem repeats that are not homologus to other repeats in the illustrated proteins. Potential N-glycosylation sites are shown in maroon colour hexagons above each open reading frame. Cys residues are shown as triangles below each open reading frame. The content of Thr is denoted by bars below each open reading frame, which are dotted where the Thr content exceed 20% and solid where the content exceeds 25%.

Modified from Dranginis *et al.,* 2007

1.1.11 The role of cell wall in yeast flocculation

The yeast cell wall plays a major role in governing the rate and the extent of flocculation (Calleja, 1987). Evidence has been reported to show that yeast flocculation occurs even in heat killed cells and in isolated cell walls.

1.1.11.1 The yeast cell wall

The cell wall is an external envelope shared by yeast and filamentous fungi that defines the interface between the microorganism and its environment. It surrounds the plasma membrane and is strategically placed at the interface between the cell and its environment. It is an extremely complex structure consisting of an elastic framework of microfibrillar polysaccharides (glucans and chitin) that surrounds the plasma membrane and to which a wide array of different proteins, often heavily glycosylated, are anchored in various ways. Intriguingly, the proteins present on the cell wall known as the cell wall proteins (CWP's) play multiple key roles in morphogenesis, adhesion, pathogenicity, antigenicity and are also promising targets for antifungal drug design (eg. against pathogenic *Candida* spp.).

In brewing yeasts, the cell wall is a hugely important and frequently underestimated organelle. It is primarily made up of an array of carbohydrates

(80-90%) with proteins embedded within it. So it's not like an 'inorganic egg shell"', rather its properties and functions keep on changing during the yeast cell's lifetime. It accounts for 15-20% of cell dry weight.

1.1.11.2 Function of the yeast cell wall

Much is known about the yeast cell wall functions, roles and responsibilities. It provides osmotic protection, acts as a selective permeability barrier and plays an instrumental role in maintaining cell shape and morphology. It provides a matrix for various enzymes involved in wall maintenance and development together with hydrolytic proteins. In pathogenic yeasts, the cell wall plays a major role in virulence, pathogenicity, antigenicity, immunomodulation of the immune response and adhesion to host substrates. In brewing, the major role that could be emphasized is its role in cell attachment to surfaces and cell-cell attachment. Finally, the role of the cell wall in flocculation has attracted more than its fair share of attention from brewing scientists.

1.1.11.3 Structure of cell wall

The yeast cell wall is every carbohydrate chemists dream! The fractionation and structural analysis of cell wall carbohydrates is usually a harsh affair involving acid and alkali extraction. But the major components of cell wall are:

(I) **Glucans**: The glucans are the major polymers in the cell wall, accounting about 30-60% of the wall. There are three classes of glucans (Fleet,1999)

1(a) Alkali insoluble acetic acid insoluble ß-(1-3) (35% of the cell wall): maintaining cell wall rigidity and shape.

1(b) alkali soluble ß-(1-3) (20% of the cell wall): playing an important role in conferring cell wall flexibility

(a) highly branched ß-(1-6) glucans are branched through ß-(1-3) linkages.: essentially playing a major role in interconnecting the cell wall polysaccharides, ß-(1-3) glucans, mannoprotein and chitin.

(II) **Mannoprotein:** The outer layer of the cell wall is composed of mannoprotein. This glycoprotein is a major player, accounting for 25-50% of the cell wall. It has been shown that the 'structural' mannoproteins are anchored to the cell wall through linkage with ß-(1-6) glucan (Cid *et al.*, 1995; Fleet and Manners, 1977). In addition to that, extracellular (periplasmic) enzymes like invertase are mannoproteins. As such the cell wall mannoproteins don't play any important role in conferring cell with cell shape or rigidity. Nevertheless, they are found to play 'interactive' roles as antigenic determinants, as receptors for 'killer toxins' and in sexual agglutination. More importantly in a brewing context, mannoproteins are the receptors in the flocculation process. Apart from roles in flocculation, mannoproteins play an important role in cell wall porosity. They are thought to obstruct diffusion through ionic interactions and the web of mannan side chains.

(III) **Chitin:** Chitin is a liner polymer of β-(1-4) linked N-acetylglucosamine. This polymer is found exclusively in the bud scars left on the mother cell surface after the cell has undergone reproduction through budding. These bud scars are used to measure cell age in *Saccharomyces cerevisiae.*

(IV) **Proteins:**

Proteins account for 5-10% of the cell wall. Broadly there are three types of cell wall proteins: (1) structural proteins (2) enzymes and (3) surface receptor proteins (see- Fig 1.3). The structural proteins include the heterogeneous mannoproteins and other proteins that are believed to be involved in the interaction with cell wall glucans. The enzymes are divided in two groups: those that are involved in cell wall morphogenesis and ones that are involved in the metabolism of nutritional substrates - for example, invertases and acid phosphatases. Finally the surface receptor proteins are the lectin-like proteins that are responsible for the phenomenon of flocculation.

The β-1,6-glucan, a flexible minor cell wall component interconnects certain cell wall proteins (CWP's), the so called glycosyl phosphatidylinositol (GPI)- CWP's, with β-1,3 glucan (~90% of GPI-CWPs) or chitin (~10% of GPI-CWPs) through a phosphodiester bridge in their GPI remnant.

The CWPs are mostly located outside the β1,3-glucan-chitin network (i.e, at the cell wall electron dense outer layer) and in minor amounts, throughout the cell wall, determining its porosity. These CWPS can be:

1. *Loosely associated, either noncovalently or through disulfide bonds, with other cell wall components.* This group of CWP's comprises

   (1) soluble precursor forms of covalently linked CWPs

   (2) proteins related to the biosynthesis and modulation of wall constituents, such as β-1,3 glucosyltransferase (Bgl2p), β-exoglucanase (Exg1p) and chitinase (Cts1p) and

(3) noncanonical proteins that are confined to the intracellular compartment because they lack the conventional secretory signal sequences.(Pitarch *et al.,* 2002 ; Klis *et al.* 2001).

2. Cell wall proteins that are covalently linked to β-1,3-glucan.

- 2(a). Directly via an alkali-labile linkage (through a O-linked side chain) such as PIR-CWP's. PIR-CWP's are highly O-mannosylated proteins with one or more internal repeat regions, a N-terminal signal peptide, a Kex2 proteolytic processing site and a C-terminal sequence with four cysteine residues at highly conserved positions (Klis *et al.* 2006; Weig *et al.* 2004).These proteins are normally located in the inner layer of the cell wall (Kapteyn *et al.,* 2000).
- 2(b) Indirectly bound by a β-1,6-glucan moiety through their GPI remnant, such as GPI-CWPS. The GPI-CWPs are highly O-glycosylated proteins with an N-terminal signal peptide, a C-terminal GPI anchor addition signal and serine and threonine-rich regions (Klis *et al.,* 2006).These CWPs are predominantly present in the outer layer of the cell wall.
- 3. Covalently anchored to the chitin by a β-1,6-glucan moiety via their GPI remnant such as some GPI-CWPs (Sestak *et al.,* 2004).Such kind of CWP-polysaccharide complex is largely found in the lateral walls or under stress conditions.

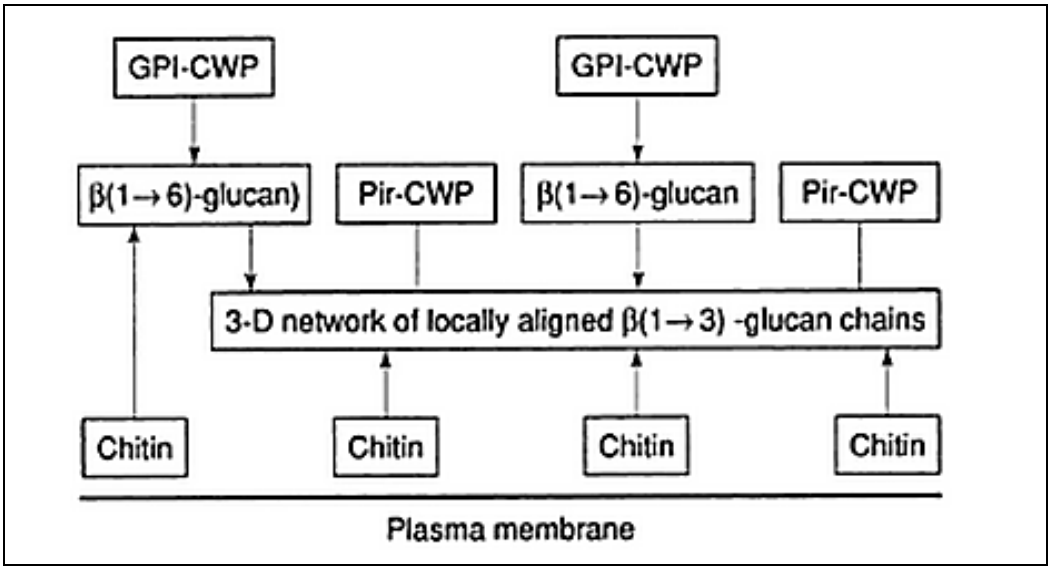


Figure 1.3 Suggested interrelationships of major wall components of *Saccharomyces cerevisiae.* There are covalent linkages between all components, to give pne macromolecular structure, as described in the text. Chitin microfibrils lie adjacent to the plasma membrane; β(1-3) and β(1-6) glucan chains are attached to the chitin; some of the cell wall mannoproteins (GPI-CWP) are attached to the β(1-6)glucan via remnants of glycosyl phosphatidylinositol anchors; cell wall proteins with internal repeats (PIR-CWP) are attached to the β(1-3) glucan.

Source: Carlile *et al.,* 2001

1.2 Aims of this thesis

1.2.1 Study rationale

The main aims of the research presented in this Thesis was to have a comparative understanding of the various factors that lead to flocculation in four industrial strains of *Saccharomyces cerevisiae* by comprehensive study on their cell surface properties, nano mechanical properties and genetic behaviour. Such information is useful for the food industries to control certain factors to

control the output and yield of the end products, thus favouring its application in food and beverage biotechnology.

It is important to study the cell wall properties of industrial yeast strains so as to have a better understanding of the phenomenon of flocculation. Cell surface parameters including cell surface hydrophobicity (CSH) and cell surface charge were investigated in these yeast strains at different (lag, logarithmic, early stationary and late stationary) phases of growth and our findings are pertinent to further understanding and potential manipulation of industrial fermentations involving *S. cerevisiae*. Thus investigations regarding the cell surface properties and flocculation behaviour of different strains of *S. cerevisiae* employed in brewing, winemaking and bioethanol industries are reflected as the main aim of Chapter three presented in this thesis.

In brewing fermentation processes, repeated pitching of yeast may lead to a loss in their flocculation ability, and this is difficult to predict (Heine *et al.* 2009). It was hypothesized that a direct determination of the cellular mannose residues or flocculin contents could provide more dynamic information regarding flocculation behaviour of industrial yeast strains. Here in, I made use of the fluorescent lectins Concanavalin A- Alexa Fluor®-350 (Con A) and *Pisum-sativum*-agglutinate-Fluorescein isothiocyanate (PSA-FITC) to analyse to investigate if their flocculation behaviour was linked to lectin receptor density and the distribution patterns of glucans and mannans on the cell wall and this was identified as the major aim of Chapter four of this thesis.

According to lectin like theory, flocculation occurs as a consequence of interaction between the specific flocculation proteins (flocculins) present only on the flocculent cells and the carbohydrate residues (receptors) of the cell walls of the neighbouring cells (Miki *et al.,* 1982). Flocculins in addition to other proteins present on the yeast cell surface play a crucial role in adhesion, communication and microbial infection (Jendretzki *et al.,* 2011). Close inspection of these proteins at the single molecule or cell level would be helpful in understanding the several physiological and biotechnological processes such as molecular recognition and cell adhesion, aggregation and flocculation, biofilm formation (Verstrepen & Klis 2006; Bauer *et al.* 2010). Thus in this context, atomic force microscopy (AFM) appears to play a crucial role, as it allows manipulations at single cell level and observing the cell surface at nanometer resolution directly on the living cell, which is not seen to be achieved by any thin section transmission electron microscopy (TEM) (Binnig *et al.* 1986; Burnham and Colton, 1989; Mizes *et al.* 1991) and this was the cardinal aim of Chapter five of this thesis.

The genetic variability of flocculation genes may have an important consequence for studies and applications targeting these genes in industrial yeasts strains with unknown genomes. This study dwelt on examining the presence of the dominant genes namely, FLO1, FLO5, FLO8, FLO9 and FLO10 by designing specific primers. *FLO*1 is the most studied gene associated to flocculation and its regulation and expression is well known (Bester *et al.* 2006 and Liu *et al.* 2007). Genetic variability related to the number of tandem repeats in this gene is responsible of the flocculation degree of yeast strains: longer repeats are associated to stronger flocculation ability (Liu *et al.*

2007 and Sato *et al.* 2002). In industrial yeasts, FLO1 has been shown to be active and regulated by FLO8. It is considered to play an important role in mannose specific flocculation, which is inhibited by mannose but not by glucose (Kobayashi *et al.,* 1996; 1998; 1999).To understand the function of FLO genes mainly, FLO1 and FLO8 in greater detail, it was necessary to investigate genes and their expression levels and this was identified as the major aim of Chapter six of this thesis.

1.2.2 Specific objectives listed by chapter

Chapter 3: Cell surface properties and flocculation behaviour of industrial strains of *Saccharomyces cerevisiae*

- To obtain greater insight into the fermentation performance of selected industrial strains
- To conduct comparative analysis of flocculation behaviour among the strains during the different phases of growth curve
- To evaluate the effect of cell surface properties like cell surface hydrophobicity (CSH) and cell surface charge (CSC) on flocculation behaviour of the selected strains of *Saccharomyces cerevisiae.*

Chapter 4: Role of cell wall polysaccharides and lectin-like receptors on flocculation of industrial strains of the yeast, *Saccahromyces cerevisiae*

- Direct determination of the cellular mannose residues and flocculin contents in industrial strains of yeast. I hope this could provide more dynamic information regarding flocculation behaviour of industrial *S. cerevisiae* strains.

- Determination of the lectins or the mannose binding sites by using Avidin-FITC probes and plotting the values using Langmuir's equation.
- Correlating the above information with the percentage flocculation ability of the strains, in order to find a connection between the flocculation ability and number of mannose sites on the cell and number of lectin binding sites on the cell surface.

Chapter 5: Cell surface elastic properties influence flocculation behaviour of industrial *Saccharomyces cerevisiae* strains.

- To understand the discrete cell adhesion forces and other nano mechanical properties for example cell surface elasticity, cell surface roughness at nano level.
- Correlate these nano-mechanical properties to the reversible adhesion phenomenon that the cell undergoes during fermentation in the presence of calcium ions known as flocculation.

Chapter 6: FLO1 and FLO8 gene expression levels governs the extent of yeast flocculation in industrial strains of *S. cerevisiae*

- To investigate the presence of 'FLO' genes, mainly, FLO1, FLO5, FLO8, FLO9 and FLO10 genes in all the four industrial strains.
- Clone and obtain sequence for the genes which has not been sequenced before in these industrial strains.
- Comparison of the expression levels of FLO8 and FLO1 by performing quantitative PCR, to determine the number of transcripts produced in real time for all the four strains.

Chapter Two

Materials and Methods

2.1 Microbiological techniques

2.1.1 Yeast cultures used in this research

The yeast cultures used in the study were obtained courtesy of Lallemand Inc. (Montreal, Canada). The four *Saccharomyces cerevisiae* strains are listed in Table 2.1. The cultures were supplied in glycerol-covered agar slopes, and sub-culturing conducted directly from these for further experimentations.

Strain Name	LYCC Number (given)
Brewing strain	**LYCCI**
Champagne strain	**LYCCII**
Wine strain	**LYCCIII**
Fuel Alcohol strain	**LYCCIV**

Table 2.1 The four industrial strains of *Saccharomyces cerevisiae* from Lallemand culture collection, employed for our study.

2.1.2 Yeast culture maintenance

The glycerol stocks of the yeasts supplied by Lallemand Inc. were streaked on YEPD agar plates (Yeast Extract, Peptone, Dextrose, Agar medium) consisting of 20g/L glucose, 20g/L peptone, 10g/L yeast extract and 20g/L agar, at 28^{o}C for 24 hours. The plates were prepared in duplicate and then stored at -20^{o}C. For testing, from these plates isolated colonies were selected and inoculated in 100ml YEPD broth (20g/L glucose, 20g/L peptone, 10g/L yeast extract) for 12-16 hours. This acted as the seed culture used as inoculum for experimental cultivations investigating different phases of yeast growth. The main culture medium was YEPD medium with the same composition but 500 ml of the media

was inoculated with the seed culture and allowed to incubate at 25^{o}C on an orbital shaker at 170 rpm. Yeast samples were collected after 2, 8, 24 and 48 hours of growth in the medium (representing lag, log, early and late stationary growth phases, respectively)*. Yeast cells were recovered by centrifugation (5000 rpm for 5 min) and washed in double distilled water prior to performing further analyses.

2.1.3 Streak plating

The glycerol slopes that were provided by Lallemand Inc were streaked for single colonies using the streak plate method on YEPGA (see Fig 2.1).

Figure 2.1 A YEPGA plate showing the growth of single colonies of *S. cerevisiaes* (Fuel alcohol strain, LYCCIV) strain in a quadrant streak pattern.

*To be discussed later in chapter three

2.1.4 Serial dilution

Serial dilutions of yeast cultures were carried out in order to count cells using a Coulter Counter and haemocytometer. Routine dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} were prepared in normal saline as shown in Fig 2.2

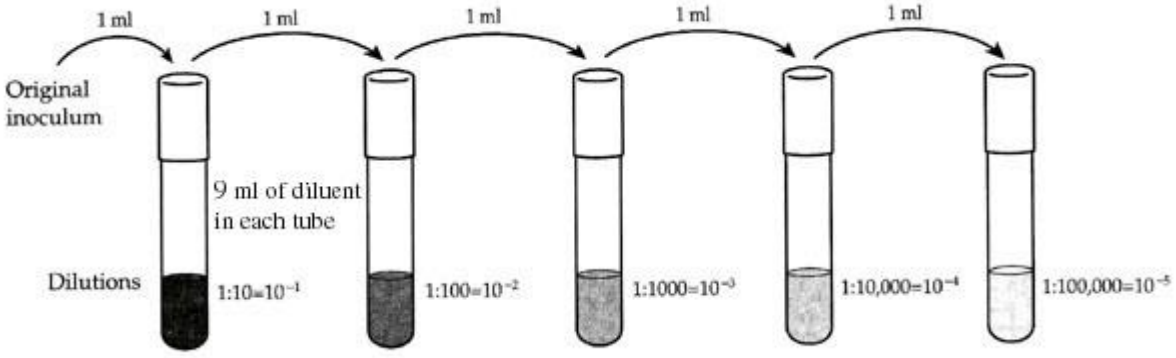


Figure 2.2 The serial dilution method followed for enumerating the cells using haemocytometer and Coulter Counter.

2.1.5 Determination of cell count

Typically, 5×10^6 cells from seed cultures were inoculated in experimental culture flasks from which cells were harvested at 2, 8, 24 and 48hrs (for analysis of lag phase, logarithm phase, early stationary phase and late stationary phase, respectively). A minimum of 1×10^7 cells/ml were harvested from each of these stages for yeast flocculation assays.

2.1.5.1 Determination of cell count using a haemocytometer

A Improved Neubauer haeomocytometer (Sussex, UK) was assembled and 10µL of yeast cell suspension pipetted into the chamber, and visualized microscopically (see Fig 2.3)

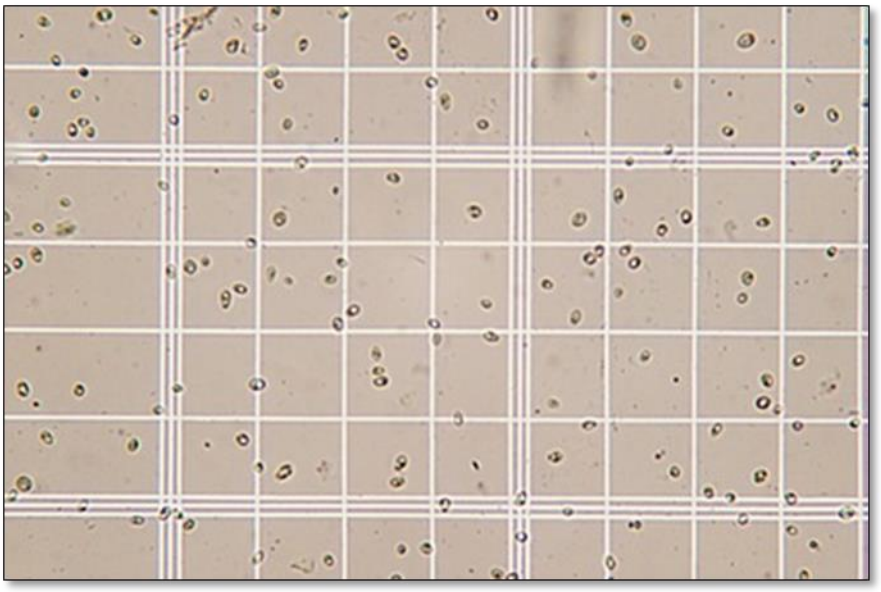


Figure 2.3 Haemocytometer image of cells of fuel alcohol strain (LYCCIV) at 40X objective.

The number of cells in five such large squares was counted and then the number of cells/ml was calculated as follows to determine the sample cell density.

The area under each large square is = 0.2 * 0.2 = 0.04 mm^2

The chamber is 0.1mm deep, so the volume under a large square = 0.04* 0.1= 0.004 mm^3

Thus, in order to get the number of cells in 1 mm^3, one divides the number of cells in each large square by 0.004 or multiply by 250. To get number per ml, multiplied by further 10^3.

Thus,

Number of cells/ml = $X/Y \times 250 \times 10^3$ **Equation 2.1**

Where X is number of cells in Y large squares

2.1.5.2 Determination of cell count using a Coulter Counter

A Coulter Counter Beckman Coulter Counter II (High Wycombe, UK) (see Fig 2.4) was used for counting yeast cells. In this instrument, a particle is pulled by vacuum through an orifice, concurrent with an electric current, and this produces a change in impedance that is proportional to the volume of the particle traversing the orifice. This pulse in impedance originates from the displacement of electrolyte caused by the particle. For the sample preparation 20uL of yeast suspension was pipetted into 20ml of Isoton to obtain a 1:1000 dilution. After obtaining a background count (Isoton alone), the number of yeast cells present in 0.5ml of suspension was determined. In order to obtain the number of cells per ml in the original culture sample, the number was multiplied by two and then with the dilution factor of 1000.

Figure 2.4 Coulter Counter used to enumerate yeast cell number/ml

2.1.6 Chemical analyses

In order to have an idea about the fermentative properties of the yeasts under study and their efficiency for industrial processes, estimation of their ethanol and CO_2 production was carried out.

2.1.6.1 Ethanol

Ethanol production by the yeasts under study was done using an instrument called Fermento Flash (Funke Gerber, 3572 –see Fig 2.5). Yeast sample supernatants were analysed to give measurements of alcohol content (v/v) and extract as well as the values derived from them: density, apparent extract, original wort and osmotic pressure.

Figure 2.5 Fermento Flash (Funke Gerber) that provided the amount of ethanol (v/v) present in media after yeast fermentation.

2.1.6.2 Carbon dioxide production

Carbon dioxide was measured using the ANKOM^{RF} Gas Measurement system (see Fig 2.6) which gives a measure of cumulative carbon dioxide gas measurement in Psi versus time. From such graphs, comparative fermentation kinetics of the yeast strains was evaluated. The system consists of RF pressure sensor modules, a "zero Remote" that measures ambient pressure, a computer interface base coordinator and operational software that can be used with any PC using a Windows XP or Vista based operating system. In our study, I inoculated yeast strains in 250 ml bottles with 100 ml of YEPD media. The RF sensor module was attached and placed in an incubator. The pressure of each bottle was then measured at selected time intervals. Pressure measurements were recorded on a standard Excel spreadsheet and gas production curves were then generated. Pressure could be outputted in psi or bar.

Figure 2.6 ANKOM^{RF} Gas measurement system, used for measurement of cumulative carbon dioxide gas measurement.

2.1.7 Yeast growth curves

100 ml of YEPG broth was prepared and each of the four strains inoculated in different flasks. The cells were allowed to grow at 30^{o}C at a shaking speed of 170 rpm for 12-18 hours after which the cell number was counted by using the Coulter Counter. Yeasts at a concentration of 5×10^{6} cells/ml were inoculated in the main fermenter containing 300 ml of media.

The main flask containing 300 ml of media (where the four phases of growth had to be monitored) were kept on a shaker incubator at 30^{o}C at 170 rpm for a period of 48 hours. The sample was taken out from the main fermenter after every two hours and the cells were then centrifuged at 5000 rpm for four min.

Each sample that was collected after every two hours was centrifuged and then washed twice with deionised water and resuspended in deionised water. The absorbance was measured for each sample at a wavelength of 600 nm.

2.2 Biotechnological techniques

2.2.1 Flocculation assay

Flocculation abilities of the yeast strains were monitored using the modified Bony method (Nayyar *et al.,* 2014). At defined periods of growth, yeast cells were harvested by centrifugation (4500×g for 5min), washed and re-suspended in de-flocculation buffer (50mM sodium acetate, pH 4.5, 5mM EDTA buffer) and washed two times. The cells were then washed twice in double distilled water. Subsequently, cells were re-suspended in flocculation buffer (50mM sodium acetate, 5mM $CaCl_2$, pH 4.5) while the culture absorbance at 600 was adjusted at 2. The cells suspended in flocculation buffer containing $CaCl_2$ were placed in

test tubes of 15 mm diameter and 50 mm height at a final absorbance at 600 of 2. The tubes were sealed and kept on the shaking incubator at 140 rpm for 30mins. After agitation, 5ml of the cell suspension was transferred to a new test tube and allowed to stand undisturbed for 6 min in a vertical position, after which, samples (1000µl) were taken from just below the meniscus and the absorbance at 600 determined spectrophotometrically (Thermo Spectronic Genesys 10UV/10 UV Scanning Spectrophotometer 10-S). The percentage of flocculated cells was calculated by subtracting the fraction of cells remaining in suspension from the total cell count.

2.2.2 Cell surface properties

2.2.2.1 Hydrophobicity assay 1

Cell surface hydrophobicity (CSH) was determined by the Hydrophobic Microsphere Assay (HMA) (Hazen and Hazen, 1992). Cells were harvested during the stationary growth phase and washed thrice with cold, sterile double distilled water. A final concentration of 4×10^6 cells were transferred to 2 ml cold HMA buffer (0.05 M $NaPO_4$, pH 7.2). Meanwhile, in a separate glass tube containing 6 µl of bead suspension was added to 2 ml of cold HMA buffer, such that the final concentration of beads was 9.02×10^8 spheres/ml. In a polypropylene tube at room temperature, 100 µl each of yeast cell and bead suspension was added and the tube left undisturbed for 2 min at 4^oC. After incubation the tube was vortexed for 30 s and 20-30 µl of the cell-bead suspension loaded on a haemocytometer. For statistical analysis, 100 cells were counted that had three or more than three beads attached to it. Determining the percentage of cells with 3 or more attached microspheres gave the Hydrophobicity Index.

2.2.2.2 Hydrophobicity assay 2

Hydrophobicity assay was also performed by Microbial Adhesion to Hydrocarbons (MATS test; Bellon-Fontaine *et al.,* 1996; Mortensen *et al.*, 2005). This assay determines the hydrophobic nature and the Lewis acid-base (electron donor/acceptor) characteristics of yeast cell surfaces. In the MATS test, the affinity of microbial cells was compared to a pair of monopolar/ apolar solvents of similar Lifshitz-van der Waals surface tension. The pair of solvents used was: (1) apolar solvent - hexadecane and the acidic monopolar solvent - chloroform, (2) apolar solvent - decane and the strongly basic monopolar solvent - ethyl acetate. Since these solvents have different surface tensions, the affinity of yeast cells to the hexadecane-chloroform and decane-ethylacetate would reflect the electron donor and the electron acceptor property of the yeast cell surface. The hydrophobic nature was judged by the affinity to apolar solvents, specifically hexadecane.

The cell surface hydrophobicity was measured using the MATS test (Bellon-Fontaine *et al.,* 1996 and Mortensen *et al.*, 2005). Yeast cells from the stationary growth phase were washed with 10 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer, 0.9% NaCl, pH 5.0 buffer and re-suspended to an absorbance of 0.8 at 400 nm (A_0). Hexadecane + chloroform and decane + ethyl acetate constituted two electron donating and accepting pairs. A 0.4 ml of each of the solvent was added to four separate test tubes, each containing 2.4 ml cell suspension and vortex-mixed for 1 min. The mixture was allowed to stand for 15 min to ensure complete separation of the two phases. Once the distinct phases appeared, 1ml of the sample was removed very carefully without

disturbing the aqueous phase and OD measured at 400 nm (A). The percentage of bound cells was subsequently calculated by:

$$\% \text{ affinity} = 1 - \left(\frac{A}{A_0}\right) \times 100 , \qquad \textbf{Equation (2.2)}$$

Where, A_0 is the absorbance at 400 nm of the cell suspension before mixing and A is the absorbance after mixing.

2.2.3 Cell surface charge

Yeast cells were cultivated and centrifuged at 4500 × g for 10 min and re-suspended in 0.02 M sodium acetate buffer (pH 4) at 5×10^7 cells/ml. The yeast cell suspension (1 ml) was then re-suspended in 1.8ml of alcian blue dye buffer solution (50 mg/L). Alcian blue is a phthalocyanine complex that has four charged sites in the molecule and is adsorbed by the negatively charged yeast cell surfaces. The suspension was incubated for 30 min at 25^{o}C on the orbital shaker at 75 rpm, centrifuged and the free dye remaining in the supernatant was determined by absorbance at 615 nm.The concentration of alcian blue was determined by reference to an alcian blue standard curve prepared from original dye/buffer solution. Thus alcian blue retention was expressed as mg of alcian blue per 5×10^7 cells/ml.

2.3 Nanotechnology Studies

2.3.1 AFM sample preparation

Air drying was used to immobilize yeasts on hydrophilic glass slides. The glass slides were made hydrophilic by immersing them in aqueous 20% H_2SO_4 for 24 h, washing five times with ultrapure water, keeping immersed in ultrapure water, and then air drying them before use. The AFM samples were prepared by

placing aliquots of 50µl (equivalent to 1× 10^8cells/ml) of yeast suspensions onto the surface of glass slides and were allowed to air dry for about 3h at room temperature and scanned by AFM (JPK atomic force microscope on Olympus IX71 inverted optical microscope – see Fig 2.7) soon after, in order to preserve the original morphology of the yeast samples under study. In order to know the differences in roughness, elastic and adhesion properties for each of the strains, the cells were scanned at bud scar (B), cytoplasm (C) and edge (E). The yeast samples were scanned in contact mode (CM) by using Si_3N_4 triangular cantilevers (Veeco, Santa Barbara, CA, USA). The Si_3N_4 cantilever's spring constant was calibrated systemically using the thermal tune method and was found to be in the range of 0.01-0.02N/m. All the images (512×512 pixels) were captured at room temperature with a scanning speed of 0.5µm/s. Since water of hydration allows the yeast cells to maintain their native structure as suggested by Canetta *et al.* (2009), thus the experiments were carried out in air at room temperature.

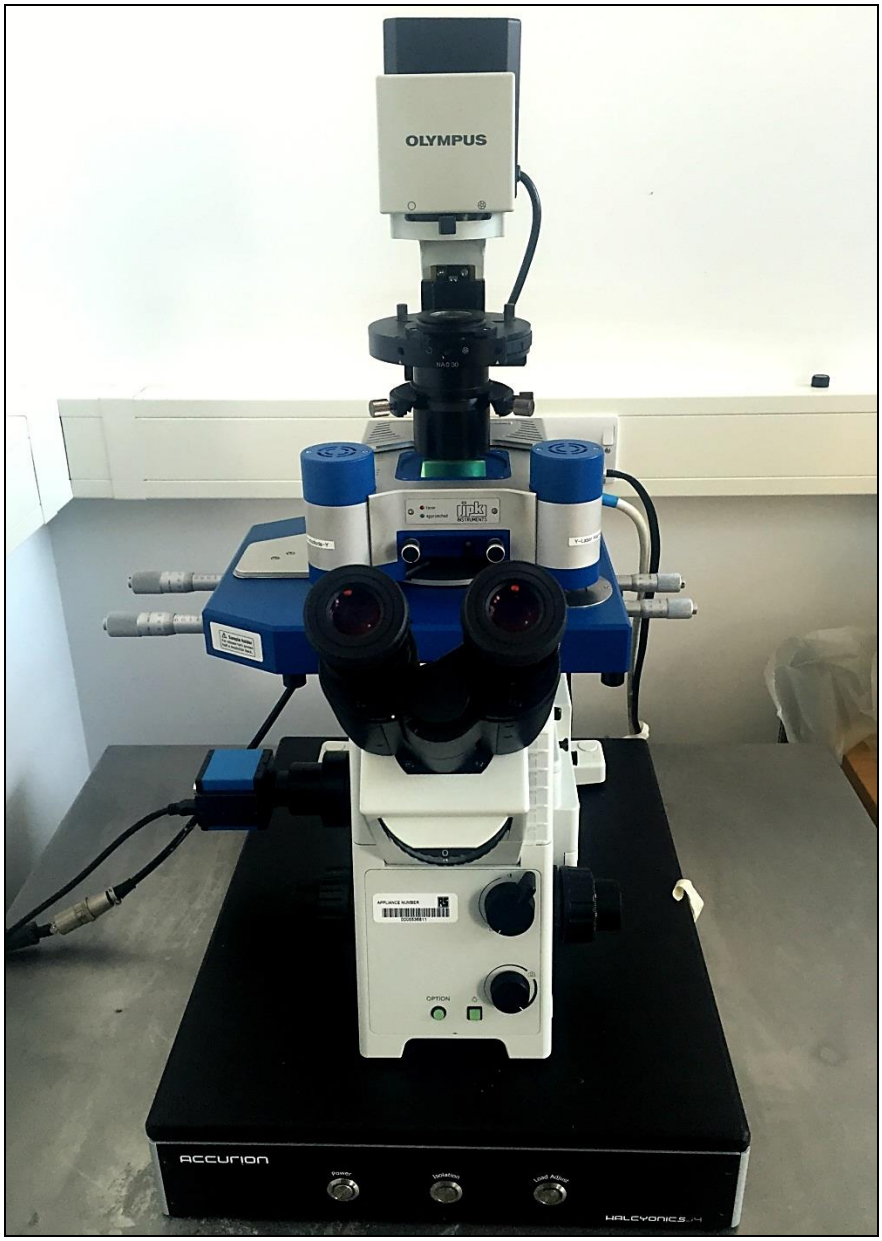


Figure 2.7 Atomic Force Microscope (JPK atomic force microscope on Olympus IX71 inverted optical microscope) used in this study.

2.3.2 Force spectroscopy experiments

After attaining the AFM images, the yeast cells on the slide were subjected to AFM spectroscopy. Fig. 1 shows a typical force spectroscopy curve. For the purpose of AFM spectroscopy, the maximal force applied to the cell was limited to 1.5 nN in order to probe the cell wall elasticity (Young's modulus) and not the

cell turgor pressure, as made at higher loading forces (Arnoldi *et al.*, 2000; Yao *et al.*, 2002). The force (F) vs. displacement curves were converted into force-distance curves and then using the Nanowizard II software, the curves were fitted to the Hertz model (Hertz, 1881), Eq. 1, taking into account a conical tip with an opening angle of 35°(α).

A simple model for describing the elastic response of a sample indented by an AFM tip is the Hertz model (see- Fig 2.8). The most appropriate geometry for the AFM tip in our case is a cylindrical cone with an opening angle α (Radmacher *et al.* 1995). The Hertz model predicts in the case of an infinite stiff tip and a soft, flat sample the following relation between indentation and loading force:

$$F = \frac{2E \tan \alpha}{\pi(1-\vartheta^2)} \delta^2 \qquad \textbf{Equation (2.3)}$$

Where, F= Force , E= Young's modulus, ϑ= Poisson's ratio, δ= Indentation (tip sample separation) and α= half cone angle

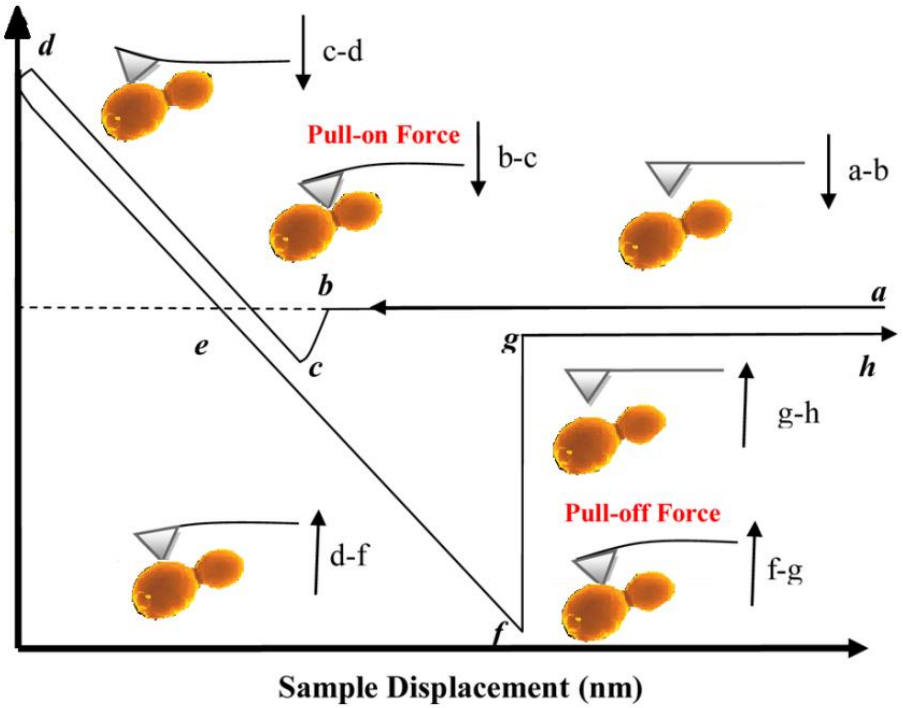


Figure 2.8 Schematic representation of atomic force microscopy used in force spectroscopy mode. Force spectroscopy gives access to force curves that can be analysed in different ways to get the values for the nanomechanical properties of the yeast cell surface. Indentation is read on the force curve and represents the Young's modulus (KPa), the area under the triangle (efg) gives the amount of work done to detach the t5ip from the point of contact and gives the adhesion energy (J) and the force required to detach is the adhesion force (nN).

2.3.3 Surface roughness and height measurement

Roughness analysis of the yeasts surface was carried out by measuring the root mean square roughness, R_{rms}, on the AFM height image. This analysis was carried out on raw AFM images (i.e., the images which were not subjected to any processing, neither flattened nor elaborated with any filter). For each of the four strains, 35 cells were selected at random and the R_{rms} was evaluated at three different areas (B, C and E) on each cell. The roughness analysis was carried using JPK software over a surface of 2.25μm^2

per cell area .The roughness values were analysed within the framework of the sampling theory by considering the yeasts as a very large population.

The roughness (RMS) was calculated according to equation (2.4):

$$Rrms = \sqrt{\frac{1}{n}\sum_{i=1}^{n} x_i^2} \qquad \textbf{Equation (2.4)}$$

The cross sectional analysis was also performed over 35 cells. Briefly, the height differences from peak to peak were taken in micrometer on the raw AFM height measured images and along the entire length of individual cells using the point-to point measurement tool. The height cross section was taken three times on individual yeast cells and statistics was applied to get the average height of the individual cells.

2.4 Biochemical studies on the *Saccharomyces* cell wall

2.4.1 Mannan and glucan staining

The following method for staining yeast cell wall glucans and mannans was modified from Heine *et al.* (2009). Yeast cells were harvested at late stationary phase of the growth curve by centrifugation at 5000rpm for 5 mins at 20^{o}C.The pellet was then washed with PBS buffer and then the cells were counted using haemocytometer to a desired concentration of 3.15 $\times$ 10^{6} cells/ml. Final concentration of the cells (1ml) was prepared in PBS and then 5µl Pisum *sativum*-agglutinate FITC Conjugate was added and incubated in dark for 25mins.The cells were centrifuged and the suspension was then transferred to 96 well plate and the fluorescence was read using a Modulus Microplate reader (Turners Biosystem). A small amount of sample was dropped on a clean slide and then the fluorescence was observed under inverted fluorescence microscope (Leica DMIRE2).The cells were then incubated with 25 µl Concanavalin A Alexa Fluor 350 for 10 mins and then centrifuged and transferred to 96 well plate. The fluorescence was noted and slides were prepared in the similar manner. The images were captured using charged coupled EMCCD camera and analysed using Andor SOLIS for imaging X-3043 software to generate the distribution of the mannans and glucans on the cell wall.

2.4.1.1 Microscopy

The stained cells were observed using an inverted fluorescence microscope (Leica DM IRE2, Germany – see Fig 2.9) and image analysis (camera: Charged coupled EMCCD iXon3, Andor, UK; software: Andor SOLIS for imaging X-

3043). The fluorescence filters used were: Zeiss filter set 02 for Alexa Fluor®-350 fluorescence (excitation G 365, BS 395, emission LP 420), Zeiss filter set 09 for FITC fluorescence (excitation BP 450-490, BS 510, emission LP 515).

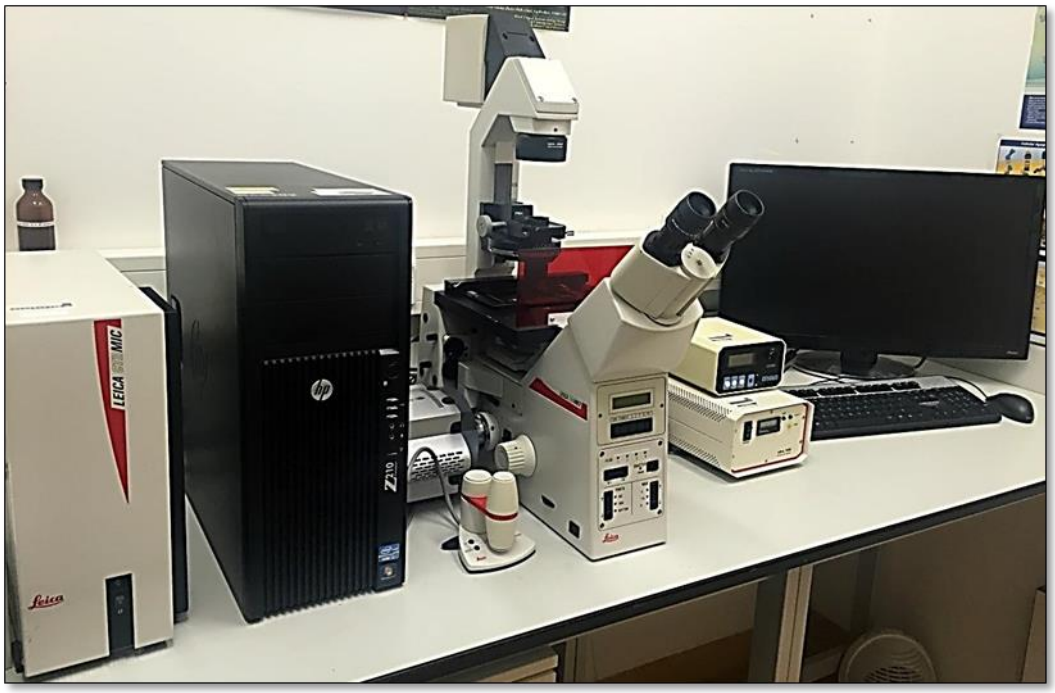


Figure 2.9 Leica DMIRE2 inverted fluorescent microscope used in this study.

The microscope incorporates a built-in Bertrand lens, 12V/100W transmitted light illumination, and a magnification changer. It currently possesses three fluorescent channels: DAPI, A488 and UV.A live cell imaging microscope configured for the acquisition of digital multi-channel fluorescence images of live cells using wide-field illumination.

2.4.2 Lectin like receptor quantification

The density of lectin like receptors present on the yeast cell surface was quantified using a Avidin-FITC probe (Sigma, England, UK). The protocol employed was modified from Patelakis *et al.* (1998) in the following manner.

