Studies on flocculation characteristics in industrial yeast strain

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by

Purvesh Jugnahkar

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Under the guidance of

Supervisor: Dr. Anand Ghosalkar

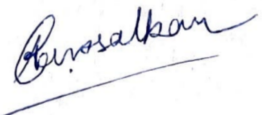
Affiliation of Supervisor

From May 2022 to Mar 2023

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled ‘Studies on flocculation characteristics in industrial yeast strain’ towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Purvesh Jugnahkar at Indian Institute of Science Education and Research under the supervision of Dr. Anand Ghosalkar, PRAJ matrix R&D, during the academic year 2022-2023.



Dr. Anand Ghosalkar

Committee:

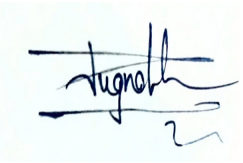
Guide: Dr.Anand Ghosalkar

TAC member: Dr.Chaitanya Athale

This thesis is dedicated to my parents

Declaration

I hereby declare that the matter embodied in the report ‘Studies on flocculation characteristics in industrial yeast strain’ are the results of the work carried out by me at the PRAJ Matrix R&D center, Pune, under the supervision of Dr. Anand Ghosalkar and the same has not been submitted elsewhere for any other degree.



Purvesh Jugnahkar
20181129
Date: April 25, 2023

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Abstract:

The ability of *Saccharomyces cerevisiae* cells to adhere to surfaces plays a significant role in defining the growth pattern of different strains under particular environmental conditions. The regulatory network that controls invasive and pseudohyphal growth, as well as biofilm formation, has been extensively studied, but other adhesion-related phenotypes, particularly flocculation, have received less attention.

In the biofuels industry, flocculation affects the efficiency of the fermentation process. Yeast strains with high flocculation efficiency can settle out of the fermentation broth more quickly, allowing for easier separation of the yeast from the biofuel product. This can result in higher yields and reduced processing time, leading to more cost-effective biofuel production.

By genetically modifying the flocculation efficiency of S. cerevisiae strains, these industries can improve their industrial processes and become more competitive in the market. This modification can lead to increased efficiency, higher yields, reduced processing time, and improved product quality. Therefore, it is of significant interest to these industries to explore and invest in genetic modification of flocculation efficiency in S. cerevisiae strains.

In this study we have tried to gain insight into the flocculation phenotype by studying various physiological and performance traits of industrial strains against their flocculant mutants. We employed a genome integrated *flo1* DNA cassette without a selectable markers.

Our preliminary data suggests that there is little to no change in ethanol making capacity between industrial strains and their flocculant mutants.

Acknowledgments

I am deeply grateful to all the people who have contributed to the successful completion of this thesis. Without their support and guidance, this work would not have been possible.

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Finally, I would like to express my heartfelt gratitude to my family and friends. Their unwavering love, encouragement, and moral support gave me the strength and motivation to overcome obstacles and complete this thesis. Their presence in my life has been a constant source of joy and inspiration.

Contributions

Contributor name	Contributor role
Purvesh Jugnahkar and Dheeraj Mahajan	Conceptualization Ideas
Purvesh Jugnahkar and Dheeraj Mahajan	Methodology
Purvesh Jugnahkar and Dheeraj Mahajan	Software
Dr. Anand Ghosalkar	Validation
Purvesh Jugnahkar	Formal analysis
Purvesh Jugnahkar	Investigation
Dr. Anand Ghosalkar	Resources
Purvesh Jugnahkar and Dheeraj Mahajan	Data Curation
Purvesh Jugnahkar	Writing - original draft preparation
Dr. Anand Ghosalkar	Writing - review and editing
Dr. Anand Ghosalkar	Visualization
Dr. Anand Ghosalkar	Supervision
Dr. Anand Ghosalkar	Project administration
Dr. Anand Ghosalkar	Funding

CHAPTER 1 : Introduction

Yeast flocculation refers to the clustering of yeast cells in asexual, reversible, and calcium-dependent aggregates, forming flocs that contain a high number of cells. These flocs then quickly settle to the bottom of the liquid growth substrate. This definition of yeast flocculation is described in scientific literature by Bony et al. (1997) and Stratford (1989a).

The way that non-identical strains of *Saccharomyces cerevisiae* grow is influenced by how well their cells can stick to surfaces, especially in certain conditions. Normally, these yeast cells grow separately in liquid and multiply by budding. But changes in the environment, like stress or lack of nutrients, can change how well the cells stick to surfaces and cause them to form groups or stick to things. This can lead to different growth patterns, like forming biofilms or invading surfaces. The have been studies that explain how some of the genes in these yeast cells affect their ability to stick to surfaces, but there is still more to learn about how these genes work.

When *Saccharomyces cerevisiae* is grown on solid surfaces, the changes in cell adhesion properties can cause it to grow in an invasive pattern, which can result in the formation of pseudohyphae. The mechanisms that control these growth patterns have been extensively studied, but less attention has been given to other adhesion-related behaviors such as flocculation (Braus et al. 2003; Reynolds and Fink 2001; Sampermans et al. 2005; Gagiano et al. 2002; Gancedo 2001; Palecek et al. 2002)..

Saccharomyces cerevisiae has several genes called FLO genes, including FLO1, FLO5, FLO9, FLO10, and FLO11. These genes each affect the properties of the cell wall in different ways. For example, the FLO1 gene helps cells stick to each other, the FLO5 gene helps cells form biofilms, and the FLO11 gene helps cells grow invasively and form pseudohyphae.

The way FLO genes are controlled is complicated and involves multiple pathways that communicate with each other, including the cAMP-PKA and MAPK pathways. Environmental factors such as the availability of nutrients and stressful situations can also affect the expression of FLO genes and their ability to adhere to surfaces.

Understanding how FLO genes work and how they are controlled is crucial for improving industrial processes that use yeast and for studying how yeast adhesion affects various traits. The goal of this dissertation is to examine specific FLO genes in detail to gain a better understanding of their function and how they are regulated.

In industrial fermentation processes that use non-flocculent strains of *Saccharomyces cerevisiae*, the yeast cells remain dispersed in the liquid medium, which makes it challenging to separate them from the final product. The separation process is typically time-consuming and costly, often involving procedures like centrifugation or filtration.

To address this issue, there is growing interest in modifying the flocculation efficiency of other industrial strains of S. cerevisiae. By doing so, these strains would become more effective in forming flocs, which are large, clumped-together groups of yeast cells. Flocs make it easier to separate the yeast cells from the fermentation broth, which would reduce production costs and increase the yield of the final product.

Genetically modifying the flocculation efficiency of S. cerevisiae strains can have a significant impact on the industrial processes that rely on these strains. For example, in the beer industry, flocculation affects the clarity and stability of the final product. Yeast strains with high flocculation efficiency can settle out of the beer more quickly, leading to a clearer and more stable product. This can also result in higher yields and reduced processing time.

Similarly, in the wine industry, flocculation can affect the clarity and quality of the wine. Yeast strains with high flocculation efficiency can settle out of the wine more quickly, leading to a clearer and more stable product. This can also result in higher yields and reduced processing time. (Chen, Y., Stowers, C., & Liu, Z. L. 2016)

In the biofuels industry, flocculation affects the efficiency of the fermentation process. Yeast strains with high flocculation efficiency can settle out of the fermentation broth more quickly, allowing for easier separation of the yeast from the biofuel product. This can result in higher yields and reduced processing time, leading to more cost-effective biofuel production. (Chen, Y., Stowers, C., & Liu, Z. L. 2016)

By genetically modifying the flocculation efficiency of S. cerevisiae strains, these industries can improve their industrial processes and become more competitive in the market. This modification can lead to increased efficiency, higher yields, reduced processing time, and improved product quality. Therefore, it is of significant interest to these industries to explore and invest in genetic modification of flocculation efficiency in S. cerevisiae strains.

In this context, this literature review aims to offer a current understanding of the physiological and genetic aspects of flocculation, both of which are crucial for implementing a successful genetic engineering approach to control flocculation.

CELL WALL ARCHITECTURE AND FUNCTION:

To make good use of the ability of S. cerevisiae to clump together (flocculate) and to understand how it works, we need to understand the structure and function of its outer layers. The ability to flocculate is actually a property of the cell wall, and experiments have shown that even when the cells are dead or the cell wall is isolated from the rest of the cell, it can still cause flocculation. This means that the cell wall is an important factor that determines how well the cells can flocculate.

The yeast cell is surrounded by a structure called the cell wall, which is made up of two layers. The outermost layer is made of a type of protein called mannoproteins, while the inner layer is made up of a type of carbohydrate called β-glucan and a small amount of chitin. These layers can be different in composition and size depending on the genetic traits of the yeast and the environment in which it grows. The cell wall is quite thick, measuring between 100-200 nanometers, and can make up to 30% of the cell's weight.

Cell wall mannoproteins-

Yeast cell wall proteins, also known as mannoproteins, are mostly made up of carbohydrates (sugars) and have a lot of mannose sugars attached to them. These mannoproteins are similar to yeast proteoglycans (Lesage and Bussey, 2006). The cell wall of S. cerevisiae contains polysaccharides (complex sugars) that bear the stress of the cell wall and provide a framework for the insertion of a layer made up of glycoproteins, which together form what is known as yeast mannan (Govender, 2009).

Mannoproteins are connected to a network of β(1,3)-glucan-chitin either indirectly through a β(1,6)-glucan moiety, or through hydrophobic interaction or disulphide bonds with other mannoproteins. S. cerevisiae has two types of modifications for cell wall proteins: O-linked mannosylation and N-linked glycosylation. Additionally, some cell wall proteins have a glycosyl phosphatidylinositol (GPI) anchor attached to them (Govender, 2009).

***O*-linked mannosylation –**

Only one to five linear mannose residues make up the short chains of oligosaccharides (fig. 5) that are connected to serine or threonine residues. The succeeding residues are connected differently ((1,3)-linked) from the first two, which are linked in a specific orientation ((1,2)-linked). These chains, which resemble rods, have the ability to lift protein domains off membrane or wall surfaces, possibly exposing them to more of the outside world. Despite the fact that these chains are very short, several studies have suggested that O-mannosylation is frequently found in CWPs, particularly those with serine/threonine-rich domains in cell wall proteins. As a result, there may be a sizable amount of O-linked mannose in the cell wall and a sizable total number of O-chains per CWP (Herscovics and Orlean, 1993).

***N*-linked glycosylation –**

N-glycosylated proteins in S. cerevisiae are proteins that have a type of sugar chain attached to a specific part of the protein called an asparagine residue (fig. 5). All these proteins start with the same basic sugar chain in the Endoplasmic Reticulum (ER). This basic sugar chain can mature and develop more complex chains that have 9 to 13 mannose sugars, called "core" sugar chains. However, some cell wall mannoproteins may further modify the core structure by adding more mannose sugars in the Golgi (Dean, 1999; Herscovics and Orlean, 1993). This process results in a backbone chain of mannose sugars that are linked together in a specific way, with up to 50 sugars extending from the core. Shorter chains of mannose sugars are then attached to the backbone, forming a highly branched structure that can contain up to 200 mannose sugars.

Yeast cell walls also have mannosyl side chains that are linked in a specific way to contribute to the negative charge of the yeast cell surface (Ballou, 1990; Dean, 1999; Herscovics and Orlean, 1993). It's important to note that lectins, proteins that help cells stick together, can attach to specific parts of the yeast cell wall sugar chains. Specifically, they bind to the ends of certain mannose sugar chains that consist of two or three mannose sugars.

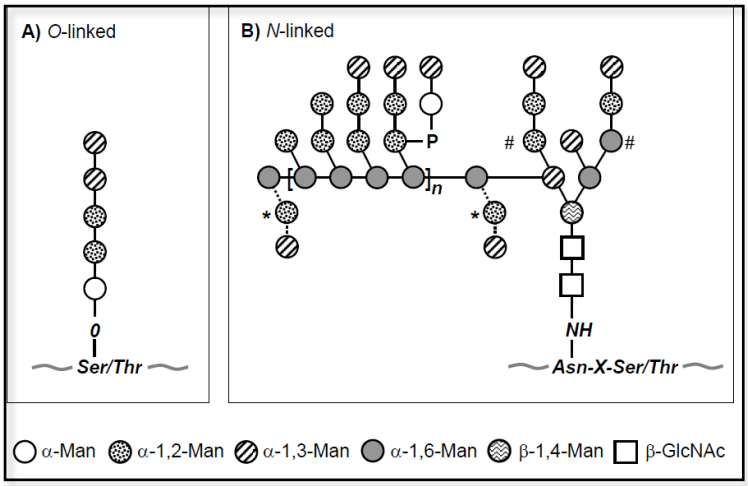

Figure 1 The process of adding sugar molecules to cell wall proteins in Saccharomyces cerevisiae can occur in two ways. A] The process of 0-linked mannosylation involves attaching small chains of a type of sugar called mannose to certain parts of the protein structure called serine or threonine residues. The length of these chains can be different, ranging from one to five mannose sugars. B] The second process is called N-linked glycosylation, which involves attaching carbohydrate side chains to the amide group of asparagine residues. The number of repeating units in the N-chains can vary, and can even be as high as 15. An asterisk is used to indicate alternative positions where an α-1,2-linked mannose can be added, which is thought to prevent elongation and is not found in the cores where outer chains are added. Additionally, some sites on the proteins can be phosphorylated, which is denoted by the # symbol. The sugars used in these processes include mannose (Man), N-acetylglucosamine (GlcNAc), and phosphate (P). (figure taken from Govender, 2009 and adapted from Herscovics and Orlean, 1993)

Cell wall anchorage of mannoproteins –

The cell wall has two types of proteins, and one type is called glycosyl phosphatidylinositol (GPI)-modified cell wall proteins (GPI-CWPs). These proteins have similar amino acid sequences, and they are grouped into different subclasses. Some of these proteins are located on the cell membrane, while others are a part of the cell wall itself.

When GPI-CWPs are made in the cell, they go through many changes as they move through different parts of the cell - endoplasmic reticulum and Golgi. These changes involve removing parts of the protein and adding a special anchor - GPI. The mature form of GPI-CWPs has only a small part of the original anchor remaining and is linked to sugar molecules in the cell wall. Although we don't fully understand their function. Some GPI-CWPs, such as Flo1p, Flo5p, Flo9p, Flo10p, and Flo11p, help the cell stick to surfaces and are involved in forming biofilms, clumps and are thought to help make the cell wall stronger.

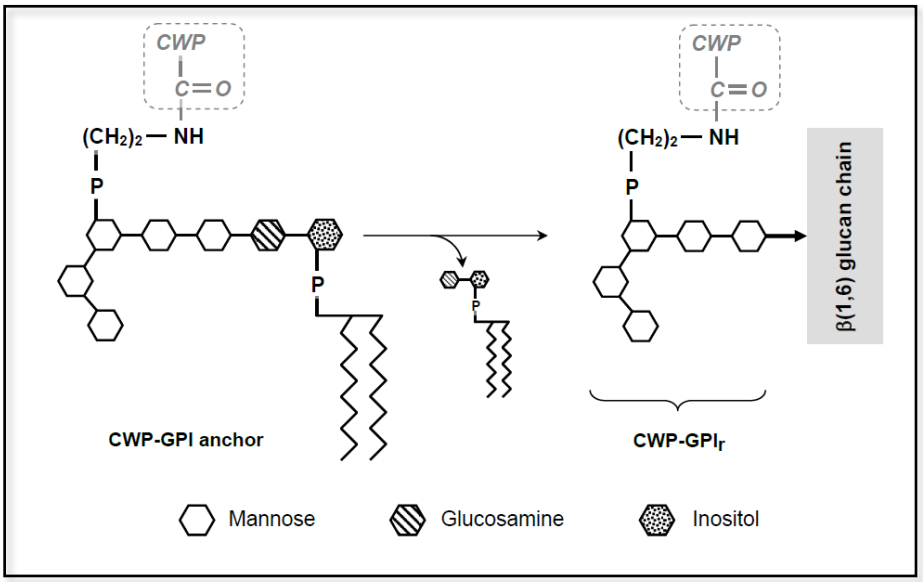

Figure 2 The cell wall of yeast cells has an inner skeletal network made of beta-glucan, and there are different types of proteins attached to it. One type is called glycosyl phosphatidylinositol (GPI)-anchored mannoproteins. These proteins are modified after they are made, and a GPI anchor is added to them. This anchor is then cleaved, and the remaining part of the protein is attached to the outer fibrillar layer of the cell wall via its mannosyl moiety. This is how certain proteins, like Flo proteins, interact with the cell wall.) (Figure taken Govender 2009, adapted from Lipke and Ovalle, 1998)

In yeast, there is another group of proteins in the cell wall called Pir-proteins. They are divided into four different subclasses and have special regions rich in serine and threonine in their structures. They are similar to GPI-CWPs because they can also have a sugar molecule, called O-mannosylation, added to them. However, unlike GPI-CWPs, Pir-proteins are spread out throughout the inner part of the cell wall and are directly attached to the 1,3-β-glucan molecules. Pir proteins are important for protecting the cell wall by controlling how things pass through it (Klis et al., 2006; Lesage and Bussey, 2006).

Functions of cell wall mannoproteins –

The outer layer of the yeast cell wall is important for regulating what goes in and out of the cell. The sugar chains on the proteins in the cell wall make it more water-retaining and can help protect against dehydration. Depending on the environment and what stage the cell is in, different proteins are added to the cell wall. This can cause different behaviors in the cell, like sticking to surfaces or forming groups. The different combinations of proteins can create many different ways that the cell behaves. (Cappellaro et al., 1994; Fidalgo et al., 2006; Govender et al., 2008; Guo et al., 2000; Ishigami et al., 2006; Lambrechts et al., 1996; Reynolds and Fink, 2001).

DEFINITION OF FLOCCULATION :

The process of flocculation in yeast cells has been observed for a long time, with the first record dating back to 1876 by Louis Pasteur. However, there was confusion in early reports as different processes like sexual agglutination, chain formation, and pseudohyphal formation were also included. To understand flocculation, it is important to differentiate between these three processes that can cause clumps of yeast cells. Sexual agglutination or yeast mating occurs when different haploid strains of S. cerevisiae exchange small peptide pheromones. This leads to changes in the cells resulting in their aggregation before fusion to form diploids. The cells stick to each other by interacting through proteins present in their cell walls. (Calleja, 1987). Chain formation happens when a budding yeast cell does not fully separate from its mother cell after division. Instead, the daughter cell remains attached and produces its own bud, and this process repeats, resulting in a chain of cells. The cells in the chain are connected by their cell walls, and all new buds grow only on one end of the chain. (Calleja, 1987; Gimeno et al., 1992; Lambrechts et al., 1996).

Flocculation is when yeast cells come together and form clumps in a liquid. It's different from other forms of yeast cell aggregation, like when cells stick together in a chain or when they mate. You can tell flocculation apart because it can be broken up by EDTA and the cells will come back together on their own after being stirred apart. Different kinds of yeast can flocculate more or less easily. Currently, flocculation is defined as the reversible, calcium-dependent, and asexual aggregation of yeast cells to form flocs containing thousands of cells that rapidly settle to the bottom of the liquid growth medium. (Govender, 2009)

THE MECHANISM OF FLOCCULATION –

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

The calcium-bridging hypothesis-

In 1964, Mill proposed the calcium-bridging hypothesis as an explanation for the mechanism of yeast floc formation. This hypothesis was widely accepted by the research community until the early 1980s. According to the theory, “flocculated cells are held together by salt bridges, in which Ca2+ ions link negative charges of two carboxyl, phosphate, and/or sulphate groups on the cell surface of interacting cells. It was also proposed that these salt bridges would be stabilized by hydrogen bonds between complementary carbohydrate hydroxyl groups at the cell wall surface. The thermal dissociation of flocs at 50-60°C supported the involvement of hydrogen bonding interactions in maintaining the bridging structure. The effect of pH on flocculation and irreversible inhibition of floc formation by 1,2-epoxypropane strongly suggested carboxyl functional groups as the most likely combine sites. This view was further supported when a correlation between the capacity for floc formation and the density of carboxyl groups on the cell surface was observed. The loss of flocculation in response to treatment with protein-denaturing agents and proteases suggested that carboxyl groups came from cell wall mannoproteins” (Govender, 2009).

Lyons and Hough (1970; 1971) presented evidence that phosphodiester groups of phosphomannan might serve as binding sites for calcium ions, providing an alternative explanation for flocculation. In the past, scientists had different theories about how yeast flocculation happens. One theory was that calcium ions create a bridge between yeast cells, causing them to clump together. This theory was widely accepted, but some scientists were unsure if it was correct. Later on, researchers discovered that the calcium-bridge theory could not explain why certain sugars could stop flocculation from happening. It also did not account for how specific types of yeast cells could interact with each other. These discoveries led scientists to doubt the validity of the calcium-bridge theory. (Calleja, 1987; Jin and Speers, 1998; Speers et al., 1992; Rose, 1993; Stratford, 1992a; Taylor and Orton, 1978).

The lectin hypothesis-

The lectin hypothesis suggests that there are specialised proteins on the surface of yeast cells that can recognize and bind to carbohydrates on other yeast cells. This interaction is facilitated by calcium ions, which help the surface proteins keep their shape. This idea was put forth by Miki and colleagues in 1982 as an alternative to the calcium-bridge hypothesis, which couldn't explain why certain sugars, such as mannose, inhibit flocculation or why the interactions between cells are so specific.

The lectin hypothesis explains that flocculation happens when two different units on the surface of yeast cells interact. These units are called flocculation receptors and are found on both flocculent and non-flocculent cells. They are made up of α-branched mannans. Evidence for this hypothesis includes experiments that show mannose can stop flocculation, yeast that don't have mannan don't flocculate, and mannan blocking with concanavalin A and chemical modification experiments. (Miki et al., 1982a; Nishihara and Toraya, 1987).

Based on studies, the proteins on the surface of flocculent yeast cells that recognise and attach to carbohydrates on non-flocculent yeast cells are composed of particular outer chain N-linked mannan side branches with just two or three mannose residues. Even a tiny number of these receptors is adequate to cause flocculation. According to research, the overall structure of these receptors is consistent across various S. cerevisiae strains, however there are minor changes in the intricate design of mannans between strains. However, the presence of these receptors has no effect on the onset of flocculation, which is the transition from single-cell yeast growth to multicellular aggregation. Furthermore, the presence of other sugars can temporarily prevent flocculation. (Stratford and Assinder, 1991).

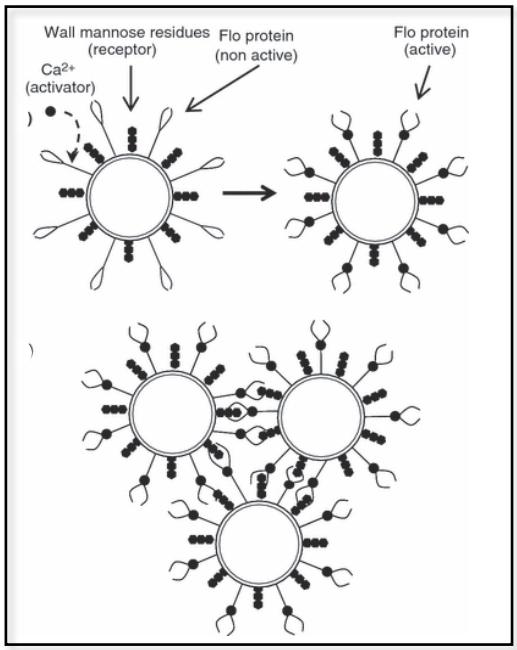

Figure 3 The lectin hypothesis is illustrated by the flocculation model, which proposes that a glycoprotein adhesin encoded by the FLO gene, called Ca2+-activated flocculin, interacts with adjacent cells by binding to mannose residues present in the mannoproteins associated with the cell wall, known as the mannan receptor. This interaction leads to the progressive formation of flocs consisting of numerous cells that bind together simultaneously. This model was proposed by Teunissen and Steensma in 1995. (Figure taken from Govender 2009)

Research has found that specific proteins on the surface of cells play a role in the flocculation process. When these proteins are damaged, cells can no longer stick together. One theory suggests that a complex of two proteins connected by a disulphide bridge is responsible for this process. Another theory suggests that a type of glycoprotein called flocculins, which are attached to the cell wall, may be responsible for binding cells together. The modified theory proposed by Teunissen and Steensma (1995) is widely accepted in the scientific community.

Studies using genome-wide predictions have found that Flo proteins have a signal at the end of the protein called GPI anchorage signal, which helps the protein to attach and go through the cell wall (Caro et al., 1997; De Groot et al., 2003; Hamada et al., 1998a; 1998b). Flocculins also have a central domain that can be modified with sugars, which help the protein form a strong, stable structure (Jentoft, 1990). Changes in the sugar recognition pattern of Flo1 protein require changes to the protein's N-terminal region that is exposed outside the cell (Kobayashi et al. 1998). There is also more evidence that Flo1p is a cell wall mannoprotein that helps cells interact with each other. (Bony et al., 1997; 1998; Javadekar et al., 2000).

Studies have shown that Flo proteins play a critical role in flocculation. In fact, when a recombinant FLO gene is added to non-flocculent S. cerevisiae strains, it can induce flocculation. While it is clear that these proteins are involved in flocculation, it is still unclear which specific lectin and ligand are involved in this interaction (Chambers et al., 2004; Cunha et al., 2006; Govender et al., 2008; Guo et al., 2000; Verstrepen et al., 2001; Wang et al., 2008; Watari et al., 1991; 1994).

FACTORS AFFECTING FLOCCULATION:

The production industry has been the primary focus of research into flocculation, and much of our current understanding of flocculation factors is derived from studies on brewer's yeast strains fig 4. presents a schematic representation of various factors that may affect flocculation. However, studies often produce contradictory results, indicating that flocculation behavior is highly dependent on multiple strain-specific factors. Despite the challenges in comparing flocculation reports due to differences in techniques and variations, most strains of S. cerevisiae exhibit flocculation under specific conditions.

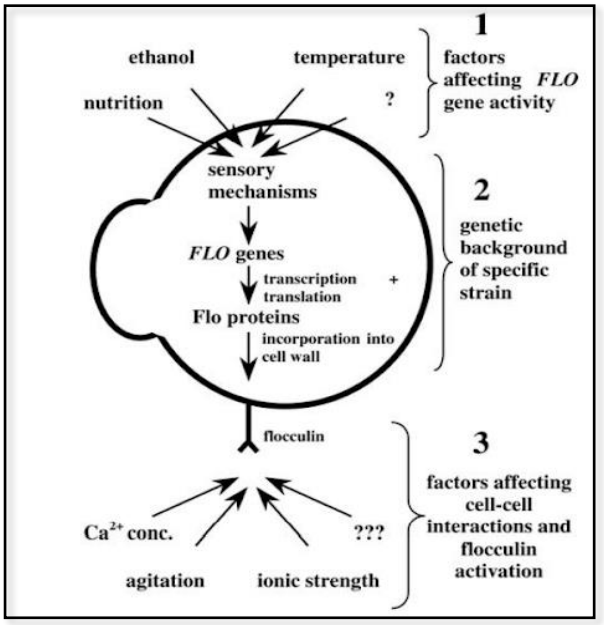

Figure 4 Factors affecting flocculation in S. cerevisiae. Three categories (1, 2 and 3) can be distinguished according to their mode of action (Figure taken from Verstrepen et al., 2003).

Sugar inhibition –

In a 1991 investigation, Stratford and Assinder discovered two types of yeast strains with differing ways of attaching to carbohydrates. The Flo1 strains were exclusively sensitive to mannose, but the NewFlo strains were inhibited by mannose, glucose, sucrose, and maltose. The Flo1 and NewFlo strains are different types of yeast that have different genes involved in how they stick together in clumps or flocculate. The Flo1 strains are more sensitive to a type of sugar called mannose, while the NewFlo strains are more sensitive to a combination of glucose and mannose(Masy et al.'s 1992). Some other strains of yeast can stick together in clumps using different methods, like interactions between molecules that don't involve mannose or calcium ions. It's important to keep in mind that research on this topic has found different results, and how yeast clumps together can vary depending on the specific strain and other factors. (Govender, 2009).

During growth, almost all Flo1 phenotype strains display constitutive expression of flocculence throughout all growth phases, regardless of the presence of nutrients (Patelakis et al., 1998; Soares and Mota, 1996; Stratford and Assinder, 1991). On the other hand, strains with the NewFlo phenotype usually flocculate exclusively in the stationary phase of yeast growth and flocculation receptors were found at all

stages of growth (Soares and Mota, 1996; Stratford, 1993), indicating that receptor availability is not the main factor for flocculation onset. Rather, it depends on the appearance of active lectins (Soares and Mota, 1996; Stratford and Carter, 1993), which are not permanently present on the yeast surface, but rather increase in amount during growth. For NewFlo strains, flocculation only occurs in the absence of glucose, sucrose, and maltose in the growth medium, as these sugars block the NewFlo flocculin binding sites and inhibit flocculation (Stratford, 1992c).

Sampermans and colleagues (2005) discovered that the process of flocculation, was linked to the amount of fermentable sugar (such as glucose, maltose or fructose) present in the growth medium. Once the sugar concentration reached a certain minimum level, flocculation would be triggered, whether the yeast were grown in a simple or nutrient-rich environment.

Research conducted by various groups of scientists including Bony et al. (1997, 1998), Javadekar et al. (2000), and Kobayashi et al. (1998) has indicated a link between the availability of Flo proteins on the yeast surface and the intensity of flocculation. Additionally, Kobayashi and his colleagues identified a novel FLO1 homologue, Lg-FLO1, which encodes a flocculin that can bind to both mannose and glucose, and is believed to be responsible for the NewFlo phenotype of most lager yeasts. In recent studies conducted by Liu and colleagues (2007a, 2007b), it was suggested that the difference between the NewFlo and Flo1 flocculation phenotypes may be attributed, at least partially, to the removal of two repeated regions located in the central domain of the Flo1 flocculin.

It has been reported that fermentable sugars play a significant role in causing the loss of flocculation in ale brewing yeasts belonging to the NewFlo phenotype, both in growing conditions and in starved cells. The NewFlo type strains show a unique induction of flocculation in response to nutrient starvation, specifically a shortage of fermentable carbon sources. This phenomenon is believed to enable yeast cells to adapt to stressful conditions by switching from non-flocculent to flocculent growth. It is suggested that flocculation may help protect cells present in the center of flocs from environmental stress or serve as a means of passive transport away from the stress.