The probe and FITC conjugates were prepared at concentrations of 1500, 1200, 900, 600, 300 and 100 µg/ml in Ca-ethanol sodium acetate buffer (pH 4.0). 20

µl of the probe-FITC conjugates was added to 2980µl of buffer respectively and mixed properly such that the final concentration of solution ranged from 10 mg/ml down to 0.66 mg/ml. The solution was then vortexed for 15 s and measured using an excitation wavelength of 494±5 nm, and fluorescence read during 1 s at 520±5 nm, using a Modulus Microplate reader (Turners Biosystem).

In order to quantify the presence of mannose receptors sites on the yeast cell surface, the cells were harvested between 12-24 hours. The method of analysis is based on spectrofluormetric measurements that generate the amount of free and bound probe. This concentration of free and bound probe on the yeast cell surface gives the estimate of the mannose binding receptors.

Briefly, the yeast cells were washed twice with distilled water and counted using a coulter counter (Beckman coulter, Germany). 2980ul of 10^6 cells/ml suspension of yeast cells in Ca-ethanol buffer (pH 4) was prepared to which 20ul of each of the probe was added, vortexed for 15 s and fluorescence intensity was noted on a using a Modulus Microplate reader (Turners Biosystem). This was repeated for all the selected probe concentrations of (1500, 900,540,324,192 µg/ml). These readings gave the probe bound to the receptor reading (A). The solution was then centrifuged for 6 min at 4,400 rpm and the supernatant was then slowly removed and the fluorescence was measured again using the Modulus Microplate reader (Reading B). For the blank determination 10^6 yeast cells/ ml were put into Ca-ethanol buffer and the volume was made up to 3 ml. In this case no probe was added. The solution was vortexed for 10 s and fluorescence reading was taken in a Modulus

Microplate reader (Reading C).The suspension was again centrifuged at 4,400 rpm for 6 min and then the readings were taken. (Reading D).

After measuring the yeast and buffer background fluorescence (free probe fluorescence intensity (B-D) and the bound probe fluorescence intensity (A-[C+(B-D)])), the actual amount of free and bound probe to the receptor was calculated in µg/ml. Further, free and bound probe concentrations were then analysed according to Langmuir equation (Knight 1970) in order to obtain the receptor density.

The bound and free probe density was then analysed according to the following Langmuir equation (Knight 1970) to obtain receptor density.

$P/x = k/x' + (1/x') P$

Where, P stands for the concentration of free probe, x is the concentration of bound probe, k is the proportionality constant and x' is the number of binding sites per molecule (number of lectin sites). After deriving the equation by P, plotting 1/x versus 1/P will give a y-intercept of 1/x'.

2.4.2.1 Statistical analyses

Statistical analysis was performed using SPSS software (version 22).One way ANOVA analysis was performed to ascertain the change in parameters in respect to yeast strains and type of sugar in the growth medium. Significance was noted using Bonferroni and Tukey's estimation. Correlation analysis was performed taking into consideration the Pearson's coefficient at two tailed level.

2.5 Molecular Studies

2.5.1 Bioinformatics tools

Ensembl

Ensembl is an online tool that was used to study the genomic sequence of FLO1, FLO5, FLO8, FLO9, FLO10 and PDA1. The complete cDNA sequence of *Saccharomyces cerevisiae* FLO genes transcripts was taken from the Ensembl and the primers were designed based on this cDNA sequence.

NCBI BLAST

BLAST (Basic Local Alignment Tool) from NCBI was used to carry out the similarity search of the primers. Primer sequences were analysed in BLAST to confirm its similarity only with the specific FLO gene of interest sequence (NCBI 2014).

Integrated DNA technologies (oligo analyzer)

Oligonucleotide analyzer tool from Integrated DNA technologies (IDT) was used to analyse the primers. Various properties of the primer including length, GC content, melting temperature, molecular weight and concentration were analysed. The primers were designed with more than 50% of GC content, melting temperature in the range of 58 -68^{0}C and no matches with other regions of the entire cDNA sequence (Integrated DNA technologies 2014).

Clustal Omega

Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to do multiple sequence alignment. The software helped to generate alignments between three or more sequences. The software was helpful in order to align

the sequences of *Saccharomyces* strains closer to strains under study to finally yield the conserved regions. These conserved regions were then targeted to prepare primers for PCR and real time PCR.

Life Technologies, Vector NTI

Life Technologies, Vector NTI, is a completely integrated suite of sequence analysis and design tools that helped to manage, view and analyse the sequences of FLO genes that I was working with. The tool also provided the flexibility to design primers for polymerase chain reaction (PCR), sequence alignment and store sequences for future references.

Primer 3 primer designing tool

Primer 3 primer designing tool (http://simgene.com/Primer3) is a free online tool to design and analyse primers for PCR and real time PCR. The software was used to design primers for real time PCR. The software's user friendly nature helped me to prepare primers which would yield a product length of less than 250 bp which are ideal for quantitative real time PCR.

2.5.2 Molecular Techniques

2.5.2.1 Isolation of genomic DNA

For the isolation of DNA the 25 mg of the yeast was added to 660ul of TE50X containing 10% SDS. It was then vortexed and incubated at 65^{o}C for 10 min whole inverting the tubes after 5 min. Then 340µl of 5M potassium acetate was added in the same Eppendorf and kept in the fridge until it solidifies, usually 15 min. After which the tubes were centrifuged at 13,000 rpm for 10 min. An aliquote of 600µl of the supernatant was then transferred to the new tube. To

this, 600ul of isopropanol was added and the tubes were mixed and inverted well with a further incubation of 10 min at room temperature. The tubes were then again centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the DNA pellet was rinsed with 100ul of cold ethanol (95%). After washing, the ethanol was discarded and the pellet was allowed to dry on the absorbent paper for 1 hr at room temperature or 10 min at 55^{o}C. Finally the pellet was resuspended in 60ul of TE1X and incubated overnight at 4^{o}C.

2.5.2.2 PCR Amplification

Two kinds of PCR amplification was performed. The former being the conventional PCR amplification for targeting the specific gene of interest from the genome, or using the template genomic DNA from the yeast samples or the template DNA from the bacterial minipreparations extraction of the isolated/cloned gene. The other PCR that was performed was the colony PCR. The technique was employed to screen for positive clones for genes of interest.

PCR reaction mix was made in PCR tubes placed on ice using commercially available PCR reagents. Typically, each PCR reaction volume was 30 uL. The following were added.

Go Taq® Flexi Buffer…… 3µL

$MgCl_2$(25mM) .....3µL (final concentration 2.5mM)

Forward Primer (5uM stocks)…… 3µL (final concentration 0.5uM)

Reverse Primer (5uM stocks)……3µL (final concentration 0.5uM)

dNTP mix (2mM each)……..3µL (final concentration 0.2mM)

DNA template……50ng

Go Taq® DNA polymerase……1 unit

Nuclease Free water……upto 30µL

2.4.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a powerful separation method frequently used to analyze DNA fragments generated by PCR amplifications, restriction digestion, genomic DNA or RNA isolation, estimation of ligation ratios and other cloning manipulations. The method could be conveniently used for determining the size of DNA molecules in the range of 500 to 30,000 base pairs.

Closely 1% solution of agarose (Biorad) was made in 1x TAE buffer in a conical flask. Agarose was fully dissolved in TAE by heating in microwave for 1 min and 30s. The agarose solution was then allowed to cool down to 40^{o}C.before beingpoured down in the gel tank cast with comb plate. After the gel solidifies, the comb plate was carefully removed and 1x TAE buffer was added in the gel tank fully immerse the solidified gel in the buffer. The sample DNA that has to be analysed was mixed with the DNA loading loading dye (Qiagen) and carefully loaded in each of the well. For the PCR amplified products, normally all the contents of the tube (30 µl) was used, while for estimating the quality of gel purified DNA normally 5µl of the sample was used. 5µl of the DNA ladder (Qiagen) was added in one of the wells to provide a reference point for the size estimation of the test DNA samples. Once the whole set up is made the gel tank was covered with a lid and the attached cables were connected with the power pack. Agarose gels were then run at 100 V for 1 h.

2.4.2.4 Visualization of the DNA bands image capturing

DNA bands within the agarose gel were visualized under UV by placing the gel on UV trans illuminator (Biorad).Before directly observing the gel in the trans illuminator the gel was first kept in the staining bath containing ethidium bromide for 15 min. Proper safety conditions were carried out and then the gel was shifted to the decolouration bath for 30 min. Once the gels are stained with ethidium bromide, the gel was placed on the UV trans illuminator and the software used to capture images was Quantity One. Depending upon the intensity of bands, appropriate exposure time was set and the image was then captured. The images were then saved in TIFF format for later analysis.

2.5.2.5 Purification of DNA from agarose gels

Purification of DNA fragments from the gel following gel electrophoresis was carried out using the gel purification kit (Qiagen).

To start with the gel was placed on the UV illuminator to visualize the DNA. With the protective clothing on and proper face mask and gloves, the bands of interest were cut with a sharp scalpel and then without much exposing them to UV , carefully and quickly transferred to the labelled Eppendorf tubes. The rest of the protocol was followed according to the manufacturer's instructions (Qiagen). Once the DNA was purified, it was run on the agarose gel to examine its quality.

2.5.2.6 Ligation of the DNA molecules

To carry out the Ligation procedure, Promega transformation kit was used. The Promega cloning vector pGem T-easy was employed. Before proceeding to the

ligation step, prior quantification of the purified DNA was required. The quantification of the DNA was done using a Nano Drop Spectrophotometer ND-1000. Based on the DNA quantity the following ligation ratios were made:

Insert: Vector

3:1

After estimating the volume of the insert and vector that would be required, the following were added in an Eppendorf tube in order to make a 10 ul ligation mixture:

Insert (Volume dependent on the Quantity of DNA fragments after purification, in my case I used 3 ul of the insert)

Vector

10x ligation buffer to the final concentration of 1x

T4 DNA ligase : 3 weiss units/ul

Nuclease free water to final volume of 10 ul.

A non-insert control was also used where nuclease free water was added with no insert added serves as a negative control, while a positive control provided by the Promega Transformation kit are also two kinds of controls that were set up with the ligation reaction. The above reaction was incubated either at room temperature for 3 h, or overnight at 4^{o}C.

2.5.2.7 Bacterial Transformation

Bacterial transformation was performed to either propagate pre-existing plasmid vectors, in which case 1µl of vector was used, or for the ligated vectors having inserts, for which 5µl ligation mix was used. In order to carry out transformation, Promega pGEM®-T and pGEM®-T Easy vector systems transformation kit (Cat. #L2001) was used. High efficiency competent cells (JM109) were supplied in the kit and were used to carry out transformation according to manufacturer's guidelines. Selection of transformants was carried out on LB/ampicillin/IPTG/X-Gal plates.

2.5.2.8 Screening of the transformed bacterial colonies and making glycerol stocks

Following the method of bacterial transformation, the growth of the transformed colonies on agar plates containing X Gal 80ug/ml, Ampicillin 100ug/ml and IPTG 0.5mM made was checked after 16 – 24 hours. The plates showed a mixed collection of both white and blue colonies. The white colonies are the transformed one and were selected and six of such colonies were streaked on a fresh plate containing X gal, IPTG and Ampicillin. After a further incubation of 16-24 h the clones that appear to be pure white were selected and further inoculated in 5 ml of LB medium containing 100ug/ml Ampicillin. Next day the tubes with the bacterial suspension were taken out and centrifuged at 14,000 rpm for 2 min. The media in the supernatant was discarded. In order to extract the transformed plasmid from the bacterial cells, Spin Miniprep kit from plasmid extraction (Qiagen) was used according to manufacturer's instructions. To screen and verify that the insert of interest is present in the vector, a PCR

amplification using T7 and SP6 primers was carried out. As I was aware that the two promoters are situated at either side of the insert, thus this way, I could get an amplified copy of the insert. After successfully transformed bacterial cultures were identified glycerol stocks were prepared with 500 ml of 20% glycerol and 500 ml of the bacterial suspension and then stored at -80^{o}C.

2.5.2.9 Bacterial plasmid extraction

Once the white colonies appear on the plate, this indicates that the cells have been transformed. After which Miniprep kit (Qiagen) was employed to extract the bacterial plasmid DNA. For techniques like propagation and clonal manipulations, plasmid Miniprep was performed.

2.5.3 Total RNA isolation

In order to prepare cDNA for the amplification of FLO1 and FLO8 by QPCR using specific relevant primers , total RNA was isolated from the four industrial strains of *Saccharomyces cerevisiae.* The total RNA for the strains was isolated using RNeasy Mini kit (Qiagen, Cat # 74104) according to manufacturer's guidelines with slight modifications. In order to extract total RNA from the cells, the yeast cells were grown on YEPG media for 36-48 hours (late stationary phase). The yeast lysate was prepared by mechanical disruption. Cells were harvested by centrifuging at 1000×g for 5 min at 4^{o}C. Not more than 5 × 10^{7} cells were mixed with 600 µl of acid washed beads (0.45-0.55 mm dia) and 600 µl of buffer RLT and centrifuged for 20 min at 4500×g. After centrifugation, the supernatant was collected into a new microcentrifuge tube. To 1 volume of homogenized lysate 1 volume of 70% ethanol was added and mixed well withoutby inversions. The sample was then transferred to an RNeasy spin

column placed in a 2 ml collection tube and centrifuged for 15s at 8000×g. The flow through was discarded. Then 700 µl of Buffer RW1 was added to RNeasy spin column and centrifuged again 15s at 8000×g. The flow through was discarded. Then 500 µl of buffer RPE was added to RNeasy spin column and centrifuged for 2 min at 8000×g to wash the spin column membrane. Lastly the RNeasy spin column was placed in new 1.5 ml collection tube and 50 µl of RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 8000×g to elute the RNA. To examine the integrity and quality of the extracted RNA, 5 µl of the RNA was run on 1.5% agarose gel in a gel tank previously washed with 10% SDS and autoclaved to remove residual ribonucleases. The resulting two bands (corresponding to 28S and 18S subunits of ribosomal RNA) ensured the integrity of the RNA isolated.

2.5.3.1 RNA quantification and determination of purity

Once the RNA was purified and checked for integrity by running it on agarose gel, it was quantified using spectrophotometric analysis. First, the RNA dilution buffer (10mM Tris.Cl, pH 7) was taken in a sterile cuvette and placed in the spectrophotometer. This was used as a reference buffer at 260nm wavelength. Next 50µl of RNA was diluted in 9950µl of RNA dilution buffer in a cuvette and its absorbance was measured at A260nm. Each reading was taken 3 times and their means calculated.

The RNA was quantified by the following formula:

$$A_{260} \times \text{Dilution factor (200)} \times 40 = x\ \mu g/ml \qquad \textbf{Equation 2.5}$$

In order to determine the purity of RNA, a ratio of the Absorbance at 260 and 280(A_{260}/A_{280}) was calculated after dilution RNA in 10mM Tris.Cl, pH 7.5. An absorbance ratio of around 2 was considered to indicate good quality RNA.

2.5.3.2 cDNA synthesis

The total RNA isolated in the above step was subjected to DNase digestion, so any DNA contamination was removed using the Qiagen RNase- Free DNase set (Cat# 79254) according to manufacturer's guideline. Known amount of RNA was used for cDNA synthesis, using RT^2 First strand kit (Qiagen, Cat # 330401) according to manufacturer's guideline.

2.5.3.3 DNA & cDNA quantification

The DNA was quantified using spectrophotometric method. First of all, 500 µl of distilled water was taken in a cuvette and placed in the spectrophotometer. The wavelength was adjusted at 260nm and the water was used to blank the spectrophotometer. Next, 10µl of DNA was diluted to 490µl of distilled water in a cuvette and its absorbance was read at 260nm. The readings were taken in triplicates and their means were calculated. The quantity of DNA was calculated according to this formula:

$$A_{260} \times \text{Dilution factor (50)} \times 50 = x\ \mu g/ml \qquad \textbf{Equation 2.6}$$

In order to determine the purity of DNA, a ratio of the Absorbance at 260 and 280 (A_{260}/A_{280}) was calculated after dilution RNA in 10mM Tris.Cl, pH 7.5. An absorbance ratio of around 1.8 was considered to indicate good quality DNA. All cDNA's were diluted 10-fold with nuclease free water and 5µl of cDNA was used in subsequent reactions for QPCR.

2.5.3.4 Primer design for QPCR

In order to know the expression of the gene of interest, optimal primers were required for Quantitative real time PCR (QPCR). The primer pairs should be able to yield a highly specific PCR product and have minimal nonspecific annealing. Three pairs of exon-exon boundary crossing primers for FLO1, FLO8 and PDA gene were designed using Primer 3 software (Broad Institute, USA) (**http://www.ncbi.nlm.nih.gov/tools/primer-blast/**).

Gene	Sequence	Annealing temperature	Transcript length
FLO1FQ1	GCGTTCAACTGTTGTGCTC	62.2	
FLO1RQ1	CCAAGAAACAGCGTTCGAG	63.6	177 bp
FLO1FQ4	TCTATCAGTAGGTGGTGCAAC	59.3	
FLO1RQ4	AGAAACAGCGTTCGAGTAAAC	60.0	190 bp
PDA1FQ1	TGAGACTTCGAAAGCCACC	63.1	
PDA1RQ1	AGTGAAACCGTGACATCTG	58.7	240 bp
PDA1FQ2	GACTTCGAAAGCCACCTTG	62.4	
PDA1RQ2	TGTGATGGCATTCTCGATACC	64.8	350 bp
FLO8F1	CCCGTGTAACAATAATACCAC	58.2	
FLO8R1	ACCCTTCGCTTTTGAGGTTG	65.2	180 bp
FLO8F2	TGGGAAGTTTCAACAAGCCG	67.1	
FLO8R2	CCAGACCGAGGTGTTGCTAT	64.0	200 bp

Table 2.2 List of primer pairs used for qPCR for FLO1, FLO8 and the housekeeping gene PDA1. The primer pairs were used on cDNA that was obtained after RNA extraction.The primers for qPCR were designed using Primer 3 online software. The primers were checked for dimer formation, GC% content etc.

Gene expression of the test genes (FLO1 and FLO8 gene) were normalized against housekeeping gene (HKG) which is constitutively expressed in all strains under the study. The reference gene should not be affected/regulated by the experimental conditions. Thus for my study, I used pyruvate dehydrogenase genes (PDA1), that is E1 alpha subunit of pyruvate dehydrogenase (PDH) complex, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA. The E1 subunit is phosphorylated and regulated by glucose (Govender *et al.* 2008).

Since the exact nucleotide sequence for FLO1 and FLO8 gene was not known for all the strains except the champagne strain, thus in order to proceed for primer designing, I first got the transcript or the CDS sequence for Champagne strain from Ensembl (http://www.ensembl.org/index.html) and then proceeded with BLAST, which gave me the list of the FLO1 and FLO8 sequence of the champagne strain and top 10 *Saccharomyces* strains were picked. The primers were designed for the conserved sequence that was obtained after multiple alignment.

Table 2.2 summarizes the primer pair sequences for the FLO1, FLO8 and PDA1 gene. Each gene was amplified in triplicates (N=3) for each of the three biological repeats of the four strains. PCR conditions* were optimized and 5µl of the amplified product was electrophoresed on a 1.5% agarose gel to verify amplicon size.

*PCR conditions remain same for all the three genes except the annealing temperature differed slightly.

Step	Temperature	Time
Initial Denaturation	94°C	30s
Denaturation	94°C	30s
Annealing*	**55°C***	60s
Extension	65°C	40s
Final Extension	65°C	10 min

35 Cycles

*Annealing temperature for PDA1, FLO1 and FLO8 gene was 55°C, 52°C and 53°C respectively.

Table 2.3 PCR conditions used for the amplification of the particular region of interest from FLO1, FLO8 and PDA1 cDNA.

2.5.3.5 Semi Quantitative qPCR (Gel densitometry analysis)

Gel densitometry analysis (Semi quantitative PCR) was used to compare the differential expression of each of the test gene with HKG between the four strains using Gel Doc™ imager (Life Technologies, Carlsbad, CA, USA).The primers (Table 2.2) were used to amplify the cDNA from all the four strains and subjected to PCR. The PCR was performed in triplicates for each of the strain and each of the gene using the conditions (Table 2.3). The final PCR product was resolved by electrophoresis on 1.5% agarose gels. The gels were photographed using Gel Doc 100 (Bio-Rad, Hercules, USA) and the images were analysed by using Band scan analyser 5.1 software.

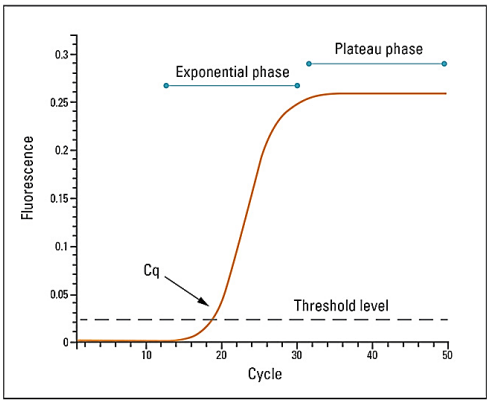
2.5.3.6 Quantitatve or realtime PCR (QPCR) using SYBR® Green Master Mix

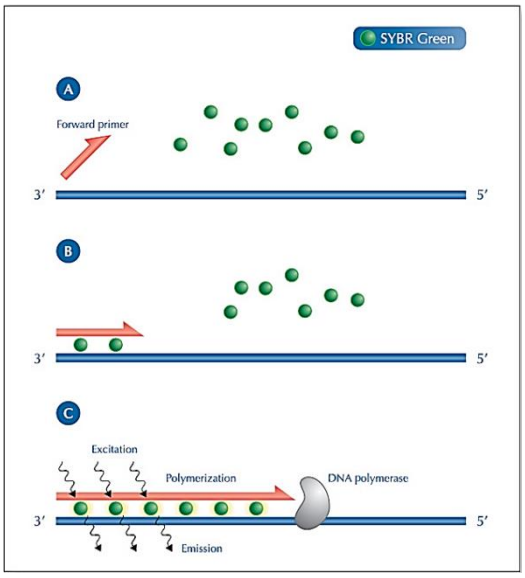
Two genes out of five genes were selected to check for the expression levels. These were FLO1 and FLO8 genes. Flo1 gene is the main flocculation gene and FLO8 gene product is required for the transcriptional activation of all the other flocculation genes namely FLO1, FLO5, FLO8, FLO9 and FLO10. Quantitaive or realtime PCR is a standard PCR with the advantage of detecting the amount of DNA formed after each cycle with either fluorescent dyes or fluorescently tagged oligonucleotides probes. In this study I used SYBR® green dye to measure the degree of fluorescence at the end of each cycle. The intensity of the fluorescence emitted during QPCR correlates to the amount of DNA product formed. Fluorescence exponentially increases as the DNA template is amplified. After a few cycles of qPCR, fluorescence surpasses a threshold level set above background fluorescence and starts to increase exponentially. Eventually the fluorescence signal levels off because the fluorescence saturates the detector of the real time PCR machine. Fluorescence is no longer related to the starting template copy major development of PCR technology that enables reliable detection and measurement of products generated during each cycle of PCR process. QPCR reactions were carried out in 96 well polypropylene plates, Stratagene (Cat. # 40133, Carlsbad, CA, USA) in a volume of 20µl and in triplicates. Briefly, the reaction mixture consisted of:

Components	Quantity
10 fold diluted cDNA	5 µl
SYBR® Green Reaction mix	10
Forward Primer (5µM)	1µl
Reverse Primer (5µM)	1µl
RNase/DNases free water	3µl
Total volume	10µl

Table 2.4 Reaction setup for performing qPCR reaction. The reaction was set up in triplicates in 96 well PCR tubes. QPCR was carried out in Stratagene MX3000P SYSTEM.

The reaction was carried out using SYBR® green master mix (Qiagen, Cat. # 204141). SYBR® green is an intercalating dye which binds to double stranded DNA and results in fluorescencent signal of intercalated dye which is several orders of magnitude higher than that of unbound dye. The working of SYBR green has been explained in Fig 2.10





a. **b.**

Figure 2.10 (a) Fluorescence Resonance Energy Transfer. The efficiency of FRET is dependent on the overlap of fluorescence of the fluorophore emission and quencher absorption spectra, as well as the physical distance between fluorophore and quencher. (b)**SYBR Green Chemistry.** A) DNA is denatured and SYBR Green molecules are free in the reaction mix. B) Primers anneal and SYBR Green molecules bind to the dsDNA. C) DNA polymerase elongates the template and more SYBR Green molecules bind to the product formed resulting in exponential increase in the fluorescence level.

The reaction was run on a MX3000P SYSTEM (Stratagene, Santa Clara, CA, USA) with the following cycling conditions: initial denaturation at 95^{0}C for 10 min followed by 40 cycles of denaturation at 95^{0}C for 30 s, annealing of 55 ^{0}C for 1 min, and a primer extension of 72^{0}C for 1 min with melting curves run after each end point amplification at 1 min for 95^{0}C, followed by 30 s increments of 1 ^{0}C from 55^0 C to 95 ^{0}C and a subsequent melting curve analysis. All reactions were run in triplicate and the mean Ct-values were used for further analysis. Three independent experiments were performed. Relative expression levels of FLO1 and FLO8 gene were calculated using the comparative cycle threshold (Ct)

method described by Pfaffl (2001) (see Fig 2.11). The housekeeping gene used was PDA1. The relative change in the quantity of the PCR product formed was directly proportional to the amount of dye incorporated which emits at 520 nm and fluorescence emitted can be detected and related to the amount of target.

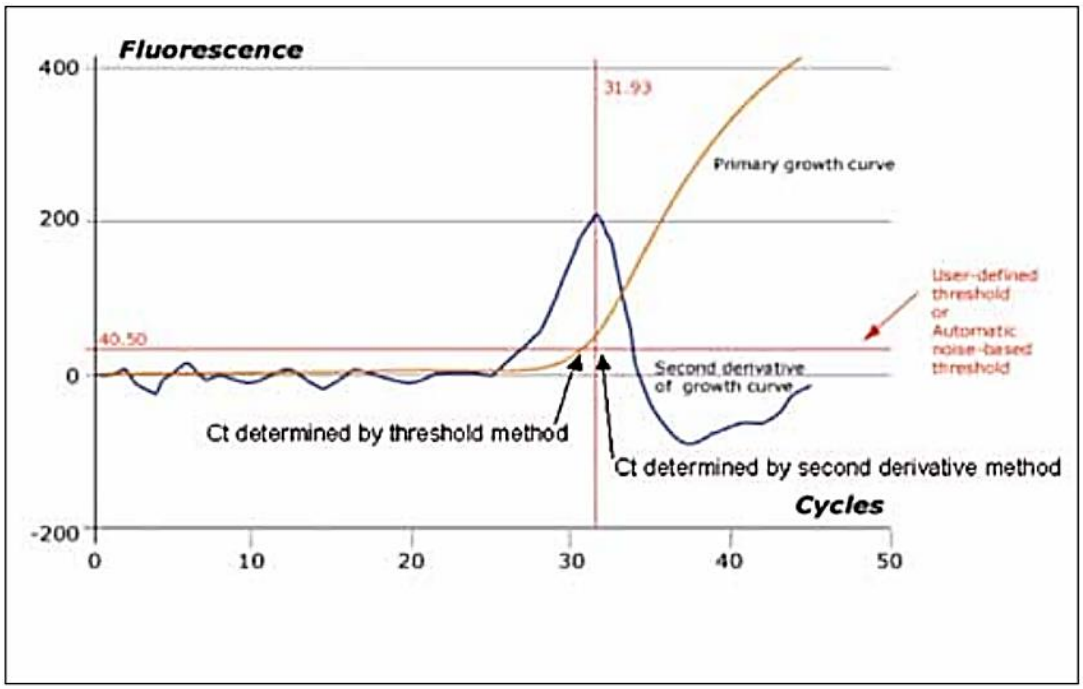


Figure 2.11 The graph explains the threshold definition and the Ct value calculation.

The threshold refers to the level of fluorescence above the baseline, at which the signal can be considered not to be background. The threshold in this study was set automatically using the calculation of threshold corresponding to the average baseline + "X" standard deviation of the baseline. The Ct value which is defined as the cycle in which there is significant increase in the reporter signal, above the threshold, that is the cycle in which the growth curve crosses the threshold. The Ct value is consequently in inverse proportion to the expression level of the gene. If the Ct value is low, it means the fluorescence crosses the threshold early, meaning that the amount of target in the sample is high. The

ROX is a fluorescent dye that is used as a passive reference for Stratagene. This dye is usually spiked into the master mix and the reporter fluorescence is normalized to the ROX signal on Stratagene Mx3000P machine.

2.5.3.7 Normalization and quantification methods

The two major methods of normalization are the absolute quantification and the relative quantification (Sellars *et al.,* 2007). In absolute quantification, the exact number of copies of the gene of interest was calculate, while in relative quantification, the expression of the gene of interest is expressed relatively to another gene. Relative quantification is the most widely used technique. Gene expression levels were calculated by the ratio between the amount of target gene and an endogenous reference gene, which is present in all samples. The reference gene has to be chosen so that its expression does not change under the experimental conditions or between different tissues (Cook and Stevenson, 2009).

Delta delta Ct ($\Delta\Delta$Ct) method is the simplest and a direct comparison of Ct values between the target gene and the reference gene. Relative quantification involves the choice of a calibrator sample. The calibrator sample can be the untreated sample (internal control or a no template control (NTC)) the time=0 sample, or any sample you want to compare your unknown to.

Firstly, the ΔCt between the target gene and the reference gene was calculated for each sample (for the unknown samples and also for the calibrator sample).

$$\Delta Ct = Ct_{target} - Ct_{reference\ gene}$$ **Equation 2.7**

Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated.

ΔΔCt value:

$$\Delta\Delta Ct = (Ct_{target} - Ct_{reference})_{calibrator} - (Ct_{target} - Ct_{reference})_{sample}$$

Equation 2.7

The normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$ and this value can be used to compare expression levels in samples.

Chapter Three

Cell surface properties and flocculation behaviour of industrial strains of *Saccharomyces cerevisiae*

Abstract

Cellular adhesion properties of yeasts depend on the characteristics of the outer layer of the cell wall. In this study, the flocculation behaviour of four industrial strains of Saccharomyces cerevisiae used for production of beer, champagne, wine and fuel alcohol was evaluated; their flocculation abilities being, 42.5%, 14.8%, 13.8% and 11.6%, respectively. The brewing yeast strain was found to be the most flocculent. Very little flocculation was observed during the lag and logarithmic phases of growth (1-15%), while during the early and late stationary phases, different strains exhibited variable flocculation patterns. Cell surface hydrophobicity (assayed using HMA and MATS) and surface charge (assayed by Alcian Blue dye retention) played important roles in dictating flocculation behaviour in different yeast strains, as did the yeast growth phase. Hydrophobicity index (HI) and % hydrophobicity of the four strains followed, respectively, the same order, viz Beer (66.6, 21.5) > Champagne (33, 10.5) > fuel alcohol (22.4, 7.4) > wine (20.5, 2.7). Our findings provide new insight into yeast cell surface properties and how these relate to behavioural characteristics of yeasts employed in industrial fermentations.

3.1 Introduction

The adhesion properties of microorganisms, which involve adhering of the microbe to other cells, tissues or solid substrates, have been the focus of wide ranging scientific and biotechnological interest (Verran and Whitehead, 2005; Verstrepen and Klis, 2006; Zhao and Bai, 2009; Kjeldsen, 2000). Adhesion properties are known to play important roles in governing many essential aspects of the life cycles of microorganisms including sexual reproduction (Chen *et al.*, 2007), cellular aggregation (e.g. flocculation), biofilm formation and invasion, and/or pathogenic behaviour (Reynolds and Fink 2001; Palmer *et al.,* 2007; Ramage *et al.*, 2009, Maury J *et al.,* 2005).

Yeast cells undergo Ca^{2+}-dependent, reversible, asexual aggregation known as flocculation. In *Saccharomyces cerevisiae*, floc formation is helpful in certain industrial fermentations such as brewing, as this aids in sedimentation of yeast cells at the bottom of cylindro-conical fermenter vessels at the end of the fermentation process (Bony *et al.*, 1997; Stratford, 1989). In some cases, co-flocculation has been reported to occur by adhesion of flocculent and non-flocculent strains of *S. cerevisiae* and lactic acid bacteria (Miki *et al.,* 1982a).

One important factor that governs the degree of flocculation is cell surface hydrophobicity which plays major roles in microbial adhesion phenomena. For example, an increase in flocculation ability is strongly correlated with an increase in cell wall surface hydrophobicity (Azeredo *et al.,* 1997). Additional factors are involved, including electron donor/acceptor properties and zeta potential (White and Walker 2011). Techniques like microbial adhesion to solvent techniques (MATS), based on cell surface affinities for a monopolar and non-polar solvent, may be used to determine the electron donor or acceptor

properties of yeast cells, whilst zeta potential can be quantified by measuring the electrophoretic mobility of cells (Vichi *et al.,* 2010).

Yeast flocculation is also governed by genetic determinants. Five flocculin genes are expressed in *S. cerevisiae*, namely, FLO1, FLO5, FLO9 FLO10 and FLO11. Flo11p exhibits a variety of roles in yeast that helps cells change and adapt during nutritional deficiencies by switching to a pseudohyphal state, enabling cells to invade substrates in response to starvation of nitrogen and glucose (Dranginis *et al.,* 2007, Saitoh *et al.,* 2005).

It is important to study the cell wall properties of industrial yeast strains so as to have a better understanding of the phenomenon of flocculation. This study thus investigated the cell surface properties and flocculation behaviour of different strains of *S. cerevisiae* employed in brewing, winemaking and bioethanol industries. Cell surface parameters including cell surface hydrophobicity (CSH) and cell surface charge were investigated in these yeast strains at different (lag, logarithmic, early stationary and late stationary) phases of growth and our findings are pertinent to further understanding and potential manipulation of industrial fermentations involving *S. cerevisiae*.

Thus, the main aim of this chapter is:-

- To obtain greater insight into the fermentation performance of selected industrial strains
- To conduct comparative analysis of flocculation behaviour among the strains during the different phases of growth curve

- To evaluate the effect of cell surface properties like cell surface hydrophobicity (CSH) and cell surface charge (CSC) on flocculation behaviour of the selected strains of *Saccharomyces cerevisiae.*

3.2 Results

3.2.1 Performance of individual strain in terms of their fermentation capability

The strains of *Saccharomyces cerevisiae* under study were industrial strains that were provided by Lallemand Inc. (Montreal, Canada). All the four strains have varied roles in industry and are employed for achieving a different end product.

I have denoted these strains on the basis of their end product for eg. LYCCI (Brewing strain), LYCCII (Champagne strain), LYCCIII (Wine strain) and LYCCIV (Fuel Alcohol strain). When observed under the microscope the cells of each of the strain had different cell morphology, size, diameter and different growth curves.

Growth curves were plotted for all the strains (see section 2.1.7). All the four strains had different growth curves, in which it was observed that the brewing strain (LYCCI) grew more slowly at the beginning but then caught up with the other strains during the exponential stage. Cultures were initiated using a starting cell density of 5×10^6 cells/ml and cells were allowed to grow in YEPG broth with growth monitored by enumerating the cells after 2 hours over a period of 48 hours. It was observed that all the strains (LYCCI, LYCCII, LYCCIII and LYCCIV) had a brief 2 hours period which was the “lag phase”. Oxygen is rapidly absorbed from the media during the lag phase in order to produce

important cell membrane constituents such as sterols and unsaturated fatty acids. It is thus important to provide enough oxygen at the beginning of fermentation and this was accomplished by shaking the flasks.

The strains exhibited steep increases in cell number/ml after 2 h and continued to increase for a further 15 h. This was considered to be the logarithmic growth phase. After 15 h the cell number remained more or less stable and thus I considered that stage to be the early stationary phase (Fig 3.1).

The accurate distinction of the phases could not be made until I plotted the cumulative CO_2 pressure curves versus time graph, which gave a better picture of when each strain entered different phases of growth. From the data I concluded that there were three phases in yeast life cycle: namely; Adaptive, Attenuative and Conditioning. The adaptive phase is the aerobic growth phase, attenuative is the anaerobic alcohol production phase, and conditioning is a seemingly quiet but nonetheless important phase in which certain primary fermentation metabolites are produced.

S. cerevisiae does not use “aerobic metabolic pathways” in high sugar media due to the Crabtree effect. This phase involves uptake of oxygen, nitrogen and sugars. With the cell membranes now permeable to sugars and to nitrogen compounds such as amino acids and small peptides, the yeast cells now have all the necessary nutrients to enter a period of rapid growth and reproduction. The growth and reproduction will continue rapidly as long as there is sufficient oxygen. When oxygen is no longer available, the yeasts will be forced to use their anaerobic metabolic pathway, which is far less efficient. The result of this shift is that growth slows dramatically, and the transition to the attenuative

phase begins. During the attenuative phase, or the beginning of the early stationary phase, is when the yeasts process sugars in media anaerobically. This is when the majority of the alcohol is produced.

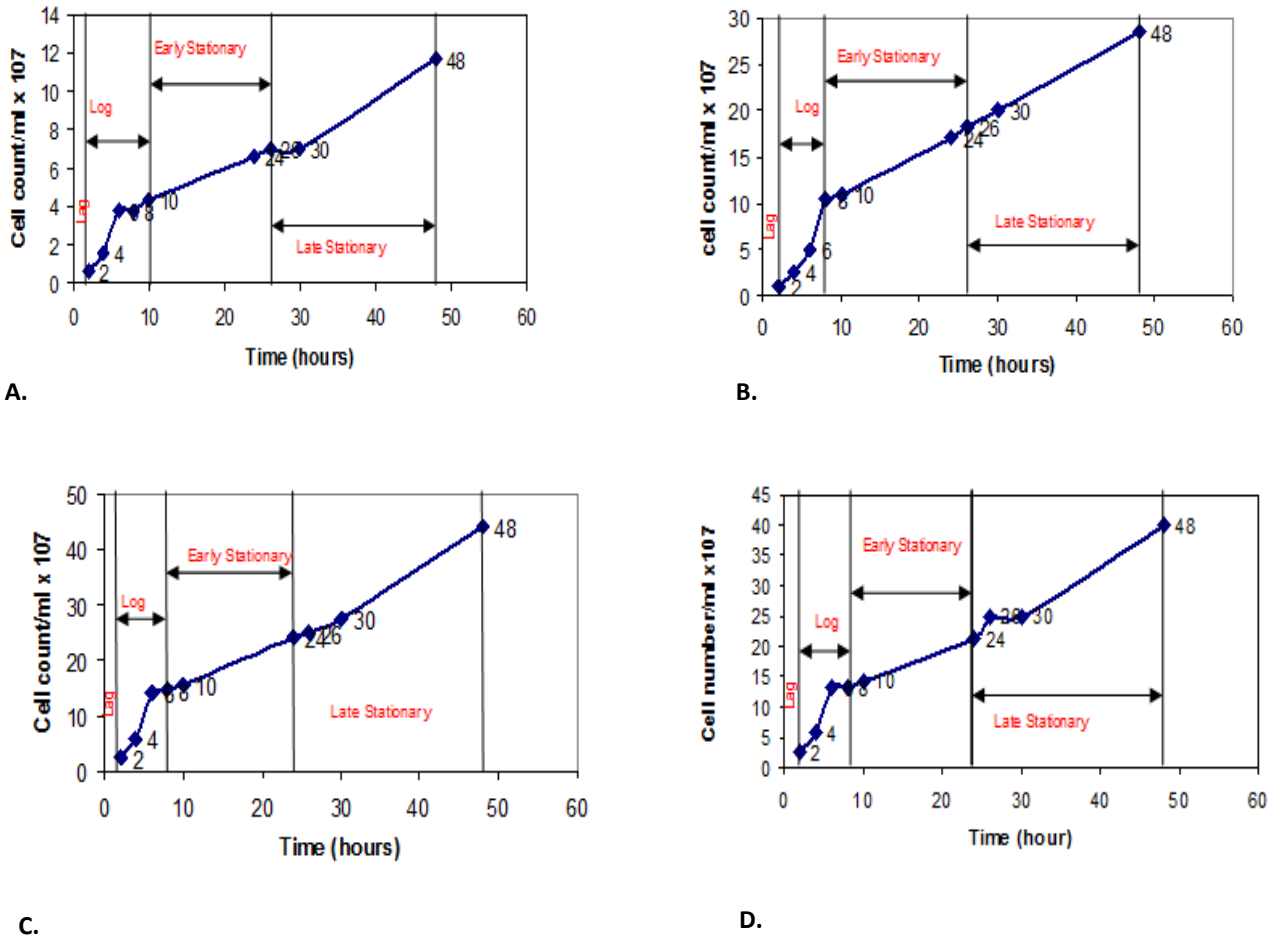


Figure 3.1 Growth curve for the strains (A) LYCCI (brewing strain) (B) LYCCII (Champagne strain) (C) LYCCIII (wine strain) (D) LYCCIV (fuel alcohol strain). Time points from 0-2 h are considered to be lag phase, 2 h onwards till 8 hours is the logarithm phase, 8-24 h is early stationary phase and 24 h onwards is the late stationary phase.

3.2.1.1 Comparative analysis of industrial yeast strains in terms of ethanol production and cumulative CO_2 production.

Fermentative characteristics of the yeast strains were obtained by estimating their ethanol production v/v and cumulative CO_2 pressure v/s time graph (see section 2.1.6). This not only helped us to achieve an understanding about their fermentative properties, but also helped in selection of suitable time points to carry out specific studies during specific phases of the growth curve. I could easily allocate specific phases the cells were for each of the strain as the curves helped us understand the proper transitioning of the cells for each of the strain.

From ethanol content (v/v) v/s time curve (Fig 3.2(A)) I concluded that the brewing strain (LYCCI) yielded the highest amount of ethanol (v/v) followed by champagne strain (LYCCII), wine strain (LYCCIII) and finally the fuel alcohol strain (LYCCIV). Maximum production of ethanol was seen only during the early stationary phase and then started reducing as the cells proceeded into late stationary phase. From Fig 3.2(B) I can see the clear transitioning of the strains through all the four major phases of growth curve namely the lag, log, early stationary and late stationary phase.

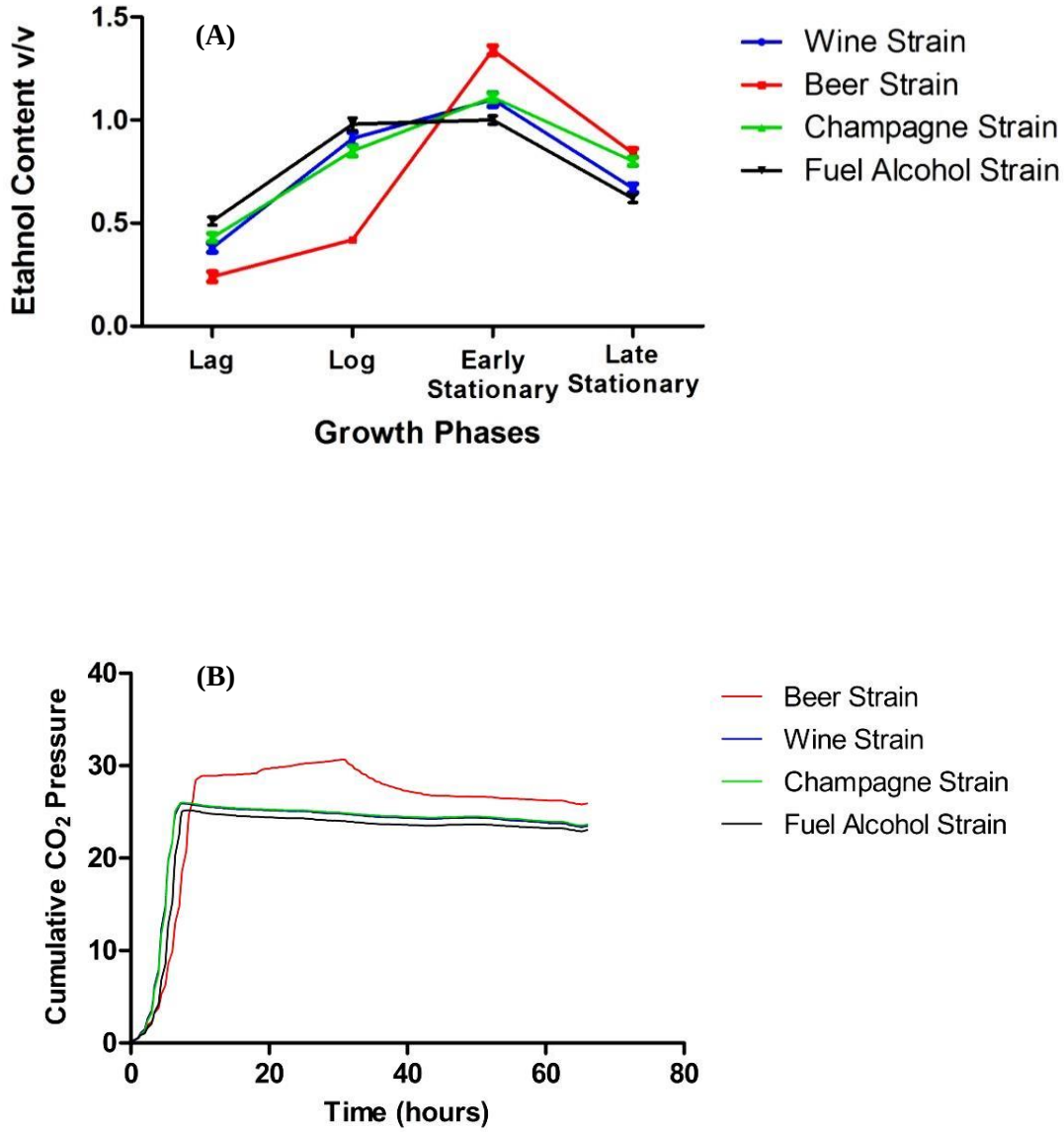


Figure 3.2(A) Ethanol production by four different strains of *Saccharomyces cerevisiae* during lag, log, early and late stationary phases of growth.