Inorganic ions –

Flocculation is a process in which small particles in a liquid come together to form larger clumps, or flocs. The role of different types of ions in this process has been studied, and researchers initially had conflicting results. However, it is now generally agreed that calcium ions play a crucial role in maintaining the active shape of the proteins that are involved in flocculation, called flocculins.

In fact, very low concentrations of calcium ions are necessary for flocculation to occur, with a concentration of 10-8 M being sufficient. Other cations, such as magnesium and manganese ions, can mimic the activity of calcium ions to some extent, but only at low concentrations. The effects of other cations on flocculation can vary depending on their concentration, the assay conditions, and the genetic background of the strains used in the experiments.

The concentration of different types of salts and ions in the liquid can affect this process. Low concentrations of some cations can enhance flocculation, while high

concentrations can inhibit it. Sodium and potassium ions can also influence flocculation, but only at low concentrations.

When the concentration of salt is low, it can lower the charge on the surface of the yeast cells and affect surface proteins, which can increase flocculence. However, at high salt concentrations, proteins can become dehydrated, which prevents calcium from binding to activate flocculins, leading to cationic inhibition.

Lectin molecules, which are involved in mannan-protein interaction, require a high water activity for optimal function. The type of surface proteins involved can also affect the process, as the NewFlo-type surface proteins are more prone to dehydration than the Flo1 type of surface proteins. Strontium and barium, acting as calcium analogues, can also inhibit flocculation in a concentration-dependent manner.

These findings help to explain why earlier studies on the involvement of cations in flocculation produced conflicting results. The concentration of the cations, as well as other factors such as the type of strain being studied, the assay conditions, and the flocculation assays used, can all affect the process.

pH –

Initially, it was believed that low pH was not important for flocculation, but that high acidity could help cells come into contact with each other and form flocs. However, further studies showed that various strains of yeast had variable tolerances to pH. Some strains could flocculate over a wide range of pH, while others could only do so at a narrower pH range. Researchers believe that adding ethanol can help induce flocculation by decreasing the repulsion between cells, making it easier for them to come into contact and form flocs. Ethanol may also help polymer chains to stick out, which could help cells come together and flocculate. (Govender, 2009). Some yeast strains called NewFlo strains can't flocculate in the usual laboratory conditions because the pH and buffering are not right. By adjusting the pH, they can be induced to flocculate in lab conditions, and a small pH change during the fermentation process can cause the cells to separate from the liquid. It is believed that changes in the electrostatic charge of surface proteins may inactivate the flocculins at certain pH levels. (Govender, 2009). Alternatively, it is possible that changes in pH directly influence FLO gene activity, inducing flocculation. Therefore, it seems that the pH range suitable for flocculation depends heavily on the specific yeast strain.

Temperature –

Temperature can affect the ability of yeast cells to flocculate or clump together. While some studies suggest that temperature has little effect as long as it stays within a certain range, other research has shown that the impact of temperature on flocculation varies depending on the specific strain of yeast. For example, some strains may flocculate better at higher temperatures, while others settle better at lower temperatures. It has also been found that some strains may not flocculate at all at certain temperatures. (Taylor and Orton, 1975; Stewart et al.1975; Stratford, 1992a).

In addition, Claro et al. (2007) recently reported that continuous mild heat shock at 37°C has a detrimental effect on flocculation phenotypic expression in a S. cerevisiae brewing strain. These inconsistent findings highlight the strain-specific nature of flocculation.

Ethanol –

Studies have shown that adding ethanol to yeast cultures can promote flocculation, which means that the yeast cells will clump together and settle to the bottom of the container. This has been observed by different researchers Amory et al., 1988; Claro et al., 2007; Dengis et al., 1995; Eddy, 1955; Jin et al., 2001; Jin and Speers, 2000; Mill, 1964; Sampermans et al., 2005).. However, some studies have found that ethanol can also inhibit flocculation, and the effect of ethanol on flocculation can vary depending on the type of yeast being used. For example, one study found that ethanol only induced flocculation in a certain type of yeast, called a top-fermenting strain (D'Hautcourt and Smart, 1999).

Several studies have shown that adding ethanol can help promote the process of flocculation in yeast (Amory et al., 1988; Claro et al., 2007; Dengis et al., 1995; Eddy, 1955; Jin et al., 2001; Jin and Speers, 2000; Mill, 1964; Sampermans et al., 2005). However, the exact way in which it works is not fully understood. One theory is that ethanol may reduce the electrical repulsion between yeast cells, allowing them to come closer together and clump. Ethanol may also affect the properties of yeast cell walls and their surface charge, making them more likely to stick together. In some cases, the presence of ethanol combined with a lack of nutrients may trigger flocculation (Sampermans et al. 2005). However, the positive effects of ethanol on flocculation depend on its concentration, and at high levels, it may actually inhibit flocculation.

GENETICS OF FLOCCULATION:

The current focus of research on flocculation genetics centers on the FLO genes, which are responsible for controlling the majority of adhesion processes. Previous studies, including a comprehensive review by Teunissen and Steensma (1995), as well as research by Lambrechts et al. (1996) and Lo and Dranginis (1996), have investigated other genes involved in adhesion. However, this study will concentrate on the FLO genes.

S. cerevisiae is a type of yeast that has five dominant FLO genes that are not linked to each other (Verstrepen et al., 2004). Four of these genes, FLO1, FLO5, FLO9, and FLO10, are located near their respective telomeres and are considered a subtelomeric gene family. The fifth gene, MUC1 or FLO11, is not located near any centromeres or telomeres but is still a dominant flocculation gene (Lambrechts et al., 1996; Lo and Dranginis, 1996). Recent studies have used genomic approaches to better understand the structure and function of these genes.

The examination of DNA sequences reveals that there are specific DNA motifs that are present in different FLO genes of S. cerevisiae. One significant motif is a repetitive sequence of around 100 nucleotides that appears in tandem in the areas encoding the central domain.

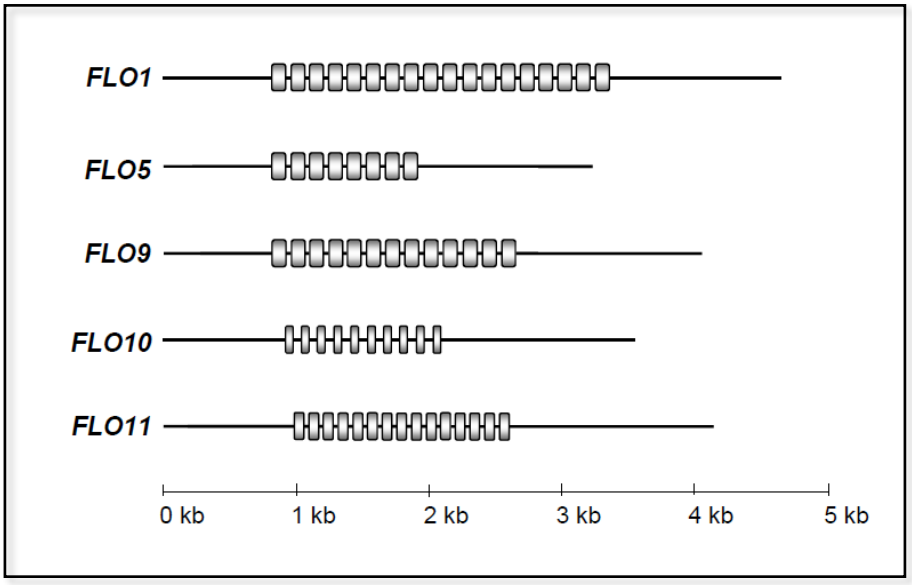

Figure 5 The FLO genes in S. cerevisiae have intragenic tandem repeats that are conserved and consist of at least 40 nucleotides. Research has revealed that the size of these repeats can differ among six various S. cerevisiae strains. Notably, FLO1, FLO5, and FLO9 have identical repeat units, as reported by Verstrepen et al. in 2005. (Figure taken from Govender 2009)

of FLO1 (18 copies), FLO9 (13 copies), and FLO5 (8 copies). However, it was observed in a comparative study of six S. cerevisiae strains that the size of these tandem repeats varied. FLO10 and FLO11 also have repetitive nucleotide sequences in the domain B encoding region, but these are unique compared to the one present in FLO1, FLO5, and FLO9. Additionally, there are other large elements

present at the beginning as well as the end of every gene that is common to many FLO genes in the S. cerevisiae laboratory strain S288C.

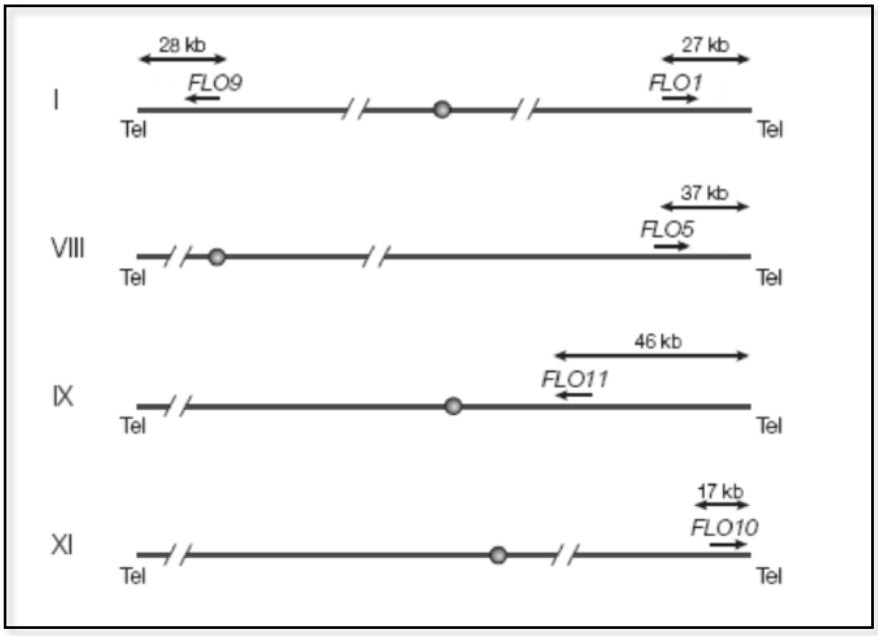

Figure 6 In the laboratory strain S288C of S. cerevisiae, the FLO genes are located on different yeast chromosomes, with the corresponding chromosome number indicated on the left, and the centromeres marked with dots. The four FLO genes located close to the telomeres are referred to as subtelomeric and are located within 40 kb of the telomeres. On the other hand, FLO11 is not categorized as either centromeric or telomeric, according to Verstrepen et al.'s findings in 2004. (Figure taken from Govender 2009)

Genetic regulation of flocculation –

There isn't much known about how other FLO genes in S. cerevisiae are controlled. Some researchers believe they are controlled by the same pathways as the FLO11 gene. Other researchers have discovered that the Flo8p protein activates the transcription of both FLO1 and FLO11, but the way each gene is regulated may differ. The Swi-Snf co-activator and the Tup1-Ssn6 co-repressor both control the FLO1 gene. The FLO1 promoter region has a putative GCN4-box motif (Teunissen et al., 1993), which may suppress FLO1 production in high nitrogen environments. Other studies have found that factors such as carbon and/or nitrogen deficiency, pH, and ionic strength all influence FLO1 expression (Jin and Speers, 2000; Soares and Seynaeve, 2000)

Besides transcriptional regulation, the activity of FLO genes in S. cerevisiae can also be influenced by other mechanisms. Recent research has found that these genes are often controlled by epigenetic processes specific to their promoters. This allows for a reversible mechanism to exist between active FLO gene expression and a silent state, enabling cells to modify the adhesive properties of their cell walls in response to extracellular cues.

Ty elements:

Ty elements are retrotransposons. The acronym “Ty” refers for “Transposons of yeast”. *Saccharomyces cerevisiae* retrotransposons and retroviruses are often compared because of the similarity between their life cycles and their mechanism of integrating cDNA into host genomes.

The genomes of many eukaryotes (organisms with cells that contain a clearly defined nucleus) contain significant amounts of retrotransposons, which are a highly distinctive group of transposable elements. Using a "copy and paste" method, retrotransposons operate. The original copy is therefore left behind, and a second copy is produced and put somewhere else in the genome. In many higher animals, this process causes the insertion of repeated DNA sequences across the genome, which is the mechanism underlying the widespread distribution of transposable elements.

The transposable DNA is transcribed into RNA as the initial step in retrotransposition. After then, the RNA fragment moves to a different region of the genome. However reverse transcriptase must copy the RNA back into DNA before it can be incorporated into the genome at the new location.

There are several different types of Ty elements that have been identified in yeast, each with their own unique characteristics and properties. Here are some of the most well-known Ty elements:

- Ty1: Ty1 is the most abundant Ty element in the yeast genome, and is present in approximately 30-40 copies per haploid genome. It has a length of approximately 6.3 kb and contains a gag-pol gene that encodes a polyprotein, as well as an env gene that encodes a surface protein. Ty1 elements can transpose via a "copy-and-paste" mechanism, where a new copy is generated and inserted at a new location while the original copy remains in place (Malik et al. 2000)
- Ty2: Ty2 is similar in structure to Ty1, but is much less abundant in the genome, with only 2-4 copies per haploid genome. It also contains a gag-pol gene and an env gene, and can transpose via a copy-and-paste mechanism. (Malik et al. 2000)
- Ty3: Ty3 is another retrotransposon found in yeast, but it differs from Ty1 and Ty2 in that it has an internal promoter that allows for its transcription from within the element itself. It is longer than Ty1 and Ty2, at approximately 9 kb, and contains both a gag-pol gene and an env gene. (Boeke et al. 1986)
- Ty4: Ty4 is the smallest of the Ty elements, at only 3.5 kb in length. It lacks an env gene, but contains a gag-pol gene and an additional ORF (open reading frame) that is unique to Ty4. Ty4 elements can transpose via a "cut-and-paste" mechanism, where the original copy is excised from the genome and a new copy is inserted at a different location. (Boeke et al. 1986)

- Ty5: Ty5 is unique among the Ty elements in that it is integrated into the rDNA (ribosomal DNA) locus of the yeast genome, rather than being scattered throughout the genome like the other Ty elements. It is also the largest of the Ty elements, at approximately 6.9 kb, and contains both a gag-pol gene and an env gene. Ty5 elements can transpose via a copy-and-paste mechanism, but they do so with a lower frequency than the other Ty elements. (Boeke et al. 1986)
- Overall, the Ty elements in yeast are an interesting and complex group of genetic elements that contribute to the diversity and evolution of the yeast genome.