Figure 3.2(B) Carbon dioxide production by four different strains of *Saccharomyces cerevisiae* depicted in terms of cumulative CO_2 pressure measured in psi.

3.2.2 Comparative analysis of industrial yeast strains in terms of their flocculation ability during different growth phases

The flocculation behaviour and cell surface properties of four different industrial strains of *S. cerevisiae* were studied. The aim of this study was to obtain greater insight into the fermentation performance of selected industrial strains and how their flocculation behaviour is affected by changes in cell surface properties which in turn are affected by changes in nutrient availability and physico-chemical conditions.

3.2.2.1 Basis for Yeast Flocculation assay

The flocculation experiments performed here were based on the use of controlled inocula, yeast growth, cell density of the suspension and the experimental temperature. Flocculation was expressed as a percentage of A_{600} as shown in equation 1.

Strains were then classified as non-flocculent (<20%), very flocculent (>85%) and moderately flocculent (20-80%) (ASBC,1986). Yeast flocculation could also be measured based on sugar de-flocculation, thermal de-flocculation, turbidity of the suspension in a glass capillary or hydrophobic interaction chromatography (Jin & Speers, 1998).

$$\% \text{ affinity} = 1 - \left(\frac{A}{A_0}\right) \times 100 \qquad \textbf{Equation 3.1}$$

Cell-cell adhesion

The fermentation performance of the industrial yeasts in YEPG was initially evaluated in small-scale fermenters. The brewing yeast strain produced the highest amount of ethanol (v/v) followed by the champagne, wine and fuel

alcohol strains. As shown in Fig 3.2(B), the CO_2 production kinetics indicated the transition of the yeast cells from one growth phase to another.

The activation and thus the expression of various flocculins resulted in the formation of macroscopic biofilms ranging in diameter from around 100 micrometres to several millimetres. Flocculation tests were carried out during all the phases of growth for the four industrial strains and it was found that all yeasts were flocculent either during the early or late stationary phases of growth. The strains exhibited significant ($p<0.05$) differences in their flocculation abilities during all the phases of growth (Fig 3.3). The brewing yeast strain was found to be highly flocculent throughout the fermentation including the late stationary phase (42.5%) when the flocculence character of the other strains diminished.

The champagne yeast strain may also be categorized as highly flocculent as it showed flocculence of about 28% during early stationary phase. The main reason for flocculation predominating during early and late stationary phase may be due to progressive crenellation and wrinkling of the cell wall during aging. This increases the potential surface area of contact compared with that of smooth younger cells and therefore promotes cell - cell adhesion (Barker and Smart, 1996).

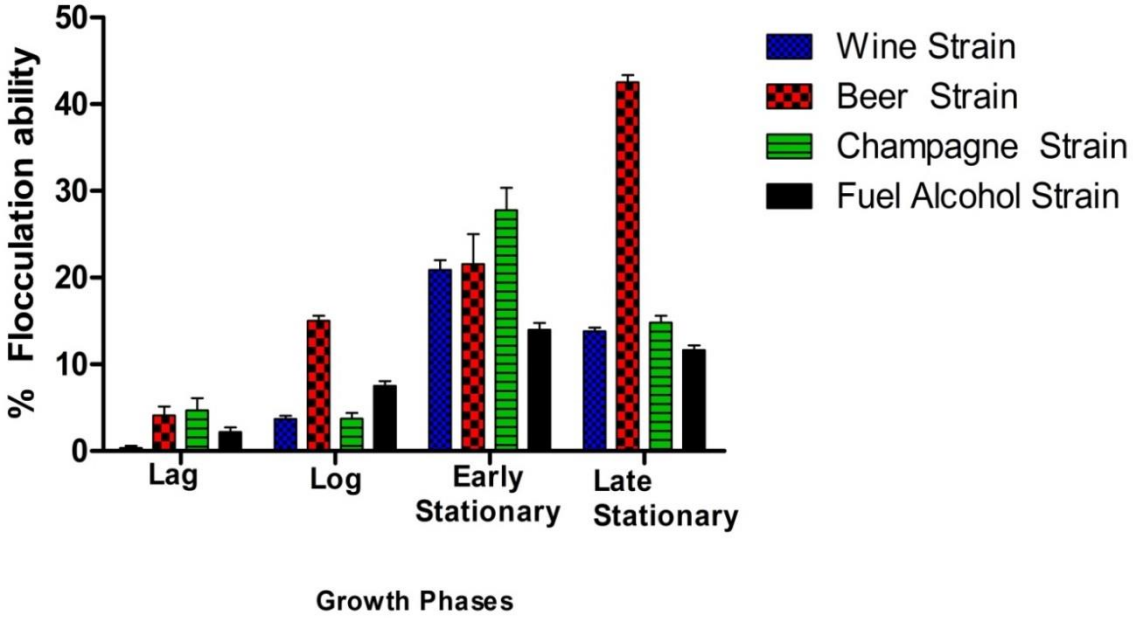


Figure 3.3 Percentage flocculation ability of different strains of *Saccharomyces cerevisiae* during different phases of growth. Champagne strain (Green) showed high flocculation during early stationary phase, while comparatively less flocculation was seen during late stationary phase. The same held true for wine strain (blue) and fuel alcohol strain (black). In contrast, beer strain (red) became more flocculent during late stationary phase. Significant difference ($p \leq 0.05$) was observed between the flocculation ability of all the strains during different phases of growth curve.

3.2.2.2 Phase wise performance of the strains

The majority of brewing yeast strains belong to the NewFlo phenotype (Stratford and Assinder 1991) and possess cyclic flocculation abilities (Soares and Mota 1996). The flocculent cells, when placed in YEPG medium, progressively lose flocculation ability and become flocculent towards the end of logarithmic phase of growth (Fig 3.3).

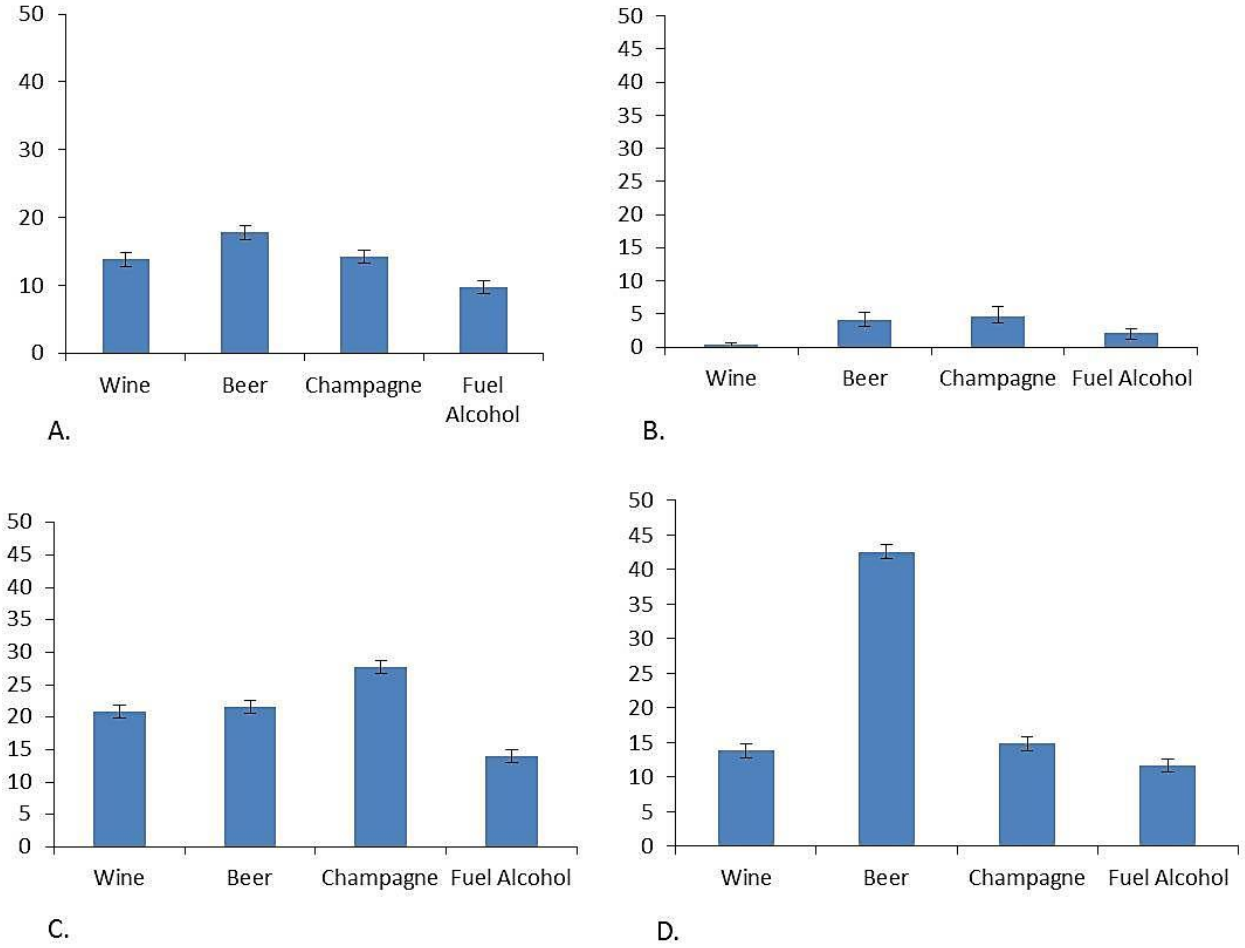


Figure 3.4 Percentage flocculation ability for the strains during different phases of growth curve. (A) Lag phase (B) Logarithm phase (C) Early stationary phase (D) Late stationary phase.

On the basis of results so obtained it is clear that LYCCII, LYCCIII and LYCCIV belonged to NewFlo type as flocculation was inhibited by mannose, glucose, maltose and fructose, suggesting proteins in these strains that were present on the cell surface were able to bind to a wider range of all these sugars, except galactose (See section 4.2.1).

3.2.3 Loss of flocculation

In the beginning of growth, the declining of flocculation of NewFlo phenotype strains can be attributed to a loss, blockage or inactivation of flocculation lectins or the receptors. However, it was shown that flocculation receptors are available in all stages of growth of these strains (Stratford 1993; Soares and Mota, 1996); this means that flocculation cycle is dependent of the presence of active flocculation lectins (Stratford and Carter, 1993).

In a general way, the triggering of flocculation of NewFlo phenotype strains occurs at the end of exponential phase of growth (Fig 3.3), when a critical nutrient, for example, sugars such as glucose, fructose or maltose (Soares and Mota, 1996; Sampermans *et al.,* 2005), in ale strains are almost depleted from the culture medium. The initial concentration of glucose of the culture medium provokes a delay in the expression of flocculation (Soares and Mota, 1996). On the other hand, cells under catabolic repression when transferred to a medium with a lower sugar concentration have shown a rapid triggering of flocculation; this fact suggests a casual link between sugar limitation and the induction of flocculation (Sampermans *et al,.* 2005). Conversely, another important point observed here is that flocculation was normally not observed during the lag phase of the growth. I assume that the majority of the cells are virgins during this phase and trying to adapt themselves to the new cultural conditions. As flocculation is more or less a stress response, the considerable % flocculation ability during the lag phase may be due to a *Hangover phase* that cells undergo when they were in a preculture state. Since the duration was 14-16 hours I can assume that some cells may have entered a senescence stage in the seed

culture conditions, which when transferred to the new medium show flocculation as they express the flocculation genes (FLO genes).

Flocculation expression is an energetic-dependent process, which requires the presence of a residual external energy source (Soares and Mota, 1996), but not an external source of nitrogen. Non-flocculent cells, in exponential phase of growth, when placed in a culture medium without carbon source do not express flocculation; on the contrary, these cells when transferred to a complete medium except nitrogen source developed a flocculent phenotype (Sampermans *et al.,* 2005).

The triggering of flocculation seems to be influenced by metabolism of the carbon source. The presence of small amount of sugars or ethanol allows the onset of flocculation while the presence of glycerol impairs the expression of flocculation (Sampermans *et al.,* 2005). Small amounts of ethanol have a positive effect on the triggering of yeast flocculation. The presence of 1% (v/v) of ethanol induces an early development of flocculation in cells growing in low amounts [0·2% (w/v)] of fermentable carbon source. This reason also partially explains the reason for the brewing strain (LYCCI) being highly flocculent compared to all other strains as it produces much higher (1.43% ethanol v/v). The shortage of nutrients combined with the presence of ethanol may be the signal that induces the onset of flocculation (Sampermans *et al.,* 2005; Claro *et al.,* 2007).

3.2.4 Cell surface hydrophobicity directly influences the degree of flocculation

3.2.4.1 Cell surface hydrophobicity

Cell surface hydrophobicity was determined by two techniques, HMA and MATS (See section 2.2.2.1 and 2.2.2.2). HMA employs latex microspheres with a diameter of 0.845 ± 0.001 µm. About 100 cells for each of the four strains were counted and percentage hydrophobicity was calculated for those cells having ≥ 3 beads attached (Fig 3.6). The reason for using 0.845 ± 0.001 µm microspheres was the ease of homogeneous suspensions as opposed to smaller microspheres (<0.845 µm dia).

Fig 3.5a shows that there were significant ($p<0.05$) differences in the hydrophobicity indices of the 4 industrial yeasts, with the brewing strain exhibiting the highest hydrophobicity index (65%). These observations were validated using both MATS test and HMA assay indicating that the brewing strain was the most hydrophobic (21.5%) toward hexadecane (apolar solvent), followed by the champagne strain (10.5%), fuel alcohol strain (7.4%) and wine strain (2.7%). In addition, the strains also showed high electron donor capacity (percentage affinity to chloroform minus percentage affinity to hexadecane). The brewing strain showed highest electron donating capacity (68.8%) while champagne strain showed the lowest (44.1%). Our studies show a direct correlation between increased CSH and initiation of flocculence during fermentation (Fig 3.8a, b). A high level of CSH facilitates higher cell-cell contact in an aqueous medium resulting in more specific lectin-carbohydrate interaction (Jin and Speers, 1998).

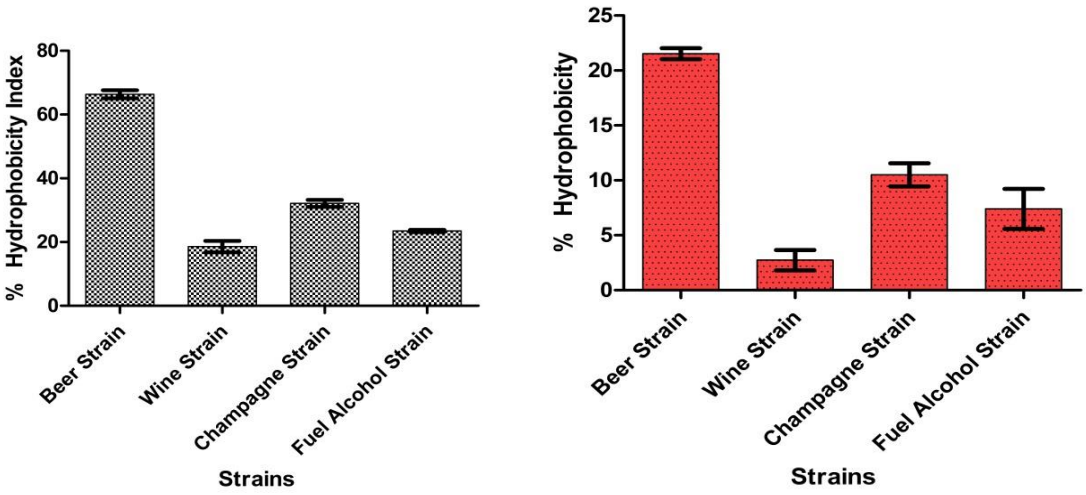


Figure 3.5a. Percentage Hydrophobicity Index (HI) of different strains of *Saccharomyces cerevisiae* by HMA test. Significant differences ($p \leq 0.01$) were observed amongst the strains. Beer strain exhibited highest %HI. Due to high CSH, more latex beads were observed to be attached to the cell surface.

Figure 3.5b. **Percent Hydrophobicity of different strains of *Saccharomyces cerevisiae* by MATS test.** Significant differences ($p \leq 0.01$) were observed between different strains. HMA and MATS tests showed similar pattern of results (compare Fig 3a with 3b).

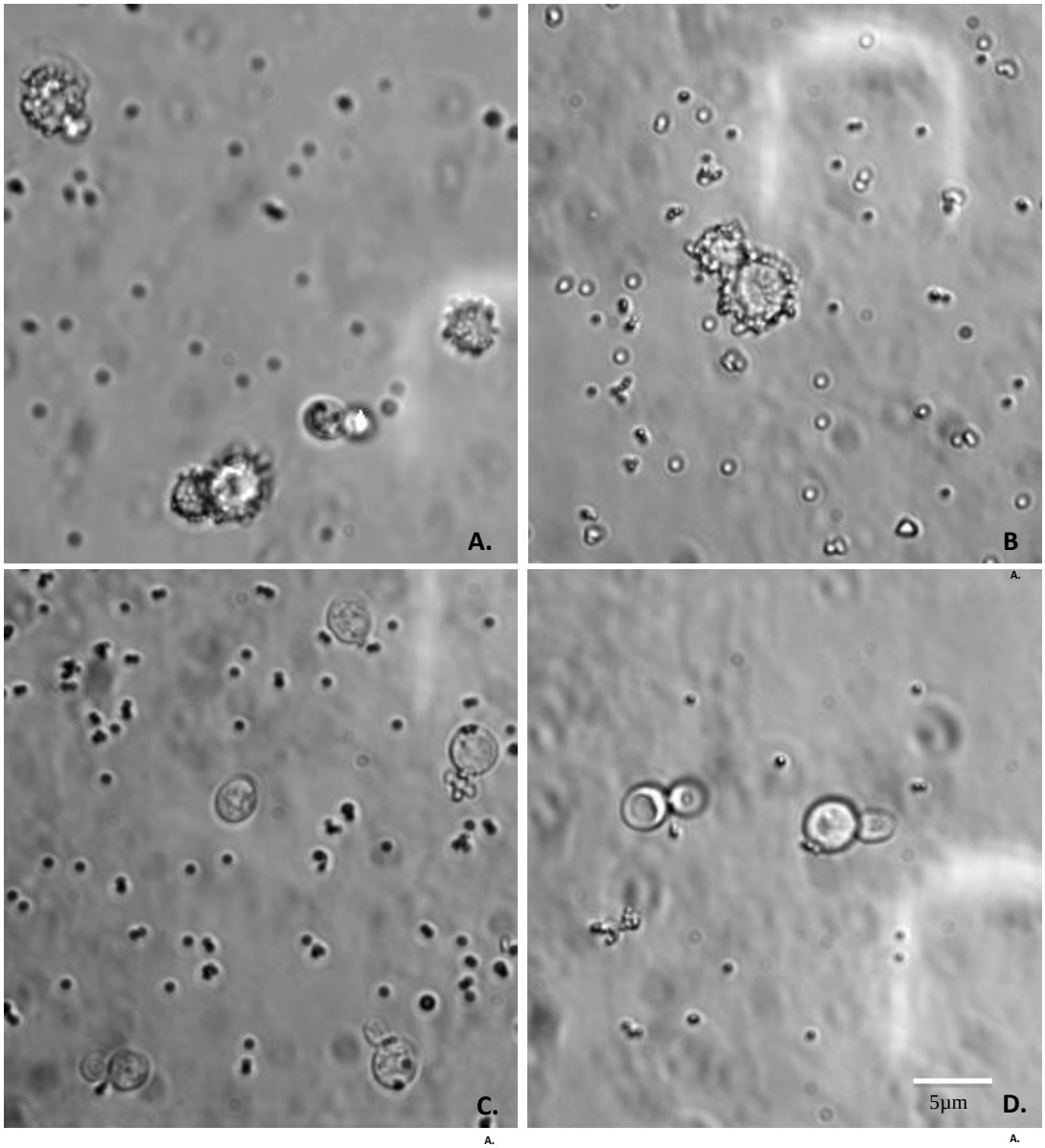


Figure 3.6 Photograph of strains (A) LYCCI (brewing strain) (B) LYCCII (Champagne strain) (C) LYCCIII (Wine strain) (D) LYCCIV (Fuel alcohol strain) at 100X objective as observed under light microscope. The small black beads are the latex microspheres which attached on the cell walls of hydrophobic yeast strains.

3.2.5 Cell surface charge

Many forces are involved in cell-cell interactions that determine the extent of attachment/adhesion of cells and also the extent of floc formation. After testing all the strains using the alcian blue retention test (see section 2.2.3), it was observed that all the strains were negatively charged during their late stationary phase. Fig. 3.7 shows that the brewing yeast strain was significantly ($p<0.05$) highly negatively charged compared to the champagne, wine and fuel alcohol strains. The presence of carboxylic and phosphodiester groups are responsible for the negative character of yeast (Jin and Speers, 1998). Cells in aqueous environments are subjected to many forces that influence cell-cell and cell-water interactions. Due to highly negative charges on the cell surface, electrostatic forces of repulsion keep cells about 10 nm from one another (Dengis *et al.,* 1995). Such forces act as a barrier to flocculation. When yeast cells age, the zymolectin biosynthesis is initiated and the CSH increases, thus hydrophobic forces come into play. These forces consist of long range Van der Waals attractions and the short range interactions, particularly hydrogen bonding (Van Oss and Giesse, 1995).

Our findings indicate that cell surface properties play important roles in determining the extent of flocculation in industrial strains of *S. cerevisiae* (Fig 3.7). This phenomenon is important in industrial fermentation processes and deeper understanding of it may lead to practical approaches (e.g. manipulation of media or physical conditions) to alter CSH and CSC, thereby providing some degree of control over the timing and extent of yeast flocculation.

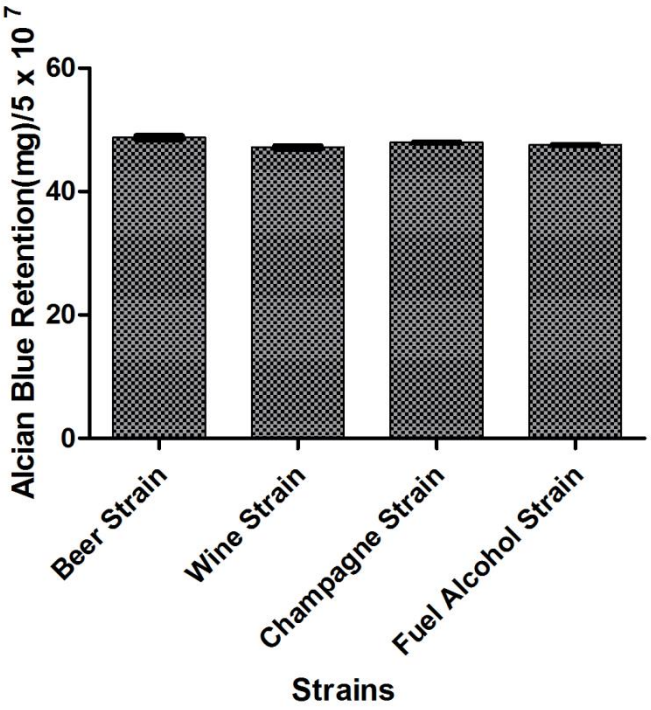


Figure 3.7. Alcian Blue retention ability of different strains of *Saccharomyces cerevisiae*. Significant differences ($p \leq 0.05$) were observed for Alcian blue retention for beer strain compared to Champagne, wine and fuel alcohol strains.

3.2.6 Correlation between the cell surface parametres and the flocculation ability of the strains

I analysed whether cell surface hydrophobicity and surface charge are important in flocculation processes of *S. cerevisiae* LYCC strains. Hydrophobicity and cell surface charge (CSC) were determined during growth in standard YEPG medium and after treatments of the yeast cells (with microsphere latex beads and non-polar and polar solvents) for cell surface hydrophobicity and Alcian blue dye test for CSC. When the interaction between flocculent yeast cells and hexadecane and with microsphere latex beads were studied by light microscopy, the yeast cells appeared to form a monolayer of cells around each of the hexadecane droplet and in case of HMA test, each

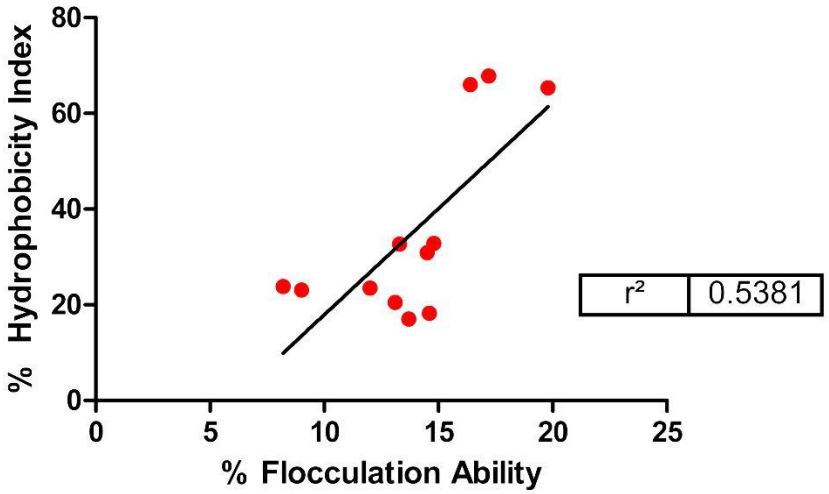
yeast cell surrounded by more than 3 latex beads. These results demonstrate that flocculent yeast cells are highly hydrophobic.

3.2.6.1 Positive correlation between cell surface hydrophobicity and flocculence

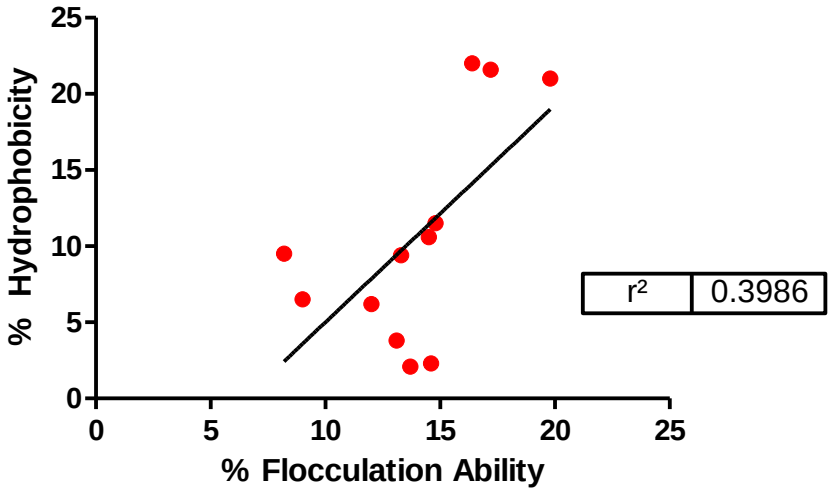
Cell surface hydrophobicity (CSH) has been reported as one of the contributors of yeast flocculation (Jin *et al.,* 1998; Straver *et al.,* 1993). In order to examine CSH, the yeast cells were harvested during the exponential and stationary phase, washed and counted for their CSH. It was observed that the CSH increased rapidly in the exponential phase but it reached the highest and stable levels in stationary phase. The reduced CSH of yeast cells in the exponential phase can be explained due to the occurrence of large number of daughter cells or virgins cells that are significantly less hydrophobic than their older counter parts (Powell *et al.* 2003). The flocculation ability of the yeast strains was found to positively correlate with CSH ($r=0.53$ (HMA) and $r=0.4$(MATS), $p \leq 0.05$). The variation in CSH partially explains the reasons for change in flocculation ability of the strains. This result also supports the previous reports on relationships between CSH and flocculation of the strains.

Cell surface charge (CSC) did not change as significantly as CSH. Apparently there were significance in the cell charge levels for the strains ($r=0.35, p \leq 0.05$) but not a direct correlation between the cell surface charge and flocculation ability was seen during growth phases for the strains. However, it was still important to recognize the possible role of (non-specific) electrostatic repulsion in flocculation, since without this repulsion in flocculation; selective cell-cell adhesion cannot function. As the cells proceeds from logarithmic phase to the stationary phase the nutrient availability in the medium decreases and thus

there is change in the surface charge on the yeast cell surface (Fischer, 1975). According to Geilenkotten and Nyns (1971) that have explained that yeast cell surface consists of phosphomannan complex overlying the protein layer. They suggested that changes in pH usually at 4.5 (when ethanol is present in medium), a pH-dependent rearrangement of the surface phosphomannan–protein complexes may be responsible for flocculation.



A.



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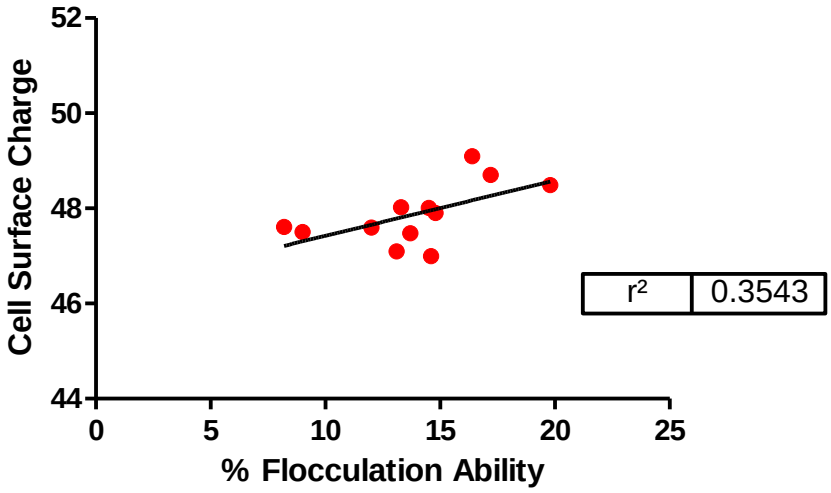


c.

Figure 3.8a. Correlation between hydrophobicity index and flocculation ability of *Saccharomyces cerevisiae*.

Figure 3.8b. Correlation between percentage hydrophobicity and flocculation ability of *Saccharomyces cerevisiae*

Figure 3.8c. Correlation between cell surface charge and flocculation ability of *Saccharomyces cerevisiae.*

Significant difference ($p \leq 0.05$) in the above figures indicates that to some extent CSH and CSC are directly correlated with Flocculation ability. These observations were made for cells in their late stationary phase.

3.3 Key Findings

Yeast flocculation, as a particular case of yeast aggregation, is a complex, fascinating and industrially relevant phenomenon. The ability of yeast cells to form flocs facilitates enormously downstream processing, especially in the brewing process. As a natural way of yeast self-immobilization, the use of flocculent strains opens the possibility of exploiting different fermentation configurations and novel fermentation designs.

At physiological pH values, the yeast cell wall has net negative charge due to the ionization of carboxyl and phosphodiester groups of cell wall proteins and phosphomannans, respectively. The repulsion of charges of the same sign prevents cells from approaching sufficiently close and thus acts as an effective barrier to cell aggregation. As a consequence, cells remain dispersed in suspension at a distance of the order of 10 nm from each other (Dengis *et al.,* 1995). The reduction of cell charge should facilitate cell–cell interactions and yeast flocculation. However, no clear relationship between yeast surface charge and the onset of flocculation was found (Dengis *et al.,* 1995).

Conversely, a positive correlation between cell-surface hydrophobicity (CSH) and flocculation was found (Jin *et al.,* 2001). CSH is partially responsible for the triggering of flocculation of brewing strains (Smit *et al.,* 1992; Straver *et al.,* 1993 and Speers *et al.,* 2006). Consistent with these results, other researchers described an increase of yeast surface hydrophobicity when Flo1, Flo5, Flo9, Flo10 and Flo11p are present in yeast cell wall (Verstrepen *et al.,* 2001b; Govender *et al.,* 2008 and Mulders *et al.,* 2009).

Brewing yeast cells (LYCCI) were found to be highly flocculent followed by Champagne strain (LYCCII), wine strain (LYCCIII) and finally fuel alcohol strain (LYCCIV). The reason being that brewing strains are usually exposed to several negative conditions such as cold-shock, nutrient starvation, osmotic stress and ethanol toxicity (Gibson *et al.,* 2007). Consequently, flocculation can act as a communitarian mechanism of survival: the external cells from the floc structure can protect the inside cells against a harmful environment by physical shielding. The possibility that flocculation can be a response to stress seems to be strain dependent.

Chapter Four

Role of cell wall polysaccharides and lectin-like receptors on flocculation of industrial strains of the yeast, *Saccharomyces cerevisae*

Abstract

Yeast flocculation is reversible aggregation of yeast cells promoted by the interaction between lectin-like protein receptors with mannose side chains on adjacent cell walls. Flocculation is also governed by several physiological factors, including the type of nutrient sugar available to yeast. Since most industrial fermentation media comprise mixtures of different carbohydrates, I aimed to evaluate the effect of sugars on yeast flocculation. I grew four industrial strains of S. cerevisiae, representing applications in the brewing, winemaking and bioethanol sectors, to late stationary phase and quantified the content of mannans, glucans and lectin-like proteins on yeast cell surfaces. I found that brewing and champagne yeast strains showed moderate to high flocculation ability when grown with glucose, fructose and galactose, but very low to no flocculation when grown with mannose. Winemaking and fuel alcohol strains showed moderate flocculation when grown on maltose and galactose and low flocculation with mannose. With regard to lectin-like receptors, I showed that their number plays a more important role in governing yeast flocculation than the mannan and glucan contents in the cell wall.

4.1 Introduction

Yeast flocculation is a type of asexual aggregation involving Ca^{2+}-dependent interaction between lectins and cell wall polysaccharides, notably mannans and glucans. Certain physical factors such as cell wall hydrophobicity, cell surface charge, cell surface topography, and cell age all contribute to the ability of yeast cells to flocculate (Amory *et al.,* 1988; Wilcocks and Smart, 1995; Straver *et al.*, 1993). Nevertheless, all factors that determine yeast cell flocculation are unknown.

Eddy and Rudin (1958) proposed the lectin hypothesis of yeast flocculation which states that in presence of calcium, cells that exhibit flocculation are able to bind highly branched mannose polymers that are located in the cell walls of the adjacent cells, leading to cell-cell adhesion (Miki *et al.*, 1982, Eddy and Rudin, 1958). Lectins are sugar-binding proteins of non-immune origin, with no catalytic activity, which play a role in cell recognition (Goldstein *et al.,* 1980). In yeast, these lectins (or flocculins) are products of a family of genes known as Flo genes. S. cerevisiae have five flocculin – encoding genes (FLO1, FLO5, FLO8, FLO9, FLO10 and FLO11). The genes FLO1, FLO5, FLO9 and FLO10 encrypt proteins related to cell-cell adhesion, while FLO11 encodes a protein responsible for cellular adhesion to substrates, diploid pseudohyphae formation and haploid invasive growth Guo *et al.*, 2000; Teunissen and Steensma, 1995; Lo and Dranginis, 1996).

Studies have shown that the N terminal part of a three-domain lectin protein is responsible for carbohydrate binding (Goosens *et al.*, 2011). This protein was found to be glycosylated at both N and O terminals and composed mainly of β-sheets. The N terminal of the protein shows high affinity binding towards

carbohydrate moieties, specifically to D-mannose, α- methyl-D-mannoside, various dimannoses, and mannans. Fluorescence microscopy has confirmed that the N terminal contains two mannose carbohydrate binding sites with different affinities. The low molecular weight mannose carbohydrates are mostly bound to the high affinity binding site of the N terminal domain while the mannans bind to the low affinity binding site. Thus, the N terminal part of the lectin is responsible for flocculation by interacting with the mannose chains.

Three types of flocculent yeast cells are known:

(i) Flo1 phenotype strains that are inhibited by mannose and derivatives

(ii) New Flo type strains, which are inhibited by mannose, glucose, maltose and sucrose, but not by galactose, and

(iii) MI (mannose insensitive) strains, in which flocculation is insensitive to mannose (Stratford and Assinder, 1991).

In brewing fermentation processes, repeated pitching of yeast leads to a loss in their flocculation ability, and this is difficult to predict (Heine *et al.,* 2009). I hypothesized that a direct determination of the cellular mannose residues or flocculin contents could provide more dynamic information regarding flocculation behaviour of industrial yeast strains. I selected 4 strains of yeast used in different fermentation applications to investigate if their flocculation behaviour was linked to lectin receptor density and the distribution patterns of glucans and mannans on the cell wall. I made use of the fluorescent lectins Concanavalin A- Alexa Fluor®-350 (Con A) and Pisum-sativum-agglutinate-Fluorescein isothiocyanate (PSA-FITC) to analyse the flocculation ability of yeast cells.

4.1.1 Fermentation of hexoses by *Saccharomyces cerevisiae*

Wild-type *S. cerevisiae* ferments glucose, the dominant sugar in all plant hydrolysates, at high rates even under anaerobic conditions. *S. cerevisiae* contains an elaborate system for hexose transport. The 32 members of the HXT (hexose transporter) family in *S. cerevisiae* differ with respect to transcriptional and posttranscriptional regulation, substrate specificity and affinity for glucose (Boles and Hollenberg 1997; Kruckeberg 1996). However, since they all transport glucose via facilitated diffusion, glucose uptake only requires a concentration gradient across the plasma membrane. After uptake, glucose dissimilation proceeds via the Embden-Meyerhof glycolytic pathway. This pathway oxidizes glucose to two pyruvate, resulting in the net formation of two ATP per glucose (Fig. 4.1). In anaerobic, fermentative cultures of *S. cerevisiae*, the NADH formed by glyceraldehyde-3-phosphate dehydrogenase is reoxidized via alcoholic fermentation. But obviously glucose is not the only carbohydrate present in the hydrolysates. In order to ferment such non-glucose carbohydrates with *S. cerevisiae*, three key criteria have to be met: (i) presence of a functional transporter in the plasma membrane,

(ii) presence of enzyme(s) that couple metabolism of the carbohydrate to the main glycolytic pathway and

(iii) maintenance of a closed redox balance.

Mannose and fructose are two isomers of glucose that occur in all plant-derived biomass hydrolysates and that can be fermented by all wild-type *S. cerevisiae* strains. Both mannose and fructose are transported by all the different members of the HXT family, although the K_m value is generally higher than that for glucose (Reifenberger *et al.,* 1997). After phosphorylation by hexokinase,

mannose-6-phosphate is isomerized to fructose-6-phosphate by phosphomannose isomerase, encoded by the PMI40 gene. Hexokinase is also responsible for phosphorylation of fructose to fructose-6-phosphate, which is subsequently metabolized through glycolysis.

Galactose, another sugar that can be fermented by S. cerevisiae, is first taken up by a dedicated member of the HXT family, the galactose permease Gal2p, and subsequently converted into glucose-6-phosphate via the Leloir pathway (Leloir, 1951; Melcher, 1997)

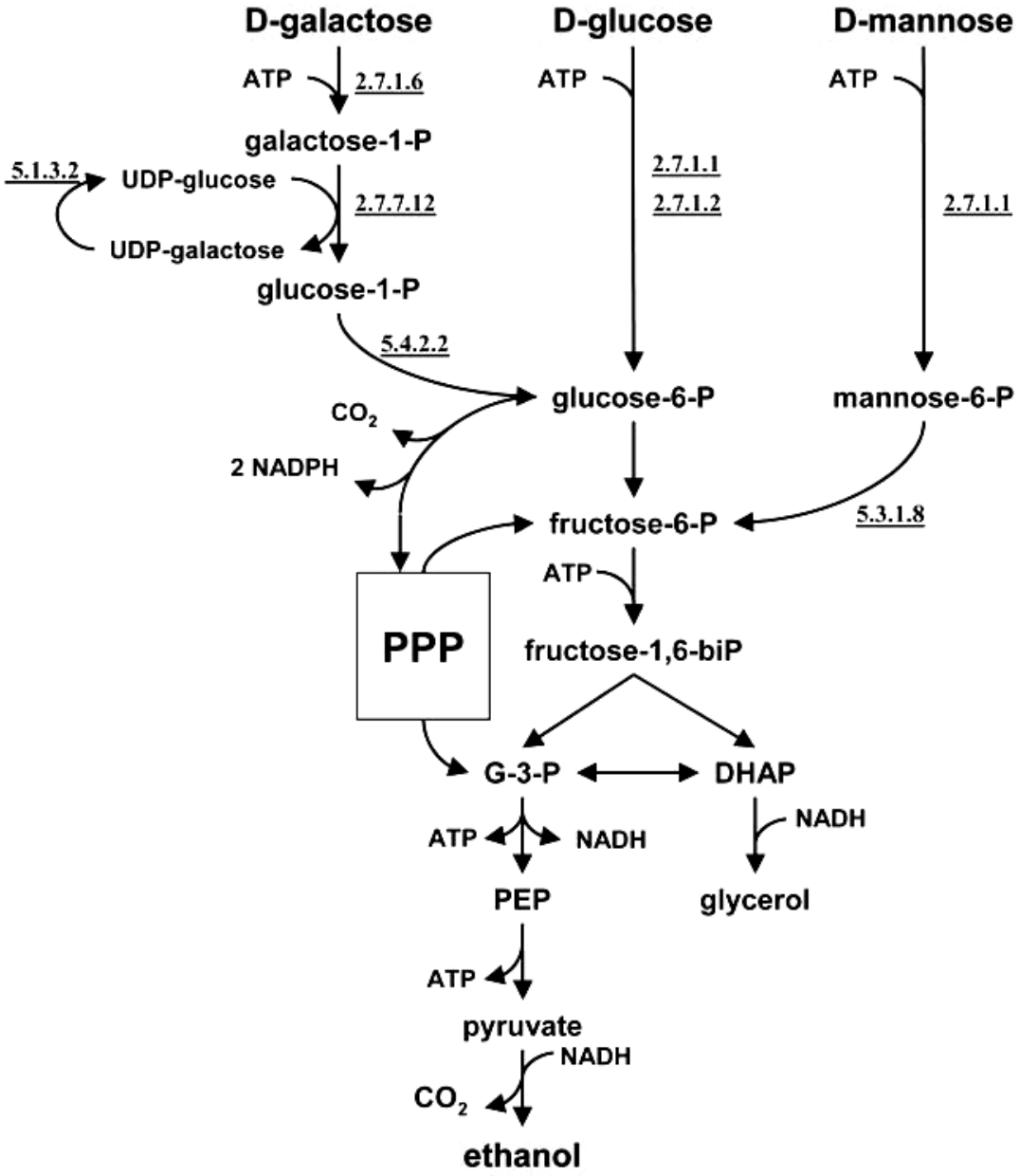


Figure 4.1 Hexose catabolism of *Saccharomyces cerevisiae*.

Glucose catabolism: 2.7.1.1, hexokinase (HXK1/HXK2); 2.7.1.2, glucokinase (GLK1); Galactose catabolism: via the Leloir pathway: 2.7.1.6, galactokinase (GAL1); 2.7.7.12, galactose-1-phosphate uridylyltransferase (GAL7); 5.1.3.2, UDP-glucose 4-epimerase (GAL10); 5.4.2.2 phosphoglucomutase (GAL5/PGM2). Mannose catabolism: 2.7.1.1, hexokinase I (HXK1); 5.3.1.8, mannose- 6-phosphate isomerase (PMI40). G-3-P, Glyceraldehyde- 3-phosphate; DHAP, dihydroxy-acetone-phosphate; PEP, phospho-enol pyruvate; PPP, Pentose phosphate
pathway.

(Source : Maris *et al.*, 2006)

Thus the main aims of this chapter are

- Direct determination of the cellular mannose residues and flocculin contents in industrial strains of yeast. I hope this could provide more dynamic information regarding flocculation behaviour of industrial *S. cerevisiae* strains.
- Determination of the lectins or the mannose binding sites by using Avidin-FITC probes and plotting the values using Langmuir's equation.
- Correlating the above information with the percentage flocculation ability of the strains, in order to find a connection between the flocculation ability and number of mannose sites on the cell and number of lectin binding sites on the cell surface.

4.2 Results

The effects of carbohydrates on the overall factors that affect the flocculation ability of industrial strains of *S. cerevisiae* are shown in Table 1. Results from flocculation assays are expressed as means (± standard deviation) of two independent experiments. In order to have a better understanding of the flocculation behaviour of industrial strains of *S. cerevisiae*, I studied the distribution patterns and semi-quantitative measurement of mannan and glucan as well as the presence of lectin-like receptors on the yeast cell walls.

4.2.1 Effect of sugars on flocculation

Fig 4.2 shows the variation in flocculation ability when the strains were grown in media containing different sugars (i.e. maltose, glucose, mannose, galactose and fructose). The interactions mediated by Flo glycoproteins can be divided into two categories namely lectin-like (cell-to-cell adhesion) and sugar-insensitive (adhesion to abiotic surfaces) adhesion phenotypes (Verstrepen and Klis, 2006). Furthermore, cell-cell adhesion phonotypes are divided into three sub types on the basis of their sensitivity towards sugars (Masy *et al.,* 1992; Stratford and Assinder, 1991). In our study, choice of yeast strain and sugar were observed to have significant effects on flocculation ability ($p \leq 0.001$). In terms of strains, it was observed that a winemaking strain (LYCCIII), exhibited a range of flocculation from 2-24%. The strain almost lost flocculation when cultured on mannose and maltose in contrast to when the yeast cells were cultured in galactose, fructose and glucose where it showed high to moderate flocculation. A fuel alcohol strain, LYCCIV, exhibited consistently weak flocculation behaviour which ranged from 10-17%, when cultured on the five selected sugars. Unlike all the other strains, there was no effect on the

flocculation when cultured on mannose. For a champagne yeast strain (LYCCII), this exhibited a range of 15-29% flocculation ability. LYCCII yeast cells flocculated more when cultured on galactose and maltose, as the cells contained a high mannan content as well as mannose binding sites on the cell wall. Lastly, the brewing strain, LYCCI, flocculated highly on fructose (53%) and glucose (43%) and lost their flocculation ability when cultured on mannose (10%). Maltose and galactose had a moderate effect on the flocculation ability of the brewing strain.

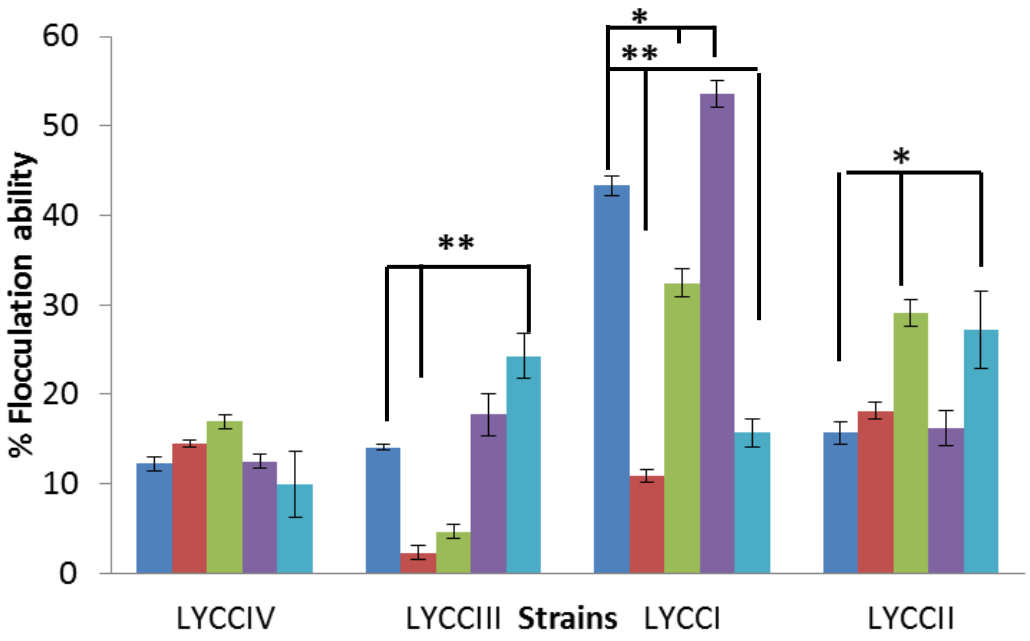


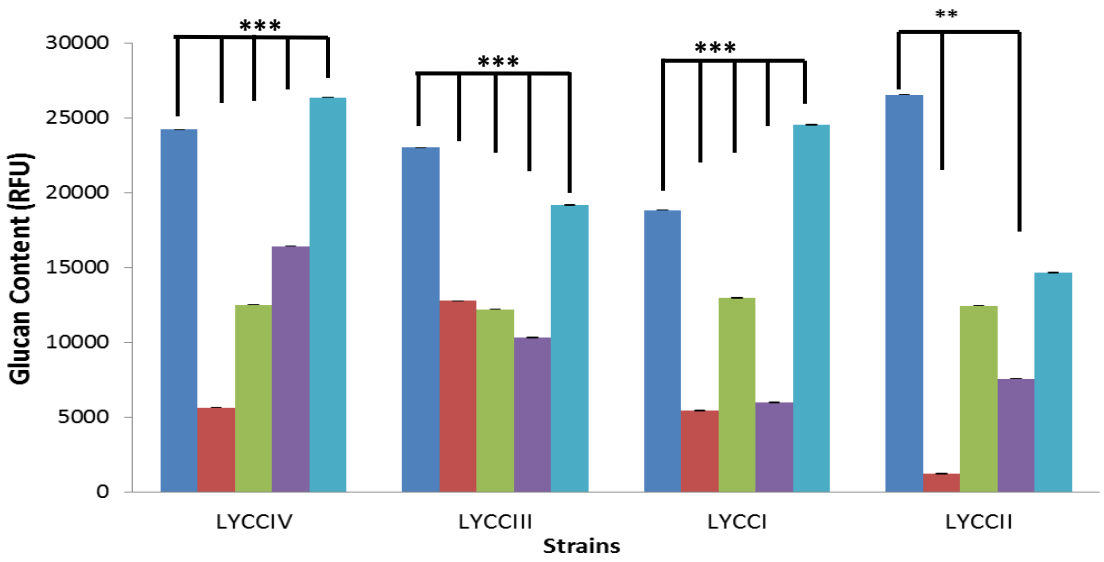
Figure 4.2 Effect of sugars on flocculation ability of four industrial yeast strains.

Representation of sugars is as follows: Glucose, Mannose, Maltose, Fructose and Galactose. Statistical significance was determined in reference to glucose for all the other sugars studied. Mean ± s.d. (n=3 on five different sugars).*P>0.05; **P>0.01 and ***P>0.001 was obtained by using one-way ANOVA (SPSS software version 22) and the data represents the least significance.

4.2.2 Effect of sugars on mannan and glucan content in yeast cell walls.

Fluorometric analysis of the selected industrial yeast strains was performed by applying fluorescent lectins ConA-Alexa Fluor and PSA-FITC to measure the levels of mannans and glucans, respectively, on the cell wall (Fig 4.3 (A), (B)). The strains were grown in media containing five different sugars (glucose, mannose, maltose, galactose and fructose) and harvested at the early stationary growth phase when a desired cell density was achieved. The protocol followed was modified from Heine *et al.* (2009). Best results were obtained when 5µg PSA-FITC per 3.15 $\times 10^6$ cells/ml was used for 25 min followed by incubation with 25µg ConA-Alexa Fluor for 10 min respectively. PSA-FITC application was done first in order to mask the effect of excess glucose residues (Fig 4.5).

From the data obtained and analysed from the spectrofluormetric findings, it was observed that the type of sugar nutrient employed governed the extent of distribution of glucans on the yeast cell wall ($p \leq 0.001$), while no such effect was observed for mannans ($p \geq 0.05$). The overall distribution of mannan remained same for all the strains when grown in different sugars. Interestingly, glucose and galactose had similar effects on cell wall glucan distribution as compared to maltose, mannose and fructose. The overall distribution pattern of mannan and glucan helped us understand the flocculation pattern of these strains grown in different sugars as these are the binding sites for the lectin like protein receptors as stated by the lectin theory (eg. Miki *et al.*, 1982).



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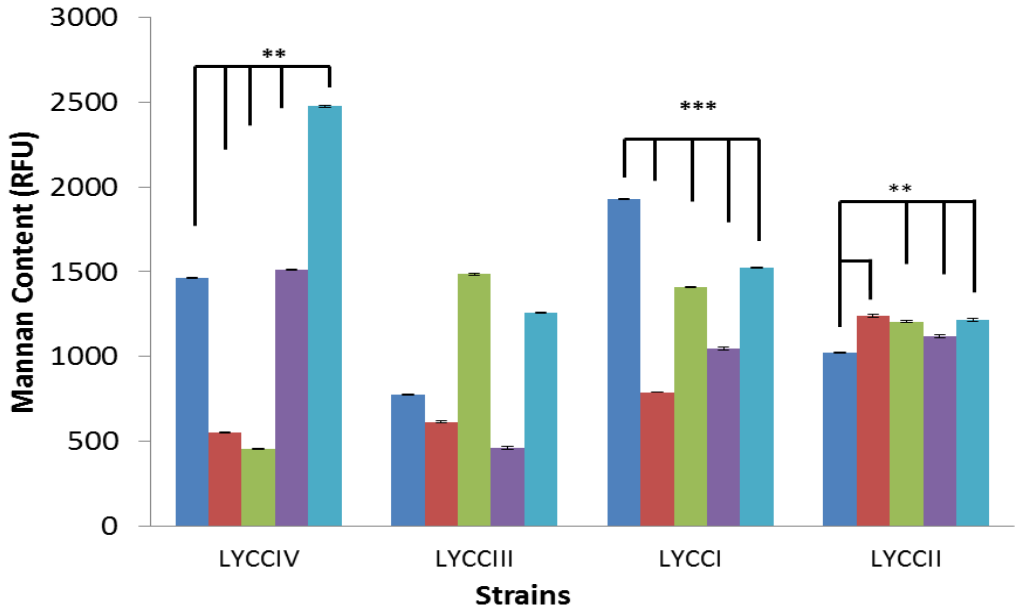


B.

Figure 4.3 Effect of sugars on (A) Glucan content (RFU) and (B) Mannan content of industrial yeast strains. Representation of sugars is as follows: Glucose, Mannose, Maltose, Fructose and Galactose. Statistical significance was determined in reference to glucose for all the other sugars studied. Mean ± s.d. (n=3 on five different sugars).*P>0.05; **P>0.01 and ***P>0.001 was obtained by using one-way ANOVA and the data represents the least significance.

4.2.3 Effect of sugar on the density of lectin like receptors.

In an attempt to quantify bound fluorescence due to lectin sites on yeast cell walls, an investigation of the amount of cell wall mannan and glucan of the four yeast strains was undertaken. Fluorescent probe intensity was unaffected by binding to the yeast cell wall and the intensity of bound probe to the yeast cell wall was similarly unaffected by the length of the binding period, or by the number of receptors occupied.

Fluorescence due to binding of the avidin-FITC complex to lectin sites provided an indication of the number of lectin sites available on the cell surface for the attachment to the neighbouring mannan residues. The bound and free probe concentrations were analysed according to the Langmuir relationship (Knight, 1970) to obtain the receptor density, and data revealed significant relationship ($p \leq 0.001$) in the four strains when grown on different sugars (Fig 4.4). This implies that the number of lectin-like receptors on the cell surface differs depending on the type of sugar available in the growth medium. In general, the brewing yeast strain LYCCI exhibited the maximum number of receptors on the cell surface, except when grown on galactose, followed by the champagne strain LYCII. Sugars in the medium may affect the transcription of FLO genes (Verstrepen and Klis, 2006). The protein products for these genes (flocculins) could either be Flo1 or NewFlo type. Flo1 type strains have flocculins that are only mannose sensitive while NewFlo has a broader sugar range for sensitivity. Thus, despite having high receptor numbers on the yeasts when cultured on mannose sugars, they exhibited weak flocculation ability.

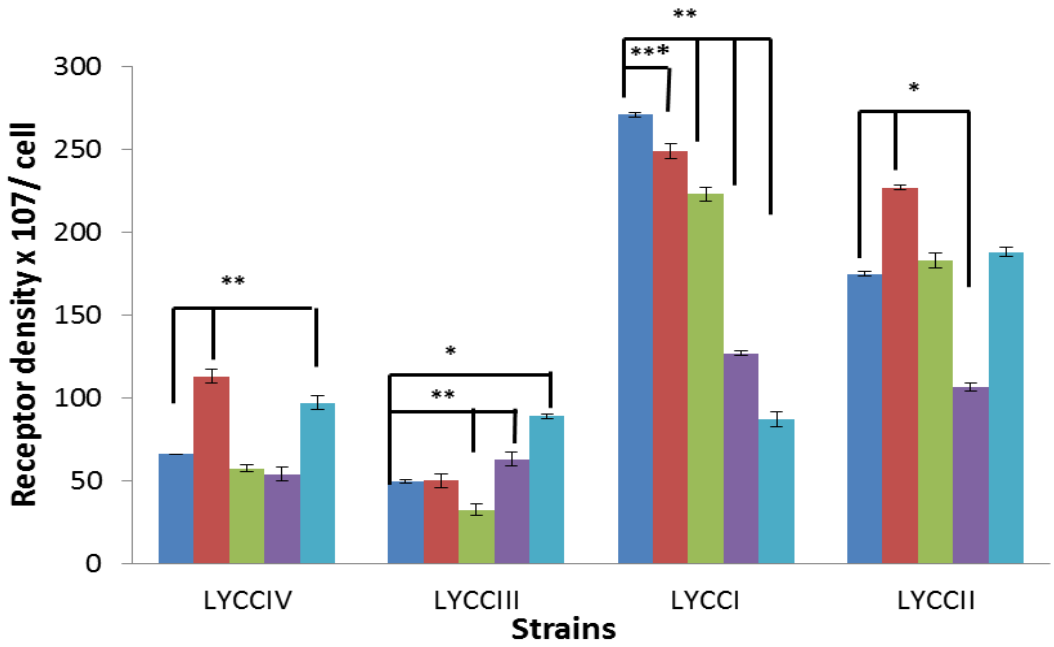


Figure 4.4 Effect of sugars on receptor density on industrial yeast cell surfaces. Representation of sugars is as follows: Glucose, Mannose Maltose Fructose and Galactose. Statistical significance was determined in reference to glucose for all the other sugars studied. Mean ± s.d. (n=3 on five different sugars).*P>0.05; **P>0.01 and ***P>0.001 was obtained by using one-way ANOVA and the data represents the least significance.

Receptor density was characterized by bound and free probe concentrations and analysed according to the Langmuir relationship. Data revealed significant ($p \leq 0.001$) relations in the four strains when grown on five different sugars suggesting that the number of lectin like receptors differ on the cell surface when the cells were cultured on different sugars.

Strain-sensitivity to different sugars is the basis of the distinction of Flo1 and NewFlo phenotypes. Furthermore, fermentable sugars, including those found in brewer's wort, induce the loss of flocculation in the early lag and logarithm phase of growth (Soares *et al.,* 2004) or in starved cells (Soares and Duarte, 2002; Soares and Vroman, 2003) most likely affecting the expression of FLO genes. Reversible inhibition of flocculation by specific sugars such as mannose, maltose, glucose or fructose, which leads to cell dispersing of the flocs and

eventually leading to loss of flocculation ability, by competitive inhibition with sugars of the yeast cell wall for lectin like receptors, has been described previously (Miki *et al.,* 1982; Stratford and Assinder, 1991; Masy *et al.,* 1992).

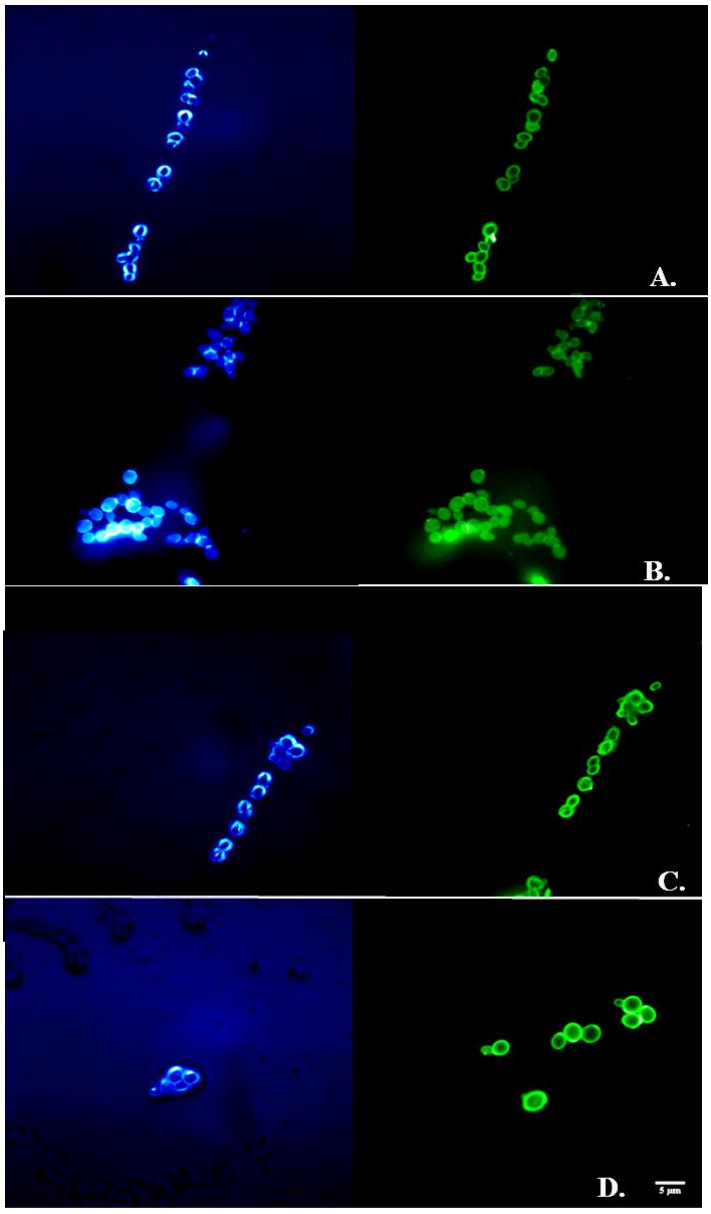


Figure 4.5 Stationary phase yeast cells of (A) brewing strain LYCCI (B) champagne strain LYCCII (C) wine strain LYCCIII (D) fuel alcohol strain LYCCIV. Mannan and glucan staining was performed using fluorescent dyes Concanavalin A-Alexa Fluor 350 (blue) and PSA-FITC (green) respectively. The cells were stained with the fluorescent dyes, incubated for 25 min and observed using an inverted microscope at 100X objective. The cells were in the range of 4-5µm.

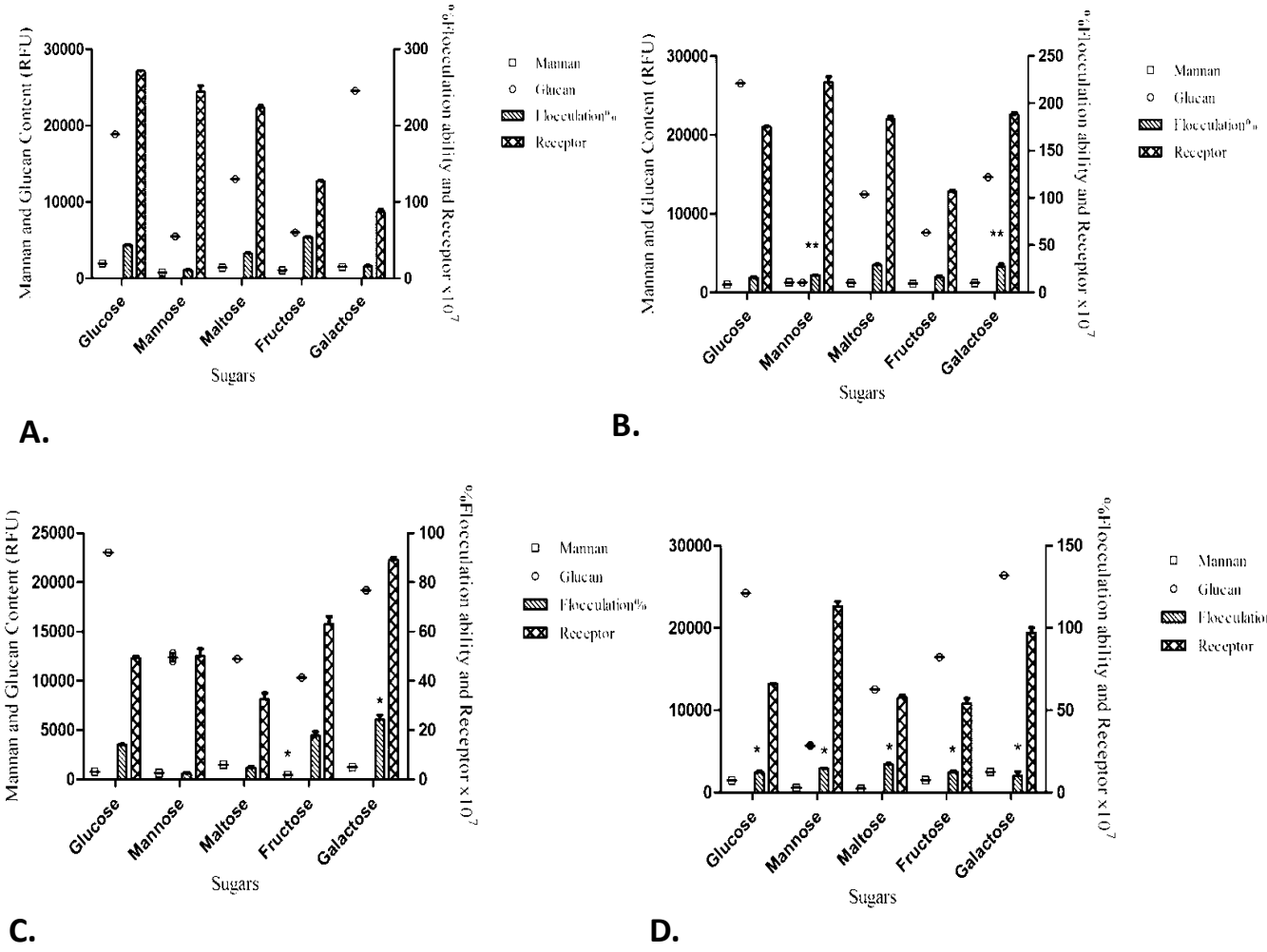


Figure 4.6 Strain wise representation of an overall effect of all the parameters studied on their flocculation ability. Representation is as follows: mannan content (O), glucan content (□), % flocculation ability (▨) and receptor concentration × 10^7/cell (▩). For each of the strains (A) brewing strain LYCCI (B) champagne strain LYCCII (C) wine strain LYCCIII (D) fuel alcohol LYCCIV, x-axis represent the 5 sugars under investigation (glucose, mannose, maltose, fructose and galactose), primary y-axis shows the mannan and glucan content in (RFU) and secondary y-axis shows the %flocculation ability and receptor concentration ×10^7.

I examined the effect of sugars commonly found in industrial fermentation media employed for brewing, winemaking, fuel alcohol production processes (mainly glucose, fructose, maltose or sucrose), as well as the other

carbohydrates like galactose, on yeast flocculation. Flocculation was determined in washed cells, in standard conditions making it possible to correlate flocculation with the presence of lectin-like cell wall receptors.

The generally accepted mechanism of yeast flocculation is lectin mediated adhesion of adjacent yeast cells to form large cell clusters. Lectins (flocculins) are required for flocculation to occur, as their presence on one cell binds to mannose residues in the cell wall of adjacent cells and so link the yeast cells into clusters that contain thousands of cells. (Stratford, 1993; Soares and Mota, 1996; Masy *et al.,* 1992; Bony *et al.,* 1998).

Maximum flocculation ability was observed for the strains when they were in the stationary phase of growth curve due to sugar depletion. I found that all the industrial yeast strains under the study belonged to the NewFlo type as flocculation behaviour in these strains was inhibited by mannose, glucose, maltose and fructose. This suggests that cell surface proteins were able to bind to a wider range of sugars, except galactose. Homologues of the FLO1 gene known as Lg-FLO1, FLONL and FLONS are believed to encode for flocculin proteins conferring the NewFlo phenotype (Kobayashi *et al.*, 1998, Liu *et al.*, 2009).

Quantification of cell wall polysaccharides and receptor sites indicated that mannan and glucan levels remained relatively constant on cell surfaces of all the strains studied (Table 1.). The main difference in flocculation ability was due to varying lectin receptor concentrations; their higher numbers on the cell surface per cell, then the higher the propensity of cells to flocculate.

4.2.4 Correlation between the cell wall mannan-glucan content, receptor binding site and flocculation ability

Yeast flocculation is generally considered to result from interactions between protein components on one cell surface and carbohydrate components on an adjacent cell surface. Thus, it is important to ascertain the effect of sugars on the cell wall polysaccharides as well as the protein or the lectin sites. Figure 4.5 shows the relationship amongst the mannan-glucan content (RFU), flocculation ability and the receptor density in yeast cells cultured with different sugars. When the data was analysed on the basis of sugars, taking each independent parameter namely (mannan content, glucan content and receptor density) vs. % flocculation ability, it was observed that sugars like maltose, fructose and galactose gave a negative correlation when plotted for mannan content (r^2 = -0.013,-0.066, -0.914, respectively) while glucose and mannan gave a positive correlation (r^2= 0.785, 0.663, respectively). Significantly less important appears to be the role of cell wall glucans in yeast flocculation. I quantified the amount of glucans in cells cultured on different sugars and their role in yeast flocculation. It was observed that glucan content vs. flocculation ability gave negative correlation for all the sugars except for maltose, which gave a positive correlation (r^2= 0.802). Finally, when the receptor density was plotted against flocculation ability, I observed a positive correlation (r^2= 0.872, 0.613, 0.938, 0.708, 0.748) for glucose, mannose, maltose, fructose and galactose, respectively.

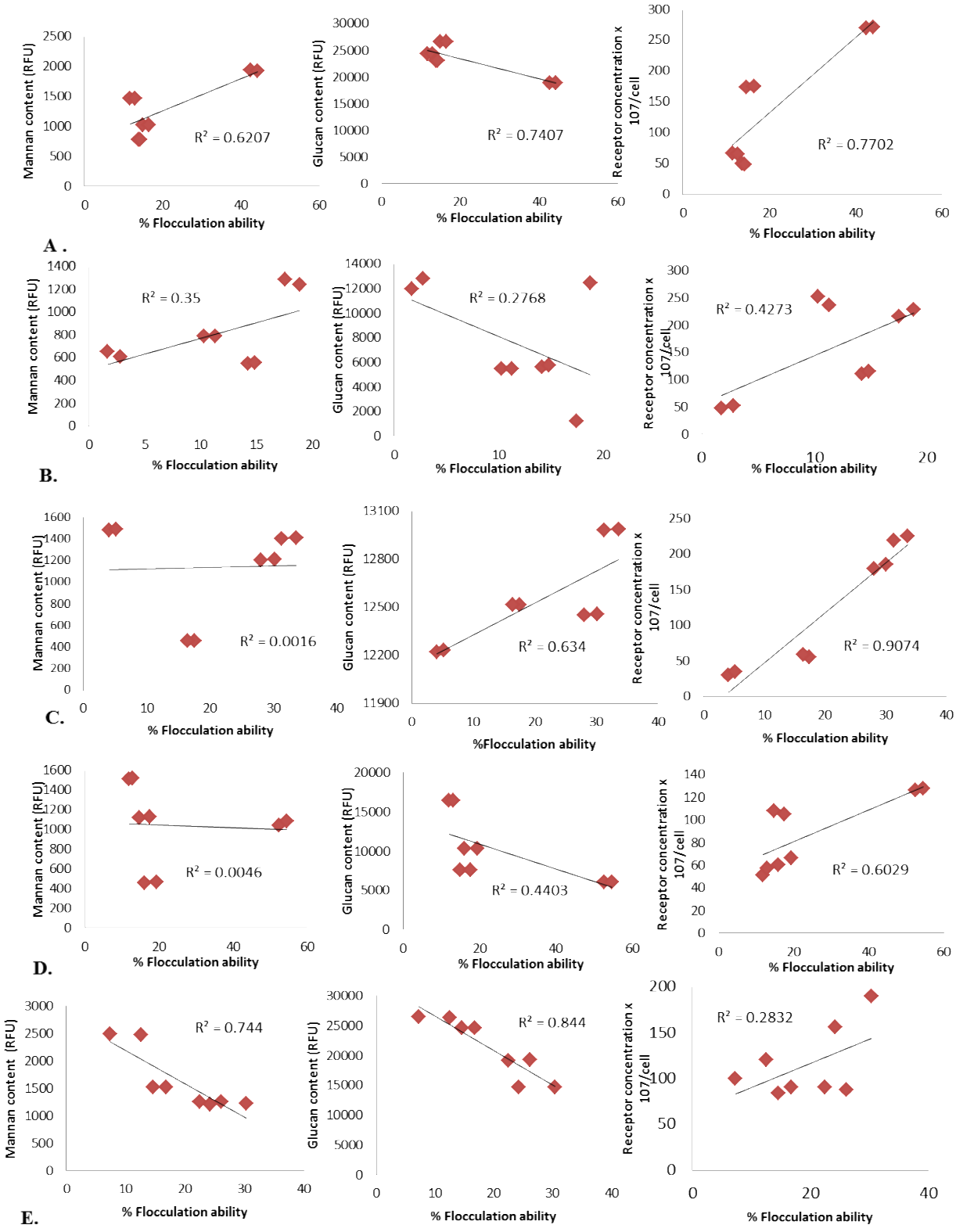


Figure 4.5 Correlation analysis performed in respect to each parameter (mannan, glucan content and receptor density) for all the strains when grown in five different sugars. (A) glucose (B) mannose (C) maltose (D) fructose or (E) galactose. Mixed response was obtained when mannan content and glucan content were plotted against % flocculation ability

respectively, while a positive indication between % flocculation ability and receptor concentration indicated that flocculation is dependent more on concentration of receptors on the cell surface, apart from being controlled by the competitive inhibition by certain sugars (eg. mannose).

4.3 Key Findings

Emergence of these receptors may be directly related to the expression levels of FLO genes. Sugars like glucose, fructose and galactose are responsible for expression of FLO genes though the Ras/ cAMP / PKA pathway (Verstrepen and Klis, 2006). In this pathway, activation of PKA, TpK2 kinases leads to inactivation Sfl1 (a suppressor of flocculation) and activates the positive regulation gene FLO8 (Gagiono *et al.,* 2003; Kim *et al.,* 2004). The FLO8 gene product acts as a transcriptional factor for other FLO genes mainly, FLO1 gene that is responsible for the formation of the lectin like receptors on the yeast cell surface (Rolland *et al.,* 2000 ; Lemair *et al.,* 2004). The pathway explains differences in the number of lectin like receptors, when the strains were grown in different sugars. In addition to the interconnection of the sugar metabolic pathways with the activation of flocculation genes, other physiological factors in the medium also influence the emergence of receptors on the yeast cell surface (eg. pH, temperature, cell age etc.) (Amory *et al.*, 1988).

Sugars	Strains	Mannans (RFU)	Glucans (RFU)	%Flocculation ability	Receptor concentration x10^7 /cell
Glucose	LYCCI	1928.5± 4.2	18852.2±3.5	43.3±1.1	271±1.4
	LYCCII	1023.7±2.0	26531.3±2.3	15.6±1.2	175±1.20
	LYCCIII	776.2±2.2	23025.4±3.0	14.0±0.3	50±1.0
	LYCCIV	1464.1±2.6	24236.9±3.6	12.2±0.8	66±0.1
Mannose	LYCCI	789.2±1.8***	5460±10.7***	10.8±0.7***	249±4.2*
	LYCCII	1240±6.5***	1250.3±4.2*	18.15±0.9	227±1.4*
	LYCCIII	614.1±7.2	12791.4±6.2***	2.25±0.8**	50±4.2
	LYCCIV	551.8±2.0***	5652.7±10.7***	14.5±0.4	113±4.2***
Maltose	LYCCI	1407.8±3.3***	12982.4±5.5***	32.4±1.6**	223±4.2
	LYCCII	1205.6±3.6***	12455.4±4.5	29±1.4**	183±4.2
	LYCCIII	1485.6±5.0	12227.8±6.2***	4.7±0.8*	33±3.5**
	LYCCIV	457.0±2.7***	12516.6±1.3***	17±0.8	58±2.1
Fructose	LYCCI	1048.7±9.6***	6011.4±18***	53.5±1.4**	127±1.4***
	LYCCII	1119.7±7.8**	7584.8±13.2*	16.2±2.0	107±2.1**
	LYCCIII	461.7±9.2	10328.2±11.1***	17.7±2.4	63±4.2*
	LYCCIV	1512.7±4.1***	16441.6±5.0***	12.5±0.8	54±4.2
Galactose	LYCCI	1523.4±1.1***	24535.6±9.4***	15.7±1.5***	87±4.2***
	LYCCII	1214.5±7.4***	14656.7±23.6	27.2±4.3*	188±2.8
	LYCCIII	1258.5±3.1	19196.2±19.4***	24.2±2.6**	89±1.4***
	LYCCIV	2475.3±7.8***	26368.5±10.4***	9.9±3.7	97±4.4***

Table 4.1 Mean values of mannan, glucan content (RFU), % flocculation ability and receptor density × 10^7/cell for the four industrial yeast strains. The industrial yeast strains were cultivated with 1% yeast extract, 2% peptone and 2% sugar (glucose, mannose, maltose, fructose or galactose). The mean contents of mannan and glucan measured with the fluorescent dyes with their standard deviation are given in relative fluorescent units (RFU). The mean

receptor concentrations × 10^7 /cell were calculated using Langmuir's equation. Statistical analyses were performed using one way ANOVA using SPSS (version 22). The p values of less than 0.05 ,0.01 and 0.001 are indicated as *, **, *** respectively, are in reference to the parameters measured when yeast is grown in glucose.

Activation of the FLO genes is not only governed by presence of glucose but is also triggered by carbohydrate depletion, which may explain the role of the glucose repression pathway, which represses FLO11 as long as glucose is present in medium. Thus, these pathways cannot be considered as single, independent entities, but rather as integrated systems working together to control adhesion (Gagiono *et al.,* 2003, Schwartz and Madhani, 2004). This explains that why strains, when cultured on glucose, fructose, galactose and maltose (to some extent) exhibit sufficient receptors on the cell surface, which in turn govern the extent of flocculation (Kobayashi *et al.* 1998). Our findings may benefit brewers, winemakers and other food manufacturers in design of fermentation media comprising sugars that would not induce premature flocculation during the early stages fermentation, resulting in better product quality and quantity.

Chapter Five

Cell surface elastic properties influence flocculation behaviour of industrial *Saccharomyces cerevisiae* strains.

Abstract

A variety of atomic force microscopy (AFM) force spectroscopy approaches have been developed for investigating native cell surfaces with high sensitivity and nanometer lateral resolution. For yeast cells, this has provided novel information on the nanomechanical properties of cell walls that play a major role in determining the flocculation characteristics. In this study, I used AFM to visualize the cell surface topography and to determine cell wall mechanical properties of different strains of Saccharomyces cerevisiae employed for brewing, winemaking and fuel alcohol production. Cell surface topography was found to correlate with the flocculation behaviour of these strains during their late stationary phase. Cell surface roughness of flocculent cells was much higher (146 ± 8 nm) compared to a weakly flocculent wine yeast strain, which had smoother surface (37.5 ± 8 nm). Nanomechanical properties of yeast cell surfaces were also investigated by AFM spectroscopy. From force-indentation curves, the Young's modulus was determined and provided insight into cell wall elasticity of the selected industrial strains. A value of ~ 1.1 MPa was obtained for the fuel alcohol strain (weakly flocculent strain) and ~ 0.4MPa for a brewing strain (highly flocculent strain). In terms of adhesion force and adhesion energy, the strains exhibited significant differences ($p \geq 0.001$). For example, the brewing strain (highly flocculent) and champagne strain (moderately flocculent) had a higher adhesion force (10 ± 0.60 nN) and adhesion energy (14.7 ± 0.7 × 10^{-15}), compared to less flocculent strains. Our findings provide evidence that yeast cell surface nanomechanical properties, including cell surface elasticity and roughness, play major roles in governing flocculation. Of lesser importance are cell surface adhesion forces and adhesion energies which display weak correlation with flocculation behaviour in Saccharomyces cerevisiae.

5.1 Introduction

The yeast cell wall is a complex carbohydrate entity, which not only protects the yeast cells from adverse conditions but also helps to maintain an optimum osmotic balance for the cell so that it could carry out its normal cellular activities. The cell wall of yeast is a mechanically and chemically resistant complex structure made up of a of microfibrillar matrix of (β- glucans, β -1,3 and β -1,6 glucans). The latter represents 50-60% of the cell wall mass, which is overlaid by highly glycosylated proteins decorated by long chains of mannose residues representing 40-50% of the cell wall mass. Another important component of yeast cell wall is chitin (~ 1-3% of the cell wall). The thickness of yeast cell wall is about 200 nm, thus utilizing AFM probes with small radius of apex and by applying a low force on the cell wall, its local mechanical properties can be investigated (Francois, 2006; Dufrêne *et al.,* 1999).

Thus, it would be interesting to understand that what forces do exactly come into play at the cell surface when the cells come close to each other during the process of flocculation visualize that what exactly is occurring at the cell surface and how much stresses impact on the biological properties of the cell wall. Moreover, it is well-known that the yeast cell surface is decorated with proteins that play a pivotal role in adhesion, communication and microbial infection (Jendretzki *et al.,* 2011). Proteins are thought to play an important role in cell wall molecular organization and remodelling, since the organization patterns differ in ways of protein attachment to the polysaccharide moiety. The first class comprises of the proteins that are bound non covalently to the β-1,3- glucan network (the SCWs family), second category are the proteins attached covalently through a remnant of the GPI anchor to β-1,6-glucans (the GPI-

CWPs) finally the third type are cell wall mannoproteins that are characterized by Protein Internal Repeat regions (PIR-CWPs or CCWs family) that are directly linked to β-1,3- glucans (Klis et al 2006). This outer layer is made of highly mannosylated proteins together with large polysaccahrides complex of 150 or more D- mannose units. The mannoprotein layer thus bears crucial biochemical and biotechnological properties, some of which are adhesion, aggregation and flocculation (Caridi, 2006; Verstrepen & Klis, 2006) as well as virulence (Francois *et al.*, 2013, de Groot *et al.*, 2008).

According to lectin-like theory, flocculation occurs as a consequence of interaction between the specific flocculation proteins (flocculins) present only on the flocculent cells and the carbohydrate residues (receptors) of the cell walls of the neighbouring cells (Miki *et al.,* 1982).Close inspection of these proteins at the single molecule or cell level would be helpful in understanding the several physiological and biotechnological processes such as molecular recognition and cell adhesion, aggregation and flocculation, biofilm formation (Verstrepen and Klis, 2006; Bauer *et al.,* 2010), resistance to antifungal drugs (Mishra *et al.,* 2007; Heinisch, 2008). Atomic force microscopy (AFM) appears to play a crucial role in the investigation of the mechanical properties of cells, as it allows manipulation at single-cell level and direct observation of the cell surface at nanometer resolution (Binnig *et al.,* 1986; Burnham and Colton, 1989; Mizes *et al.,* 1991).

5.1.1 Basic principle for AFM:

The technique was introduced by Binnig and Quate in 1986. Since the technique is based on the measure of interactive forces between the sharp tip

and the sample, AFM belongs to the scanning probe microscopes family, wherein the microscope scans the sample, while maintaining constant given parameter. This allows us to scan living cells close to their physiological environment. Deflections of cantilever (typically 100-400µm in length are noted as the tip (10-100 nm radius of curvature) scans over the surface of the cell to produce a three dimensional image of the surface. In force spectroscopy mode, the cantilever and tip are successively approached and retracted from the surface. The tip is approached to the surface sample and then retracted in the Z- direction; forces versus distance (F-D) curves are recorded. In a biological sample the F-D curves consist in non-contact and deformation component. Force distance curves are obtained and fitted to models (normally hertz) describing the nanomechanics of indentation, giving the elastic properties of the surface (Figure 5.1). The retraction curve also provides relative quantitative information on adhesion or interaction events. This is because when the AFM tip is in contact with the sample, higher force is required to disrupt the interaction during the retraction of the tip from the sample, which results in the measurement of adhesion force.

Thus, the main aim of this chapter is:-

- To understand the discrete cell adhesion forces and other nano mechanical properties for example cell surface elasticity, cell surface roughness at nano level.
- Correlate these nano-mechanical properties to the reversible adhesion phenomenon that the cell undergoes during fermentation in the presence of calcium ions known as flocculation.

5.2 Results

The main aim of this study was to understand the discrete cell adhesion forces and other nanomechanical properties for example cell surface elasticity, cell surface roughness at nano level and correlate it to the reversible adhesion phenomenon that the cell undergoes during fermentation in the presence of calcium ions known as flocculation. Thus in order to achieve this, the flocculation pattern of the strains was studied during all the phases of growth curve and then the AFM analysis was performed only on the samples prepared from late stationary phase of the growth cycle as they exhibited maximum flocculation behaviour.

5.2.1 Imaging live yeast cells at high resolution

Yeast cells for each of the strain were scanned in contact mode (CM) by using Si_3N_4 triangular cantilever with spring constant of 0.01 N/m in air. The samples for AFM studies were prepared by immobilizing the yeasts on hydrophilic glass slides. The entire procedure followed is explained in (section 2.3). The main aim for imaging was to investigate the differences in the cell wall morphology at the nanoscale level. The cells of each strain were air dried in order to preserve the natural morphology of the cells. Small imaging forces (0.1-0.5 nN) were used to obtain images of small areas on the air dried slides without detaching the cells or altering the surface morphology significantly.

Both height as well as error images are shown in (Figure 5.1). Note that the border of the cell was surrounded by an artefactual structure resulting from the contact between the AFM probe and the glass slide. From the data it was clear that the brewing strain LYCCI had the roughest cell wall (146 ±8 nm), followed

by the champagne strain LYCCII (95.1 ± 8nm), fuel alcohol strain LYCCIV (73.5± 5 nm) and finally wine strain LYCCIII (35.7± 8 nm). The surface roughness pattern could be correlated (R^2 = 0.846) to the flocculation pattern observed for the strains (Figure 5.3). It was observed that the higher the surface roughness of the cell surface, the larger the frictional force between the two surfaces of yeasts coming together. Thus, more sites for anchorage to hold on each other's surface and this could explain the formation of stable flocs in the fermenter and the increase in the flocculation ability for the strain (Ahimou F *et al.,* 2003; Dague *et al.,* 2010). Cross sectional analysis was also performed on the height images and along the entire length of individual cells. From the data I observed that brewing strain LYCCI were much bigger cells (2.1± 0.05µm) followed by fuel alcohol strain LYCCIV (1.1±0.1 µm), champagne strain LYCCII (0.9 ± 0.1 µm) and wine strain LYCCIII (0.6 ± 0.05 µm).