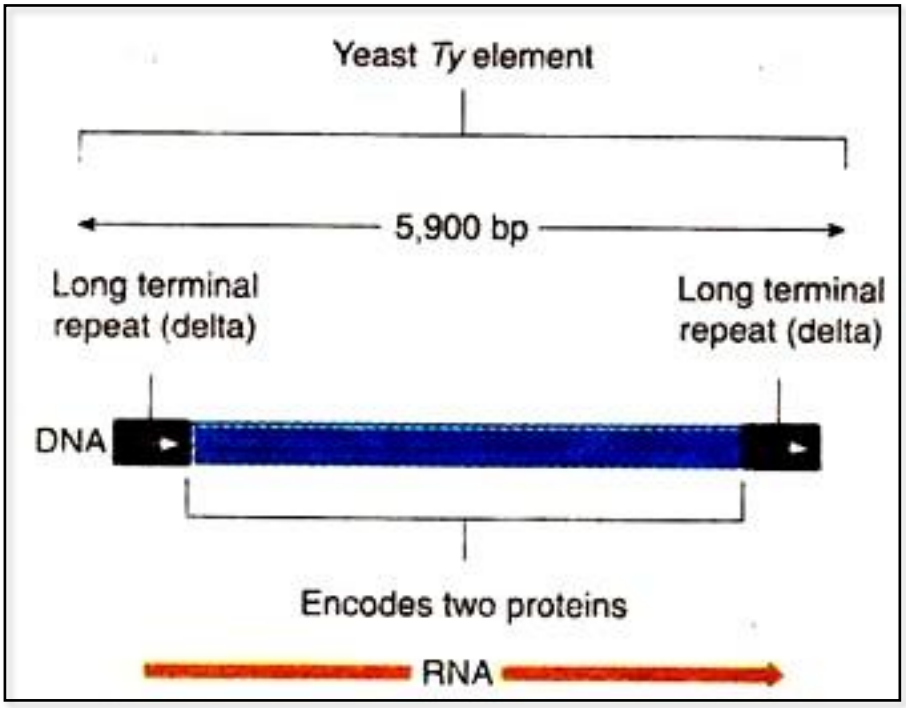

Figure 7. The Ty transposable element in yeas. Image taken from https://www.notesonzoology.com

TEF1 promoter:

The TEF1 promoter is a well-characterized promoter in the yeast *Saccharomyces cerevisiae*. It regulates the transcription of the TEF1 gene, which encodes the translation elongation factor 1-alpha, an essential protein involved in protein synthesis.

The TEF1 promoter contains several conserved elements that are important for its activity. These include a TATA box, which is recognized by RNA polymerase II and is important for transcription initiation, as well as upstream activating sequences (UAS) that bind transcription factors and regulate the level of gene expression. (Mumberg,1995)

The TEF1 promoter is constitutive, meaning that it drives steady-state expression of the TEF1 gene under normal growth conditions. However, its activity can be

modulated by various environmental and physiological cues, such as glucose concentration, oxygen availability, and temperature.

Because of its strong and reliable activity, the TEF1 promoter has been widely used as a tool for gene expression in yeast. It has been used to drive the expression of a variety of genes of interest, including fluorescent proteins, enzymes, and transcription factors, for both research and industrial applications. (Mumberg,1995)

PRM9 terminator:

The PRM9 terminator is a sequence that is found at the 3' end of the PRM9 gene in the yeast *Saccharomyces cerevisiae*. It serves as a signal to terminate transcription of the PRM9 gene and is an important component of the genetic machinery that controls gene expression in yeast.

The PRM9 terminator sequence is composed of about 100 base pairs and contains a number of conserved elements. These elements include a polyadenylation signal, which directs the addition of a poly(A) tail to the mRNA transcript, and a stem-loop structure that is thought to be involved in the termination of transcription (Tan-Wong SM, 2012)

The function of the PRM9 terminator is to ensure that transcription of the PRM9 gene stops at the appropriate site, so that the mRNA transcript that is produced is complete and can be properly translated into the PRM9 protein. Without a terminator sequence, transcription could continue past the end of the gene, resulting in the production of incomplete or truncated transcripts that would be non-functional or potentially even harmful to the cell. (Tan-Wong SM, 2012)

The PRM9 terminator is also useful as a tool for genetic engineering in yeast. It can be used as a component of artificial gene constructs to ensure proper termination of transcription of the gene of interest, and to prevent read-through transcription into downstream sequences. Additionally, the PRM9 terminator has been used as a model system for studying the mechanisms of transcriptional termination in yeast and other organisms. (Tan-Wong SM, 2012)

Chapter 2: Material and methods

Strains:

Table 1. This table contains all the strains used in the course of this study.

Name	Genotype	Property	Type
CEN PK 2.1.C	MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289, MAL2-8c SUC2	Non-flocculating	laboratory
PRY-E	MATa/α prototroph, industrial strain	Non-flocculating	Industrial
PRY-G	MATa/α diploid, industrial strain	Flocculating	Industrial
Praj IBA Yeast (PRY-98)	MATa/α diploid, industrial strain IBA producing yeast	Non-flocculating	Industrial
PRY-X	MATa/α diploid, industrial strain	Non-flocculating	Industrial

Primers used in this study:

Table 2 This table contains all the primers used throughout the course of this study.

Name	Sequence 5’ to 3’	Description
Delta up F	GTCATCGATGTTAGAGGAAGC	Homologous to 250bp Ty1 element
Delta up R	AAATCTGGAAGAGTAAAAAAGGAGTAGAAACATTTTG AAGCTATGACAGGCGCTACCATGAGATATATG	Homologous to 250bp Ty1 element
TEF F	CAACAATTATCTAATTACCCACATATATCTCATGGTAG CGCCTGT CATAGCTTCAAAATGTTTCTAC	TEF1 promoter forward primer
delta dwn R	GACCAACCAGATGGATTGGC	250 bp downstream homology reverse primer
TEF flo1 R	AAGTGTAAAGACTGCCAAAAACATATAGCGATGAGGC ATTGTCATAAAACTTAGATTAGATTGCTATGC	TEF1 promoter reverse with flo1 homology

flo1 F	GCAAGAATCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTTTATGACAATGCCTCATCGC	Flo1 forward primer with TEF promoter homology
flo1 R	TACCTTGCCTGGTAAAGTTGTGTGCTAGTGTCTCCCGTCTTCTGTTTAAATAATTGCCAGCAATAAGG	Flo1 reverse primer with PRM9 terminator homology
PRM flo1 F	GGTTTAAGTGTCTTCATTGCGTCCTTATTGCTGGCAATTATTTAAACAGAAGACGGGAGACAC	PRM9 forward primer with flo1 homoogy
Delta 12 F	TCAACAATGGAATCCCAAC	Ty1 forward primer
Delta 21 R	GTGGATTTTTATTCCAAC	Ty1 reverse primer

DNA manipulation and cassette generation conditions:

To ensure proper amplification Q5® High-Fidelity 2X Master Mix was employed for all *flo1* related amplification which was amplified from freshly extracted gDNA from CENPK 2.1.C laboratory strain. The details of the PCR conditions are stated in the table below:

Table 3 This table contains the detail of PCR reaction of flo1 gene

Component	**Volume in test samples (ul)**	**Volume in control sample (ul)**
HS polymerase	25	10
Primer F (100 uM)	2.5	1
Primer R (100 uM)	2.5	1
CENPK 2.1.C gDNA	2.5	0
Water	17.5	8
Total	**50**	**20**

All routine PCR amplifications were performed using high-fidelity PrimeStar HS PCR mastermix, TaKaRa BIO, USA (catalog no.).

Table 4 This table contains the details of the plasmids used in the study.

Plasmid/strain	Description	Source
pUC 19::HygR	Hygromycin resistant gene (HPH) This antibiotic resistance marker is flanked by attB and attP sites recognized by bacteriophage phiC31 integrase	Praj plasmid library

Yeast transformation:

A 100 mL YPD media was inoculated using culture of yeast grown overnight at OD600 0.2. The culture was incubated at 30°C and 150 rpm until the cells reached OD600 0.7-0.9, after which they were harvested by centrifugation. The cells were then suspended in a fresh electroporation buffer and left at room temperature for 1 hour. After centrifugation and washing with cold water and sorbitol, the cells were resuspended in 100 µl of ice-cold 1 M sorbitol and labeled as competent cells. To transform the cells, 80 µl of competent cell suspension was mixed with 4 pmol of DNA cassette fragments and incubated on ice for 5 minutes.The mixture was then taken in electroporation cuvette with 4-mm gap and given an electric pulse of 1.5 kV, 25 µF, 200 Ω with Gene Pulser Xcell electroporation system (Bio-Rad, USA)—for S. cerevisiae. After the pulse, the cells were immediately transferred in ice bath. The cells were recovered in 3-ml ice cold YPD and incubated at 30 °C, 150 rpm overnight. The culture was then plated on YPD agar with respective antibiotic for selection (extracted from Mahajan et al, 2019).

PCR confirmation of strains using delta mapping:

Transformants were screened for cross strain contamination using delta region amplification. Wildtype parent strains were delta amplified to confirm the transformants with their parent strains. High-fidelity PrimeStar HS PCR mastermix, TaKaRa BIO, USA (catalog no.) was employed for the amplification using delta 12 and delta 21 primers. Multiple PCR amplified products were obtained in every PCR reaction and pattern similarity was used to differentiate between different transformants and their parent strain.

Flocculation assay:

To quantitatively evaluate the flocculation of yeast cells, a 10 ml YPD media culture was prepared in a 25 ml culture tube and incubated at 30 °C and 150 rpm. The entire culture was vortexed for 1 minute, and 1 ml of it was collected to measure the OD600, which was noted as 'F1'. After allowing the tube to rest for 5 minutes, another 1 ml of supernatant was taken and the OD600 was measured, which was noted as 'F2'. This method was adapted from Dahiya et al, 2016. Flocculation ability (F) was calculated using following equation:

$$F(1-F2/F1) \times 100$$

Fermentation trial of IBA yeast:

The yeast strains used in the experiment were cultured twice before fermentation. In the first pre-culture, yeast cells were cultured in Erlenmeyer flasks containing sterile YPD medium (yeast extract 1% w/v, peptone 2% w/v, and dextrose 2% w/v; pH 5–5.5) at 30 °C for 18–20 hours with shaking at 150 rpm. A loop of glycerol stock was used to inoculate the first pre-culture. For the second pre-culture, a sterile medium consisting of 5% w/w sugar in the form of sugarcane molasses B, 0.5 g/L urea, and pre-culture 1 as inoculum (10% v/v) was used. The second pre-culture had a working volume of 40% in Erlenmeyer flasks and was cultured for 18–20 hours until reaching a cell density of approximately 2.5–3.5 × 108 cells/mL. The shake flask fermentation was carried out in Erlenmeyer flasks with a working volume of 50%. The fermentation medium, consisting of 19% w/w sugar in the form of sugarcane molasses, 0.5 g/L urea, and inoculum (pre-culture 2) 10% v/v, was sterilized. The fermentation was carried out at 30 °C with shaking at 150 rpm. Samples of the fermentation broth were collected at different time points until 48 hours and analyzed for sugar, ethanol and byproducts. (adapted from Mahajan et al, 2019).

Fermentation trial of PRY-E and PRY-X with molasses B:

The yeast strains were grown twice in advance before fermentation. In the first pre-culture, yeast cells were grown in an Erlenmeyer flask containing sterile MGYP medium (Malt extract 0.3% w/v, yeast extract 0.3% w/v, peptone 0.5% w/v, and glucose 1% w/v; pH 4.5-5) at 30 °C for 18–20 hours with shaking at 150 rpm, until the approximate OD600 was between 15–20. Glycerol stock was inoculated in pre-culture 1. The second pre-culture was in a sterile medium consisting 5% w/w sugar from sugarcane molasses B, 0.5 g/L urea, and pre-culture 1 was used as an inoculum (10% v/v). Pre-culture 2 had a working volume of 40% in an Erlenmeyer flask and was cultured for 18–20 hours until the approximate cell density was 2.5–3.5 × 108 cells/mL. Shake flask fermentation was done with a working volume of 50% in an Erlenmeyer flask. The fermentation medium was sterilized and consisted of 19% w/w sugar in the form of sugarcane molasses, 0.5 g/L urea, and inoculum (pre-culture 2) 10% v/v. Fermentation was carried out at 30 °C under shaking conditions of 150 rpm. Samples of fermentation broth were taken at different time points up to 48 hours and analyzed for residual sugar, ethanol, glycerol, and acetic acid (adapted from Mahajan et al, 2019).

Fermentation trial of PRY-E, PRY-X and PRY-G using cane syrup:

The yeast strains used in the experiment were pre-cultured twice before fermentation. To prepare pre-culture 1, yeast cells were grown in an Erlenmeyer flask with sterile MGYP medium (Malt extract 0.3% w/v yeast extract 0.3% w/v, peptone 0.5% w/v, and glucose 1% w/v; pH 4.5-5) at 30 °C for 18-20 hours with shaking at 150 rpm until the approximate cell density reached 15-20. Glycerol stock was inoculated in pre-culture 1. The second pre-culture was in a sterile medium consisting 5% w/w sugar from sugarcane syrup B, 140 mg/L urea, 56 mg/L DAP, 7 mg/L MgSO4, and 7 mg/L ZnSO4, and pre-culture 1 was used as the inoculum (10% v/v). The pre-culture 2 was then cultured in an Erlenmeyer flask at a working volume of 40% for 18-20 hours until the approximate cell density reached 2.5-3.5 x 108 cells/ml.For fermentation, a working volume of 50% was used in an Erlenmeyer flask with the sterilized fermentation medium, which contained 20% w/w sugar in the form of sugarcane syrup, 560 mg/L urea, 224 mg/L DAP, 28 mg/L MgSO4, and 28 mg/L ZnSO4, and inoculum (pre-culture 2) 10% v/v. The fermentation was carried out at 30 °C with shaking at 150 rpm. Samples of the fermentation broth were collected at different time points up to 48 hours and analyzed for residual sugar, ethanol, glycerol, and acetic acid (adapted from Mahajan et al, 2019).

Analytical methods:

- High-performance liquid chromatography (HPLC):

The collected samples of the fermentation mixture at different time intervals were analyzed for the presence of sugars (sucrose, glucose, and fructose), ethanol, and glycerol using a high-performance liquid chromatography machine. To measure the concentration of sugars, they passed the samples through a column called Aminex 87 Na+ (300 × 7.8 mm) and maintained the column temperature at 85 °C. The mobile phase used was 0.01 M of Na2HPO4 with a flow rate of 0.6 ml/min. They detected the sugars using a Refractive Index Detector (RI detector) that was kept at a temperature of 40 °C. The researchers calculated the total sugar concentration in the molasses by adding the concentrations of sucrose, glucose, and fructose. To determine the concentration of ethanol, glycerol, and acetic acid, they loaded the samples onto an Aminex 87 H+ column, maintained at a temperature of 55 °C, and used 0.005 M of H2SO4 as the mobile phase. They also used an RI detector to detect these compounds, with the same mobile phase flow rate of 0.6 ml/min (adapted from Mahajan et al, 2019).Yields were computed on *w*/*w* basis.

- Gas Chromatography:

Samples of IBA fermentation broth collected at different time points were analyzed for Iso-butanol content by gas chromatography. To determine iso-butanol concentrations in the samples, they were loaded on DB-624 (60m x 0.32mm x 1.8um) column. The temperature of the oven was 40^{o}C for 5 mins, then 10^{o}C/min to 240^{o}C for 5 mins. Nitrogen gas was used as the carrier gas at 3ml/min flow rate. The injection volume was 1.0 ul with air flow of 280 ml/min, hydrogen flow 45 ml/min and made up by nitrogen by 27 ml/min flow rate (extracted from Mahajan et al, 2019).

qPCR protocol:

LUNA qPCR Master Mix, forward and reverse primers, template DNA, nuclease-free water were used for the qPCR reaction. The reaction mix was homogenized well by pipetting and gentle vortexing. Bio-Rad CFX96 Real Time PCR Machine was employed to perform the qPCR reaction. Initial denaturation was kept at 95°C for 1-3 minutes. Amplification temperature was set at 95°C for 15-30 seconds, followed by 60°C for 30-60 seconds, for a total of 35-40 cycles. Melting curve analysis was set in the range of 60°C to 95°C with a temperature increase of 0.5°C per second. The annealing temperature for the primers was optimized based on the melting temperature of the primers and the template DNA.

CHAPTER 3: RESULTS

- Flo1 cassette generation –

Flo1 gene was amplified from freshly isolated gDNA from CENPK 2.1.C-laboratory strain. The size of the gene to be PCR amplified was calculated theoretically using yeast genome database which in turn came out to be 4.6 kB.

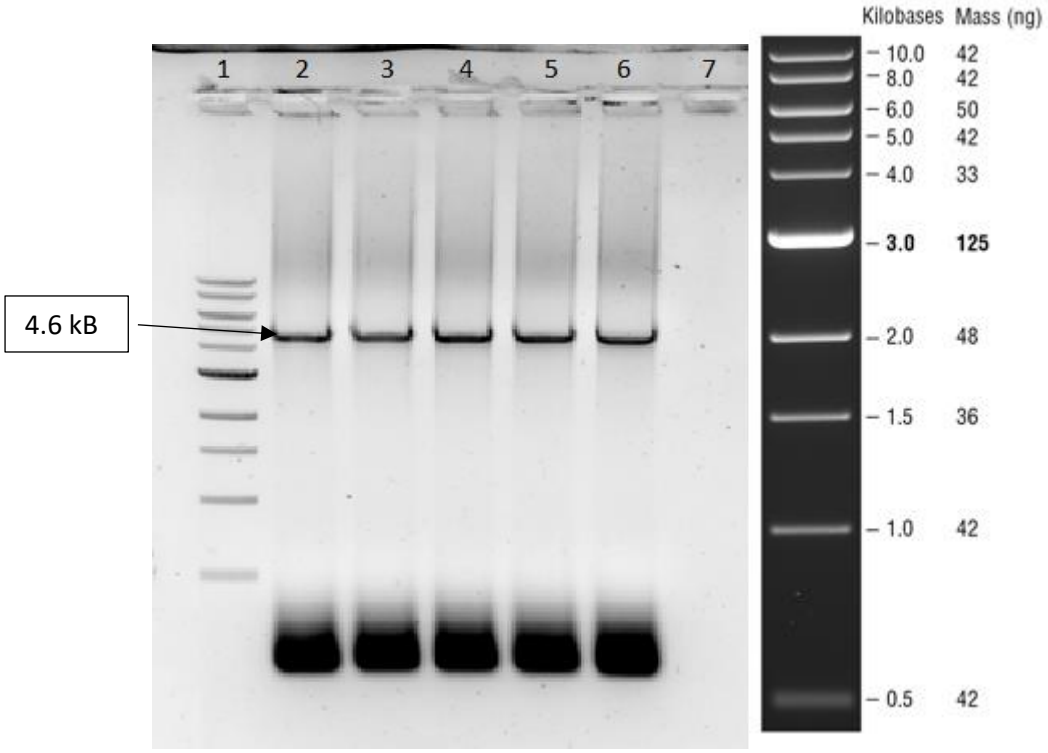

Figure 8. 1% agarose gel picture of Flo1 PCR product amplified out of CENPK 2.1.C gDNA. Well 1 was loaded with 1kB NEB DNA ladder while well 2, 3, 4, 5, and 6 contained flo1 PCR products.

The PCR product was gel extracted using MN TaKARA BIO column-based gel extraction kit. And was used as one of the templates for *flo1* transformation cassette. It was observed during the PCR amplification process that due to the size of the gene containing multiple repeats, the amplification obtained through each PCR reaction was fairly low and hence multiple PCR reaction were needed to be setup to compensate for the weak amplification.

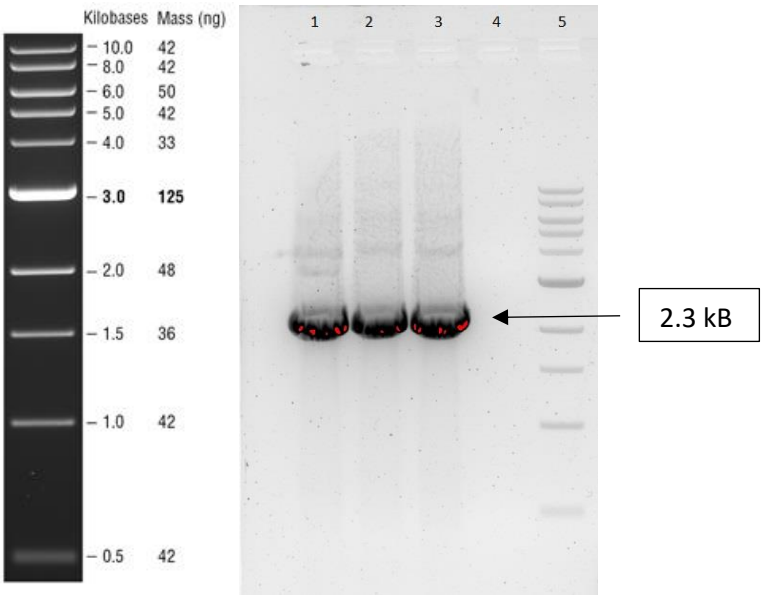

Figure 9. 1% agarose gel containing PRM9-attB-HygR-attP-downstream homologous region. Well 1, 2 and 3 contain the PCR ligated product of PRM9-attB-HygR-attP-downstream homologous region ligation reaction. Well 4 is empty and well 5 contains 1 kB NEB DNA ladder

PRM9-attB-HygR-attP-downstream homologous region were PCR ligated using high fidelity Primestar HS PCR mastermix TAKARA BIO. PRM9 terminator 250 bp which was amplified out of CENPK 2.1.C gDNA. The phiC31 site containing hygromycin B resistance gene was amplified out of Praj plasmid library, PPL::attB-HygR-attP whose expected size was 1.75 kB. Downstream homologous region to Ty1 element was selected to be 251 bp and was amplified out of CENPK 2.1.C gDNA. PCR ligation performed on these three fragments yielded a product of 2.3 kB which was gel eluted and used for *flo1* cassette transformation.

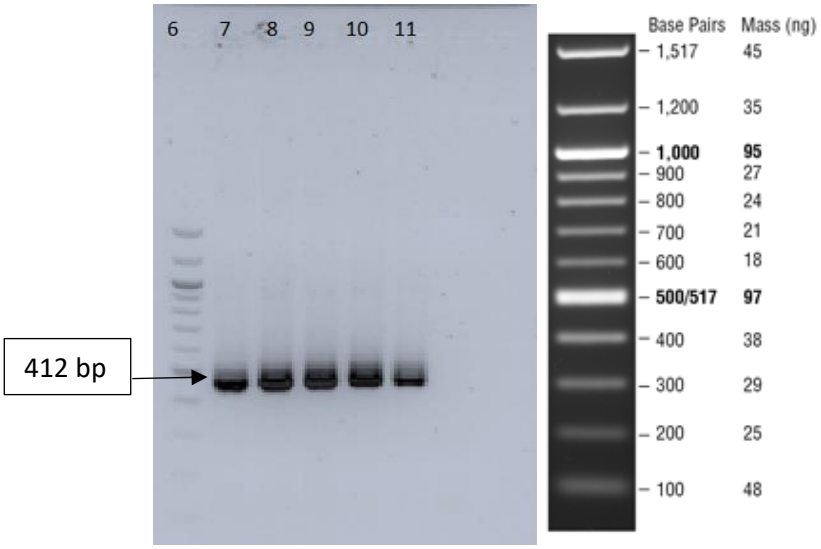

Figure 10. 1% agarose gel picture containing TEF1 promoter. Well 6 contains 100 bp NEB DNA ladder. Well 7, 8, 9, 10 and 11 contain PCR product of TEF1 promoter amplification PCR.

TEF1 promoter had the estimated size of 412 bp and was PCR amplified using high fidelity Primestar HS PCR mastermix. The PCR product was run on 1% agarose gel for size confirmation and later gel eluted to be ligated with 250 bp upstream homologous region of Ty1 element. The PCR ligation of 250 upstream homology and TEF1 promoter were used as one of the three fragments in *flo1* cassette transformation.

Yeast Transformation:

- CENPK 2.1.C *flo1* – 52 colonies were repatched onto hygromycin B containing YPD plates for confirmation and incubated at 30^{o} C for 48 Hrs. 50 out of the 52 colonies repatched showed growth confirming *flo1* cassette transformation. 18 of these colonies were further inoculated into YPD media for visual assessment of flocculation phenotype. All 18 confirmed colonies displayed varying degree of flocculation phenotype. Three out of these 18 colonies i.e. colony 15, colony 16 and colony 17 were selected for varying degrees of flocculation and further confirmation was done by employing Mat a and Mat α PCR confirmation. Since CENPK 2.1.C was the only haploid strain used in the study, a single band in the PCR product of mating type PCR was employed as an indicator for parent strain confirmation.

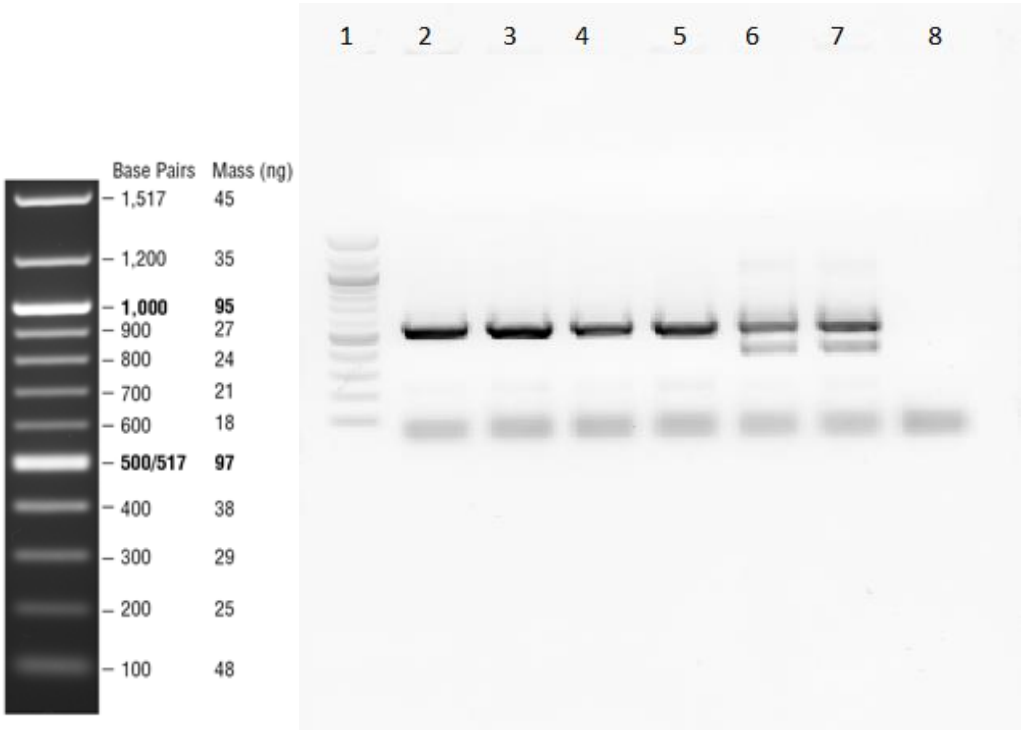

Figure 11. 2% agarose gel containing mating type PCR for CENPK 2.1.C flo1 transformants, ER and GR. Well 1 was loaded with 100 bp NEB DNA ladder. Well 2 was loaded with mating type PCR product from wildtype CENPK 2.1.C. Wells 3, 4 and 5 were loaded with mating type PCR product of colony 15, 16 and 17 respectively. Well 6 and 7 were loaded with ER and GR respectively. Well 8 was loaded with No template reaction mixture and used as a negative control for the reaction.

Since only single band was observed for the mating type PCR reaction for all 3 colonies, they were confirmed to be CENPK 2.1.C.

- PRY-E *flo1* - 42 colonies were repatched onto hygromycin B containing YPD plates for confirmation and incubated at 30^{o} C for 48 Hrs. All 42 colonies repatched showed growth confirming *flo1* cassette transformation. 21 of these colonies were further inoculated into YPD media for visual assessment of flocculation phenotype. All 21 confirmed colonies displayed varying degree of flocculation phenotype. Three out of these 21 colonies i.e. colony 24, colony 29 and colony 36 were selected for varying degrees of flocculation and further confirmation was done by employing delta mapping PCR reaction. Since each strain displayed different pattern of Ty1 region amplification gDNA of colony 24, 29 and 36 were isolated and delta mapping PCR amplification was performed using delta 12 and delta 21 primers.

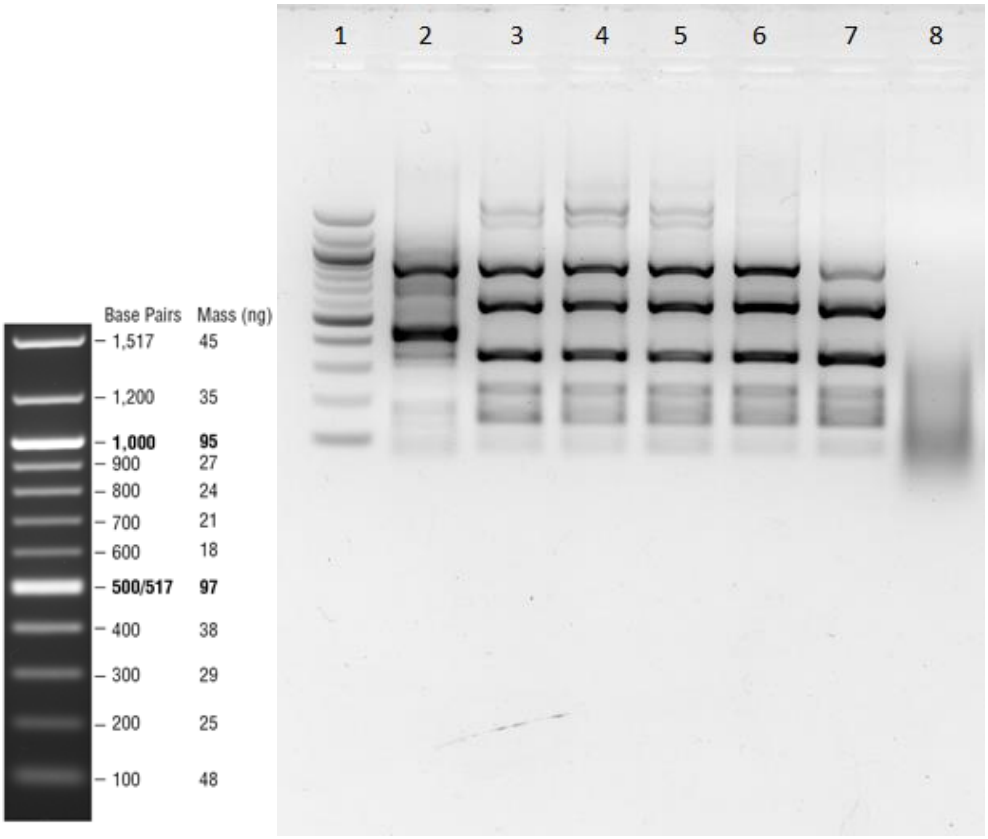

Figure 12. 2% agarose gel picture containing ER wildtype, ER flo1 transformants and BY4741 delta region amplification. Well 1 was loaded with 100 bp NEB DNA ladder. Well 2 was loaded with PCR product of delta region amplification performed on BY4741 gDNA. Well 3 contained PCR product of delta region amplification using ER wildtype gDNA used as positive control. Well 4, 5 and 6 was loaded with PCR product of delta region amplification from colony 24, 29 and 36 respectively. Well 7 was loaded with PCR product of delta region amplification using ER viability gDNA and used as control. Well 8 was loaded with No template reaction mixture and used as a negative control for the reaction.