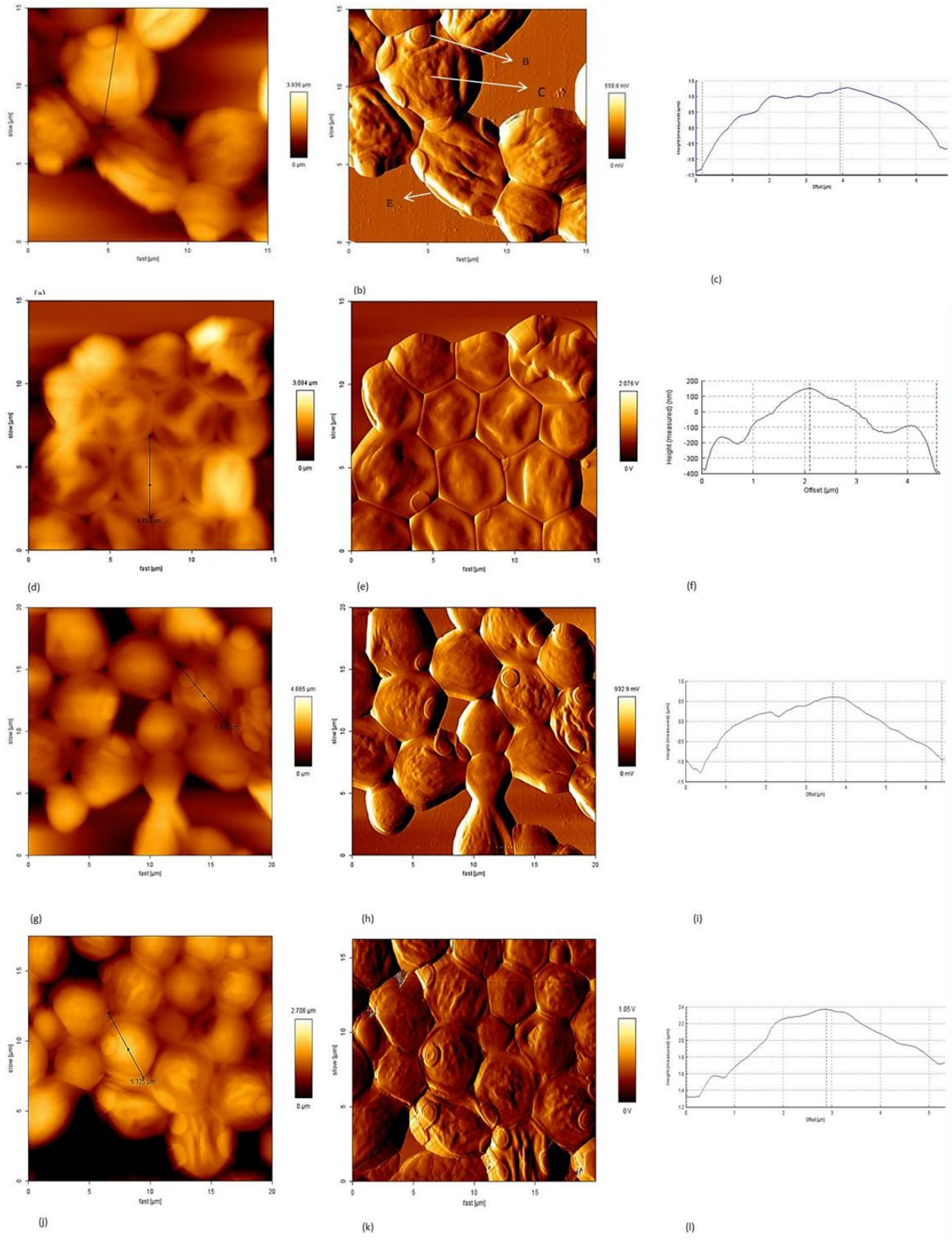


Figure 5.1 AFM images of the surface of four industrial strains of *Saccharomyces cerevisiae*. (a,d,g,f) represent the height images. (b,e,h,k) represent the error signal images and (c,f,i,l) are the height measurement taken using the JPK Nanowizard software.

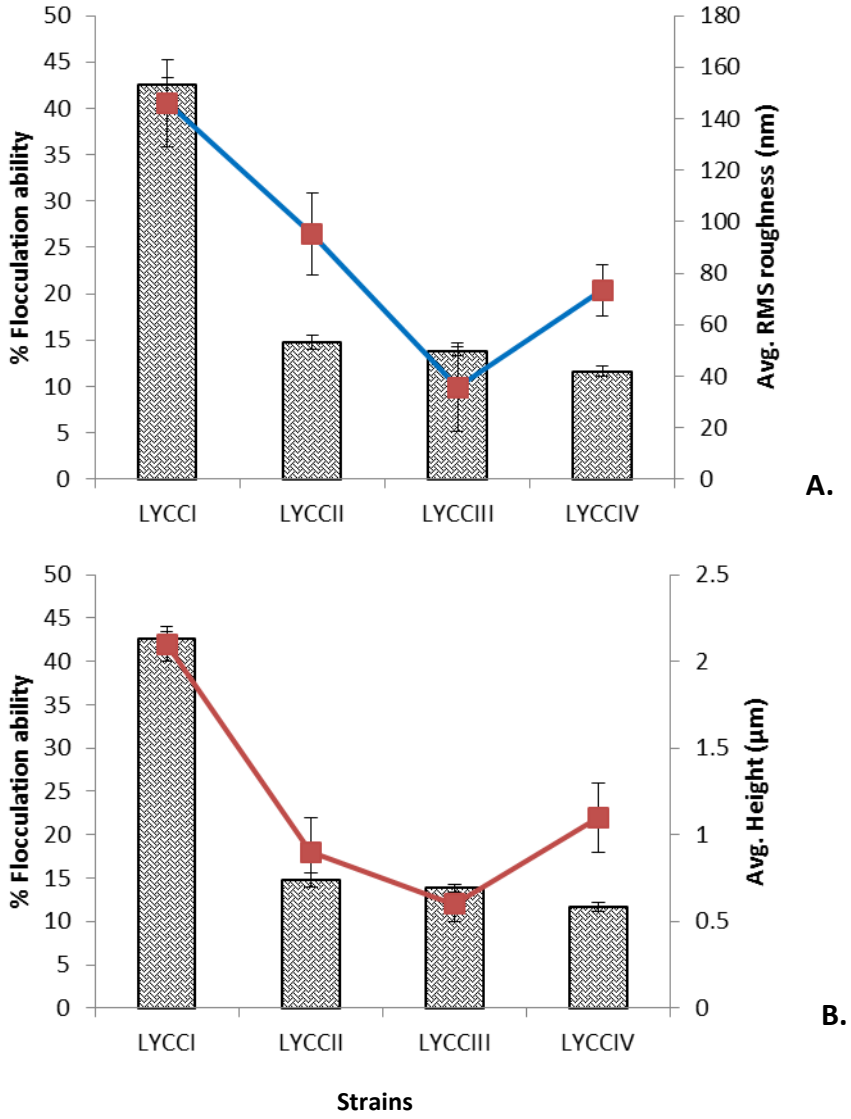


Figure 5.2 Effect of (A) RMS roughness (nm) , (B) Cell height measurement (µm) on the % Flocculation ability of four *Saccharomyces cerevisiae* strains during their late stationary phase. Statistically significant direct relationship ($p \leq 0.001$) explained that more the roughness and size the cell, higher is their % flocculation ability. The columns represent the % flocculation ability, while (■) are the plotted secondar axis parametres.

5.2.2 Effect of Young's modulus on the flocculation behaviour

Flocculation tests were carried out during all the phases of growth for the four industrial strains and it was found that all yeasts were flocculent either during the early or late stationary phase. The strains exhibited significant ($p < 0.05$)

differences in their flocculation abilities during all the phases of growth (Figure 5.3). The brewing yeast strain LYCCI was found to be highly flocculent throughout the fermentation including the late stationary phase (42.5%) when the flocculence character of the other strains diminished. The order followed champagne strain LYCCII (14.8%), wine strain LYCCIII (13.8%) and finally fuel alcohol strain LYCCIV (11.6%) (Nayyar *et al.,* 2014).

The mean Young's modulus was calculated by performing AFM force spectroscopy experiments. Analysis of more than 100 cells for each strain at three different areas, that is, budscar (B), cytoplasm (C) and edges (E) respectively revealed the overall difference in the elasticity of the strains over these areas. The curves were fitted using the conical model using a value of 35° for the half opening angle From the fit and assuming a Poisson's ratio of 0.5, which is expected for soft biological materials, I deduced the Young's modulus. The higher Young's modulus, the lower the elasticity of the material.

From the data (Figure 5.3), I found that the strains were significantly different in their cell wall elasticity ($p \leq 0.001$). To know if the number of bud scars on the cell wall contributed to the change in the elasticity of the cell wall, the force spectroscopy measurements were performed at bud scar (B), cytoplasm (C) and edges (E). Analysis revealed that there was no significant change in elasticity ($p \geq 0.05$). Brewing strain LYCCI was found to be quite elastic (389 ± 7 kPa), followed by champagne strain LYCCII (685 ± 7 kPa), wine strain LYCCIII (787 ± 9 kPa) and finally fuel alcohol strain LYCCIV (1152 ± 11 kPa). The rather large standard error on the obtained average values reflects variability of the measurements across the surface of the same cell as well as variability associated with independent cell cultures.

5.2.3 Effect of Adhesion force and adhesion energy on flocculation behaviour

The adhesion force gives a measure of maximum adhesion of the cell onto the AFM probe. In the current study I could only gather information about the maximum adhesive force that the yeast cell wall exerted on the cantilever. Linking the AFM probe with the lectins specific to mannans, could give the quantitative measurement of the adhesive forces between the mannans and the lectins and also the number of lectin binding sites on the yeast cell wall. But in the present study, our work is restricted on the combined adhesive force that comes into play when another surface comes in contact. Thus, this relation indicates that the higher the adhesion forces (nN), the higher the adhesion force between the yeast cells and thus higher would be the stability of the flocs in the fermenter. The force of adhesion was found to be higher at the edges or the point of contact between the cells for brewing strain LYCCI (10± 0.60 nN) and followed by champagne strain LYCCII (9 ± 0.60 nN), fuel alcohol strain LYCCIV (7 ± 0.63 nN) and wine strain LYCCIII (6± 0.65 nN).

From the statistical analysis performed on the data, I observed that the strains differed significantly in their overall adhesion force on the cell wall ($p \leq 0.001$). Adhesion forces differed for the strains at different areas when analysed ($p \leq 0.001$). Cytoplasm region showed almost consistent adhesive force for the four strains while the adhesive force for the brewing strain LYCCI was found to be significantly different at the bud scar (B) and edge (E) region to the other three strains (Fig 5.1).

Adhesion energy measurements give the amount of work or energy required for the cell to detach from the AFM probe. It was observed that wine making strain LYCCIII had maximum adhesion energy (22.5±0.6 × 10^{-15} J) followed by brewing strain LYCCI (14.7 ±0.7 × 10^{-15} ±J), fuel alcohol strain (10.5±0.5 × 10^{-15} J) and champagne strain LYCCII (9.1 ±0.5 × 10^{-15} J). The differences in the adhesion energy were observed more significantly at the cytoplasm and edges ($p \leq 0.001$) of the cells of respective strains and to a lesser extent on the bud scar region of the cell ($p \geq 0.05$).

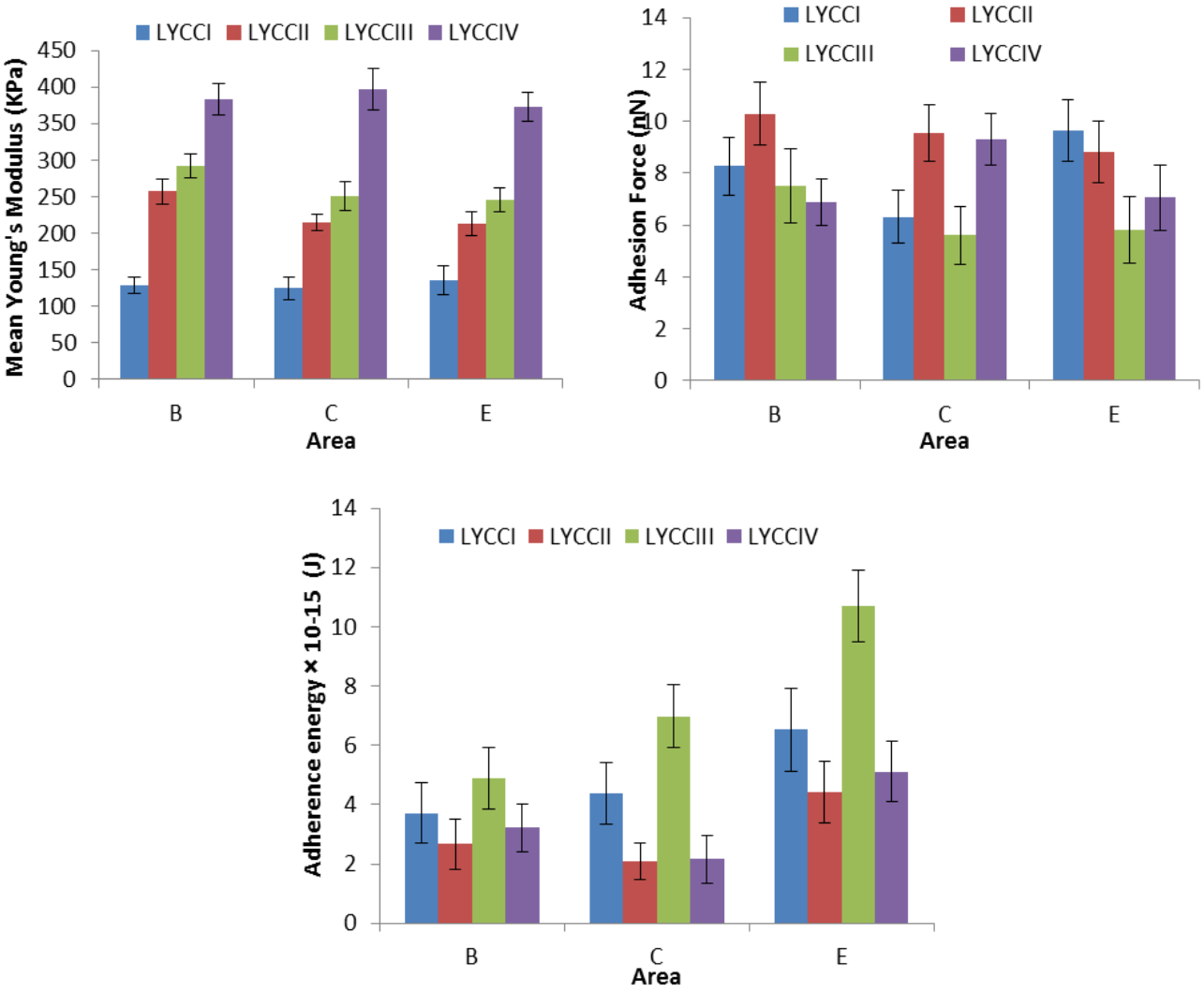


Figure 5.3 Nanomechanical properties analysis of yeast cell wall from four industrial strains of *Saccharomyces cerevesiae.* 100 force curves were converted into indentation curves and fitted to the Hertz model. The Young's modulus, adhesion energy and adhesion force for each condition was measured for each force curve and the data was collected at Bud scar (B), Centre (C) and Edge (E). Overall, brewing strain LYCCI was found to be most elastic and had the maximum adhesion force, while LYCCII, champagne strain had the maximum adhesion energy.

5.2.4 Correlation studies between the percentage (%) flocculation ability and the different nano mechanical properties studied

The differences in the cell wall architecture of strains provoked strong nanomechanical changes of the cell wall, accompanied by significant topological change, it was interesting to plot flocculation ability and surface roughness vs. parameters (like for e.g. elasticity modulus, adhesion energy and adhesion force) to investigate a possible correlation between these biophysical parameters. As shown in Figure 5.4, a negative correlation between %flocculation ability and elasticity could be drawn. When using data from cells at late stationary phase this correlation was relatively strong ($r = -0.824$, $p \leq 0.001$),, while a strong positive correlation could be drawn between %flocculation ability and mean surface roughness ($r = 0.846$, $p \leq 0.001$). These findings seems to show that the higher the surface roughness of the cell wall, the higher the flocculation ability of the respective strains during fermentation. One of the reasons could be a better and stable anchorage that the rough surfaces provide in holding the flocs in the liquid medium.

As we are aware of the inverse of the Young's modulus and elasticity thus, from the data it was inferred a positive correlation between %flocculation ability and elasticity of the cell wall. This is suggestive of flexibility of cell wall as one of the

important contributors towards aiding in higher flocculation ability of the yeast strain during fermentation.

In contrast to the strong correlation observed between %flocculation ability vs. elasticity and surface roughness measurement, there was a weak positive correlation between %flocculation ability vs. adhesion force and adhesion energy ($r = 0.354$, $p \geq 0.05$), suggesting that the forces of attraction and repulsion played a lesser role in governing the flocculation ability as the cell wall topography especially cell wall elasticity and cell surface roughness played.

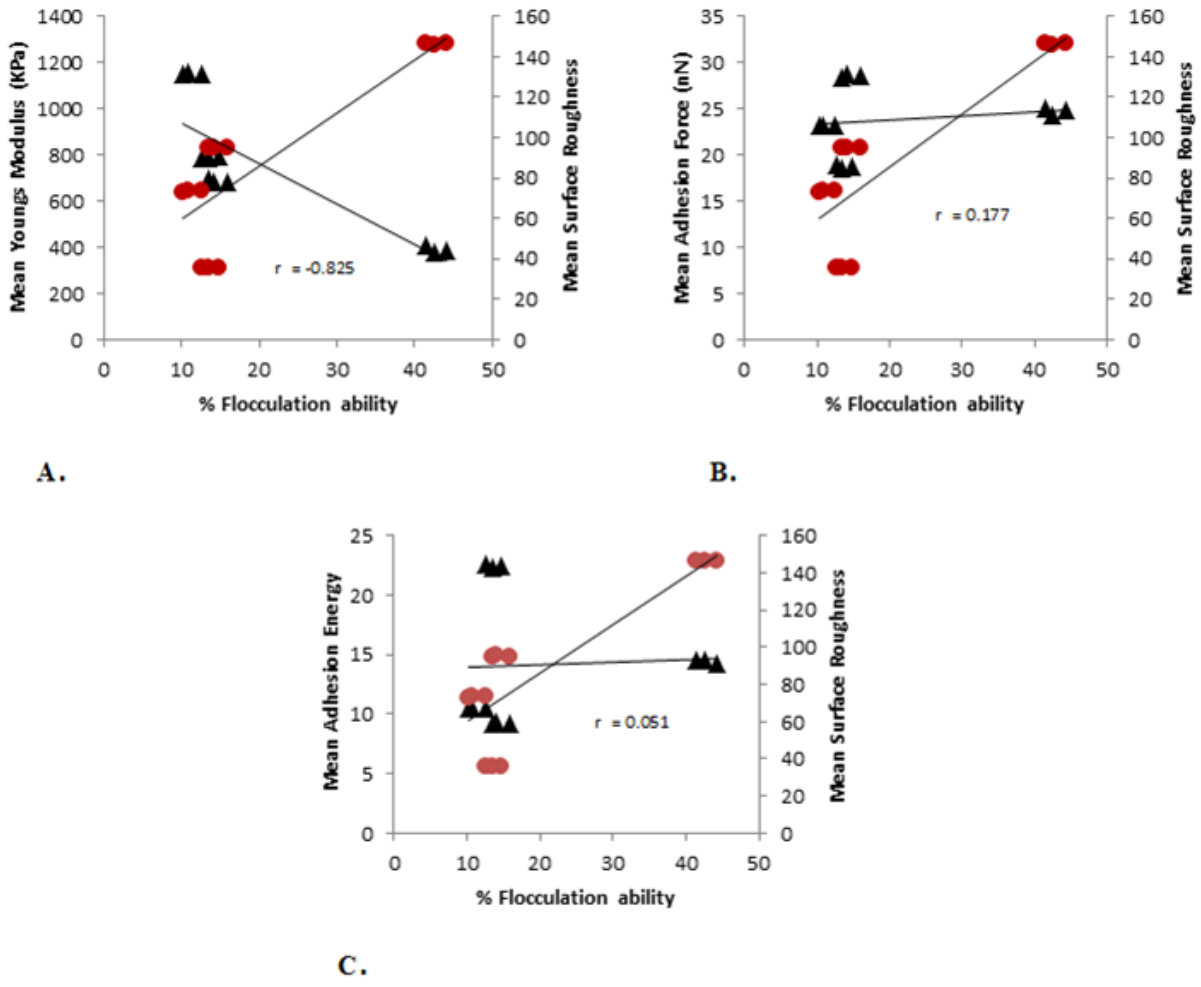


Figure 5.4 Correlation studies between the percentage (%) flocculation ability and the different nanomechanical properties studied. (●) represents a positive correlation (R^2= 0.846) between %flocculation ability and the mean

surface roughness. (A) (▲) Correlation between % Flocculation ability v/s mean Young's modulus (R = -0.826), which states a statistically significant positive correlation between % Flocculation ability and Elasticity, as p≤0.001. (B), (C) (▲) Correlation between % Flocculation ability v/s Adhesion force (R= 0.177) and % Flocculation ability v/s Adhesion energy (R= 0.051) show a positive correlation which is statistically insignificant (p≥0.05).

5.3 Key Findings

Parameters	LYCCI			LYCCII			LYCCIII			LYCCIV		
	B	C	E	B	C	E	B	C	E	B	C	E
Young's modulus (KPa)	129 ±11	124 ±15	135 ±20	257 ±17	214 ±11	213 ±16	292 ±17	250 ±17	245 ±16	383 ±21	397 ±28	372 ±19
Adhesion Energy × 10^{-15} (J)	3.7 ±1	4.4 ± 1	6.5 ±1.4	2.7± 0.8	2.0 ± 0.7	4.4 ± 1	4.9 ± 1	7.0 ± 1	10.7 ± 1.2	3.2 ± 0.8	2.2 ± 0.8	5.1 ± 1
Adhesion Force (nN)	8.2 ±1	6.3 ±1	9.7 ±1	10.3 ±1.2	9.5 ±1	8.8 ±1	7.5 ±1.4	5.6 ±1	5.8 ± 2	7.0 ± 0.9	9.3 ±1	7.0 ±1.3
Surface Roughness (nm)	146 ±17			95 ±16			36 ±17			73.5 ±10		
Height (µm)	2.1 ±0.1			0.9 ±0.2			0.6 ±0.1			1.1 ±0.2		
% Flocculation ability	42.5 ±0.8			15 ±0.8			14 ±0.4			12 ± 0.5		

Table 5.1 Measure of each of the parameters (Young's modulus, Adhesion force, Adhesion energy) at three different areas namely bud scar (B), cytoplasm (C) and edge (E). Measure for surface roughness, Height measurements and % flocculation ability were done on the overall cell surface.

Analysis revealed that there was not much significant change in elasticity (p≥0.05) at bud scar, cytoplasm and at edges, but significant difference in their cell wall elasticity (p≤ 0.001) overall. I observed that the strains differed significantly in their overall adhesion force on the cell wall (p≤0.001). Adhesion forces differed for the strains at different areas when analysed (p≤ 0.001). The differences in the adhesion energy were observed more significantly at the

cytoplasm and edges ($p \leq 0.001$) of the cells of respective strains and to a lesser extent on the bud scar region of the cell ($p \geq 0.05$).

Exploration of biochemical properties of cell surface using AFM

The yeast cell wall is a dynamic organelle that has to adapt to the changing environment. The outer layer of the yeast cell wall is made of highly mannosylated proteins decorated with large polysaccharides complex of 150 or more D-mannose units (mannan layers). Chemical and genetic studies pioneered by Ballou and collaborators have largely elucidated the structure of these polysaccharides attached to cell wall proteins (Ballou, 1990). This mannoproteins coat bears important biochemical and biotechnological properties, such as adhesion, aggregation and flocculation (Caridi, 2006; Verstrepen and Klis, 2006) as well as virulence (de Groot *et al.,* 2005; de Groot *et al.,* 2008). Expression of these properties can be exerted through various types of interactions that can involve hydrophobic or electrostatic forces, or specific receptor-ligand binding forces. These forces can be measured using AFM tips.

I conclude that AFM shares a lot of similarity with scanning electron microscopy (SEM) and transmission electron microscopy (TEM), but is advantageous in yeast cell wall research, by being a force spectroscopy that produces a qualitative description of the ultrastructure of cell wall (Osumi, 1998). Information on integrity and local nanomechanical properties of the microbial ultrastructure were obtained using AFM. Morphological changes in the yeast cell surface for different strains, at nanoscopic level were observed by

immobilizing the cells on hydrophilic slides by air drying for as long as 5h (Canetta *et al.,* 2006a, 2006b, 2009).

The topological changes were quantified in terms of changes in roughness, which was found to be maximum for the brewing strain LYCCI, which was also found to be highly flocculent (Table 5.1). The dramatic change in roughness may have some consequence on the adherence capacity of yeast to material surface (Gollardo-Moreno *et al.* 2004, Mercier-Bonin *et al.,* 2004). Also it is clear from our observation that the elasticity of the cell wall, as determined by force-indentation curves, is directly related to the flocculation patterns for the strains. This explains why strains with higher mean Young's modulus, adherence energy and adhesion force exhibited higher % flocculation ability. Altogether these changes help us understand the rigidness of the cell wall which could help us relate to the reasons contributing to different flocculation patterns for the strains.

Also, it was concluded from the data that was obtained for the adhesion force and adhesion energy suggesting difference in the stretching of the macromolecules between the four yeast strains. Based on these data, it could be further suggested that the much longer rupture distances on *S. cerevisiae* may reflect the stretching of both mannans and the polypeptidechains of the mannoproteins. The different physical properties of the mannoproteins between the four strains may explain why the surface of LYCCI was more hydrophobic than that of other *S cerevisae* strains.

In conclusion, the cell wall elasticity is predominantly dependent upon the modular architecture of the cell wall, which is largely dependent on the cross-

links between chitin and β-glucan. Our results indicate an important role of cell wall elasticity and cell surface roughness in governing the extent of flocculation of specific strains during the fermentation. As a corollary, cell adhesion force and adhesion energy, which is mainly dependent on cell wall composition, is probably not an important factor in cell flocculation. In the near future, it would be interesting to investigate how the modular architecture takes place at the nanoscopic level, by the use of functionalized AFM tips with either specific cell wall-perturbing drugs or antibodies targeted against cell wall remodelases.

Chapter Six

FLO1 and FLO8 gene expression levels governs the extent of yeast flocculation in industrial strains of *Saccharomyces cerevisiae*

Abstract

In Saccharomyces cerevisiae, a group of structurally related, cell-wall associated proteins encoded by the FLO gene family are directly responsible for many of the cellular adhesion phenotypes displayed by this organism. In this chapter, I tried to detect the presence of the dominant flocculation genes, namely FLO1, FLO5, FLO8, FLO9 and FLO10 in four industrial yeast strains, using the primers based on S288c, whose sequence is known. Detecting these genes in the industrial yeast genomes validates their presence and role in flocculation, but the expression levels of only FLO1 and FLO8 gene was determined using quantitative PCR. FLO1 encodes a dominant flocculation factor and appears to be exclusively required for cell-cell adhesion. Induction of flocculation is abolished by deletion of FLO1, the dominant flocculation gene. This explains that flocculation is not just the structural parameter of the yeast cell, but genetic as well. In order to activate the transcription of FLO1, Flo8p is required, which acts as a transcriptional activator of FLO1. In this study, I tried to correlate the differential expression of FLO1 and FLO8, to the percentage flocculation ability of the strains, explaining the molecular basis of the flocculation behaviour of these strains which governs their performance during fermentation. I concluded that the strains show presence of different truncated forms of FLO1 and the internal tandem repeats affected the flocculation ability of the strains, thus making brewing strain LYCCI having FLONL as highly flocculent amongst all the strains. The absence of bands for FLO10 (B) and FLO5 (A) when amplified using different forward primers and same reverse primer suggested polymorphism in the FLO genes, which has been previously been reported only in FLO1. Lastly results from quantitative PCR analysis suggested that higher expression of truncated form of FLO1 (FLONL) with higher number of internal tandem repeats in brewing strain LYCCI makes it highly flocculent strain, followed by champagne strain which shows high expression levels of truncated form FLO1NS gene with lesser tandem repeats as the second most flocculent gene amongst the industrial strains.

6.1 Introduction

Flocculation is a cooperative protection mechanism that shields cells from stressful environments (Smukalla *et al.,* 2008). Flocculation in *S. cerevisiae* is mediated by specific cell surface proteins, known as flocculins, or zymolectins or adhesins, which are capable of binding directly to mannose residues present on the cell wall of adjacent yeast cells (Stratford, 1989).As there are two main flocculation phenotypes, based on sugar inhibitions: Flo1-type which is mannose sensitive only and NewFlo-type which is sensitive to glucose, maltose, sucrose as well as mannose (Stratford and Assinder, 1991; Masy *et al.,* 1992). These two types of flocculation are not only just influenced by the environmental conditions, but genetic conditions also play an important role in controlling the type expressed. Different chromosomal genes, FLO1, FLO5, FLO8, FLO9, FLO10, FLO11, FLONS, FLONL and Lg-FLO1 related to flocculation of *S. cerevisiae* have been identified and all described as dominant genes (Govender *et al.,* 2008, Liu *et al.,* 2007 and Verstrepen *et al.,* 2004). The Flo11p flocculin enables yeast to adapt to a changing nutritional environment by switching to a pseudohyphal mode of growth. Of these, only FLO11 is expressed in most laborartory strains of *S cerevisiae*, where it exhibits a profusion of phenotypes. FLO11 expression is required for flocculation in S. *cerevisiae* var. *diastaticus* (Lo and Dranginis, 1996) and for invasion of substrates and formation of pseudohyphae in Σ1278b strains (Lo and Dranginis, 1996; Lipke and Kurjan, 1992) *FLO*1 is the most studied gene associated to flocculation and its regulation and expression is well known (Bester *et al.,* 2006 and Liu *et al.,* 2007). Genetic variability related to the number of tandem repeats in this gene is responsible of the flocculation degree of yeast strains: longer repeats are

associated to stronger flocculation ability (Liu *et al.,* 2007 and Sato *et al.,* 2002). Verstrepen *et al.* (2005) demonstrated that size variation of *FLO*1 induced phenotypic alterations of adhesion, flocculation and biofilm formation. Proteins encoded by these *FLO* genes share a common modular organization that consists of three domains as reported by Li *et al.* (2013): the C- terminal domain is modified by the secretion machinery of the cells and finally anchors the adhesion to the yeast cell wall (Pittet and Conzelmann, 2007). The central region acts a spacer that improves the accessibility of the N- terminal binding domain outside of the yeast cell wall. It is rich in serine and threonine regions (Bony *et al.,* 1997; Breinig and Schmitt, 2002). Finally the N terminal domain is responsible for formation of amyloid responsible for the flocculation (Ramsook *et al.,* 2010; Bauer *et al.,* 2010). The central domain contains many tandem repeat regions of DNA sequence that due to their instability can drive slippage and recombination reactions within and between *FLO* genes, with the generation of novel *FLO* alleles, conferring to yeast cells diversity and variety in flocculation ability (Verstrepen *et al.,* 2004 and Verstrepen *et al.,* 2005). The mutational frequency of tandem repeats, which is at least 100 times higher than normal point mutation, explains that industrial yeast strains may thus have their personal small reservoir of different adhesion- encoding genes that differs from the FLO gene family described in the sequenced *S. cerevisae* S288C strain.

FLO1, present on chromosome I is the best-known flocculation gene in yeast. It contains an open reading frame of 4614 bp encoding for a protein of 1537 amino acids, which shares the common three-domain structure with other flocculation proteins. The large central domain of flocculin Flo1 contains: eighteen tandem repeats of 45 amino acid residues (repeat unit A), two repeats

of 20 amino acid residues (repeat unit B), three repeats of 51 amino acid residues (repeat unit C) and three repeats of 9 amino acid residues (repeat unit D). This makes FLO1 unstable in genetics and evolves in nature rapidly, even converting the Flo1 type in NewFlo type phenotypes (Verstrepen *et al.,* 2005). Sequence analysis indicated that the deletion in these truncated forms occurred only in the tandem repeat unit A of FLO1. Meanwhile, the number variation of repeats in unit A of FLO1 influences the degree of flocculation, the more the repeats, the stronger the flocculation (Verstrepen *et al.,* 2005). FLONL and FLONS are the two popular known truncated forms of FLO1 (Liu *et al.,* 2007). The full length of FLONS and FLONL was 3843 and 4293bp, respectively. Moreover it was found that there was no obvious difference in the mRNA transcripts level of the two gene. FLONL and FLONS genes presented an ORF of 1686bp and 3396bp respectively. Flo1p is anchored in the cell wall by a non-covalent stabilization (Bony *et al.,* 1997); the hydrophobic C-terminal region of Flo1 protein corresponds to a GPI-anchor signal addition (Watari *et al.,* 1994). Deletion of this hydrophobic region impairs the anchorage of protein to cell wall and results in the loss of flocculation (Bony *et al.,* 1997). The deletion of N-terminal region impairs the development of a flocculent phenotype (Bony *et al.,* 1997). However, the N- terminal of Flo1 protein contains the sugar recognition domain, which is important for flocculation definition of Flo1 to NewFlo phenotypes (Kobayashi *et al.,* 1999).

Flocculation is controlled by many genes of which the FLO1, FLO8 and FLO11 play an important role. The global repressors of Tup1p and Ssn6p are responsible for repression of FLO1 transcription (Teunissen *et al.,* 1995). Flo8 was originally reported to be the dominant gene which confers haploid cell

specific flocculation ability and also encodes for the putative activator of FLO1 (Kobayashi *et al.,* 1996). Flo8p activates transcription of genes required for filamentous growth and adhesion, such as the cell wall adhesin genes FLO1, FLO10, and FLO11, and the glucoamylase gene STA1.Flo8p activity is regulated by the cAMP-protein kinase A (PKA) pathway. Phosphorylation of Flo8p by PKA (Tpk2p) promotes Flo8p binding to FLO11 promoter.

In industrial yeasts, FLO1 has been shown to be active and regulated by FLO8. It is considered to play an important role in mannose specific flocculation, which is inhibited by mannose but not by glucose (Kobayashi *et al.,* 1996; 1998; 1999). The FLO8 gene is present on chromosome V of the *S. cerevisae* genome and encodes one of the key transcriptional activators of FLO genes (Lie *et al.,* 1996). Kobayashi *et al.* (1996) reported that Flo8p are mainly localized in the nucleus and are required in diploid filamentous growth, haploid invasion and flocculation. Flo8p has similar binding sites on FLO1 and FLO11 promoters as shared by the negative regulators of flocculation Ssn6p, Srb8p and Tup1pSsn6p-Tup1p act as a complex in transcriptional repression (Keleher *et al.,* 1992), in repressing the transcription of FLO1.

The genetic variability of flocculation genes may have an important consequence for studies and applications targeting these genes in industrial yeasts strains with unknown genomes. I therefore designed primers to detect specific flocculation genes namely FLO1, FLO5, FLO8, FLO9 and FLO10. All the yeast strains showed the presence of the 5 dominant flocculation genes. I then tried to sequence the most important two genes which I selected for our study, FLO1 and FLO8 in the four industrial strains. The complete sequence

was not easy to obtain due to the presence of tandem repeats in the genes specially in FLO1 but still our major aim to have an idea about the expression level in the strains and correlating it with the their physical aspects seen in the flocculation behaviour was achieved by performing quantitative PCR.

To understand the function of FLO genes mainly, FLO1 and FLO8 in greater detail, it is necessary to investigate genes and their expression levels. The complete *S. cerevisiae* genome sequence has been determined, which makes it possible to carry out a comprehensive analysis of mRNA (Lashkari *et al.,* 1997; DeRisi *et al.,* 1997).

Thus, the main aims of this Chapter are:

- To investigate the presence of 'FLO' genes, mainly, FLO1, FLO5, FLO8, FLO9 and FLO10 genes in the four industrial yeast strains.
- Clone and obtain sequences for the genes which have not been sequenced before in these industrial strains.
- Examine and compare the expression levels of FLO8 and FLO1 among the four yeast strains by performing quantitative PCR.

6.2 Results

6.2.1 Identification and amplification of major FLO genes in the industrial yeast strains

The four strains under study are employed for different industrial applications. In order to understand the physiological behaviour of the yeast strains under fermentation conditions, it's equally important to study the genetic aspects of the strains (See Table 6.1). As it's already reported that the laboratory strains have "Flo" gene family, that consists of dominant genes like FLO1 and FLO5 (sharing 96% homology with FLO1), FLO9 (sharing 96% sequence homology with FLO1) and FLO10 (sharing 58% homology with FLO1) that were later identified on the basis of sequence homology to FLO1. The other dominant gene FLO11 shares a sequence homology of 37% with FLO1, thus sharing a distant homology with FLO1, while having a high degree of homology with STA1 gene. Strains that exhibit the four dominant FLO structural genes, FLO1, FLO5, FLO9 and FLO10, belong to the FLO1 phenotype. While later it was realised that replacement of FLO1 gene, that is Lg-FLO1 exists in cells in addition to the other genes in NewFlo phenotype strains (Kobayashi *et al.* 1998).

Gene (under study)	Length (bp)
FLO1	4808
FLO5(A)	3629
FLO5(B)	3429
FLO8	2383
FLO9	3303
FLO10(A)	3670
FLO10(B)	3222

Table 6.1 Dominant FLO genes for this study. The lectins that play an important role in flocculation are a product of these FLO genes. The table shows the dominant FLO genes and their length found in a laboratory strain SC288c.

6.2.1.1 DNA isolation

Isolation of yeast genomic DNA was carried out using the procedure described in section 2.4.2.1.

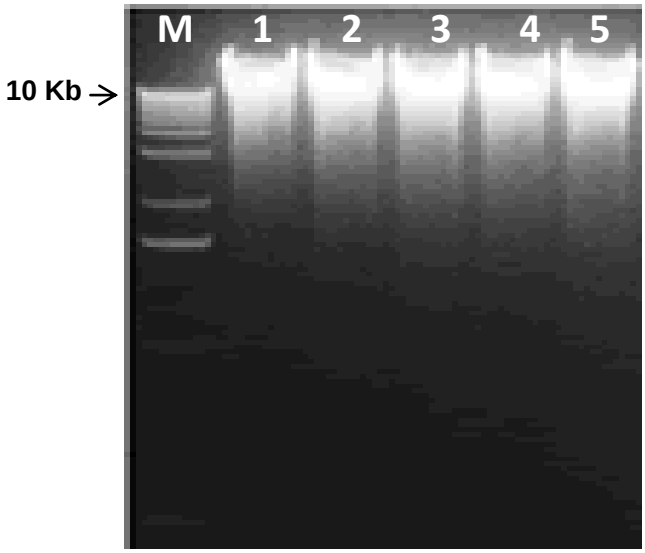


Figure 6.1 Isolation of genomic DNA from the four industrial strains of *Saccharomyces cerevisiae*.10µl of isolated genomic DNA was run on 1% agarose gel along with 1Kb DNA ladder (Qiagen) to check for its integrity. Lane 1,2,3,4,5 represents the genomic DNA bands from brewing strain LYCCI, champagne strain LYCCII, wine strain LYCCIII , fuel alcohol strain LYCCIV and unknown strain used as a control respectively.

6.2.1.2 Primer design

Since the strains under study were industrial strains and the exact sequence for the genes was not known, thus I progressed with designing primers keeping the FLO gene sequences of S288C as the template and designed the primers for FLO1, FLO5, FLO8, FLO9 and FLO10.

The genes were amplified in fragments and the length of each fragment was around 1000 bp. Only those primers that were 18-22 nucleotide long, had a melting temperature between 52-58^{o}C, GC content between 40-60% and gave no formation of secondary structure were selected. Care was taken while designing the primers was to avoid any repeats especially AT rich repeats at the end of the primer. Table 6.2 gives a list of all the primers that were used to amplify the genes in the industrial strains.

Gene	Fragme -nts	Primer combination	Length (bp)	Sequence
FLO1		**F1:R1**	**4804**	**FLO1F1**:ACTACTGCCTACATATTTATTCGGAAG
				FLO1R1:AGCAAAGAAAAGATACACAGATACG
FLO5	**(A)**	**F1:R5**	**827**	**FLO5F:** GTCCTTCTTTGGGTTAGAAAATAGCGG
	(B)	**F2:R3**	**1383**	**FLO5F1**:GGTAGAGAAGTTCCTCTGGTC
				FLO5R5:GATAATAGTAGCCTGCATACATG
	(C)	**F4:R1**	**1467**	**FLO5F2**:AGAGATTCTTCTGATGAATGG
				FLO5R3:TAATAATTGGACGCGAAGAC
				FLO5F4:AGTGAGGGTCTAATCAGCAC
				FLO5R: GAAATTTCACCATCGCGATACACCA
				FLO5R1:GAACTACTGTTTTCGTCTCAGC
FLO8		**F:R**	**2383**	**FLO8F:**GAATAGTTCGTATCCAGATTCAATTCCT
				FLO8R:GCCTTGGGAATTAATAAAATTGAAATC
FLO9	**(A)**	**F1:R3**	**1580**	**FLO9F:** TACCATACGATTGCCAGCAATACGG
	(B)	**F3:R**	**1670**	**FLO9F1**:AGTAACTAGCGTAGTTTGTTGC
				FLO9R3:AACTGCTAGCACCATAATAACTAC
				FLO9F3:CATTTCAGTGGATGTAGATGTG
				FLO9R:GCACATTATCGTGCCAAATTATTCT
FLO10	**(A)**	**F1:R4**	**1054**	**FLO10F:** GGTTGTTGTGATCCGTCACGTGTAT
	(B)	**F3:R3**	**653**	**FLO10F1**:ATCTGTAGCTAATGTTGCTCTAG
				FLO10R4:ATGAGGTTGCATATGGAGTG
	(C)	**F4:R1**	**1144**	**FLO10F3**:ACCGAAACTGAGTCTACCAG
				FLO10R3:AGCTGTGAAATATTTGTTCG
				FLO10F4:CCAACTAATTTGATTACCAGC
				FLO10R1:ACGAGGCTGTTGAGACTGTG

Table 6.2 List of primers used to amplify the genes of interest using normal PCR conditions. The genes were amplified in fragments as they were long. Forward and reverse primers were designed for every 500 bp through the entire length of each of the gene. Selected combinations of forward and reverse primers gave us the fragments of the gens which were collated to get the whole gene product.

6.2.1.3 Amplification

In order to carry out the PCR reaction, I used two types of DNA Polymerase Taq. Initial set of PCR amplification was carried out using the DNA Polymerase MyTaq™ (Bioline). Since this Taq does not amplifying fragments up to 5 Kb with good efficiency, thus I tried to amplify the genes in fragments. As can be seen the genes and their primers used for amplification are mentioned in Table 6.2. Later I used LongAmp® DNA Taq (New England Biolabs), which gave us the genes in their full length. In this case only the first forward (F1) and last reverse (R1) primer was used to amplify the whole gene of interest.

Initial Denaturation……95^{o}C for 10 mins

Denaturation……..95^{o}C for 1 min

Primer Annealing……...47^{o}C/50^{o}C* for 1 min } 35 cycles

Extension……72^{o}C for 4 min

Final Extension……...72^{o}C for 10 min

*The conditions for amplification used for gene amplification for genes FLO1, FLO10 and FLO8 were carried out at 47^{o}C. Annealing for FLO9 and FLO5 were carried at 50^{o}C with MyTaq™ (Bioline).

Conditions used for gene amplification with LongAmp® DNA Taq (NEB):

Initial Denaturation……94°C for 1 min

Denaturation……..94°C for 30s

Primer Annealing…….....47°C for 1 min } 35 cycles

Extension……65°C for 5 min

Final Extension……...65°C for 10 min

6.3.1.3.1 ***PCR amplification for whole gene fragments***

The list of primers used for amplifying the specific genes in fragments have been mentioned in Table 6.3 and in order to amplify whole genes using LongAmp® DNA Taq, primers were used in combinations. I tried to detect whole genes with some upstream and downstream sequences. However, in some cases the genes due to their long sizes were amplified using different sets of primers which included the whole coding sequences but the upstream and downstream sequences differed. For FLO10 (A) primer combination of **FLO10F:FLO10R** gave a PCR product of 3670bp (CDS+ 514 upstream+ 327 downstream sequences). FLO10 (B) was obtained using **FLO10F1:FLO10R** in combination to yield a PCR product of 3222bp (CDS + 61 upstream+ 327 downstream sequences). For FLO5 (A) primer combination of **FLO5F:FLO5R** gave a PCR product of 3629bp (CDS+ 406 upstream + 226 downstream sequences) and primer combination **FLO5F1:FLO5R** gave FLO5 (B) 3429bp product (CDS + 206 upstream+ 226 downstream). In case of FLO1, **FLO8 and FLO9**, only one whole gene product was obtained. Primer combination **FLO9F:FLO9R** was used to obtain a fragment of 3303bp (CDS+ 160 upstream

sequence). For FLO1 primer combination used was **FLOF1:FLO1R1** which was targeted to obtain a product of 4808bp (CDS+ 149 upstream +45 downstream), while for FLO8, **FLO8F:FLO8R** gave a product of 2383bp (CDS). Figure 6.2 shows the presence of FLO1/FLO5/FLO8/FLO9/FLO10 genes in the four industrial strains.