The above gel picture (fig 12.) was used to confirm parent strains for the *flo1* transformants. As can be observed from the fig 12 that each strain displays a different pattern of Ty1 element amplification and hence was employed for the confirmation of strains.

- PRY-98 *flo1* - 4 colonies were repatched onto hygromycin B containing YPD plates for confirmation and incubated at 30^{o} C for 48 Hrs. All 4 colonies repatched showed growth confirming *flo1* cassette transformation. All 4 of these colonies were further inoculated into YPD media for visual assessment of flocculation phenotype. 3 out of 4 confirmed colonies displayed varying degree of flocculation phenotype. These three colonies i.e. colony 1, colony 2 and colony 3 were selected for varying degrees of flocculation and further confirmation was done by employing delta mapping PCR reaction. Since each strain displayed different pattern of Ty1 region amplification gDNA of colony 24, 29 and 36 were isolated and delta mapping PCR amplification was performed using delta 12 and delta 21 primers.

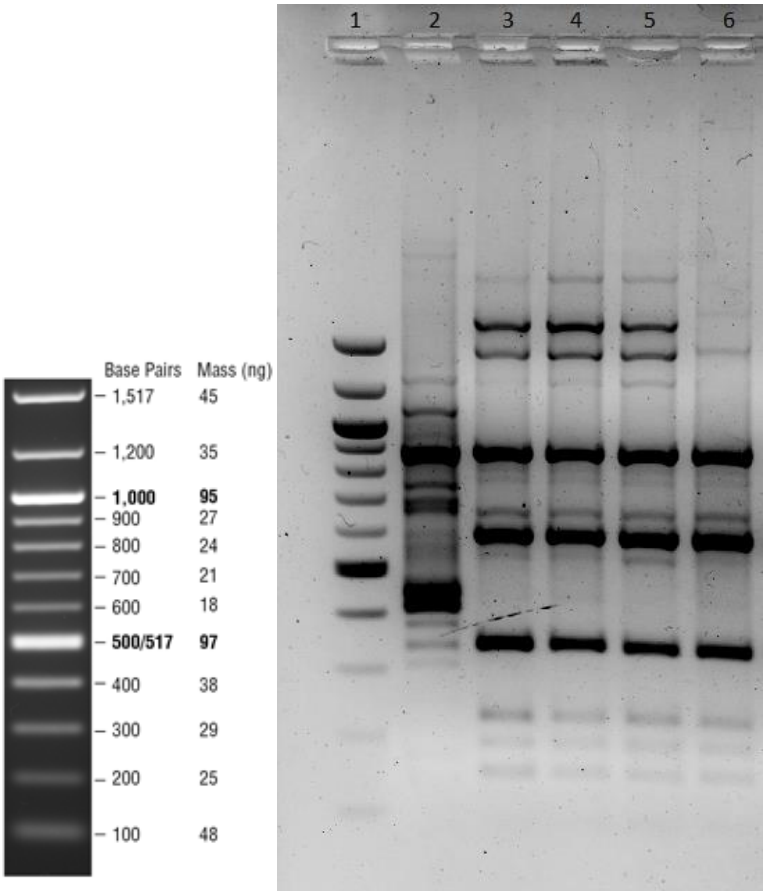

Figure 13. 2% agarose gel picture containing PRY-98 wildtype, PRY-98 flo1 transformants and BY4741 delta region amplification. Well 1 was loaded with 100 bp NEB DNA ladder. Well 2 was loaded with PCR product of delta region amplification performed on BY4741 gDNA. Well 3, 4 and 5 contained PCR product of delta region amplification using colony 1, 2 and 3. Well 6 contained PCR product of delta region amplification using PRY-98 wildtype.

PRY-X *flo1* – 16 colonies were repatched onto hygromycin B containing YPD plates for confirmation and incubated at 30^{o} C for 48 Hrs. All 4 colonies repatched showed growth confirming *flo1* cassette transformation. All 4 of these colonies were further inoculated into YPD media for visual assessment of flocculation phenotype. All 16 confirmed colonies displayed varying degree of flocculation phenotype but only 2 colonies displayed prominent flocculation phenotype. These two colonies i.e. colony 15 and colony 16 were selected for varying degrees of flocculation and further confirmation was done by employing delta mapping PCR reaction.

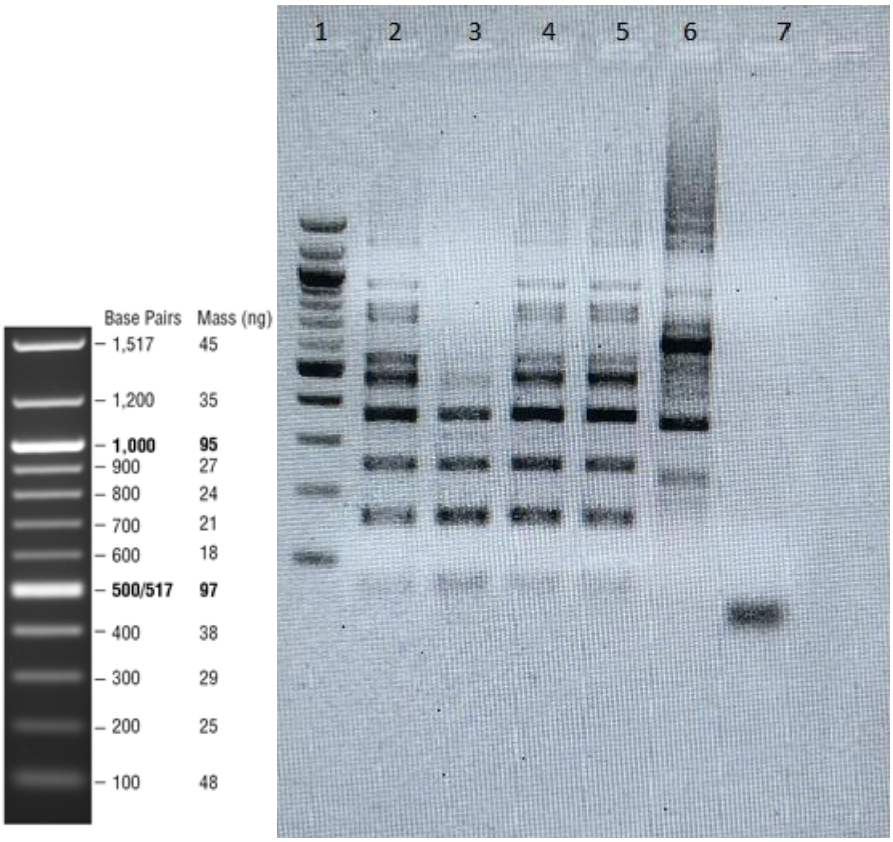

Figure 14. 2% agarose gel picture containing PRY-X wildtype, PRY-X flo1 transformants and BY4741 delta region amplification. Well 1 was loaded with 100 bp NEB DNA ladder. Well 2 was loaded with PCR product of delta region amplification performed on PRY-X wildtype gDNA. Well 3, 4 and 5 contained PCR product of delta region amplification using colony 15, 16 and viability respectively. Well 6 contained PCR product of delta region amplification using BY4741. Well 7 has no template PCR reaction for negative control.

Flocculation assay:

Flocculation efficiency for our *flo1* cassette containing strains were compared against their wildtype strains. The number of cells settled in 5 mins were calculated in %.

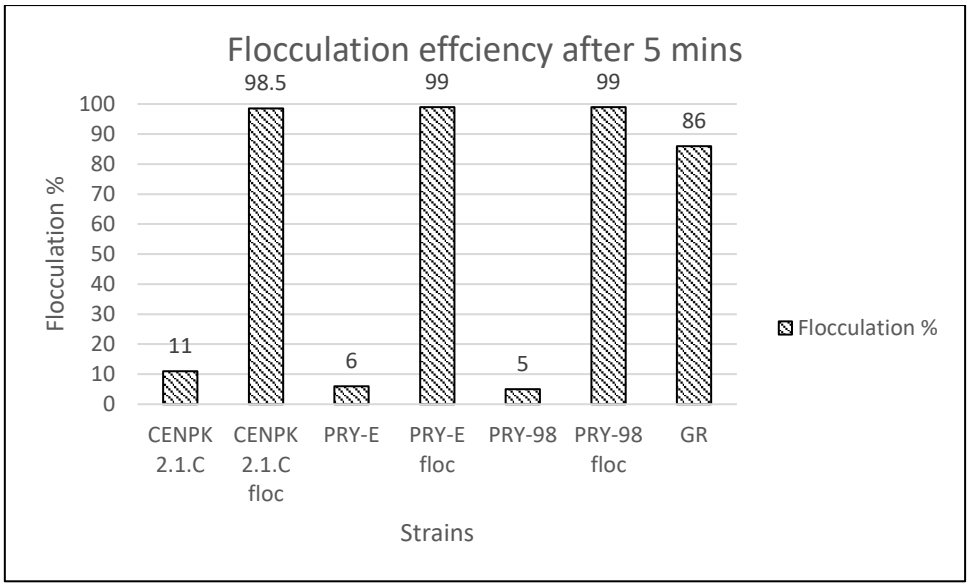

Figure 31 This graph depicts the percentage efficiency of flo1 transformed cells to settle down and form flocs.

IBA fermentation:

Iso-butanol fermentation was performed using 6 Erlenmeyer flask – C1, C2, C3, C7, C8, C9 contained PRY-98 wildtype strain while F4, F5, F6, F10, F11, F12 were inoculated with PRY-98 flo1 colony 36 and their OD600 was normalized during inoculation of main fermentation. Samples were taken at different time points (16Hrs, 24hrs and 40Hrs) and analyzed for iso-butanol levels and sugar residue to determine the length of fermentation run.

- 16 hrs sample data -

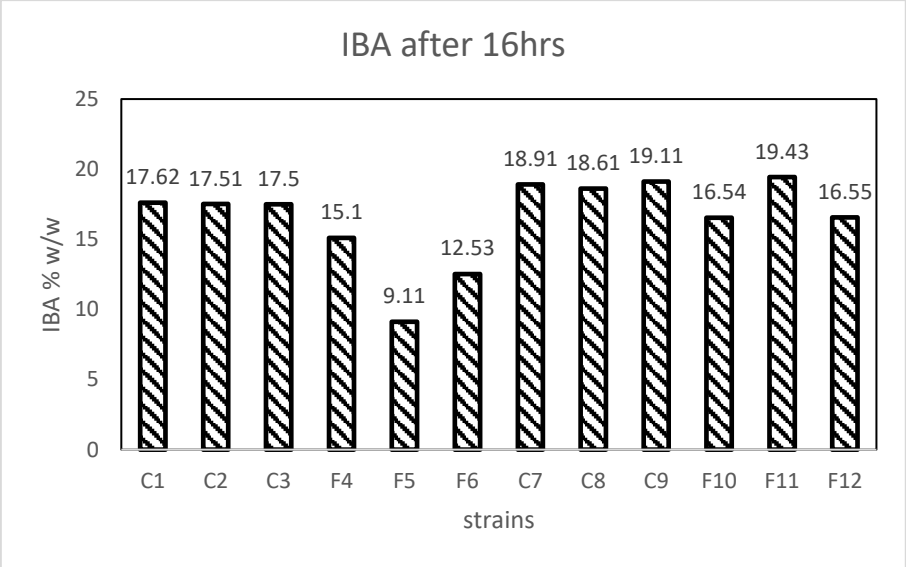

Figure 15. Isobutanol production graph for each flask after 16 Hrs. C1, C2, C3, C7, C8 and C9 were wildtype PRY-98 strain. F4, F5, F6, F10, F11, and F12 was PRY-98 flo1 transformant colony 36.

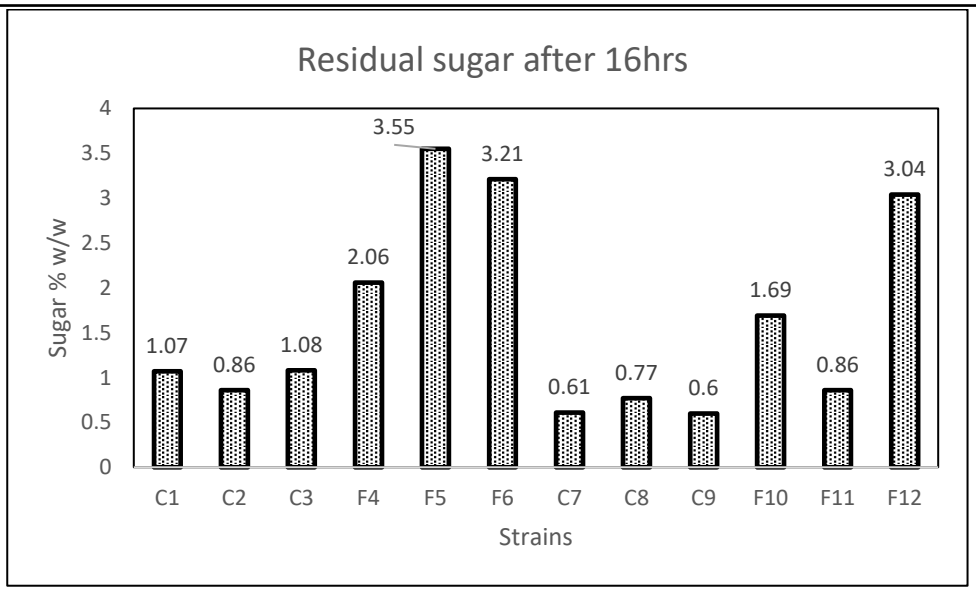

Figure 16. Total fermentable sugar levels i.e. sum of sucrose, glucose and fructose levels is indicated as residual sugar in the above graph. C1, C2, C3, C7, C8 and C9 were wildtype PRY-98 strain. F4, F5, F6, F10, F11, and F12 was PRY-98 flo1 transformant colony 36.

F5 displayed the least amount of IBA but contained the most amount of residual sugar remaining. Since there was residual sugar left in the broth this trend was not attributed as an anomaly. All other samples displayed comparable values of iso-butanol production.

- 24hrs sample data -

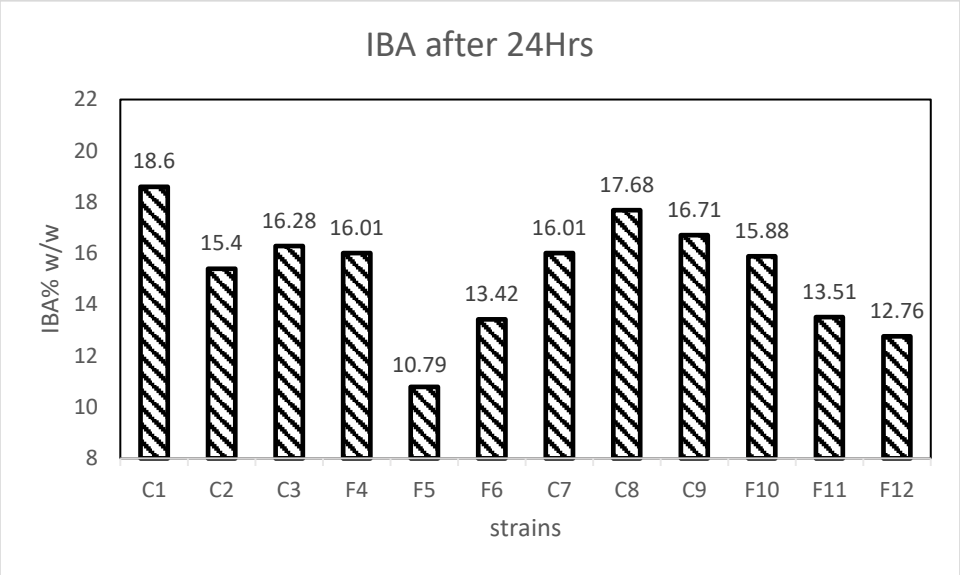

Figure 17. Isobutanol production graph for each flask after 24 Hrs. C1, C2, C3, C7, C8 and C9 were wildtype PRY-98 strain. F4, F5, F6, F10, F11, and F12 was PRY-98 flo1 transformant colony 36.

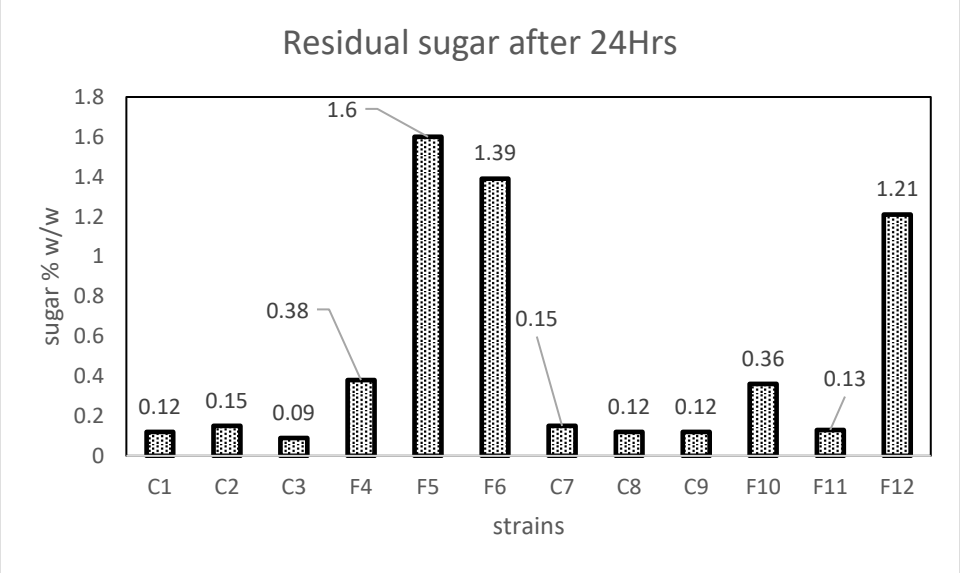

Figure 18 Total fermentable sugar levels i.e. sum of sucrose, glucose and fructose levels is indicated as residual sugar in the above graph. C1, C2, C3, C7, C8 and C9 were wildtype PRY-98 strain. F4, F5, F6, F10, F11, and F12 was PRY-98 flo1 transformant colony 36.

The drop in iso-butanol values from 16hrs to 24hrs could indicate the reversal of the cycle for the production of iso-butanol. This in turn could be reflected in the form of other by-products like Diacetyl, ethanol or glycerol. But even after 24hrs the consumption of sugar was in proportion to production of IBA or other by-products.

- 40Hrs sample data-

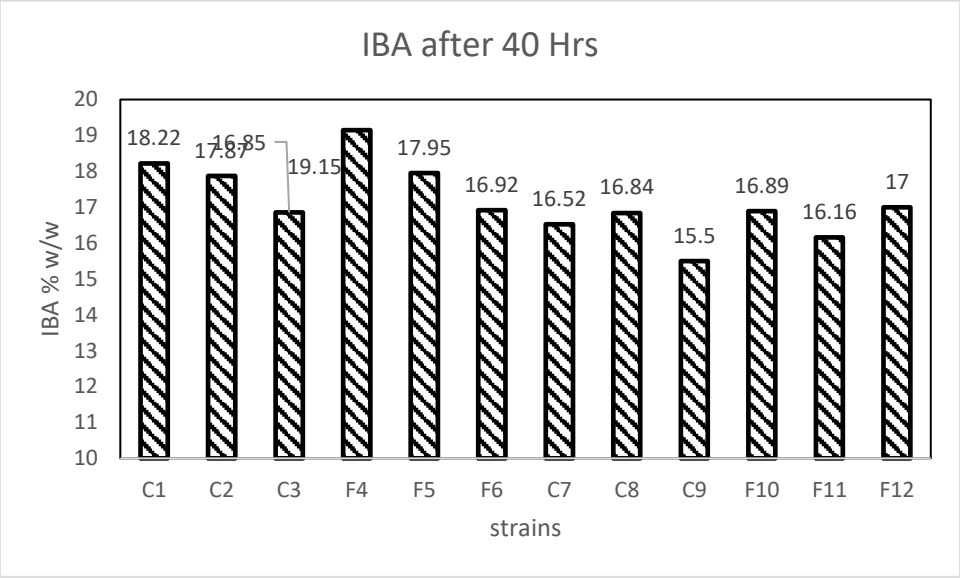

Figure 19 Isobutanol production graph for each flask after 40 Hrs. C1, C2, C3, C7, C8 and C9 were wildtype PRY-98 strain. F4, F5, F6, F10, F11, and F12 was PRY-98 flo1 transformant colony 36.

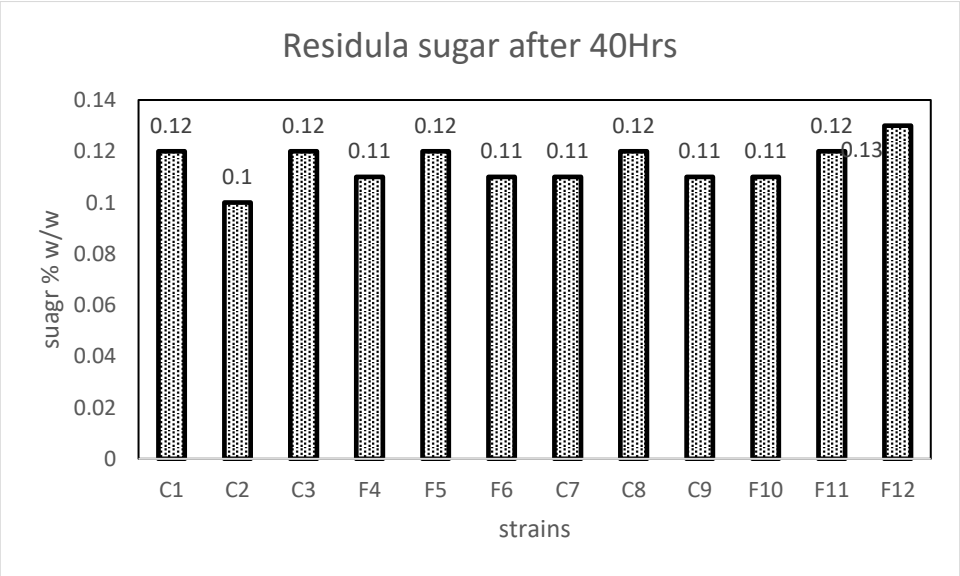

Figure 20. Total fermentable sugar levels i.e. sum of sucrose, glucose and fructose levels is indicated as residual sugar in the above graph. C1, C2, C3, C7, C8 and C9 were wildtype PRY-98 strain. F4, F5, F6, F10, F11, and F12 was PRY-98 flo1 transformant colony 36.

After 40 Hrs most of the sugar in the broth was consumed by the cells. This marked the end of the fermentation run. Looking at the 40 hrs iso-butanol levels it is clear that there is little to no difference in production efficiency of PRY-98 even after transformation of *flo1* cassette.

Molasses B fermentation-

Ethanol fermentation of mollasses B was performed using 3 erlenmeyer flask each for every strain. 4 strains were used in the study – ER, ER floc(colony 24), XP, XP floc(colony 16). OD600 was normalized for each strain at the start of the main fermentation and the total sugar at the start of the main fermentation was measured to be 19% w/w. Samples were taken at 16, 20 and 24Hrs and were analyzed using HPLC.

- 16Hrs sample data-

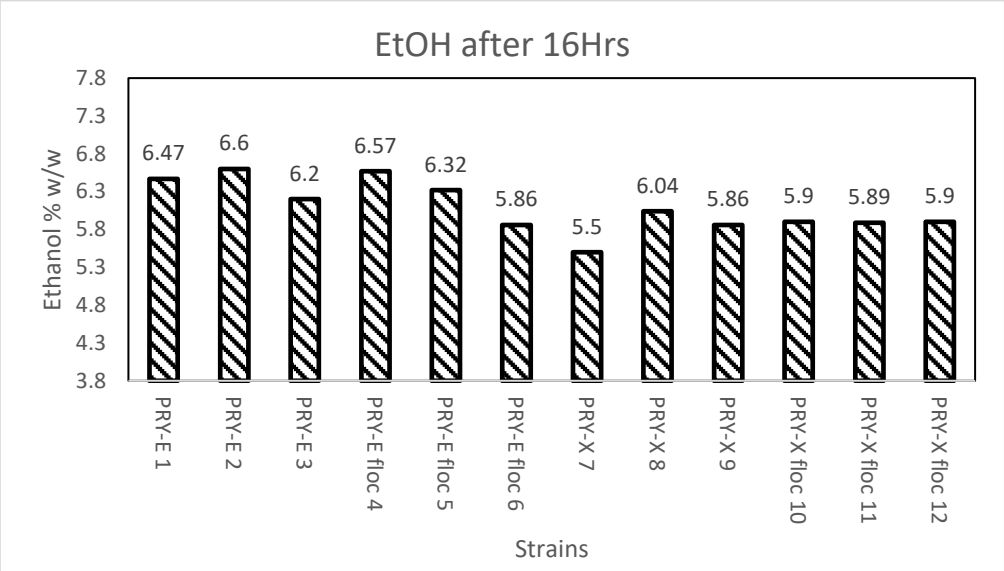

Figure 21 Ethanol percentage w/w for all the strains

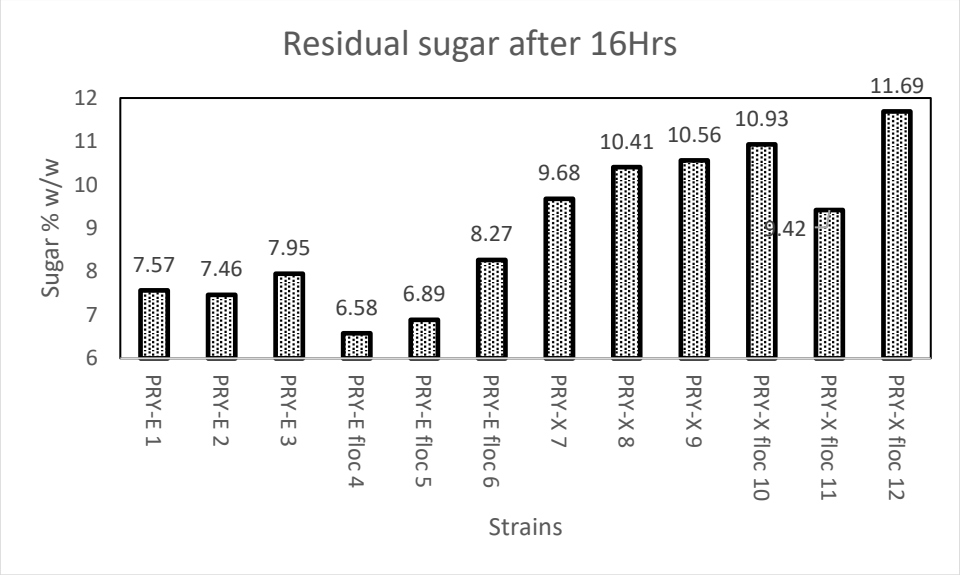

Figure 22 Total fermentable sugar levels w/w for all flasks

The 16Hr samples displayed conventional trend of ethanol production against the consumption of sugar. It was also observed that PRY-X and PRY-X floc have slower rate of sugar consumption and ethanol production.

- 20Hrs sample data -

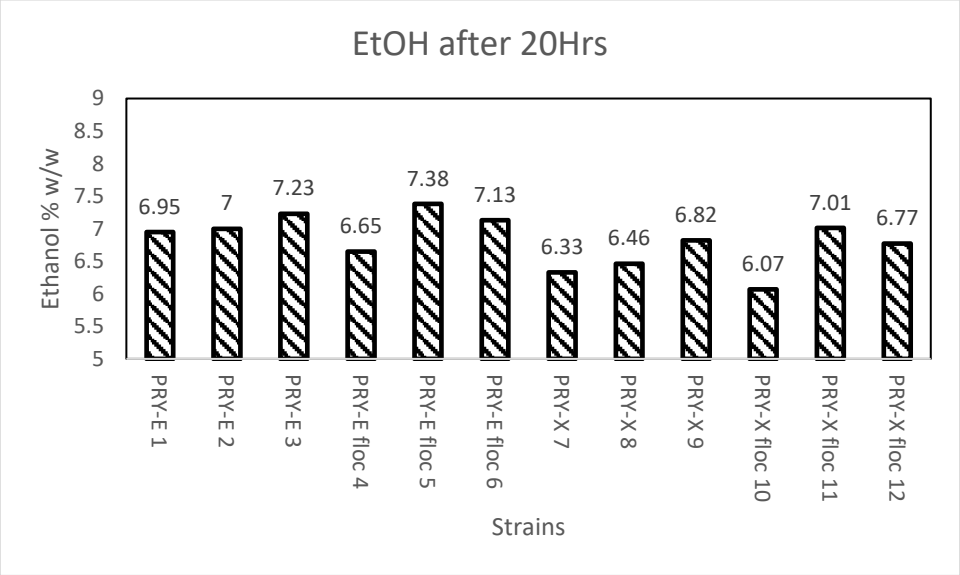

Figure 23 Ethanol percentage w/w for all the strains

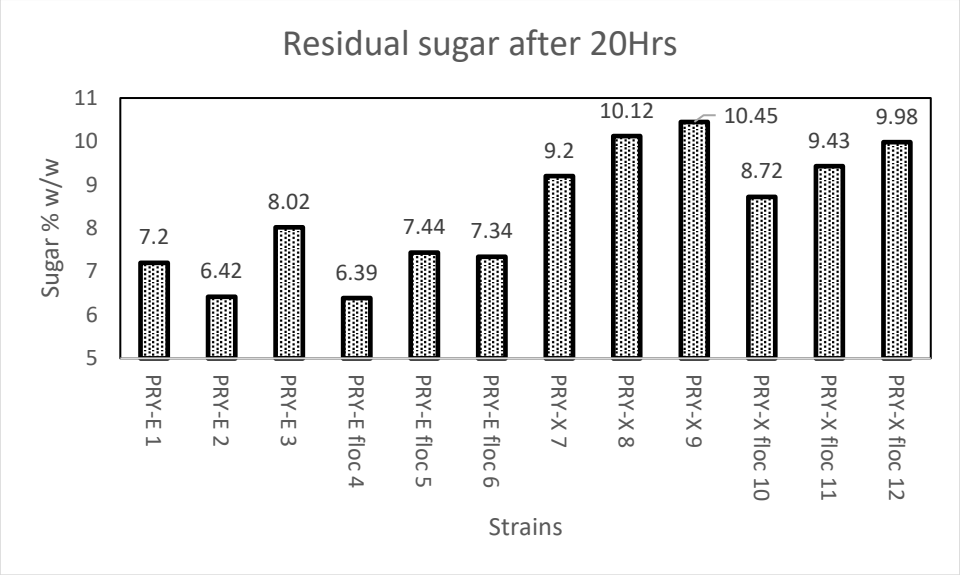

Figure 24 Total fermentable sugar levels w/w for all flasks

The 20Hr samples displayed conventional trend of ethanol production against the consumption of sugar. It was also observed that PRY-X and PRY-X floc have slower rate of sugar consumption and ethanol production.