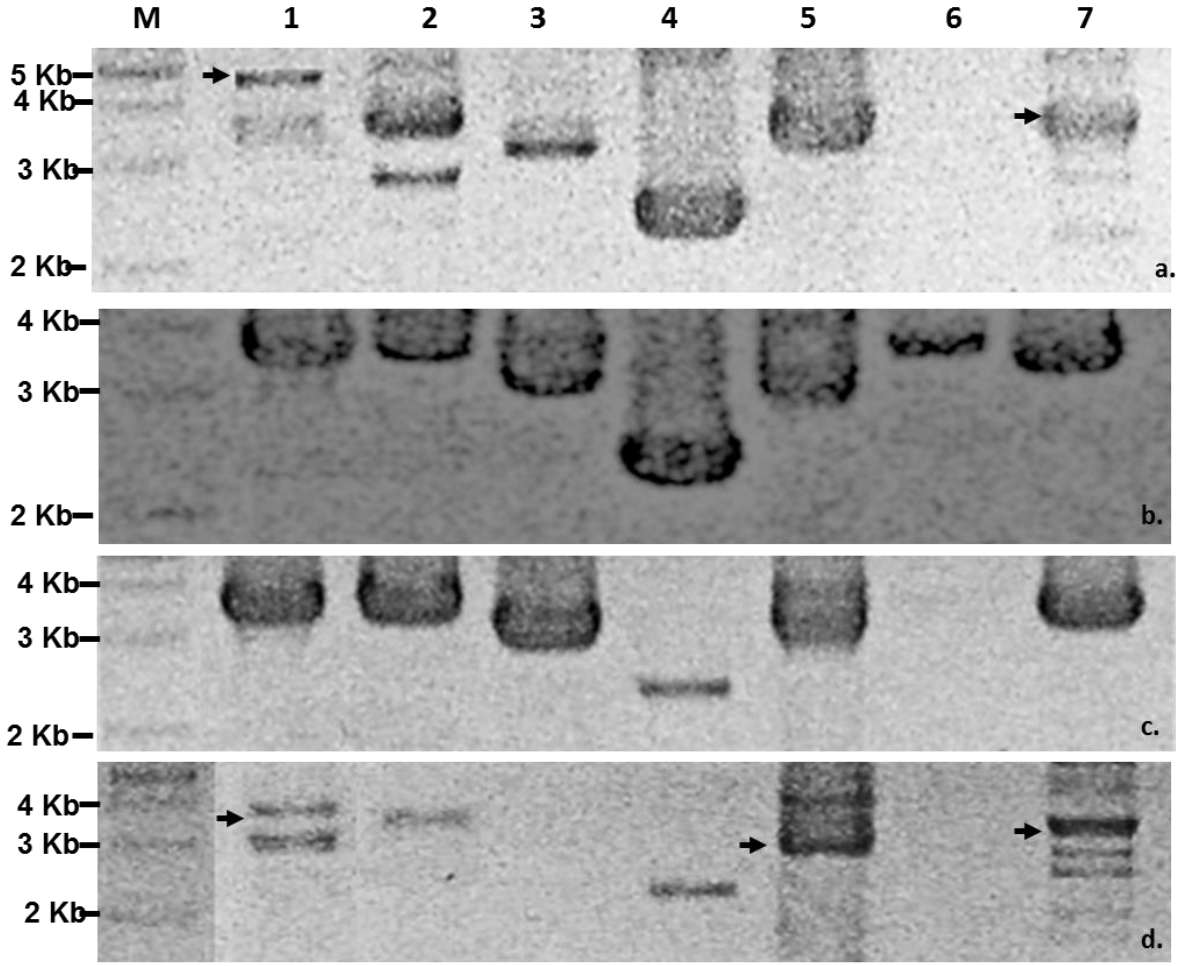


Figure 6.2 Detection of genes FLO1, FLO10A, FLO10B, FLO8, FLO9, FLO5A and FLO5B in industrial yeast strains of *S. cerevisiae* by PCR using their respective primers. (a) Brewing strain (LYCCI) (b) Champagne strain (LYCCII) (c) Wine strain (LYCCIII) (d) Fuel alcohol strain (LYCCIV). 10µl of PCR product was run on 1% agarose gel along with 1Kb marker (lane M). Lane 1, 2, 3, 4, 5, 6, 7 represent amplified product for genes FLO1, FLO10A, FLO10B, FLO8, FLO9, FLO5A and FLO5B respectively. In case of presence of nonspecific PCR products, arrows represent the specific bands.

All the five genes of interest namely FLO1, FLO5, FLO8, FLO9 and FLO10 were found to be present in the four industrial strains. Figure 6.2 shows the presence of bands on the gel for the specific genes, however, the presence of either of the forms (A) and (B) for FLO10 and FLO5 confirms the presence of FLO10 and FLO5 genes respectively in the genome of these industrial strains. The result of these amplifications allowed us to identify the presence of truncated versions of FLO1 gene in the strains. These were the FLONS, FLONL and FLO1M genes. FLONS lost two internal repeated regions, whereas one repeated sequence was inserted into the middle repetitive sequence of FLO1 gene and FLO1M was also reported to have deletion in middle region. (Liu et al 2007). FLONS (3.8 Kb) , FLONL (4.3 Kb) and FLO1M (3.1Kbp) were derived from FLO1 gene. Brewing strain LYCCI showed the presence of two fragments, one at 4.3 Kb and another at 3.8 Kb, which confirmed the presence of FLONL and FLONS respectively .However champagne strain LYCCII and wine strain LYCCIII showed the presence of only one band at 3.8 Kb, which indicates the presence of only FLONS in their genome. Fuel alcohol strain LYCCIV also showed the presence of two bands at positions 3.8 Kb and at 3.1 Kb, indicating the presence of FLONS and FLO1M in its genome. From the experiment, it can be explained that why brewing strain LYCCI has higher flocculation ability as compared to other three strains, as higher the number of tandem repeats present, higher is the flocculation ability. Also it explains the NewFLO phenotype of these strains, as repeated deletions in the FLO1 gene caused the flocculation phenotype conversion from FLO1 to NewFlo (Teunissien *et al.* 1993a, Watari *et al.* 1989 and Liu *et al.* 2007).

In the case of FLO10(A), all the strains gave a band at 3.6Kb, however, the same didn't hold true for FLO10(B) where all the strains gave a band at 3.2 Kb except fuel alcohol strain LYCCIV. Moreover a similar situation was observed for FLO5(A) and (B) genes , where only champagne strain gave the band amongst all the other industrial strains. In the study I used different primer combinations to detect the presence of the genes in strains and moreover, the primers were designed keeping the laboratory strain S288c in mind. The reverse primer in case of both the sets of FLO10 and FLO5 genes were kept same and the only difference was at the forward primer. Thus it indicates a probable presence of polymorphism within 400 bp and 200 bp to the coding sequence of FLO10 and FLO5 gene respectively within the strains. Lane 4 and 5 (Fig 6.2) showed the presence of FLO8 and FLO9 gene at 2.4 Kb and 3.3 Kb in all the four strains. Lane 6,7 showed the presence of FLO5 (A) and FLO5(B) respectively. However, it was observed that all the strains showed the presence of 3.6 Kb fragment, but only champagne strain showed the presence of 3.4 Kb fragment. As there is only a difference of 200 bp upstream of the coding sequencing compared to the attained PCR product in case of FLO5(A) explaining the brewing strain LYCCI, wine strain LYCCII and fuel alcohol strain LYCCIV could probably harbour a mutation in approximately 200 bp upstream to the coding sequence thus not providing any site for primer binding while PCR amplification.

6.3.1.3.2 PCR amplification results for of fragments obtained with MyTaq™

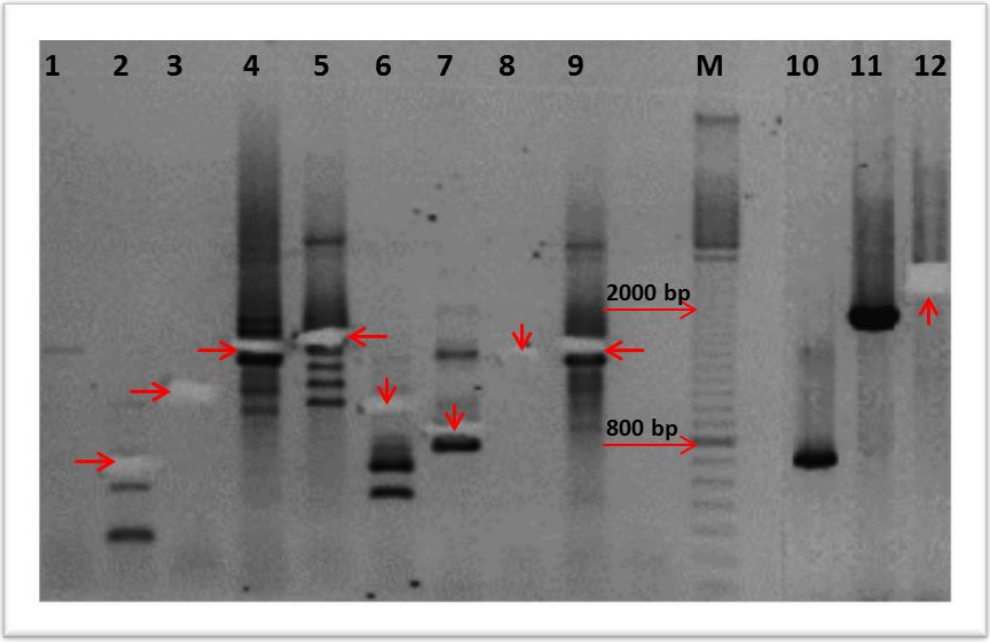


Figure 6.3 (a) Detection of genes fragments for FLO1, FLO10, FLO8, FLO9 and FLO5 in brewing yeast strains of *S. cerevisiae* LYCCI by PCR using their respective primers. Lane represents the gene fragments obtained in ascending order, FLO10A, FLO10B, FLO10C, FLO9A, FLO9B, FLO9C, FLO5A, FLO5B, FLO5C, FLO1, FLO8A, FLO8B respectively. In case of presence of nonspecific PCR products, arrows represent the specific bands.

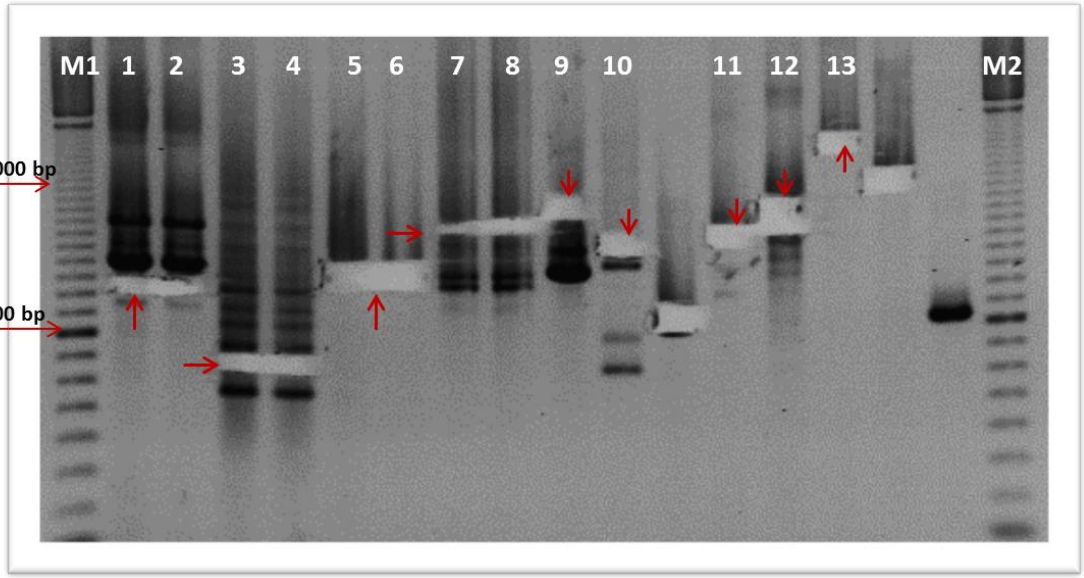


Figure 6.3 (b) Detection of genes fragments for FLO1, FLO10, FLO8, FLO9 and FLO5 in champagne yeast strains of *S. cerevisiae* LYCCII by PCR using their respective primers with MyTaq™ . 10µl of the sample was run

along with 100bp marker on 1% agarose gel. Lane represents the gene fragments obtained in ascending order, FLO10A (L1,L2), FLO10B (L3,L4), FLO10C (L5,L6) ,FLO9A (L7,L8), FLO9B (L9) , FLO9C (L10) , FLO5B (L11), FLO5C (L12), FLO8B (L13) respectively.

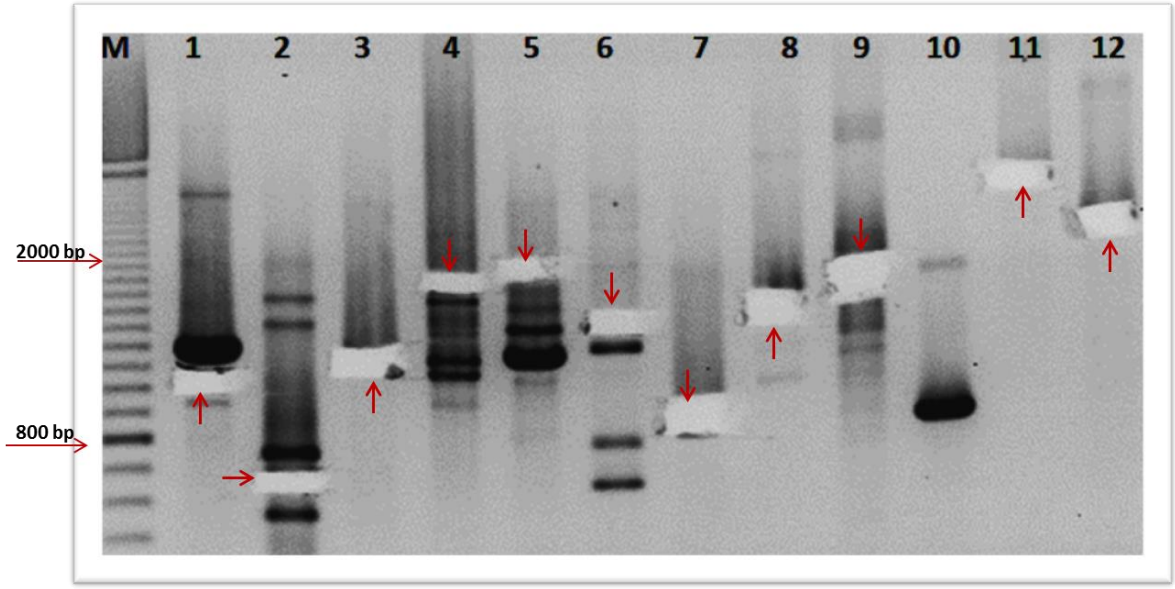


Figure 6.3 (c). Detection of genes fragments for FLO1, FLO10, FLO8, FLO9 and FLO5 in wine yeast strains of *S. cerevisiae* LYCCIII by PCR using their respective primers with MyTaq™.10µl of the sample was run along with 100bp marker on 1% agarose gel. Lane represents the gene fragments obtained in ascending order, FLO10A, FLO10B, FLO10C, FLO9A, FLO9B, FLO9C, FLO5A, FLO5B, FLO5C, FLO1, FLO8A, FLO8B respectively.

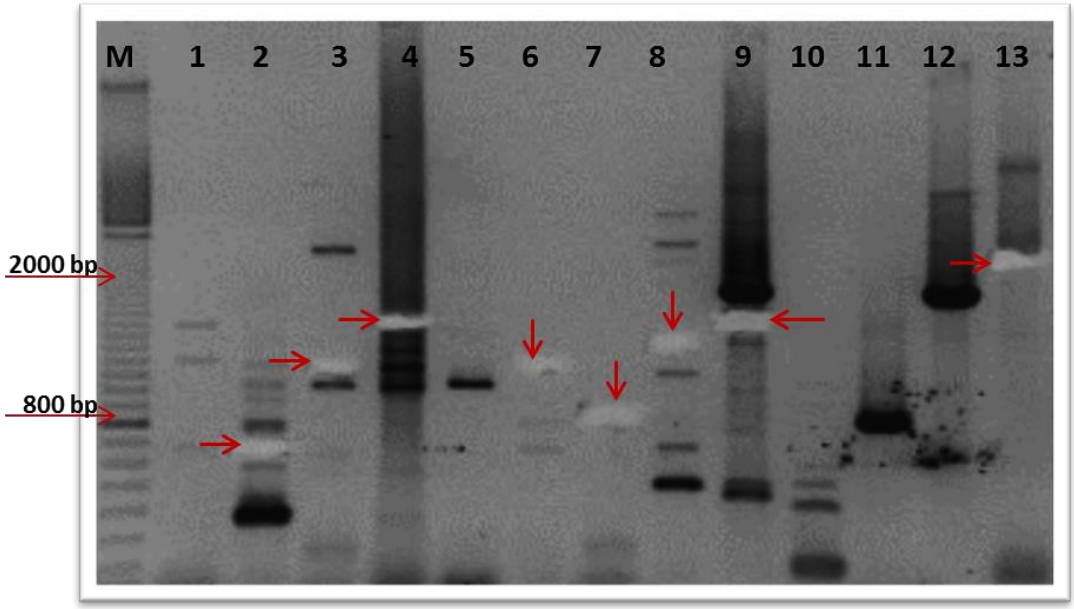


Figure 6.3 (d) Detection of genes fragments for FLO1, FLO10, FLO8, FLO9 and FLO5 in fuel alcohol yeast strains of *S. cerevisiae* LYCCIV by PCR using their respective primers with MyTaq™ . Lane represents the gene fragments obtained in ascending order, FLO10A, FLO10B, FLO10C,FLO9A, FLO9B, FLO9C, FLO5A, FLO5B, FLO5C, FLO1, FLO1P (promoter), FLO8A, FLO8B respectively.

6.2.1.4 Transformation and cloning

I obtained the PCR products for both the whole genes for FLO1, FLO5, FLO8, FLO9 and FLO10 as well as the fragments of these genes. The fragments of the genes were ligated in pGEM Teasy vector. However, not all the fragments were efficiently cloned into. This was revealed by gel images obtained after miniprep followed by PCR using the primers specific for T7 and SP6.

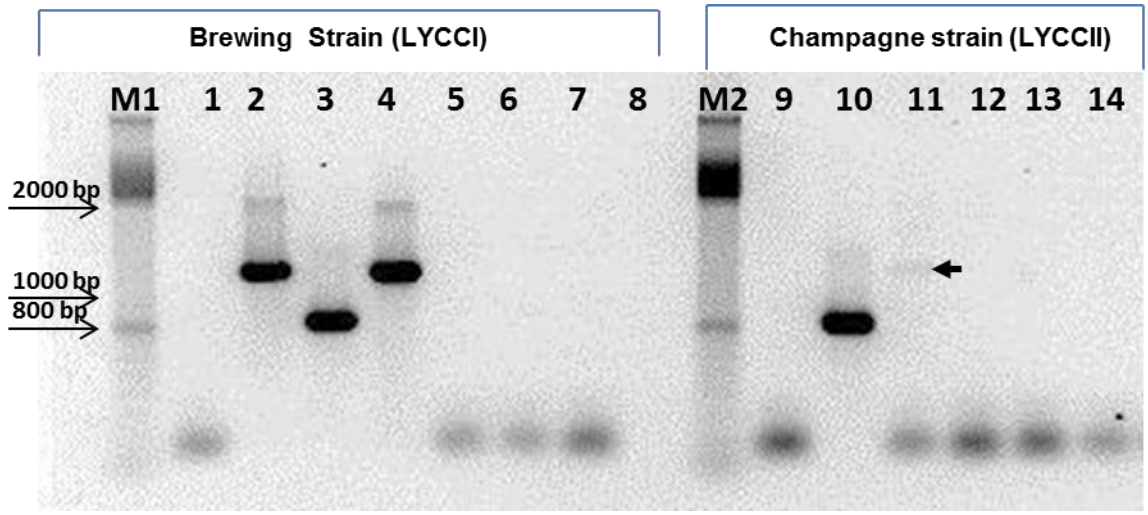


Figure 6.4 (a) The presence of desired fragments (inserts) from the vector after transformation of E.coli JM109 competent cells with T7 and SP6 specific primers. 10µl of the product was run along with 100bp marker on 1% agarose gel. Each lane for brewing strain, LYCCI, represents the following inserts in ascending order, FLO8, FLO10A, FLO10B, FLO10C, FLO9A, FLO9C, FLO5B, FLO5C respectively. Each lane for the champagne strain, LYCCII, represents the following inserts in ascending order, FLO8, FLO10B, FLO9A, FLO9B, FLO5B and FLO5C respectively.

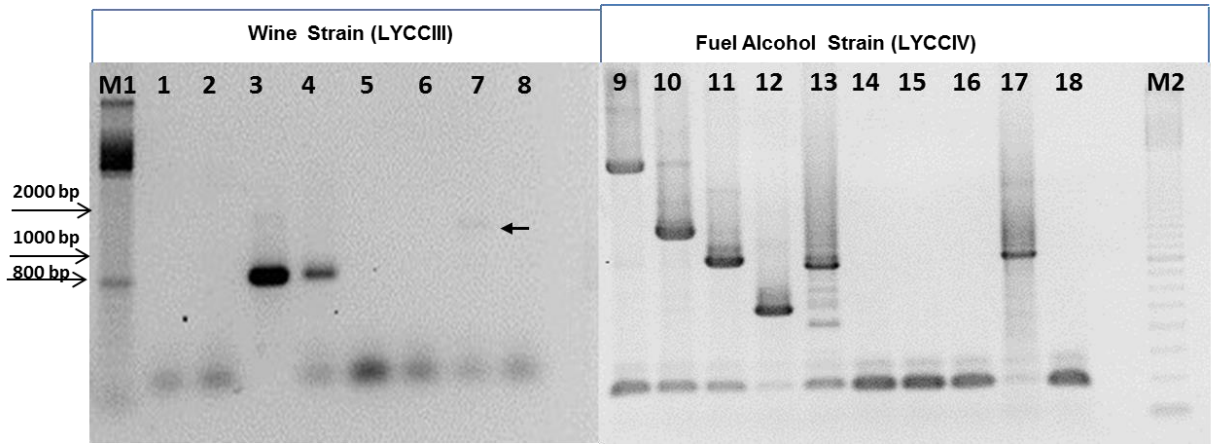


Figure 6.4 (b) The presence of desired fragments (inserts) from the vector after transformation of E.coli JM109 competent cells with T7 and SP6 specific primers. 10µl of the product was run along with 100bp marker on 1% agarose gel. Each lane for wine strain, LYCCIII, represents the following inserts in ascending order, FLO8, FLO10A, FLO10B, FLO10C, FLO9A, FLO9C, FLO5B, FLO5C respectively. Each lane for the fuel alcohol strain, LYCCIV, represents the following inserts in ascending order, FLO8, FLO10C, FLO10B, FLO10A, FLO9A, FLO9B, FLO5B, FLO5C, Positive control and negative control respectively.

From Fig 6.4 (a) and (b), it's observed that not all the fragments were transformed efficiently in the yeast strains. Transformation was only observed for fragments FLO10 (A), (B) and (C) in brewing strain LYCCI, fragments FLO10B, FLO9A for champagne strain LYCCII, fragments FLO10A, FLO10B and FLO9C and fragments FLO8, FLO10C, FLO10B, FLO10A and FLO9A for fuel alcohol strain LYCCIV. Troubleshooting was carried out and perhaps mainly the ligation reaction was not carried for sufficiently long time to allow proper ligation. Another reason observed was that short fragments of less than 1000bp, in most of strains showed the presence of band after PCR with T7 and SP6 primers. Therefore, the construct size could have affected the transformation efficiency. Alternatively it could be due to variable PCR conditions.

6.2.2 DNA sequencing of the selected genes of interest: FLO1 and FLO8

Figure 6.4 (a) and (b) shows that not all the fragments were successfully transformed and cloned. Due to time constrains, I selected only two genes for sequencing i.e FLO1 and FLO8 genes for the four industrial strains. The presence of FLO1 and FLO8 genes was done by making use of the commercial sequencing service and then performing blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP__4932__9518) of the PCR product of these two genes for the four industrial strains.

6.2.2.1 DNA sequencing for FLO1

6.2.2.1.1 Brewing Strain, LYCCI

For FLO1- Forward Primer (F1)

TTTTACCAGTATTGAAGTTCCTCNACATAAAAGGGGTNCTACGAAGTAAGTAAGATCTT
CGTAACAAGACANAANGGGNCTNGGCGGGAGNNATATAGTTGTGTCCCGTTAGGAANGG
TCGNNTNTTGGATATTGGGGGCCCTGGTGATTTATTTGGTTTCTATACTACCCCAACAA
ACGTAACC

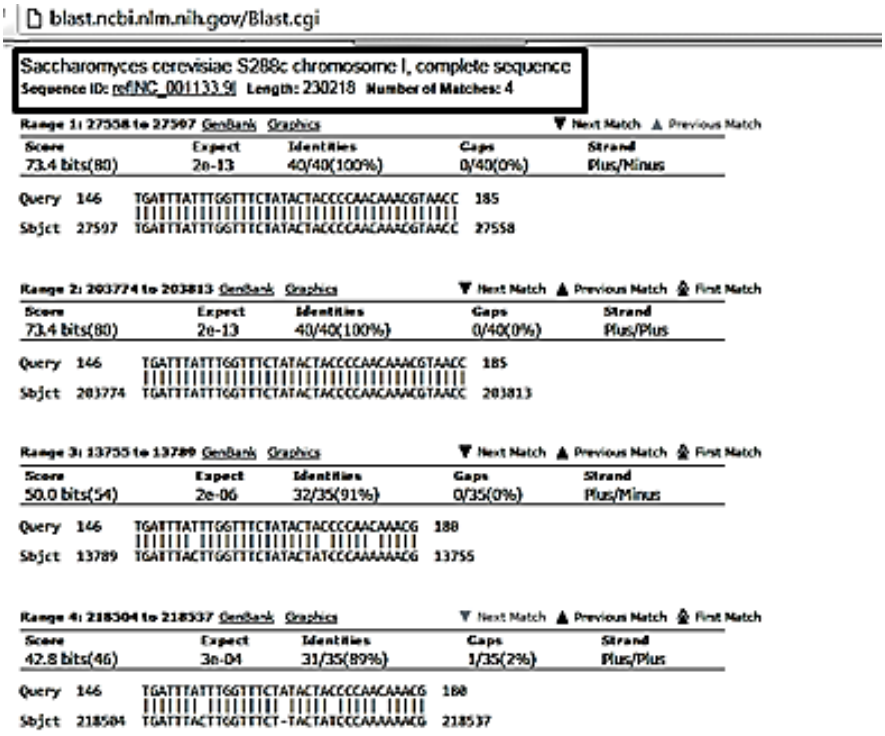


Figure 6.5 (a) The BLAST result of the DNA sequence obtained for brewing strain, LYCCI for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

For FLO1- Reverse Primer (R1)

TGCTGGGACGATAACNNNAGTGACTTATTGGCTTTCAAAATCTGATCCGGCTGGAACAC
AGACGGCTTCCGCGACCGATGTGATTGGTCNNNCAGCAGTAGTCTTGTTTCTGTATGAA
ACGTGGCAACACCAA

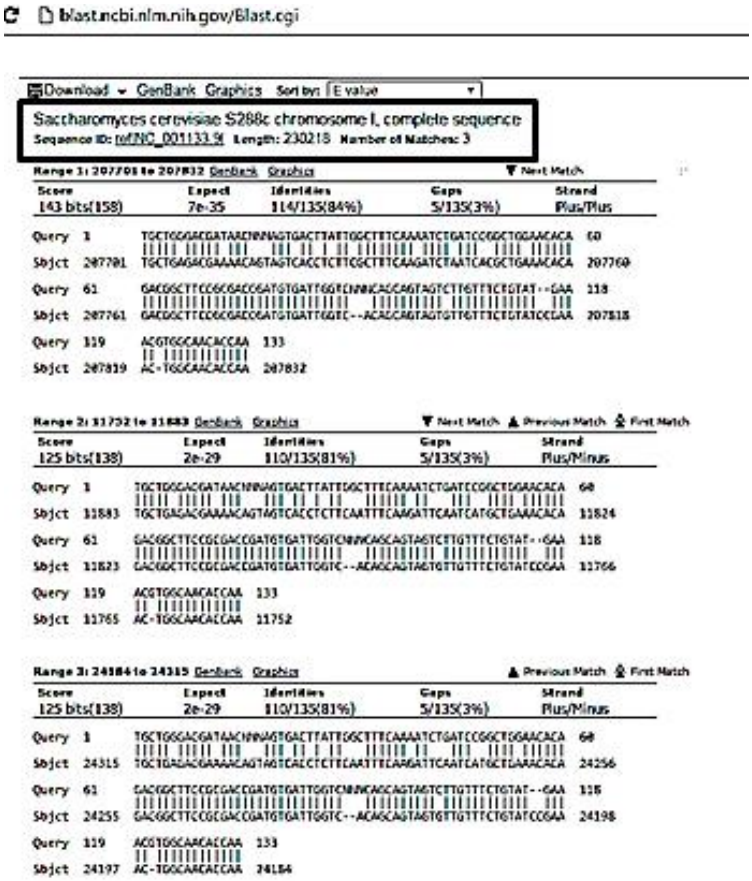


Figure 6.5 (b) The BLAST result of the DNA sequence obtained for brewing strain, LYCCI for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

6.2.2.1.2 Champagne Strain, LYCCII

For FLO1- Forward Primer (F1)

TTCCTTGTCCTCAAGAAGATTCCTATGGAAACTTNCATGTACGTTCACCTTTCGGGTTT
TTGGCTTGTGGGGGGGCGTGTTGCCGTNGAGNAGATGCACCCCTATTGTGTTGNCAGNG
GCNTACNTCNCNATNTAGATTTTGGGNTAGGCGGTGTT

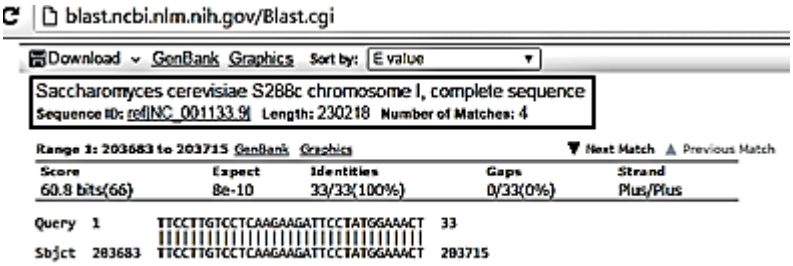


Figure 6.6 (a) The BLAST result of the DNA sequence obtained for champagne strain, LYCCII for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

For FLO1- Reverse Primer (R1)

ACGACGCCTCTAACCATTATAATATTGCCAGCAATAAGGAGCATGAAGACCNGCAGTTN
CNGGTTCTTGACGATGTCGCAACAGCCTNNNAGCACACCANNAAGCAGCTTCTTAGGAT
ATNNNACAGCTTCTTTAGAAATTTCAACGTATGCTGGCAGTGCCAACGCTACTGGCCGG
TAGTGGGTTTA

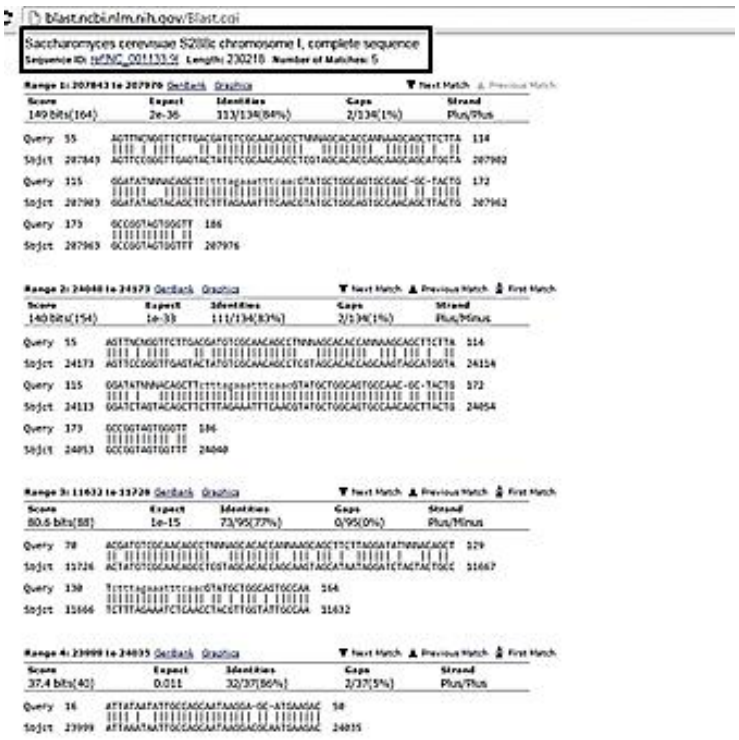


Figure 6.6 (b) The BLAST result of the DNA sequence obtained for champagne strain, LYCCII for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

6.2.2.1.3 Wine Strain, LYCCIII

For FLO1- Forward Primer (F1)

CCAGAGGAAAAGTGGGANNNATATAAATTTTTACNNNNATTCATTGAAAGACATAT
CCACATACAATAATGCAGCATATATGGATAATGGATATGCCTCAAAAACCAAAACT
AGTTCTGTCGGAGGAAATTATGATATCTCGNNNNATTATAATATTCCCTGTGTTAG
TTCATCCGGCACATTTCCAGAGACTCAAGAAGAT

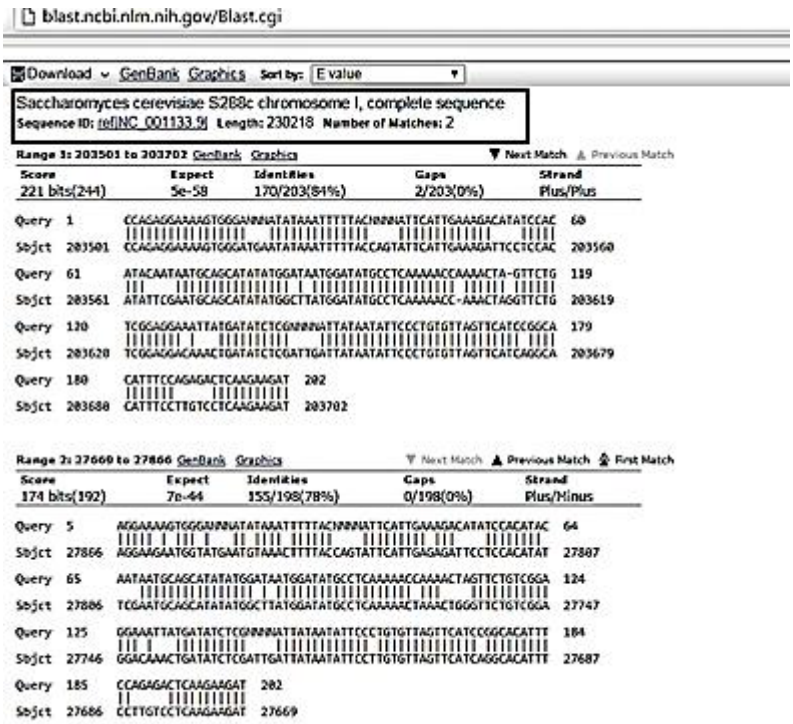


Figure 6.7 (a) The BLAST result of the DNA sequence obtained for wine strain, LYCCIII for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

For FLO1- Reverse Primer (R1)

GCTGGTTCTGTCTAGCTTACTGCAAAATGTGGCGCATTGAGACCTTTGACAAGTGCTCGGGGA
AACGCCTGATGCCGCATCTCCATCCTTCTGGGTGCGGTCTTTTCCAACCCGGCAATTCTTGCG
AGAGTCATCTCTGCAATAAATCCATNNNNCCAATGATTGTTTCCACAGCTACTCGTACTGTTA
GCGGCGGTCACAACA

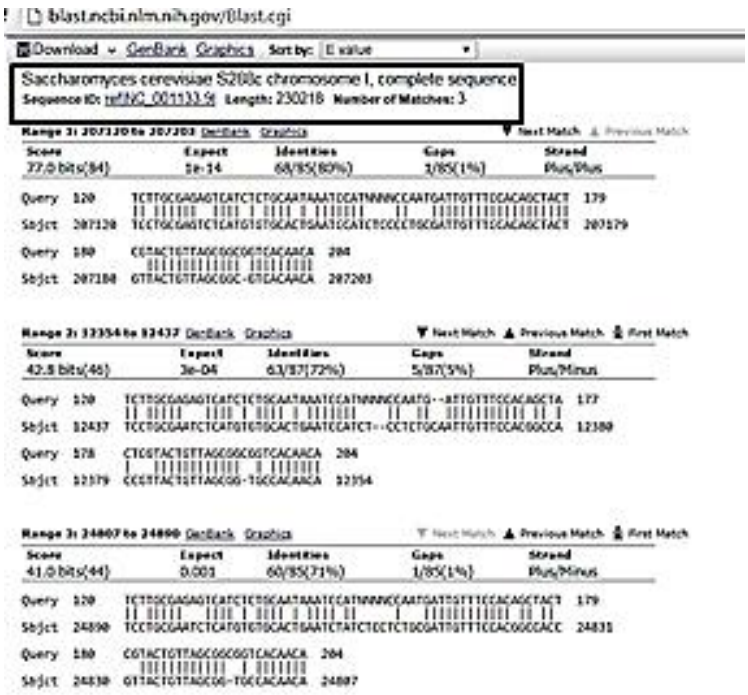


Figure 6.7 (b) The BLAST result of the DNA sequence obtained for wine strain, LYCCIII for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

6.2.2.1.4 Fuel Alcohol Strain, LYCCIV

For FLO1- Forward Primer (F1)

TTGAATATAATACTCCCTGCGTTAGCTTAGCAGGCACATTTGCTTTTCCTCAAGAAGATTC
CTATNNAANCTGGGGATGCANAGGAATGGGTGNGCATTCTGATAGTCAAGGAATTGCANNN
CACATTATCGGAATTTTGGGATTGGGNGGTGACAGTGCGAATACTGNCGGGCCGTGGTCGT
GTTAAGNAAGGGNNTTCGGTNTCGANTAAGTGGANGGCCNTAG

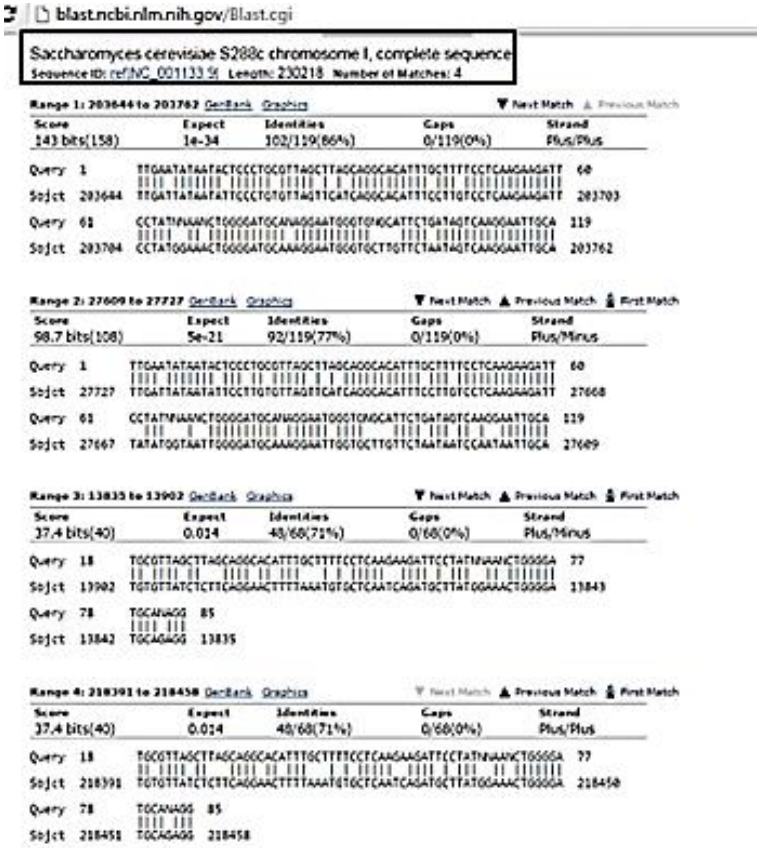


Figure 6.8 (a) The BLAST result of the DNA sequence obtained for fuel alcohol strain, LYCCIV for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

For FLO1- Reverse Primer (R1)

CTAAGAAGCGGAATTCATTAATGGAGGCCGACACAATGACAGTTTCATCAGTTGGCAAGCC
ATTGTAATATTCCCTGTGTTAGTTCATCAGGCACATTTCCTTGTCCTCAAGAAGATTCCTA
TGGAAACTGGGGATGCAAAGGAATGGGTGCTTGTTCTAATAGGTTCCAGTGACAGTAGCCA
TTTCAGTGGATGTAGATGTAAATTACCGGTCATGGTGCGGTTGTAGTATTCGGCGTGTCTG
TGTTGGTGTGTTGACAACAATGATGGTCGCTCACTTACACAGCGCTCGTCTGGTAACAGC

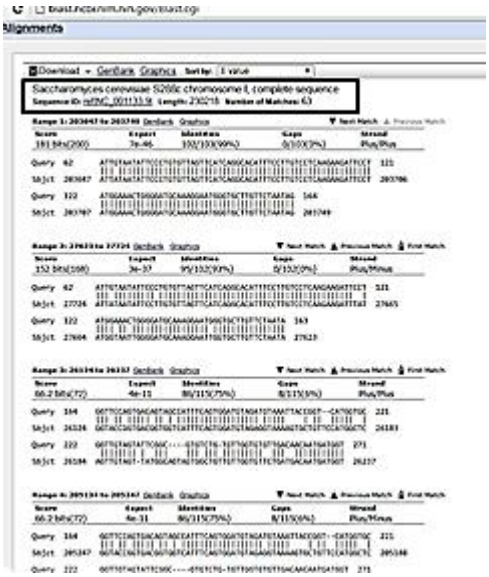


Figure 6.8 (b) The BLAST result of the DNA sequence obtained for fuel alcohol strain, LYCCIV for FLO1 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome I confirms the presence of FLO1 gene.

Thus, from (Fig 6.5-6.8) BLAST on sequencing results pointed chromosome I (with maximum hits), indicated that the gene so amplified and sequenced was for FLO1 gene.