- 24Hrs sample data-

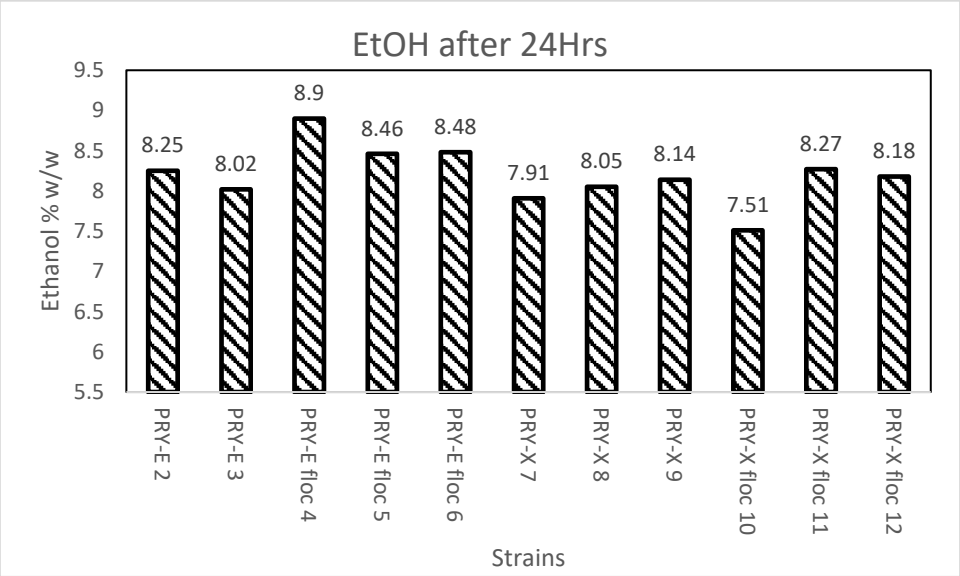

Figure 25 Ethanol percentage w/w for all the strains

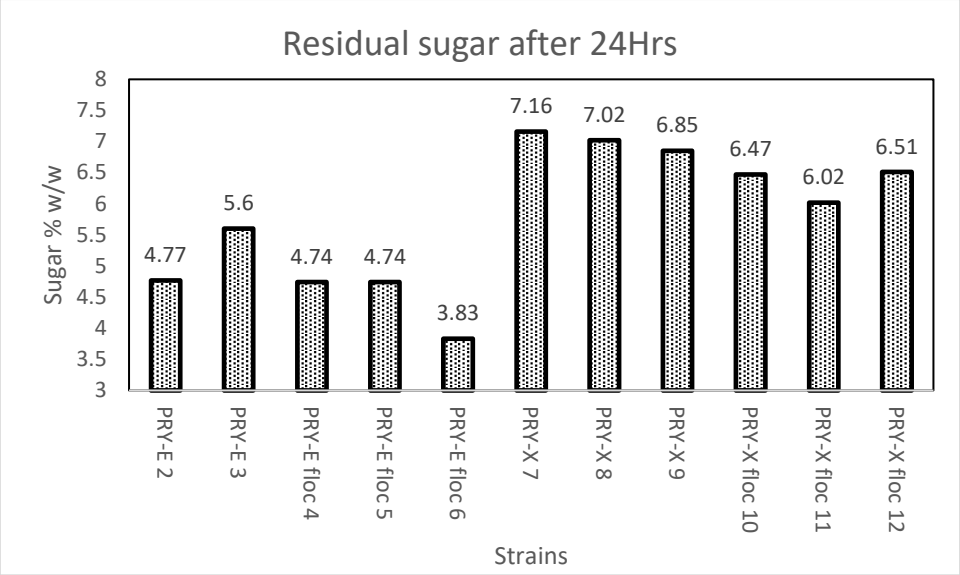

Figure26. Total fermentable sugar levels w/w for all flasks

Fermentation process was stopped after 24Hrs and samples were analyzed using HPLC for ethanol and by-product formation. Ethanol levels were also confirmed using distillation and Anton Paar. The distilled broth yielded comparable levels of ethanol produced i.e. ~ 10% v/v. ER and XP wildtypes as well as *flo1* transformants displayed comparable amount of ethanol.

Cane syrup fermentation:

Ethanol fermentation of cane syrup was performed using 3 erlenmeyer flask each for every strain. 5 strains were used in the study – ER, ER floc(colony 24), XP, XP floc(colony 16) and GR. OD600 was normalized for each strain at the start of the main fermentation and the total sugar at the start of the main fermentation was measured to be 22% w/w. Samples were taken at 28, and 48Hrs and were analyzed using HPLC.

- 28Hrs sample data-

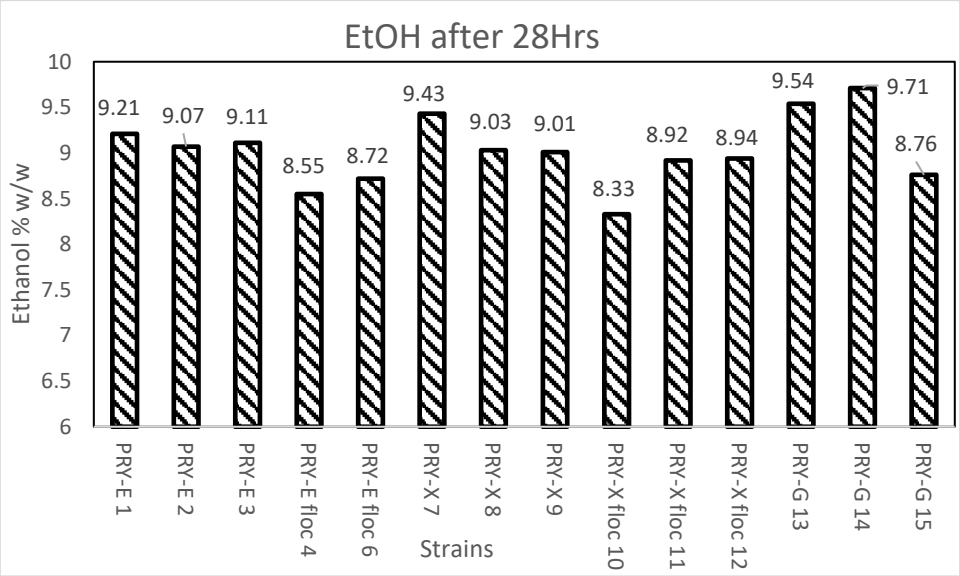

Figure 27. Ethanol percentage w/w for all the strains

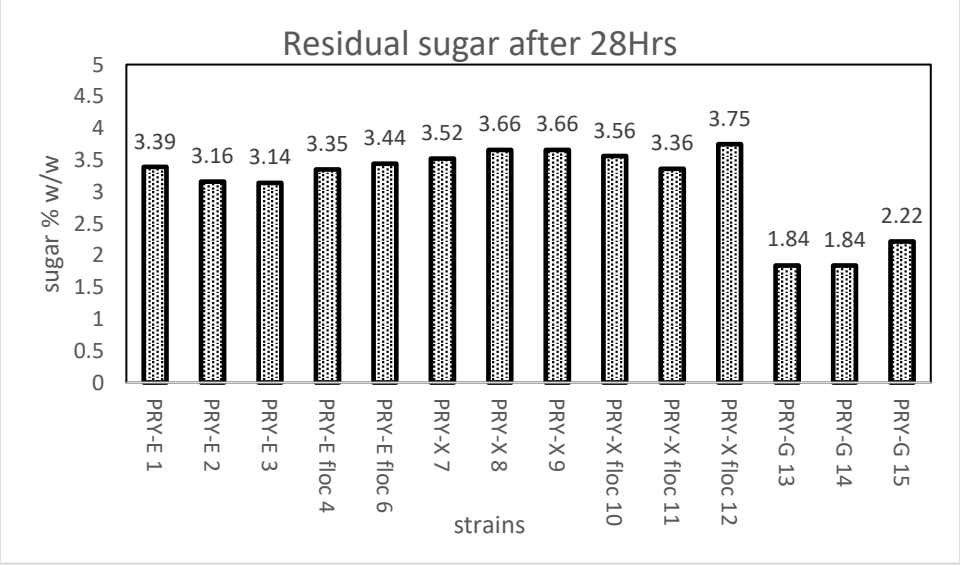

Figure 28. Total fermentable sugar levels w/w for all flasks

The 28Hr samples displayed conventional trend of ethanol production against the consumption of sugar. It was also observed that PRY-G has higher rate of sugar consumption and ethanol production.

- 48Hrs sample data-

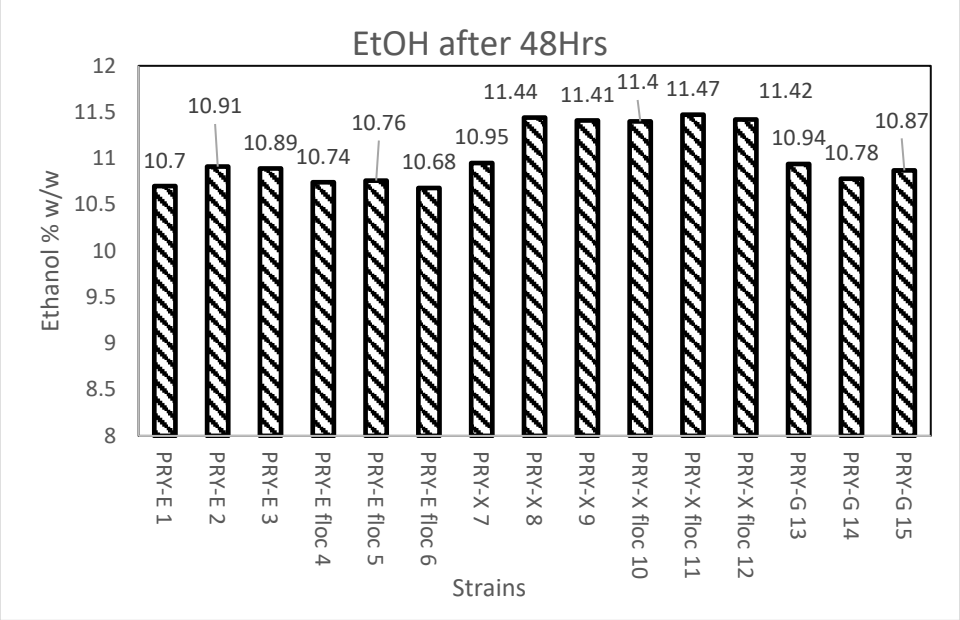

Figure 29 . Ethanol percentage w/w for all the strains

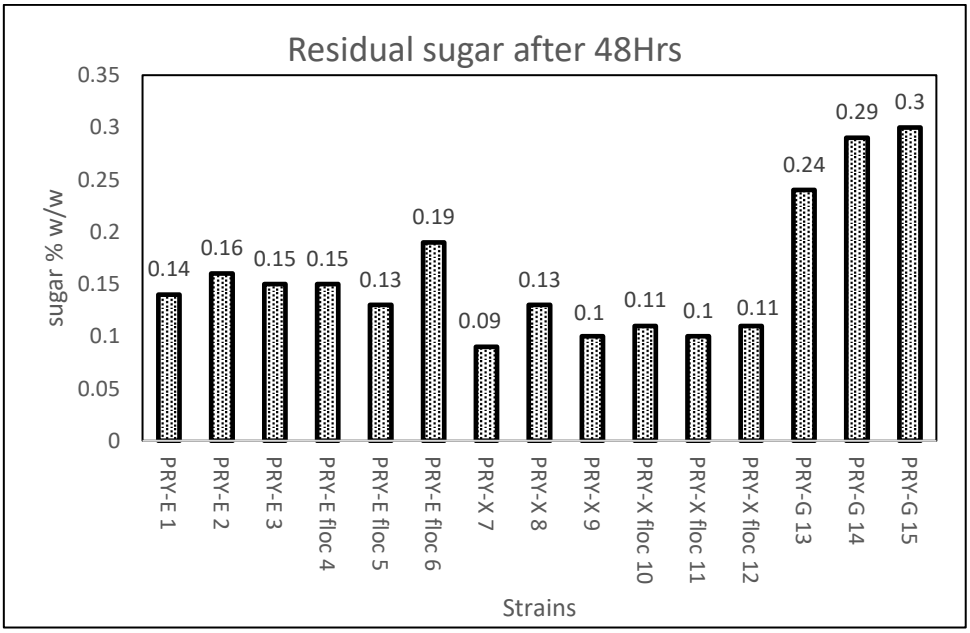

Figure 30 Total fermentable sugar levels w/w for all flasks

Fermentation process was stopped after 48Hrs and samples were analyzed using HPLC for ethanol and by-product formation. Ethanol levels were also confirmed using distillation and Anton Paar. The distilled broth yielded comparable levels of ethanol produced i.e. ~ 13% v/v. XP wildtypes as well as *flo1* transformants displayed higher amount of ethanol production as compared to ER and PRY-G amount of ethanol i.e. ~ 14%.

CONCLUSION AND OUTLOOK:

In industrial fermentation processes that use non-flocculent strains of *Saccharomyces cerevisiae*, the yeast cells remain dispersed in the liquid medium, which makes it challenging to separate them from the final product. The separation process is typically time-consuming and costly, often involving procedures like centrifugation or filtration.

In the biofuels industry, flocculation affects the efficiency of the fermentation process. Yeast strains with high flocculation efficiency can settle out of the fermentation broth more quickly, allowing for easier separation of the yeast from the biofuel product. This can result in higher yields and reduced processing time, leading to more cost-effective biofuel production.

The understanding of the roles and regulation of FLO genes and their encoded proteins is important for improving industrial processes that rely on yeast, as well as for understanding the basic biology of yeast adhesion and its impact on various phenotypes.

Through this study we were able to gain insight into the process of flocculation and the introduction of it into different strains helped us visualize more cost-efficient models for ethanol and bio-fuels production. The tendencies of cells to settle down in a matter of minutes can be used for conceptualization of better continuous fermentation process and the robust nature of this flocculation phenotype has opened new line of thinking for the future of industrial ethanol production.

The strains can be further optimized by testing the varying degrees of flocculation and finding the most feasible percentage. qPCR study for *flo* genes would be able to give us a better insight and future direction in regards to the correlation between copy number and fermentation efficiency of a strain while bearing the burden of an extra cell wall protein.

Through this study we were able to obtain a genome integrated *flo1* mutant with varying degrees of flocculation which are anti-biotic marker free. Further research to find optimum feedstock and fermentation conditions for the *flo1* mutant industrial strains would award us with lesser carbon footprint and better energy efficiency.

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