6.2.2.2 DNA sequencing results for FLO8 gene

6.3.2.2.1 Brewing strain

For FLO8- Forward Primer (F1)

GCCCAGTAACCGTAATGGCAGCCAGTATAACAAGATTTGCAGAGCAATATTGCAATGGC
AACAAATAGTGAACAGCAGCGACAACAACAGCAGCAGCAGCAACAGCTGCAACAGCAGT
GGATAACTTACCTACCNNGGAATATTNNGTTNNNATTCTTGTATCTTTGATTATTTATT

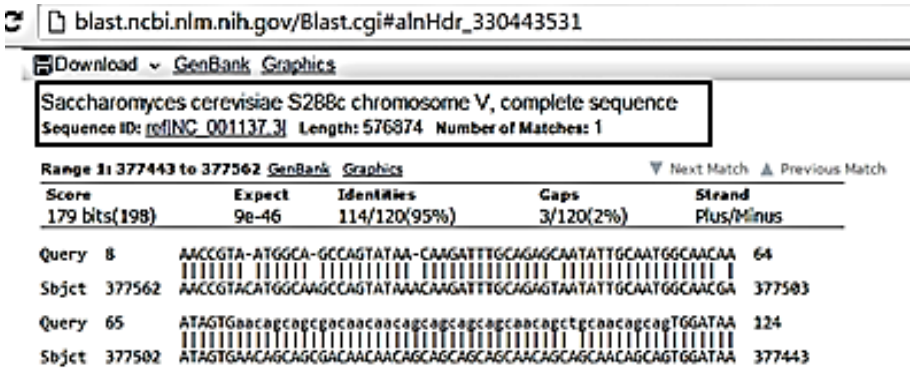


Figure 6.9 (a) The BLAST result of the DNA sequence obtained for brewing strain, LYCCI for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Reverse Primer (R1)

TTTTTCCATTTGTATCAGTAATTGAGTCGTATCCGGTCCTTGGTCTTCAACCATACCAA
TATTCCCAAACTTCAAGTTCTGAGAGTGTACATTCTGATTTTCATTAGTACTGGTTTGC
TGAGGACCCAAAGTTGTACCC

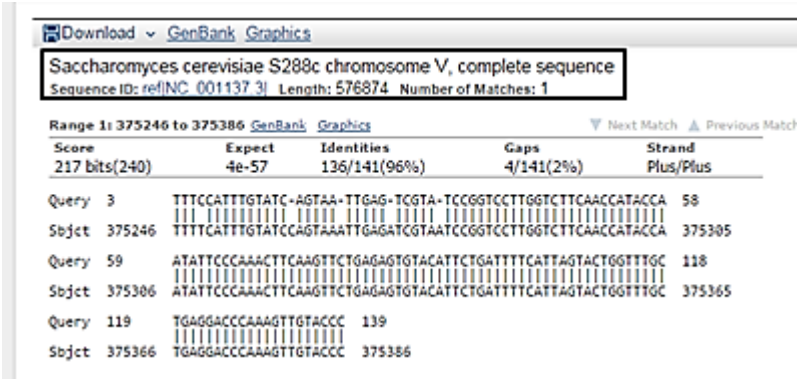


Figure 6.9 (b) The BLAST result of the DNA sequence obtained for brewing strain, LYCCI for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Reverse Primer (R2)

GAACATCAGTGAGCTTCTCACGATGGGCTAGATTTGGATTAGAATAAAAACGTCCTGGC
CAACGGAAGAGCGTGAACGGGGCGTCCTTTACTTTGAAGATCGCGTCACCGTCCGGCGG
GCAGCCAGCAATGAAGTAGTCCATTTTAACAACTTCGTGGCACGGGTAAACTTTGGTGG
TGATACGCGGGATGTCCGGGTGGAACGGAACAACCGCTTTCGCACCCGGAACCGCGGTC
GCAGAGTTAACGTACGCTTCCGCCAGGCAGTCTTTCAGTTCGAAAACGTTACGCATCGC
CGGACACCACCCCAACCGCGCACGCACAAAGAGATCAGGAGTCCAGTTTCCGGAGTGTT

CCGGGTTCATGTTTCTTCAAAAAACACCACCTTCACGAACCGATCGCGCTCTTCCGGGA
TACGTTTGATGTCGTCACAATAACGCACAGGATACGTTTCCTC

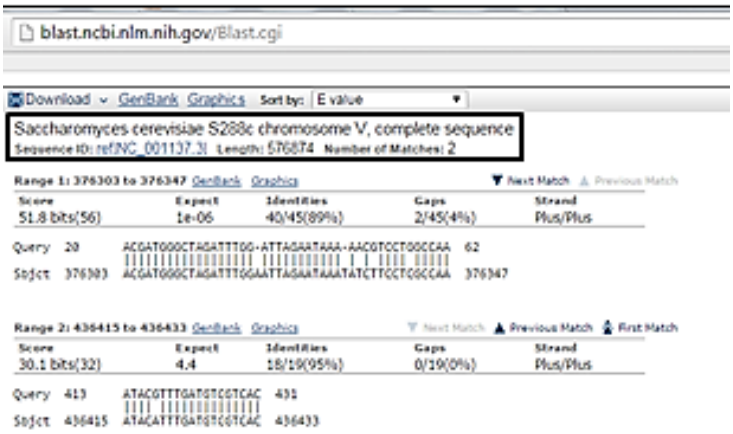


Figure 6.9 (c) The BLAST result of the DNA sequence obtained for brewing strain, LYCCI for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

6.2.2.2.2 Champagne strain

For FLO8- Forward Primer (F1)

CCAGGCTCTCGTAATGGCAGCCAGTATAAACAAGATTGCAGAGTATATTGCAATGGCAA
CGAATAGTGAACAGCAGCGACAACAACAGCAGCAGCAGCAACAGCAGCAACAGCAGTGG
ATAAATCAACCTACGGCGGAAAATTCGGATTTGAAGGAAAAAATGAACTGCAAGAATAC
GCTCAATGAGTACATATTTGACTTTCTTACGAAGTCGTCTTTGAAAAACACTGCAGCAG
CCTTTGCTCAAGATGCGCACCTAGATAGAGACAAAGGCCAAAACCCAATCGACGGACCC
ACCTCTAAAGAAA

Figure 6.10 (a) The BLAST result of the DNA sequence obtained for champagne strain, LYCCII for FLO8 gene obtained after DNA sequencing.

The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Forward Primer (F2)

TACGAGCTAGCTAAGCCGGTCTAGTGGCTAAGAGACGAAGAAAAAATAATACCGCTACA
GTTTCCGCGGGATCGACGAACGCTGGTTCGCCAAATATTACCACACCAGGCTCAACAAC
AAGTGAACCCGCTATGGTAGGTTCAAGAGTAAATAAGACTCCAAGATCAGATATTGCTA
CTAACTTCCGCAATCAAGCAATAATATTTGGCGAGGAAGATATTTATTCTAATTCCAAA
TCTAGCCCATCGTTGGATGGAGCATCACCTTCCGCTTTAGCTTCTAAACAGCCCACAAA
GGTAAGGAAAAATACAAAAAAGGCATCCACCTCAGCTTTTCCAGTAGAGTCTACGAATA
AACTCGGTGGCAACAGCGTGGTGACAGGTAAAAAGCGCAGTCCCCCTAACACTAGAATG
TCGAGGAGGAAATCCACTCCTTCTGTTATTCTGAATGCTGATGCCACTAAGGATGAGAA
TAATATGTTAAGAACATTCTCGAATACTATTGCTCCGAATATTCATTCCGCTCCGCCCA
CTAAAACTGCGAATTCTCTCCCTTTTCCAGGTATAAATTTGGGAAGTTTCAACAAGCCG
GCTGTATCCAGTCCATTATCTTCAGTGACAGAGAGTTGCTTCGATCCAGAAAGTGGCAA
GATTGCCGGAAAGAATGGACCCAAGCGAGCAGTAAACTCAAAAGTTTCGGCATCATCCC
CATTAAGCATAGCAACACCTCGGTCTGGTGACGCTCAGAAGCAAAGAAGTTCTAAGGTA
CCAGGAAACGTGGTTATAAAGCCGCCACATGGGTTTTCAACCACCAATTTGAATATTAC
TTTAAAGAACTCTAAAATAATCACTTCACAAAATAATACAGTATCCCCAAGAATTGCCG
AAATGGGGGAAAAACATATTGGAAGCCGCAAGTAAGGCAATGATTCAAGAAATAGGTAA
AGGCAATCGTAACACATTTATCTACTCCATAAGGAAAAAATAGCGCAATCTCTCATAAG
CCAACGGCATCGATT

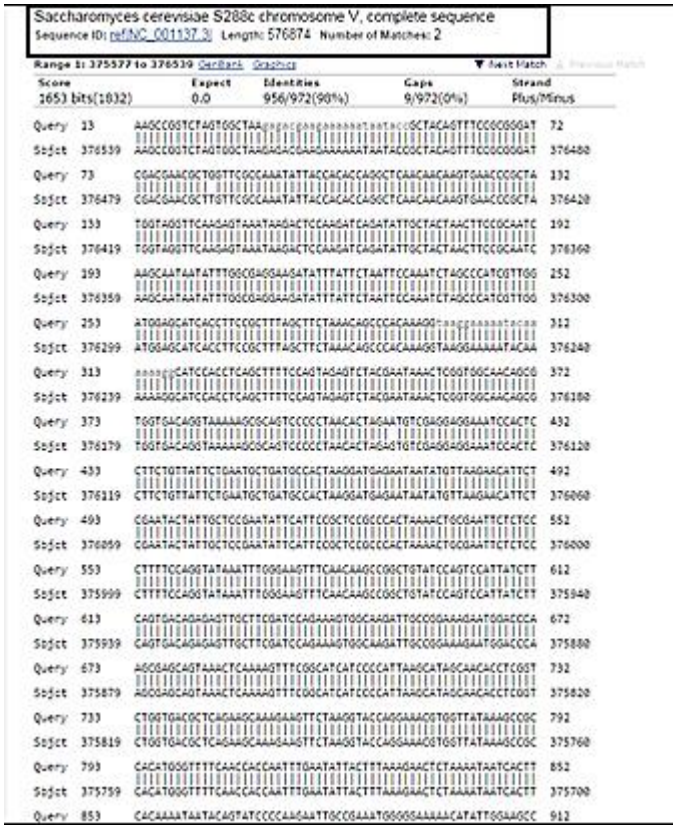


Figure 6.10 (b) The BLAST result of the DNA sequence obtained for champagne strain, LYCCII for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Reverse Primer (R1)

TATTTCTTTGATCAGTAAATTGAGATCGTAATCCGGTCCTTGGTCTTCAACCATACCAA
TATTCCCAAACTTCAAGTTCTGAGAGTGTACATTCTGATTTTCATTAGTACTGGTTTGC
TGAGGACCCAAAGTTGTACCCACATTGGATGAGGGCTGAATTAATGTGTTATCATTATC
GCCACTATTTGTAGATGCAGAGGTTTCATCAGCAACATTTGAATTCTCGTTTGGTGTTC
TATTGTTAGAAGCATAAGCTTGATTAGGAAACAACAAAGAACTTGAATTTTTGAGGGCG
TCAAAATCATATCCTTGATTATTACTACTCGGCTTTTTTTCCTCTGGAGTAGATAATGT
GTTACGATTGCCTTTACTACTTCTTGAATCATTGCCTACTTGCGCCTCCAGTATGTTTC
CCCCATTCGGCAATTCTTGGGATACTGTATTATTCTGTGAAGTGATTATTTTAGAGTTC
TTTAAAGTAATATTCAAATTGGTGGTTGAAAACCCATGTGGCGGCTTTAGAACCACGTT
TCCTGGTACCTTAAACTTCTTTGCTTCTGAGCGTCCTGCAGACCG
AGGAGTTGCTACTGCT

Figure 6.10 (c) The BLAST result of the DNA sequence obtained for champagne strain, LYCCII for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Reverse Primer (R2)

CTAAGGGTAGCTCATCACGATGGGCTAGATTTGGATTAGAATAAATATCTTCCTCGCCA
AATATTATTGCTTGATTGCGGAAGTTAGTAGCAATATCTGATCTTGGAGTCTTATTTAC
TCTTGAACCTACCATAGCGGGTTCACTTGTTGTTGAGCCTGGTGTGGTAATATTTGGCG
AACCAGCGTTCGTCGATCCCGCGGAAACTGTAGCGGTATTATTTTTTCTTCGTCTCTTA
GCCACTAGACCGGCTTTAACTTTACCCTTCGCTTTTGAGGTTGCAGATCTACTTCTTGT
AGATTTTGACGTCGGGACATTATTTGGTTTATCTGTTGAATGCATAGTTTTTAAACTTT
TAGGTTGGTTCACTGGAGATTTGTTATTAGTTGTATTATTTGTGGTATTATTGTTACAC
GGGTTTCCCACTGGATTTTCCGTAGTGGGTTGCATTGGATAATTATAGACTGGCCAGCC
AGTAACGTTTTGACTCCGATTCTGGGTTGGCCCTACATTTGTAAAATCGCCGGAAGGCG
GCGCTGTGGGAGCAACAGCAGTTGCACCAGTCGTGGGGTTTGCATTATTGTATGGTGGA
ATGGAAAAATTATTAACGATAGGGTTACCAACCATAGGAATTGGTATAGGGTTCATATT
AACATTGCGCATTTGAACCGCAGGTGCCATAGGATTTCCTAGCATCATAGCAGCCAAGT
GCATGGGGTCTATGTCCTCGTTACTATATTCCCCTCTTCGTTCTGCATCGTGTTGTAGC
CTTGCCGCATGAACAGCCAAGCTTCTATATATTTGTTCCTGCCTTTGTTCTTGAAGAAC
TAGTTGATAATATTGCTGAGCGAACTCTGAGCCACCTCTGGAAGAACTGGTATTAAAGA
TGTCCCAGAATATTTGCCACCATTCATACAAAAAGCCTTGAGGTGGATCTACTACCTTC
GAGAACGTATTCTGGTTACCATTGTTTTCTTTAGATTTGGGTCCCGCCAATTGGGTTTT
GGCCTTTGTCTCTTTCTAGGTGCGCATCTTGAACAAAGGCTGCTGCAGTGTTTTTCAAA
AACAACTTCCTAAAAAAGTCAAATATGTACTCATTGAGCGGTTTCTTGGCGTTCCTTT

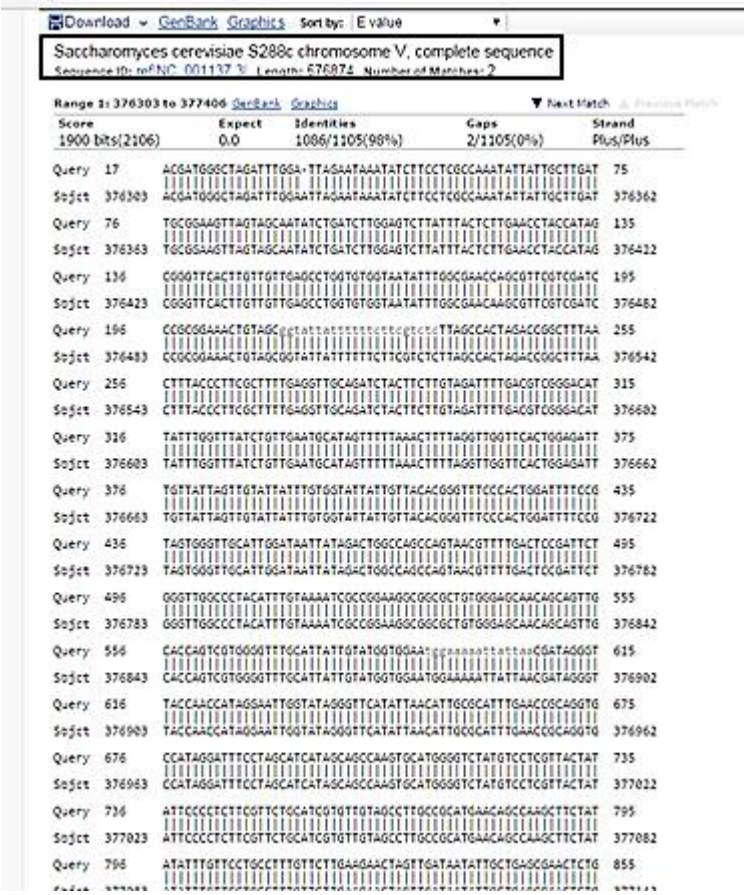


Figure 6.10 (d) The BLAST result of the DNA sequence obtained for champagne strain, LYCCII for FLO8 gene obtained after DNA sequencing.

The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

6.2.2.2.3 Wine strain

For FLO8- Forward Primer (F2)

CAAAAATGTAATTTCCTTAAGGAGGAACAAAGGCGAAAAAAAATGTCTCGTAAACTGGT
TGTTCATCATCCGGGTACCACGAATAGATCAAGCAATAGTTGTGTTCGCCTGGACGACG
ACCCCATGCACTTGGCTAAANTGATGCTAGGCTATGGCACCTGCGTTCAAATGCGCAAT
GTTAATATGAACCCTATACCAATTCCTATGGTTGGNNNNNTATCGTTAATCGTATTTCC
ATTCCACCATACAATAATGNNNNNCCCACGACTGGTGCTATCGATGTTGCTCCCACAGC
GCCGCCTTTTAATGATTTTAC

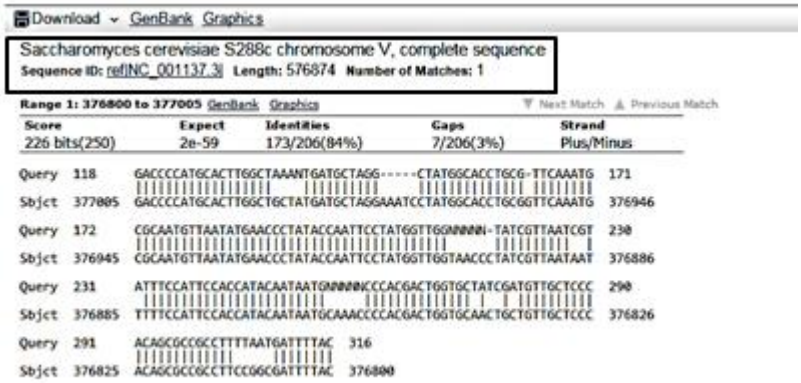


Figure 6.11 (a) The BLAST result of the DNA sequence obtained for wine strain, LYCCIII for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Reverse Primer (R2)

CCTCAAAAATTTTTTTAGCGAGTTATGCAAAATGTGGCGCATTGAGACCTTTGACAAGT
GCTCGGGGAAACGCCTGATGCCGCATCTCCATCCTTCTGGGTGCGGTCTTTTCCAACCC
GGCAATTCTTGGAGTGACCGAGGTCGCGACC

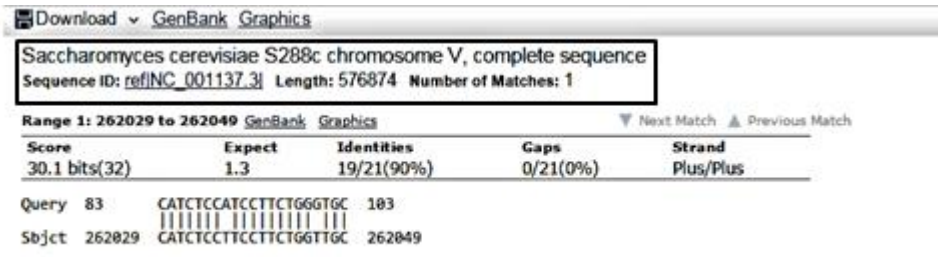


Figure 6.11 (b) The BLAST result of the DNA sequence obtained for wine strain, LYCCIII for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

6.2.2.2.4 Fuel Alcohol strain

For FLO8- Forward Primer (F1)

AATGGTAACCAGAATACGTTCTCGAAGGTAGTAGATACATTCTTATTCGANGGTCCTGA
ATGAACCATGGTATGTTTTGGTAGCAANNNNNCTGGGACATCTTTAAATANNNTATANA
TGGCAAGCAGAATAAACCAAT

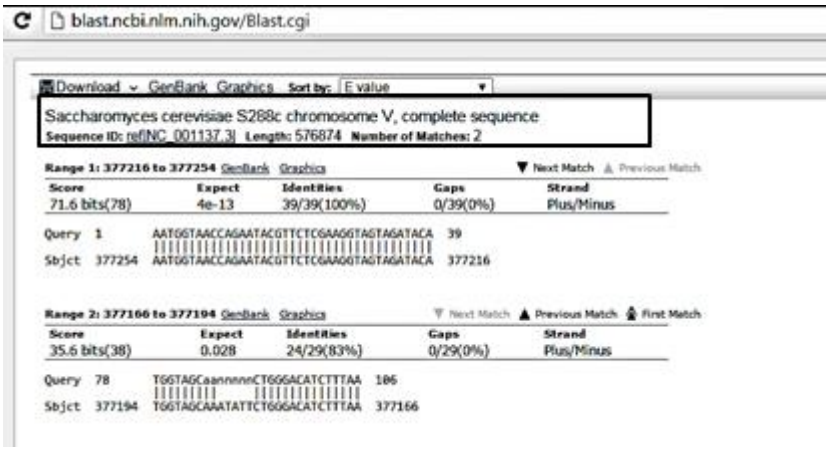


Figure 6.12 (a) The BLAST result of the DNA sequence obtained for fuel alcohol strain, LYCCIV for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

For FLO8- Reverse Primer (R2)

CTAAGAAGCGGAATTCATTAATGGAGGCCGACACAATGACAGTTTCATCAGTTGGCAAG
CCATTGGTTCCANNNHCAGTAGCCATTTCAGTGGATGTAGATGTAAATTACCGGTCATG
GTGCGGTTGTAGTATTCGGCGTGTCTGTGTTGGTGTGTTGACAACCTTATGCTTCTAAC
AATAGAACACCAAACGAGAATTCAAATGTTGCTGATGTCTGCATCTACAAATAGTGGCA
ATGATGGTCGCTCACTTACACAGCGCTCGTCTGGTAACAGC

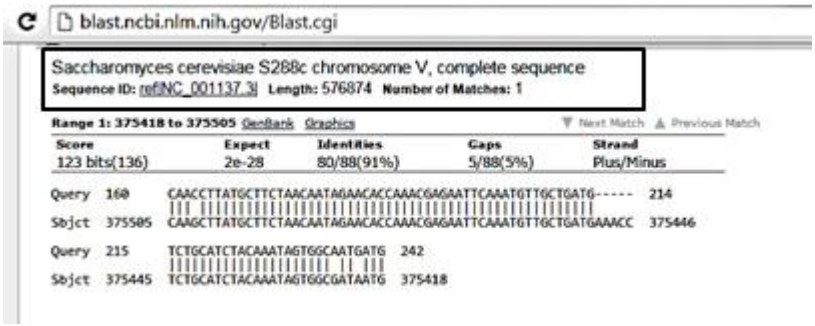


Figure 6.12 (b) The BLAST result of the DNA sequence obtained for fuel alcohol strain, LYCCIV for FLO8 gene obtained after DNA sequencing. The maximum identity to *S. cervisiae* chromosome V confirms the presence of FLO8 gene.

Thus, from (Fig 6.9-6.12) BLAST on sequencing results pointed chromosome V (with maximum hits), indicated that the gene so amplified and sequenced was for FLO8 gene.

6.2.3 Gene Expression levels for FLO1 and FLO8 genes

6.3.3.1 RNA isolation from the four industrial strains

In order to prepare cDNA for the amplification of FLO1 and FLO8 primers using QPCR, total RNA was isolated from the four industrial strains of *Saccharomyces cerevisiae* (see Fig 6.13)

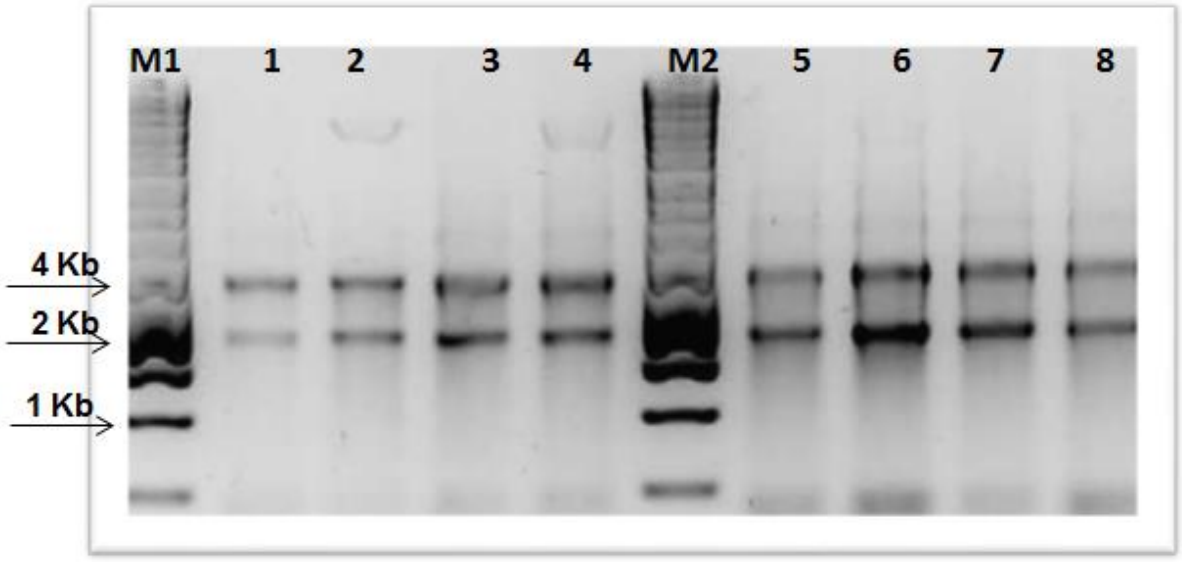


Figure 6.13 Two distinct bands (18S, below and 26S, above) observed after RNA isolation of the four industrial strains. 5µl of the sample was run in each lane on 1.5% agarose gel along with 1 Kb ladder. The lanes represent the isolates in duplicates: (L1,L5) Brewing strain, (L2,L6) Champagne strain, (L3,L7) Wine strain and (L4,L8) Fuel alcohol strain.

6.2.3.2 Primer designed for QPCR

Gene	Sequence	Annealing temperature	Transcript length
FLO1FQ1	GCGTTCAACTGTTGTGCTC	62.2	
FLO1RQ1	CCAAGAAACAGCGTTCGAG	63.6	177 bp
FLO1FQ4	TCTATCAGTAGGTGGTGCAAC	59.3	
FLO1RQ4	AGAAACAGCGTTCGAGTAAAC	60.0	190 bp
PDA1FQ1	TGAGACTTCGAAAGCCACC	63.1	
PDA1RQ1	AGTGAAACCGTGACATCTG	58.7	240 bp
PDA1FQ2	GACTTCGAAAGCCACCTTG	62.4	
PDA1RQ2	TGTGATGGCATTCTCGATACC	64.8	350 bp
FLO8F1	CCCGTGTAACAATAATACCAC	58.2	

FLO8R1	ACCCTTCGCTTTTGAGGTTG	65.2	180 bp
FLO8F2	TGGGAAGTTTCAACAAGCCG	67.1	
FLO8R2	CCAGACCGAGGTGTTGCTAT	64.0	200 bp

Table 6.3 List of primer pairs used for qPCR for FLO1, FLO8 and the housekeeping gene PDA1. The primer pairs were used on cDNA that was obtained after RNA extraction. The primers for qPCR were designed using Primer 3 online software. The primers were checked for dimer formation, GC% content etc.

6.2.3.3 Quantitative and realtime PCR (QPCR) using SYBR® Green Master Mix

Table 6.3 summarizes the primer pair sequences for the FLO1, FLO8 and PDA1 gene. Each gene was amplified in triplicates (N=3) for each of the three biological repeats of the four strains. PCR conditions* were optimized and 5µl of the amplified product was electrophoresed on a 1.5% agarose gel to verify amplicon size.

*PCR conditions remain same for all the three genes except the annealing temperature differed slightly.

Step	Temperature	Time
Initial Denaturation	94°C	30s
Denaturation	94°C	30s
Annealing*	**55°C***	60s
Extension	65°C	40s
Final Extension	65°C	10 min

35 Cycles

*Annealing temperature for PDA1, FLO1 and FLO8 gene was 55°C, 52°C and53°C respectively.

Table 6.4 PCR conditions used for the amplification of the particular region of interest from FLO1, FLO8 and PDA1 cDNA.

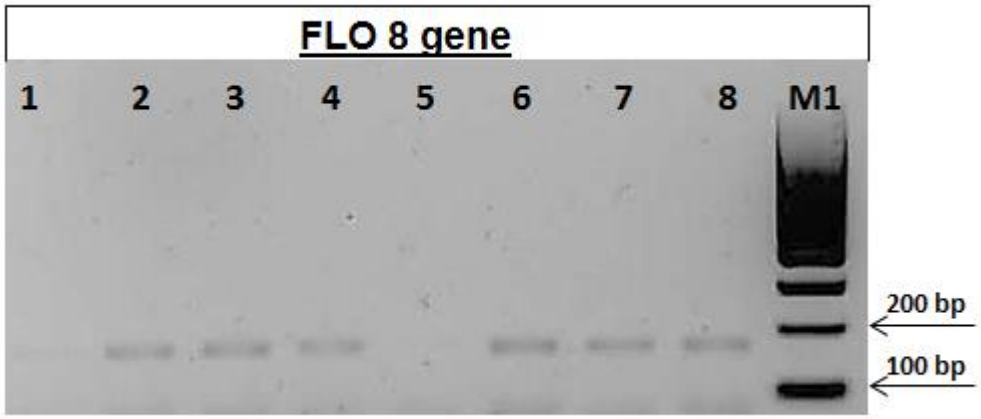


Figure 6.14 (a) FLO8 gene PCR product obtained after using the QPCR primers on the cDNA isolates from the four industrial strains. 10µl of the sample was run on 1.5% agarose gel along with 100 bp ladder. The sample was loaded in duplicates and the arrows represent the specific band at (180bp). Lane (L1,L5) represents fuel alcohol strain LYCCIV, lane (L2,L6) wine strain LYCCIII, lane (L3,L7) champagne strain LYCCII and lane (L4,L8) brewing strain LYCCI.

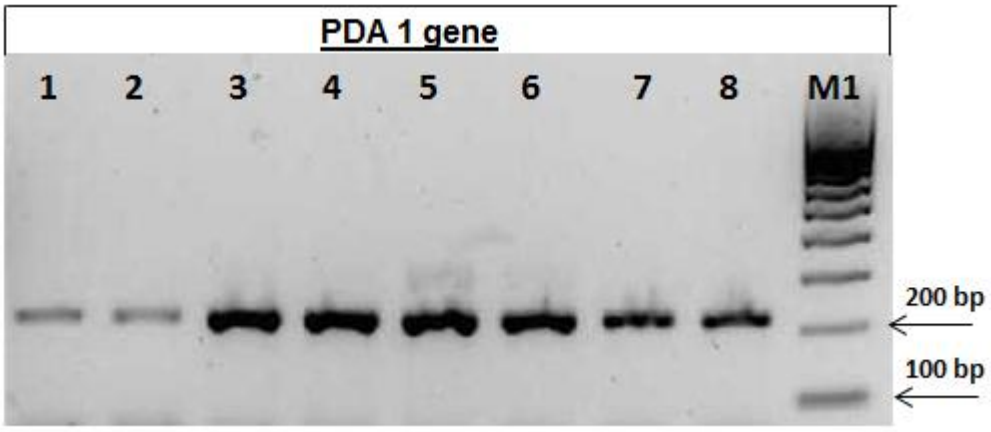


Figure 6.14 (b) PDA 1 gene PCR product obtained after using the QPCR primers on the cdNA isolates from the four industrial strains. 10µl of the sample was run on 1.5% agarose gel along with 100 bp ladder. The sample was loaded in duplicates and the arrows represent the specific band at (240bp). Lane (L1,L2) represents fuel alcohol strain LYCCIV, lane (L3,L4) wine strain LYCCIII, lane (L5,L6) champagne strain LYCCII and lane (L7,L8) brewing strain LYCCI.

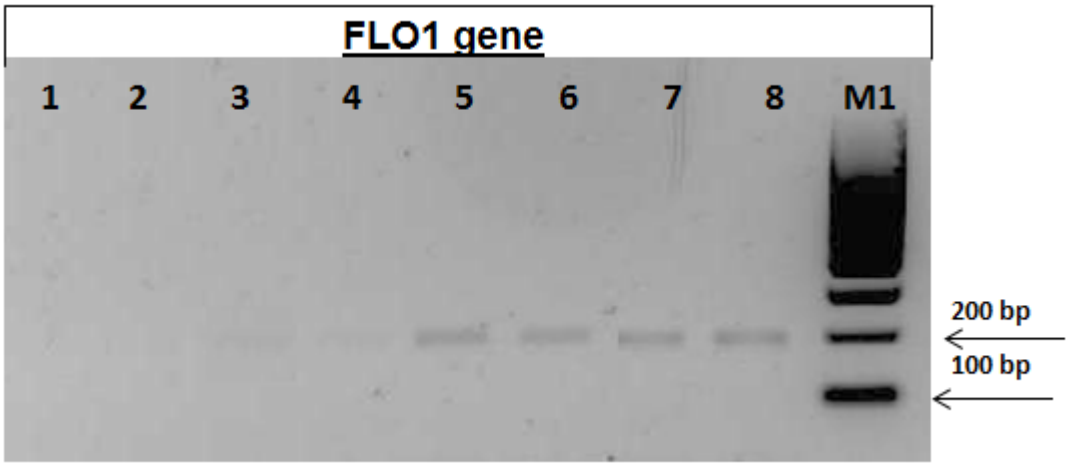


Figure 6.14 (c) FLO1 gene PCR product obtained after using the QPCR primers on the cdNA isolates from the four industrial strains. 10µl of the sample was run on 1.5% agarose gel along with 100 bp ladder. The sample was loaded in duplicates and the arrows represent the specific band at (190bp). Lane (L1,L2) represents fuel alcohol strain LYCCIV, lane (L3,L4) wine strain LYCCIII, lane (L5,L6) champagne strain LYCCII and lane (L7,L8) brewing strain LYCCI.

Gel densitometry analysis (Semi quantitative PCR) was used to compare the differential expression of each of the test gene with HKG between the four strains using Gel Doc^{TM} imager (Life Technologies, Carlsbad, CA, USA).The primers (Table 2.2) were used to amplify the cDNA from all the four strains and subjected to PCR. The PCR was performed in triplicates for each of the strain and each of the genes using the conditions (Table 2.3). The final PCR product as shown in (Fig 6.14(a),(b) and (c)) was resolved by electrophoresis on 1.5% agarose gels. The gels were photographed using Gel Doc 100 (Bio-Rad, Hercules, USA) and the images were analysed by using Band scan analyser 5.1 software.

6.2.3.4 Relative quantification of FLO1 and FLO8 genes

Quantification of expression levels of each gene, FLO1 and FLO8, were assessed by both the gel Densitometry (Biorad, Hercules, CA, USA) and real time PCR machine MX3000P system (Stratagene, Santa Clara, CA, USA). Figure 6.15 shows the relative gene expression levels for the two genes under study.

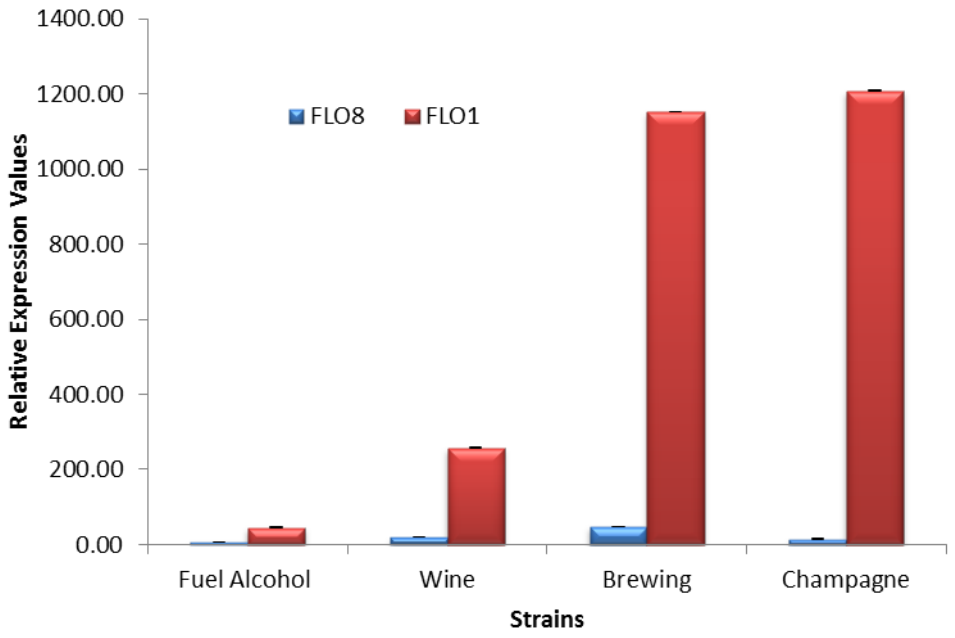


Figure 6.15 Relative QPCR expression of FLO1 and FLO8 genes in selected industrial strains normalised with PDA1 (housekeeping gene). Samples were taken during the late stationary phase. The relative expression value for each sample was defines as $2^{-\Delta Ct}$ where Ct (target)represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data were normalized to the relative expression value of the housekeeping gene PDA1 in each respective sample, thus normalized relative expression for the target gene as $2^{-\Delta Ct\ (target)}/2^{-\Delta Ct\ (PDA1)}$. Data are presented as Mean ± SE of three replicates (N=3).

To investigate the expression levels of truncated forms of FLO1 genes and FLO8 genes, the LYCCI, LYCCII, LYCCIII and LYCCIV cells were harvested in their stationary phase. RNA isolation was carried out, followed by cDNA synthesis as explained in section (2.4.3.2). It was assumed that nutrient starvation and oxidative stress would have induced the expression of flocculation genes namely FLO1 and its derivatives as well as FLO8 genes in these strains (Sampermans *et al.,* 2005). FLO1 is the most studied gene related to flocculation (Russell *et al.,* 1980). It encodes a large flocculation protein rich in Ser/Thr amino acids (Watari *et al.,* 1994). High number of tandem repeats present in the central domain of FLO1 gene makes it genetically quite unstable.

Therefore, we normally encounter the truncated forms of FLO1 in these industrial strains. As suggested by Liu *et al.*(2007) both the truncated forms of FLO1 gene produced the same transcript levels indicating significantly no contribution of the upstream sequence of FLONL. The product of FLO1 are thus important in order to study the cell adhesion properties as these proteins endowed with calcium dependent lectin activity help cell–cell adhesion events during yeast flocculation (Verstrepen *et al.* 2003). Bester *et al.* (2006) observed that FLO8 gene product Flo8p extensively plays an important role in regulating FLO1 gene apart from FLO11 and STA genes. The housekeeping gene selected was PDA1 gene which encodes for the protein product pyruvate dehydrogenase (acetyl-transferring) subunit E1 alpha. E1 alpha subunit of pyruvate dehydrogenase (PDH) complex catalyses the direct oxidative decarboxylation of pyruvate to acetyl-CoA. The protein product of PDA1 gen is active in phosphorylated form and is regulated by glucose.

From the data, it's clear that the levels of both the genes were up-regulated as compared to the housekeeping gene. Figure 6.15 shows the levels of FLO1 and FLO8 genes normalized to the housekeeping gene. The transcription levels of FLO1 were higher than FLO8 in all the industrial strains. These transcription levels are strongly correlated with the percentage flocculation ability in all strains. Brewing strain (LYCCI) and champagne strain (LYCCII) exhibited higher levels of FLO1 gene expression compared to wine strain (LYCCIII) and fuel alcohol strain (LYCCIV) strain. Moreover, it is clear from the data that even though direct correlation was observed between the FLO8 and FLO1 expression levels in all the strains (i.e higher expression levels for FLO1 were observed with higher expression levels of FLO8), it was not the case for the

champagne strain LYCCII. FLO1 expression was found to be independent of FLO8 expression level which is believed to be one of the transcriptional activator for FLO1 gene. This could be due to three main reasons, firstly as both FLO8 and MSS11 genes are critical for the process of flocculation in addition to presence of FLO1 (Fichtner *et al.,* 2007). Bester *et al.* (2006) also confirmed this by northern blot analysis and identified FLO1 as the main target gene for Flo8p and Mss11p with regards to flocculation, and that the levels of FLO1 transcription can in all cases be broadly correlated with the levels of flocculation. Thus chances are in case of champagne strain LYCCII, the high levels of FLO1 might be primarily due to Mss11p and not Flo8p. Meanwhile, it was also observed that increased copies of MSS11 can support transcription of flocculation genes (FLO1) even in lower expression levels of Flo8p protein. The expression levels of MSS11 were not studied here, thus it cannot be confirmed. Another reason for higher FLO1 expression levels in champagne strain LYCCII even with lower expression level of Flo8p compared to brewing strain LYCCI could be due to the regulation of FLO1 gene by those involved in chromatin remodelling, including the Swi-Snf co activator and the Tup1p-Ssn6p co-repressor complexes (Fleming and Pennings, 2001). Lastly another reason for higher expression of FLO1 levels in champagne strain LYCCII could be due to greater stability and half-life of FLO1 mRNA in the champagne strain LYCCII over the other three industrial strains.

6.3 Key Findings

Flocculation is an attractive property of *Saccharomyces cerevisiae*, which plays important roles in the fermentation industry and in environmental remediation. The process of flocculation is mediated by a family of cell surface flocculins.

Amongst the dominant flocculation genes are FLO1, FLO5, FLO8, FLO9, FLO10, FLO11, FLONS, FLONL and Lg-FLO1. Proteins encoded by these FLO genes share a common modular organization that consists of three domains: an amino-terminal lectin domain which protrudes from the cell surface and is responsible for the binding to carbohydrate, a central domain that is extremely rich in serine and threonine residues, and a carboxyl-terminal domain containing a glycosyl phosphatidylinositol anchoring sequence (Pittet and Conzelmann, 2007; Li *et al.,* 2013). DNA sequence responding to the central domain contains many tandem repeat regions, which are highly dynamic components of yeast genome. These repeats drive slippage and recombination reactions within and between FLO genes, leading to the generation of novel alleles or pseudogenes, which endows yeast cells with diversity and variety in flocculation ability.

The result of PCR amplification helped us to identify the major dominant genes in the four industrial strains. Truncated forms of FLO1 were observed in the strains, since the complete length of FLO1 was difficult to amplify due to its genetic instability. While the brewing strain showed the presence of both FLONL and FLONS, the champagne and wine strains showed the presence of only FLONS sequence which is expected to be 3.8Kbp compared to the 4.3Kbp of FLONL sequence. FLO1M (3.1Kb) was found to be present in addition to FLONS in fuel alcohol strain. This data strongly suggests that higher the tandem repeats present in FLO1, higher is the flocculation ability of the strain, explaining why brewing strain LYCCI is highly flocculent as compared to the fuel alcohol strain LYCCIV. Thus the presence of repeats in FLO adhesins, enables *Saccharomyces* to adapt its adhesion behaviour, finding an optimal balance

between the adherent cells and free cells that can escape from the mass and explore new surfaces (Verstrepen *et al.,* 2005 and Liu *et al.,* 2007). In addition to variations in the flocculation ability of the industrial strains due to the variation in tandem repeats in FLO1 region, I also observed flocculation phenotype conversion from Flo1 to NewFlo (Liu *et al.,* 2007) explaining that all the industrial strains belong to NewFlo type of flocculation phenotype.

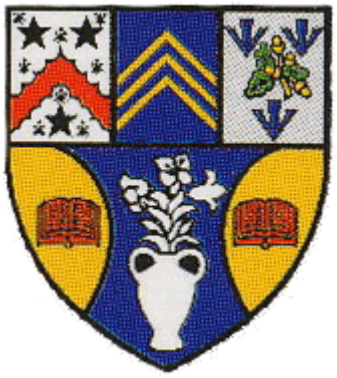
Another important point to be noted from the PCR amplification experiments was the absence of FLO10(B) band for champagne strain and the absence of FLO5(A) band in brewing, wine and fuel alcohol strain. Since the primers were designed keeping the laboratory strain S288c sequence of FLO10 and FLO5 in mind, wherein the primers chosen for PCR amplification of FLO10 (A), FLO10 (B), FLO5 (A) and FLO5 (B) had only differences in few sequences upstream to the coding sequence. It was concluded that the strains that did not give any band for FLO10(B) and FLO5(A) probably had mutations in upstream region, thus not providing the adequate sites for primer binding and hence amplification of the genes.

The analysis of *FLO*1 gene expression using quantitative PCR method demonstrated a correlation with flocculation data of the strains. The high level of flocculation of the strain LYCCI (brewing) correlated with the high level of *FLO*1 gene expression. A correlation between the *FLO*1 gene expression and the ability of other strains to flocculate in industrial substrates was also determined. This enables predicting the flocculation ability using a molecular method, which allows for a quick, economically desired and reasonable selection of industrial strains. From Fig (6.15) it is clear that the levels of both genes (FLO1 and FLO8) were up-regulated as compared to the housekeeping gene. Higher levels

of FLO1 gene in the brewing strain along with the presence of FLONL sequence (which is believed to contain more repeat sequences as compared to FLONS) makes the brewing strain highly flocculent compared to the other industrial yeasts under study. According to Liu *et al.* (2007), FLONL protein possesses the intact N- terminal of the FLO1 protein, but has lost most of the C-terminal sequence. However, not just deletion in repeat units in the middle caused conversion in flocculation type, but also affected the sugar binding affinity. Strains carrying FLONL exhibited weaker inhibition on flocculation as compared to the strains that carried only FLONS form. The internal repeat deletion activated a latent, high affinity conformational state of FLON proteins for both the C-1 hydroxyl group of glucose and the C-4 hydroxyl group of galactose. Thus the higher affinities of the FloNL protein implies that the C- terminal in the Flo1p can also influence the sugar binding strength, despite the indirect demonstration that the N terminal region of Flo1p contains the sugar recognition domain (Wang D *et al.,* 2008; Liu *et al.,* 2007 and Verstrepen *et al.,* 2005). Thus I concluded that the strains showed presence of different truncated forms of FLO1gene and the internal tandem repeats affected the flocculation ability of the strains, thus making brewing strain LYCCI having 4.2 Kb of FLONL as the most highly flocculent amongst the strains. The absence of bands for FLO10 (B) and FLO5 (A) when amplified using different forward primers and same reverse primer suggested polymorphism in the FLO genes, which has been previously been reported only in FLO1 gene (Kobayashi *et al.,* 1996; Javadekar *et al.,* 2000).

Lastly quantitative PCR analysis also helped us understand the reason behind different degree of flocculation exhibited by these industrial strains due to

differential gene expression levels. The two dominant genes were subjected to QPCR analysis. The results suggested higher expression of the truncated form of FLO1 gene (FLONL), with higher number of internal tandem repeats, makes the brewing yeast strain LYCCI highly flocculent. This was followed by the champagne strain (being the second most flocculent yeast under study) which shows high expression levels of the truncated form of the FLO1NS gene with lesser tandem repeat.



Chapter Seven

Discussion and Conclusion

7.1 Discussion

This research work investigated four industrial strains of *Saccharomyces cerevisiae* in terms of their physiological and genetic profiles which are believed to lead differences in their flocculation behaviour. The research also highlighted the dynamic nature of *Saccharomyces* cell wall and the need to acquire more information in terms of its nano mechanical properties, such as elasticity and surface roughness, which were found to contribute profoundly in terms of governing flocculation in industrial strains. Overall, this study aimed to provide a clearer understanding of the cell wall properties in industrial yeast strains so that successful interventions may lead to more successful fermentations processes of value to the food and beverage industry.

The current research work was carried out to further our knowledge on the comparative understanding of the flocculation behaviour of industrial strains employed in industry for different applications, mainly brewing, wine making and bio-ethanol production, specifically gaining an overall understanding on their cell surface properties (in terms of CSH and CSC), nano mechanical properties, some biochemical aspects of the cell wall (for e.g. quantifying the lectin-like receptors and mannan sites per cell) and lastly understanding the gene expression levels by quantifying the expression of mRNA. Subsequently, additional knowledge gained in these domains were further elaborated and transformed into the generation for a prospective predictive strategy to handle some of the most concerning problems faced by the food and beverage industry nowadays with flocculation at its nodal point. This research work produced a comparative study of the four industrial strains of *Saccharomyces cerevisiae* in terms of their physiological and genetic profiles which are believed to lead

differences in terms of their flocculation behaviour. This research also highlighted the dynamic nature of *Saccharomyces* cell wall and the need to acquire more information in terms of its cell wall nano mechanical properties mainly elasticity and surface roughness levels which were found to contribute profoundly in terms of controlling the flocculation behaviour in these industrial strains. Altogether this study aims to have precise and clearer understanding of the cell wall properties of these industrial strains so that a successful intervention could be conceptualized and then actualized to achieve better quality end products which are cost effective to food and beverage industry.

7.1.1 Cell surface properties, like CSH and CSC contribute towards high flocculating nature of brewing strains (LYCCI) compared to *Saccharomyces* strains employed for wine making (LYCCCIII), champagne (LYCCII) and bio-ethanol (LYCCIV).

Since cellular adhesion properties of yeast depend on the outer layer of the cell wall (Coltri *et al.,* 2006; Singh *et al.,* 2011; Javadekar *et al.,* 2000). This section of study reported on investigation of physiological parameters as well as the cell surface properties that helped us to evaluate the flocculation ability of the strains during their growth (Fig 3.3). Brewing yeast cells LYCCI (42.5%) were found to be highly flocculent followed by Champagne strain LYCCII (14.8%), wine strain LYCCIII (13.8%) and finally fuel alcohol strain LYCCIV (11.6%). The reason being that brewing strains are usually exposed to several negative conditions such as cold-shock, nutrient starvation, osmotic stress and ethanol toxicity (Gibson *et al.,* 2007). Consequently, flocculation can act as a communitarian mechanism of survival: the external cells from the floc structure can protect the inside cells against a harmful environment by physical shielding.

The possibility that flocculation can be a response to stress seems to be strain dependent. Very little flocculation was observed during the lag and logarithmic phases of growth (1-15%), while during the early and late stationary phases, different strains exhibited variable flocculation patterns. Thus our study supports the finding of Speers *et al.* (2010) and Soares and Vroman (2003) indicating the importance of flocculation more towards the brewing strain as the brewing strain disperses, replicates, ferments as single cells and then flocculates rapidly following the depletion of nutrients (sugars in wort). This gives rise to early or premature flocculation which leaves unattenuated sweet beer, whilst late or poor flocculation requires yeast cells to be removed by filtration or centrifugation. Thus the flocculation characteristics of yeast strains are of major significance in brewing making it highly necessary to have a fit for purpose yeast for modern brewing industry with strong flocculation characteristics towards the end of fermentation (Jin and Speers, 1998; Soares, 2010; Stewart and Russell, 1981)

As suggested by Panteloglou *et al.* (2012), physiological state of cells is one the four main factors in addition to genetic background (presence of flocculation (FLO) genes and their regulatory elements, wort nutritional status (free amino acids and divalent cations), environmental conditions (temperature, pH, presence of alcohol, osmotic pressure etc.) which were found to affect the extent of flocculation in a particular strain. Thus on similar lines, it was observed from our studies that Cell surface hydrophobicity (assayed using HMA and MATS) and surface charge (assayed by Alcian Blue dye retention) played important roles in dictating flocculation behaviour in different yeast strains, as did the yeast growth phase. Hydrophobicity index (HI) and % hydrophobicity of

the four strains followed, respectively, the same order, viz Beer (66.6, 21.5) > Champagne (33, 10.5) > fuel alcohol (22.4, 7.4) > wine (20.5, 2.7).

Cell surface hydrophobicity (CSH) is one of the factors that governs the degree of flocculation in microbial adhesion (Nayyar *et al.,* 2014; Potter *et al.,* 2015; Jin *et al.,* 2001). Cell surface hydrophobicity was determined by two techniques, HMA and MATS. While HMA employs latex microspheres and informs only about the overall hydrophobic nature of the cell wall in a population. About 100 cells for each of the four strains were counted and percentage hydrophobicity was calculated for those cells having ≥ 3 beads attached (Fig 3.6). Additional factors involved, including electron donor/acceptor properties and zeta potential were determined using MATS test (White and Walker 2011). Techniques like microbial adhesion to solvent techniques (MATS), based on cell surface affinities for a monopolar and non-polar solvent, may be used to determine the electron donor or acceptor properties of yeast cells. When the interaction between flocculent yeast cells and hexadecane and with microsphere latex beads were studied by light microscopy, the yeast cells appeared to form a monolayer of cells around each of the hexadecane droplet and in case of HMA test, each yeast cell surrounded by more than 3 latex beads. These results demonstrate that flocculent yeast cells are highly hydrophobic.

Yeast cell surface charge was found to be negative using Alcian blue retention test, but whether the degree of negativity of the cell surface affected the flocculation ability in the strains is still not very clear. From our data it was observed that the brewing strain LYCCI was highly negatively charged compared to the champagne, wine and fuel alcohol strains. The presence of

carboxylic and phosphodiester groups are responsible for the negative character of yeast (Jin and Speers, 1998).

Thus to sum up on the cell surface properties, it could be concluded that as suggested by Jin *et al.,* (2010) and co-workers, cell surface hydrophobicity (CSH) has been reported as one of the contributors of yeast flocculation and I further conclude that a positive correlation between CSH and flocculation explains the reasons for change in flocculation ability of the strains. Meanwhile, Cell surface charge (CSC) did not change as significantly as CSH. Apparently there were significance in the cell charge levels for the strains but not a direct correlation between the cell surface charge and flocculation ability was seen during growth phases for the strains.

7.1.2 Sugars in the media show variability in flocculation pattern for the four industrial strains. Number of lectin-like receptors influences the flocculation behaviour more than the mannans and glucans present on the Saccharomyces cell wall.

In this section of research work, I tried to culture *Saccharomyces* strains in five different sugars namely, glucose, mannose, maltose, fructose and galactose. As observed in Chapter three, I have already seen effect on flocculation when the cells of each of four strains were cultured in glucose, thus in the current study, effects of other sugars was explained in Fig 4.2, 4.4 and 4.4 in reference to when the cells were cultured in glucose (standard). I hypothesized that a direct determination of the cellular mannose residues or flocculin contents could provide more dynamic information regarding flocculation behaviour of industrial yeast strains. Thus I investigated if the flocculation behaviour in these four

industrial strains was linked to lectin receptor density and/or distribution patterns of glucans and mannans on the cell wall. I made use of the fluorescent lectins Concanavalin A- Alexa Fluor®-350 (Con A) and *Pisum-sativum*-agglutinate-Fluorescein isothiocyanate (PSA-FITC) to analyse the flocculation ability of yeast cells.

It would not be justified to make exact classification of the four strains in Flo-1 type or NewFlo type phenotype, just on the basis of their flocculation performance on test sugars (glucose, mannose, maltose, fructose and galactose), as in here , I examined the effect of sugars on the three parameters (for e.g. mannan content, glucan content and receptor density/ cell) which are believed to contribute to the flocculation profiles of the strains under study, however, once the cells of particular strains were harvested from the growth media containing different sugars, the flocculation was determined in washed cells, in standard conditions making it possible to correlate flocculation with the presence of lectin like receptors. Genetic factors also play an important part in defining the overall profile of the strain and thus the classification of the strains was made, only once some knowledge about dominant FLO genes was attained about the strain from Chapter six of this thesis.

The strains displayed a more or less NewFlo type phenotype as they were seen to be equally sensitive to all the sugars and not just mannose, unlike the characteristic property observed in Flo-1 type strains. But still the clear distinction could be made only after some genetic tests because as much as flocculation is a cell surface property, but however, the phenomenon of flocculation is also under the control of certain proteins known as flocculins/ zymolectins that are poduct of FLO genes (Holle *et al.,* 2012).

This effect on the flocculation ability of the strains was widely because the sugars had a significant effect on the receptor number/ cell. The presence of active lectins on the yeast cell surface could be explained due to fluorescence observed when the cells were treated with Avidin-FITC probe in the presence of Ca^{2+} in the solution. This study also indicated that mannan and glucan levels remained relatively constant on cell surfaces of all the strains studied (Table 4.1). The overall distribution of mannan remained same for all the strains when grown in different sugars. Interestingly, glucose and galactose had similar effects on cell wall glucan distribution as compared to maltose, mannose and fructose.

As summed up by Singh *et al.* (2012), lectins play an important role in flocculation as their presence on the flocculated cells helps to promote the aggregation of yeast cells by binding to the non-reducing termini of α- (1-3)-linked mannan side ranches (Stratford and Assinder, 1991; Stratford and Carter, 1993; Taylor and Orton, 1978). Since much of flocculation in case of Saccharomyces cerevisiae was observed during the stationary phase (Stratford and Carter, 1993), thus I selected cells of the four industrial strains in their stationary phase to measure the active lectins as well as to quantify the mannan and glucan content. Brewing strain LYCCI was found to harbour maximum number of active receptors/ cell on its cell wall. This was much clearer from the correlation analysis performed (Fig 4.7) which explained that glucan content vs. flocculation ability gave negative correlation for all the sugars except for maltose, which gave a positive correlation, whereas, the same held true for mannan content vs. flocculation where a negative correlation was observed for sugars like maltose, fructose and galactose. It was only the receptor density

when plotted against flocculation ability, gave a positive correlation for all the sugars, strongly suggesting the role of receptors on the cell surface playing a dominant role in governing the flocculation of the strains.

However, it is equally important to point out the strong influence of different carbon source on the cell wall composition of yeast. The data so obtained gave a semi quantitative measure of glucan and mannan by employing Concanavalin A- Alexa Fluor®-350 (Con A) and *Pisum-sativum*-agglutinate-Fluorescein isothiocyanate (PSA-FITC) respectively on the yeast cells. The results indicated a significant difference in the amounts of mannan and glucan content in the cell wall of the strains when ther were cultured on culture medium containing different sugars. This was found to be in agreement with some of the work by Aguilar-Uscanga and François, (2013) who measured the polysaccahrise composition of *Saccahromyces cerevisiae* cell wall under various growth conditions. Thus it would be better to conclude that the kind of carbon source employed for the culture of these industrial strains influences the amounts of lectin like receptors and cell wall composition of glucans and mannans for each of the industrial strains, however, it's only the active lectin like receptors that play an important role over the cell wall composition of mannan and glucans in governing the different patterns for flocculation in these industrial strains of *Saccharomyces cerevisiae.*

7.1.3 Cell surface topography parameters play an important role in governing the extent of flocculation. Higher the cell surface elasticity and surface roughness, higher are the possibilities of the cell exhibiting flocculence in media.

Research in this part of thesis was conducted by using Atomic force microscope (AFM) in order to investigate the nanomechanical properties of the yeast cell surfaces. The tool is extremely beneficial as it's been used to investigate the native cell surfaces with high sensitivity and nanometer lateral resolution. The technique helped us to understand that what forces do exactly come into play at the cell surface when the cells come close to each other during the process of flocculation visualize that what exactly is occurring at the cell surface and how much stresses impact on the biological properties of the cell wall. Atomic force microscopy (AFM) makes it possible to observe, manipulate and explore the cell surface at a molecular resolution, and therefore has produced a wealth of new opportunities in cell biology, including understanding the nanoscale organization and dynamics of cell membranes and cell walls, measuring cell mechanics and cell adhesion, unraveling the molecular elasticity of cellular proteins and the mechanisms by which they assemble into nanodomains in the membrane (Müller and Dufrêne, 2011; Müller *et al.*, 2009). In this Commentary, I explain the basic principles of AFM, discuss the strategies employed for imaging live cells, provide the discreet role of these nanomechanical properties in governing the extent of flocculation and provide a critical evaluation of the potential and limitations of the technique.

Soon after its invention, AFM became a valuable tool for imaging cells (Butt *et al.,* 1990; Radmacher *et al.,* 1992). However, AFM imaging of single cells requires their firm attachment to a surface, which is not always a simple task. A straightforward approach is to exploit the ability of animal cells to spread and adhere to solid supports (Radmacher *et al.,* 1992). In my study, I fixed the yeast cells directly on the glass slide which were made hydrophilic by immersing in

conc. Sulphuric acid (Canetta *et al.*, 2009). Although a lot of research on nanomachanical properties of the yeast cell surface has been done (Binnig *et al.,* 1986; Müller and Dufrêne, 2011; Müller *et al.,* 2009; Dufrêne, 2008,Dufrêne, 2008b; Engel and Gaub, 2008).

One of the attempts of this study was to see if the cell surface elasticity varied at the three different areas on the cell surface i.e. cell centre (C), edges (E) and at the bud scar (B). AFM imaging and AFM spectroscopy studies revealed that there was not much significant change in elasticity over the three specific areas studied for each of the strain, but overall, the strains differed in their elasticity patterns suggesting that more elastic a particular strain is higher are the chances it would flocculate. As much as a lot of literature points out the stiff nature of the bud scare, it was seen after the AFM analysis that the young's modulus was not very significantly different at the bud scar compared to the edge and the centre of the cell. Another important deduction was made on the cell surface roughness patterns for the strain (Fig 5.3). Higher the surface roughness of the cell surface, larger the frictional force between the two surfaces of yeasts coming together. Thus, more sites for anchorage to hold on each other's surface and this could explain the formation of stable flocs in the fermenter and the increase in the flocculation ability for the strain.

An important note here is that in the current work I tried to measure the forces between the yeast cell surface and the tip of cantilever and not between the yeast cell surface and a probe/cell attached to the tip of cantilever. In the present study, our work is restricted on the combined adhesive force that comes into play when another surface comes in contact. Here, the study regarding the adhesive forces between the cell surface and cantilever (i.e. adhesion force and

adhesion energy) by no means suggests the forces in reality that plays an important role between cell-cell adhesions. The forces mentioned (adhesion force and adhesion energy) are clearly representing the forces involved in cell-surface adhesion. Linking the AFM probe with the lectins specific to mannans, could give the quantitative measurement of the adhesive forces between the mannans and the lectins and also the number of lectin binding sites on the yeast cell wall (Dufrêne, 2015).

From the data, I observed that cytoplasm region showed almost consistent adhesive force for the four strains while the adhesive force for the brewing strain LYCCI was found to be significantly different at the bud scar (B) and edge (E) region to the other three strains (Fig 5.1), suggesting another reason for brewing strain, LYCCI to be highly flocculent amongst the industrial strains studied. Thus, I could conclude that there was a positive correlation between %flocculation ability and elasticity of the cell wall. This is suggestive of flexibility of cell wall as one of the important contributors towards aiding in higher flocculation ability of the yeast strain during fermentation.

The dramatic change in roughness may have some consequence on the adherence capacity of yeast to material surface (Gollardo-Moreno *et al.* 2004, Mercier-Bonin *et al.,* 2004). Also, it was concluded from the data that was obtained for the Adhesion force and adhesion energy suggesting difference in the stretching of the macromolecules between the four yeast strains. Based on these data, it could be further suggested that the much longer rupture distances on *S. cerevisiae* may reflect the stretching of both mannans and the polypeptidechains of the mannoproteins. The different physical properties of the mannoproteins between the four strains may explain why the surface of LYCCI

was more hydrophobic than that of other *S cerevisae* strains. Our results indicate an important role of cell wall elasticity and cell surface roughness in governing the extent of flocculation of specific strains during the fermentation. As a corollary, cell adhesion force and adhesion energy, which is mainly dependent on cell wall composition, is probably not an important factor in cell flocculation.

Undoubtedly, the main limitation of AFM imaging in yeast biology is their rather slow temporal resolution. Hopefully, current efforts in developing high-speed AFM techniques should soon provide access to millisecond resolutions using live cells.

7.1.4 Elevated relative expression levels of FLO1 and higher number of intergenic tandem repeats made the brewing strain, LYCCI, highly flocculent amongst the four industrial Saccharomyces strains, while FLO8 mediated activation of FLO genes is strain specific.

As much as natural flocculation provides brewers with a cost effective way to separate yeast cells from green beer at the end of fermentation (Powel and Diacetis, 2007). Correct floc formation at the end of fermentation is a vital phenomenon for brewers (Verstrepen *et al.,* 2003) as late or weak flocculation makes it necessary for the industries to employ expensive filters and centrifugation systems for separating the yeast from the end products. As learnt from the literature the process of flocculation is mediated by a family of cell surface flocculins. Amongst the dominant flocculation genes are FLO1, FLO5, FLO8, FLO9, FLO10, FLO11, FLONS, FLONL and Lg-FLO1 (Govender *et al.,* 2008, Liu *et al.,* 2007 and Verstrepen *et al.,* 2004).

In this part of current research, novel and important findings on the genetic profiles of the strains, mainly in terms of presence of dominant FLO genes and understanding the relative expression levels of two of dominant genes (FLO1 and FLO8) in these industrial strains was made. Such a genetic profile in addition to the physiology and cell surface properties (as studied in Chapter 3-5) contributed to the variation in the flocculation ability in industrial strains of *Saccharomyces.*

The genetic variability of flocculation genes may have an important consequence for studies and applications targeting these genes in industrial yeasts strains with unknown genomes. I therefore designed primers to detect specific flocculation genes namely FLO1, FLO5, FLO8, FLO9 and FLO10. All the yeast strains showed the presence of the 5 dominant flocculation genes. The result of PCR amplification revealed:

- That one of the main reasons for higher flocculation rates in brewing yeast strains due to presence of both truncated forms of FLO1 namely, FLONL and FLONS, the champagne and wine strains showed the presence of only FLONS sequence which is expected to be 3.8Kbp compared to the 4.3Kbp of FLONL sequence. FLO1M (3.1Kbp) was found to be present in addition to FLONS in fuel alcohol strain. This data strongly suggests that higher the tandem repeats present in FLO1, higher is the flocculation ability of the strain
- In addition to variations in the flocculation ability of the industrial strains due to the variation in tandem repeats in FLO1 region, I also observed flocculation phenotype conversion from Flo1 to NewFlo (Liu *et al.,* 2007)

explaining that all the industrial strains belong to NewFlo type of flocculation phenotype.

- Another important point to be noted from the PCR amplification experiments was absence of FLO10 (B) band for champagne strain and absence of FLO5(A) band in brewing, wine and fuel alcohol strain. Since the primers were designed targeting the laboratory strain S288c, thus suggesting probable presence of polymorphism within 400 bp and 200 bp to the coding sequence of FLO10 and FLO5 gene respectively within the strains.

Due to time constraints, I was only able to obtain the sequence results for FLO1 and FLO8 gene for the four industrial strains. I wished to move ahead with the quantitative PCR (QPCR) analysis for FLO1 and FLO8 gene as discussed FLO1 gene is dominant gene responsible for flocculation in Saccharomyces and FLO8 being one of the transcriptional activator, made it necessary for selecting these two genes for QPCR analysis. With the help of bioinformatics software, it was already known that the location of FLO1 and FLO8 gene was Chromosome I and Chromosome V respectively. The sequences so obtained from the sequencing services were subjected to BLAST and the results identified FLO1 and FLO8 gene. I was not able to sequence the whole gene, and only first few 250-500 bp were only sequenced as the PCR product was directly used as a template and moreover FLO1 gene contained a lot of internal tandem repeats.

The analysis of FLO1 and FLO8 gene expression using quantitative PCR method demonstrated a correlation with flocculation data of the strains. The high level of flocculation of the strain LYCCI (brewing) correlated with the high level of *FLO*1 gene expression. A correlation between the *FLO*1 gene

expression and the ability of other strains to flocculate in industrial substrates was also determined (Fig 7.1). Higher levels of FLO1 gene in the brewing strain along with the presence of FLONL sequence (which is believed to contain more repeat sequences as compared to FLONS) makes the brewing strain highly flocculent compared to the other industrial yeasts under study. According to Liu *et al.* (2007), FLONL protein possesses the intact N- terminal of the FLO1 protein, but has lost most of the C-terminal sequence. However, not just deletion in repeat units in the middle caused conversion in flocculation type, but also affected the sugar binding affinity. Strains carrying FLONL exhibited weaker inhibition on flocculation as compared to the strains that carried only FLONS form.

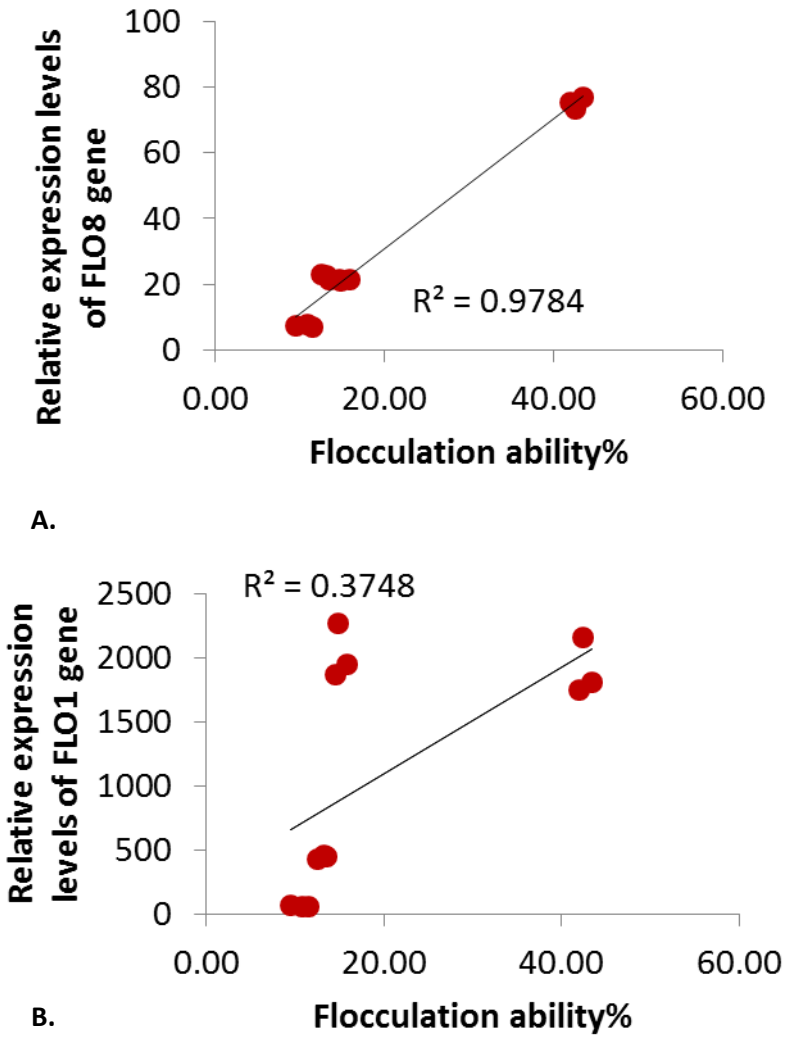


Fig 7.1 Correlation pattern observed between the relative expression of FLO1 and FLO8 gene vs. %flocculation ability.

In terms of FLO8 gene relative expression, direct correlation was observed between the FLO8 and FLO1 expression levels in all the strains (i.e higher expression levels for FLO1 were observed with higher expression levels of FLO8), except for champagne strain LYCCII. In champagne strain, FLO1 expression was found to be independent of FLO8 expression level which is believed to be one of the transcriptional activator for FLO1 gene. This was concluded to be probably due to the following reasons:

- As studies by Fichtner *et al.* (2007) and Bester *et al.* (2006) suggest a the role of both FLO8 and MSS11 genes are critical for the process of

flocculation in addition to presence of FLO1. Thus chances are in case of champagne strain LYCCII, the high levels of FLO1 might be due to primarily due to Mss11p and not Flo8p. The expression levels of MSS11 were not studied in this study, thus it cannot be confirmed and completely stated as the sole reason for higher FLO1 expression even with low expression of FLO8.

- Another reason for higher FLO1 expression levels in champagne strain LYCCII even with lower expression level of Flo8p compared to brewing strain LYCCI could be due to regulation of FLO1 gene by those involved in chromatin remodelling, including the Swi-Snf co activator and the Tup1p-Ssn6p co-repressor complexes (Fleming and Pennings, 2001).
- Lastly another reason for higher expression of FLO1 levels in champagne strain LYCCII could be due to greater stability and half-life of FLO1 mRNA for champagne strain LYCCII over other three industrial strains.

7.2 General conclusions

The current study of yeast flocculation in industrial yeast strains covered aspects ranging from nano mechanical to biochemical and molecular properties of the yeast cell wall and how it plays a major role in controlling the phenomenon of yeast flocculation. Even though just a structure on the yeast cell which envelops the yeast cell and protects the yeast cell from environmental stress and osmotic pressures, it resides within the proteins which promotes the adhesion between two yeast cells. A lot of laboratory strains have been studied extensively in past, but this is one of the comparative studies covering in depth knowledge on various analysis of the yeast cell wall to produce an overall

picture of the factors that contribute towards the flocculating nature of industrial yeast strains.

One of the major challenges for the yeast biologist and industries today is to identify the prime reasons for flocculation and devising strategies to have a fit for purpose yeast for the modern brewing industry which exhibits strong flocculation characteristics towards the end of fermentation (Verstrepen *et al.,* 2003). Thus the flocculation characteristics of yeast strains are of major significance in brewing as well as food and beverage industry as the number of suspended yeast cells in wort during both primary and secondary fermentation affects the speed of fermentation, flavour formation, maturation and filtration. Thus I conclude from this study:

Factors which influence flocculation of commercial yeast strains

During a particular industrial fermentation process, flocculation can be affected by multiple parameters. For a given strain, flocculation depends on a combination of four main factors: (1) genetic background (presence of flocculation (FLO) genes and their regulatory elements), (2) wort nutritional status (in particular the content and profiles of sugars), (3) environmental conditions (shearing and adhesive forces) and (4) physiological state of cells (cell surface hydrophobicity, cell surface charge.).

For industrial yeast strains, the establishment of a universal and reliable test for flocculation would be very worthwhile. Towards such goals, the sharing of information and samples between industry research labs is needed to further our understanding of industrial yeast flocculation mechanisms and to prevent premature flocculation. Finally, developments in knowledge of the genetic and

epigenetic regulation of flocculation in commercially relevant lager brewing strains should help to explain apparent inconsistencies observed in the incidence of this phenomenon.

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Appendix - I

Journal Articles and Conference Abstracts Publications from this thesis

Published Paper

Nayyar, A., Walker, G. M., Canetta, E., Wardrop, F., & Adya, A. K. (2014). Cell surface properties and flocculation behaviour of industrial strains of Saccharomyces cerevisiae. *International Journal of Applied Microbiology and Biotechnology Research 2 (6)*

Nayyar, A., Walker, G. M., Wardrop, F., & Adya, A. K. (2014). Flocculation in industrial strains of *Saccahromyces cerevisae*: role of cell wall polysaccharides and lectin-like receptors. ***Journal of Applied Microbiology***

Submitted Paper

Nayyar, A., Walker, G. M., Canetta, E., Wardrop, F., & Adya, A. K. (2014).Cell surface elastic properties influence flocculation behaviour of industrial Saccharomyces cerevisae strains. ***Yeast***

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Cell surface properties and flocculation behaviour of industrial strains of *Saccharomyces cerevisiae*

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ABSTRACT

Cellular adhesion properties of yeasts depend on the characteristics of the outer layer of the cell wall. In this study, the flocculation behaviour of four industrial strains of *Saccharomyces cerevisiae* used for production of beer, champagne, wine and fuel alcohol was evaluated; their flocculation abilities being, 42.5, 14.8, 13.8 and 11.6%, respectively. The brewing yeast strain was found to be the most flocculent. Very little flocculation was observed during the lag and logarithmic phases of growth (1-15%), while during the early and late stationary phases, different strains exhibited variable flocculation patterns. Cell surface hydrophobicity [assayed using hydrophobicity microsphere assay (HMA) and microbial adhesion to solvent (MATS)] and surface charge (assayed by Alcian Blue dye retention) played important roles in dictating flocculation behaviour in different yeast strains, as did the yeast growth phase. Percentage hydrophobicity index (HI) and % hydrophobicity of the four strains followed, respectively the same order, viz Beer (66.6, 21.5) > Champagne (33, 10.5) > fuel alcohol (22.4, 7.4) > wine (20.5, 2.7). Our findings provide new insight into yeast cell surface properties and how these relate to behavioural characteristics of yeasts employed in industrial fermentations.

INTRODUCTION

The adhesion properties of microorganisms, which involve adhering of the microbe to other cells, tissues or solid substrates, have been the focus of wide ranging scientific and biotechnological interest (Verran and Whitehead, 2005; Verstrepen and Klis, 2006; Zhao and Bai, 2009; Kjeldsen 2000). Adhesion properties are known to play important roles in governing many essential aspects of the life cycles of microorganisms including sexual reproduction (Chen et al., 2007), cellular aggregation (e.g. flocculation), biofilm formation and invasion, and/or pathogenic behaviour (Reynolds and Fink 2001; Palmer et al., 2007; Ramage et al., 2009, Maury J et al., 2005).

Yeast cells undergo Ca^{2+}-dependent, reversible, asexual aggregation known as flocculation. In *Saccharomyces cerevisiae*, floc formation is helpful in certain industrial fermentations such as brewing, as this aids in sedimentation of yeast cells at the bottom of cylindro-conical fermenter vessels at the end of the fermentation process (Bony et al., 1997; Stratford, 1989). In some cases, co-flocculation has been reported to occur by adhesion of flocculent and non-flocculent strains of *S. cerevisiae* and lactic acid bacteria (Miki et al., 1982a).

One important factor that governs the degree of

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VIII Division of Biotechnology and Forensic Sciences, School of Contemporary Sciences, University of Abertay Dundee, Bell Street, Dundee DD1 1HG, Scotland, UK. Communicated by Ashok K Adya <A.Adya@abertay.ac.uk>.

The following will be presented at the 30th International Specialised Symposium on Yeast in Stará Lesná, June 18-22, 2013.

1 Nayyar A, Walker G, Canetta E, Wardrop F, Adya A. 2013. Understanding cell-surface structure-function relationships in industrial yeasts.

Cell surface adhesion properties of yeasts are crucial for many biological processes, such as sexual reproduction, tissue or substrate invasion, biofilm formation and flocculation. Understanding and controlling this latter phenomenon is of commercial interest to yeast biotechnology industries. For example, flocculation in brewing yeasts can determine the degree of attenuation of the wort. Early or premature flocculation is a common cause of 'hung' or 'stuck' fermentations giving rise to exceedingly sweet beer, whereas a lack or delay in flocculation can cause beer clarification problems. In this study we used a modified flocculation assay [1] to determine flocculation capabilities of four industrial yeast strains employed for winemaking, fuel alcohol, brewing and champagne production. We also investigated cell surface hydrophobicity characteristics in these yeast strains and were able to correlate flocculation behaviour with hydrophobicity as determined using the Hydrophobicity Microsphere Assay (HMA Assay) and the MATHS test (Microbial Adhesion to Hydrocarbons). It was found that the highly flocculent beer producing yeast strain with 42% flocculation ability exhibited concomitantly high cell-surface hydrophobicity index of 66%. Adhesion (adhesion force and energy) and elastic (Young's modulus) properties, and ultra-structure of cell walls (surface morphology and roughness) of the same yeast strains were then investigated at the nanoscale using Atomic Force Microscopy (AFM). This work is providing new information regarding surface morphology, nanomechanical properties of yeast cell walls and their physiological behaviour. This work will hopefully lead to greater understanding about the onset of yeast flocculation, and the various factors that may be responsible for the process in industrial fermentations.

[1] Bony, M., Barre, P. & Blondin, B. 1998. Distribution of the flocculation protein, Flop, at the cell surface during yeast growth: the availability of flop determines the flocculation level. Yeast, 14: 25-35.

Poster at ISSY 2013

Structure –Function Relationship in Industrial Yeast Strains

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INTRODUCTION

- Adhesion properties of yeasts are crucial for many essential biological processes such as sexual reproduction, tissue or substrate invasion, bio-film formation and others.
- Yeast flocculation is 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).
- Adhesion properties applicable in improving the yeast biotechnology are dependent directly or indirectly on characteristics of cellular surface, usually the outer layer of the cell wall.
- Exploring the cell wall in-depth, especially its nanoscale structure would be helpful in gaining insights into the process of flocculation and it's various aspects.

AIMS

- **To gain insight into the yeast cell wall and correlate its structural aspects to the functional properties for various biotechnological processes, especially fermentation**
- **To understand how ultra-structure and nano-mechanical characteristics are linked to functional properties of yeast**

Four *Saccharomyces cerevisiae* yeast strains studied according to their technological usage:

- Beer Strain
- Wine Strain
- Champagne Strain
- Fuel Alcohol Strain

Flocculation Assay

Flocculation Ability for the four industrial strains was measured by the method provided by Bony et al (1998) with some modifications. Relative differences in the initial (A_0) and final absorbance (A) obtained by suspending the yeast cells in the flocculation buffer determine their percentage flocculation ability.

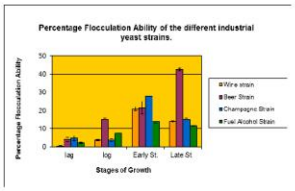

Fig 5. Flocculation ability of the yeast strains at different phases of the growth curve. Cells show less flocculation ability during lag and log phase and high flocculation ability during the early and late stationary phase. The Beer strain was found to be more flocculant, while wine strain was least flocculant.

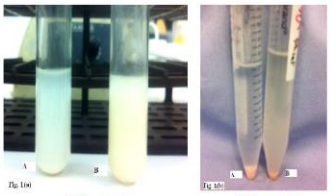

Fig 6 (a, b). Tubes A – Cells suspended in flocculation buffer containing calcium. Tubes B – Cells suspended in flocculation buffer containing EDTA.

Hydrophobicity Microsphere Assay

Cell surface hydrophobicity was determined by Microsphere Latex Bead Assay using beads with a diameter of 0.845 ± 0.001 µm. 100 cells for each of the four strains were counted and % age hydrophobicity calculated for those cells having ≥ 3 beads attached to it.

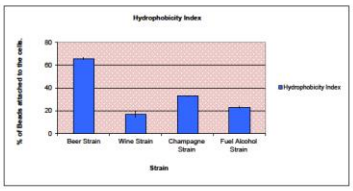

Fig 7. Hydrophobicity Index directly represents the Cell Surface Hydrophobicity (CSH). Beer Strain shows the maximum CSH levels, while the Wine Strain shows minimum CSH.

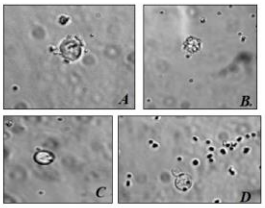

Fig 8. Latex beads attached to (A) Beer Strain, (B) Champagne Strain, (C) Fuel Alcohol Strain, (D) Wine Strain.

Atomic Force Microscopy (AFM)

To extract information on the mechanical features of the cell wall, AFM force spectroscopy experiments were performed. These experiments yield force-distance curves. The tip interacts with the cell surface of the sample and gives insight about various forces that operate at the atomic level.

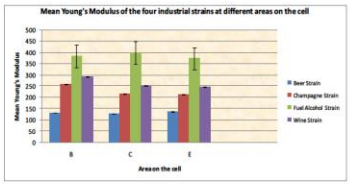

Fig 1. Mean Young's modulus (Y) for the four strains determined by AFM gives the measure of elasticity. Lower the Y, higher the elasticity. Beer strain shows minimum value for Y, suggesting elastic nature of its cell wall.

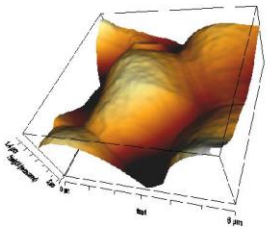

Fig 2. A 3D- rendered AFM image of a single yeast cell (Beer Strain).

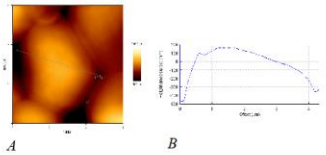

Fig 3. (A) AFM image taken in contact mode for Beer Strain; (B) Height measured of a single yeast cell.

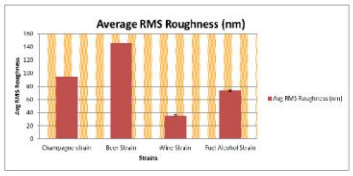

Fig 4. Higher the RMS surface roughness of the cell wall, higher the probability of floc formation and higher floc retention in the liquid medium.

CONCLUSIONS

Flocculation ability of different yeast strains is determined by physical parameters, such as its growth phase and initial seeding cell concentration.

The flocculation ability of a given strain is related to the Cell Surface Hydrophobicity (CSH), cell surface roughness (RMS), and elasticity of the cell wall.

In particular, (i) the higher the CSH and RMS roughness, the higher the flocculation ability, (ii) the higher the elasticity of the cell wall, the better the stability of the flocs of yeast in its growth medium.

FUTURE DIRECTIONS

To apply some molecular biology tools, such as PCR, Western blotting, gene sequencing, etc complementarily to the biotechnology and nanotechnology techniques to understand molecular level interactions between cell wall carbohydrates and lectins that lead to flocculation. We also envisage usage of Raman Spectroscopy technique.

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- Bony, M., Barre, P. & Blondin, B. (1998) Distribution of the flocculation Protein, Flop, at the cell surface during the yeast growth. *Yeast*, **14** : 25-35.

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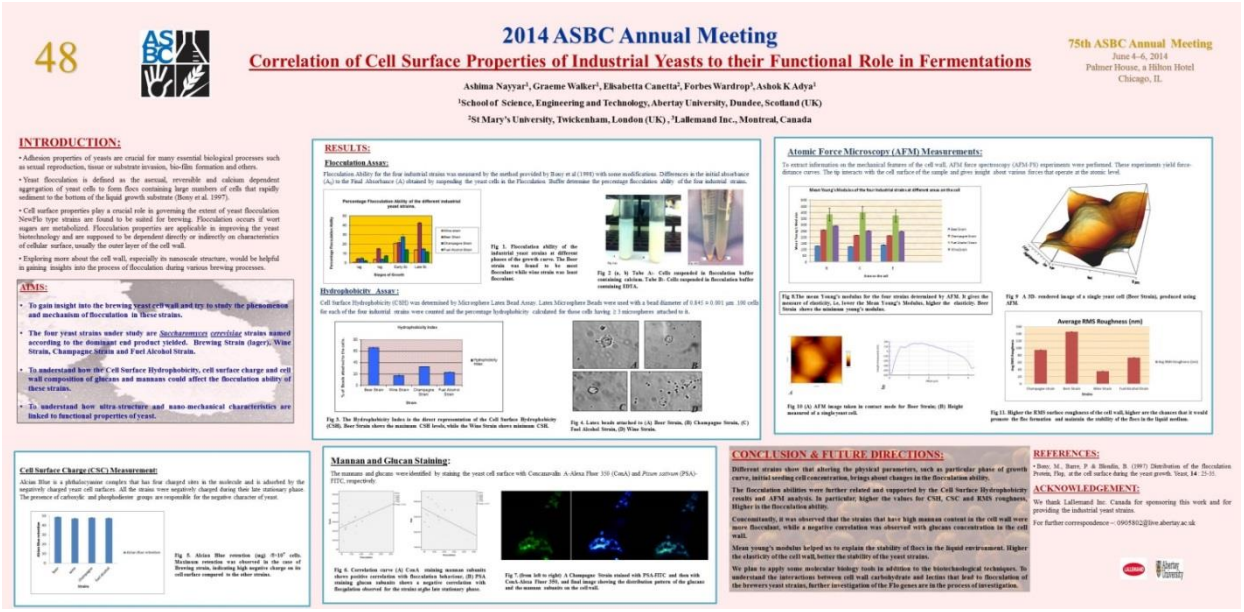
V Division of Biotechnology and Forensic Sciences, School of Contemporary Sciences, University of Abertay Dundee, Bell Street, Dundee DD1 1HG, Scotland, UK. Communicated by Ashok K Adya <A.Adya@abertay.ac.uk>.

Paper to be presented at the The American Society of Brewing Chemists (ASBC) meeting in Chicago in June 2014.

1 Nayyar A, Walker GM, Canetta E, Wardrop F and Adya AK Correlation of Cell Surface Properties of Industrial Yeast Strains to their Functional Role in Fermentations

Adhesion properties are known to play important roles in governing many essential aspects of the life cycles of microorganisms like sexual reproduction, cellular aggregation during processes such as flocculation and bio-film formation, invasion and/or pathogenic behaviour, and many others. Adhesion properties, by far, are dependent on the characteristics of the cellular surface, usually the outer layer of the cell wall. Microorganisms can adjust their adhesion properties by changing the structure of their external cell surface. Flocculence, is the ability of the yeast cells to flocculate under optimal conditions, which is a cell wall property independent of its environment. Thus, when we study flocculation we need to consider the cell wall properties The flocculation behaviour of four industrial *Saccharomyces cerevisiae* strains expressing either *Flo1* or *NewFlo* phenotype were examined. These were strains employed for brewing, champagne production, winemaking and fuel alcohol production. The behaviour of brewing and champagne strains differed in terms of their cell-surface hydrophobicity, cell-surface charge, and presence of adhesins and cell-wall binding sites (mannose residues) which likely impinge on their flocculation behaviour. The brewing yeast strain exhibited the highest degree of flocculation amongst all the strains, and it was accompanied with a concomitantly high hydrophobicity index of 66%. This supports our hypothesis that cell surface hydrophobicity plays a major role in controlling yeast flocculation behaviour in the fermenter. Equally important is cell-surface charge which were shown in highly flocculent brewing strains to possess very high negative charge. From the studies, it was observed that high cell surface hydrophobicity, bonds between the adhesins and mannose residues (stabilised by Ca^{2+} ions) and finally the surface topography of yeast strains are responsible for maintaining flocs during the fermentation process. We have additionally observed that in contrast to wine and fuel alcohol yeast strains, brewing and champagne strains exhibit increased cell-wall mannose concentrations from the early stationary phase to the late stationary phase. This correlates with simultaneous increase in flocculation ability. Brewing yeasts may therefore be characterised by a high density of mannose residues on their outer cell-walls. In addition, we found that the brewing yeast strain studied had a high lectin density (3.65×10^6 lectins /cell) compared with the champagne strain (2.44×10^6 lectins/cell). Yeast adhesion properties and cell wall physiology were further investigated at the nanoscale using Atomic Force Microscopy (AFM). For example, surface roughness, Young's Modulus, and adhesion energy of industrial yeast strains determined by AFM provided new information regarding yeast cell walls and physiological behaviour. The work will further aid in greater understanding about the onset of yeast flocculation, and the vital role that cell surface hydrophobicity, cell surface charge, surface topography together with the density of adhesins on the yeast cell surface play for the brewing processes in fermentation.

Poster presented at ASBC, Chicago 2014



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2014 ASBC Annual Meeting

Correlation of Cell Surface Properties of Industrial Yeasts to their Functional Role in Fermentations

75th ASBC Annual Meeting
June 4–6, 2014
Palmer House, a Hilton Hotel
Chicago, IL

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