Change in Flocculation Level of a Targeted Yeast Strain

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BIOL 324

4/28/22

Abstract

Flocculation, which is the clumping of yeast cells, is a necessary component in the brewing industry, as the level of flocculation determines the yeast content and flavor of a beer. Modifying yeast strains is a tool used to control flocculation levels and alter a beer's characteristics. The purpose of this experiment was to increase flocculation level of yeast strain WLP570 through a promoter swap of the GAL1 within the *FLO11* gene. The *FLO11* gene affects cell adhesion and flocculation ability while GAL 1 regulates the gene's expression. It was hypothesized that the promoter swap of the target strain WLP570 would lead to an increase of flocculation level due to the GAL 1 increasing *FLO11* expression and as a result increasing flocculation level. The goal is to distinguish what level of flocculation the altered strain reaches and compare to the controls to determine its new level. This was done through genomic modification to gain control of expression of a gene using PCR, gel electrophoresis, yeast transformation and an assay. We anticipated an increase in flocculation level.

Introduction

An essential component in any brewers process of crafting the perfect strain, yeast flocculation is the formation of flocs through cell adhesion. This reversible process uses lectin-like proteins, called flocculins, which protrude from the cell wall, to bind to mannose residues of neighboring yeast cells through calcium ion activation (Verstrepen et al., 2003). Resulting in the separation of the flocs from the medium they're in, these clumps of cells either go through sedimentation or rise to the surface, depending on which type of yeast strain they are. This separation from the flocs determines the flavor and yeast content of the beer, with the complex process relying on specific flocculant genes to ensure the best outcome.

These flocculant genes, also called FLO genes, can help dictate the flocculation ability of the yeast. The FLO genes include *FLO*1, *FLO*5, *FLO*8, *FLO*9 and *FLO*11. Most of these genes are very similar to another, such as the FLO1, FLO5 and FLO9, while FLO8 differs from the others as it is responsible for encoding the transcriptional activator of the FLO genes. Absence of the FLO8 gene means that flocculation is unable to occur due to transcription of the FLO genes not being possible (Vestrepen et al., 2003). The *FLO11* gene is unique in that where other FLO genes are adjacent to their telomeres, *FLO11* is neither next to a telomere nor a centromere, where close proximity to teleomers often results in mutation and genetic recombination (Halme et al., 2004). This is important to note as those genes closer to telomeres are more likely to be transcriptionally repressed through telomeric silencing, meaning that gene expression is reduced or does not occur.

For studying FLO genes and how they impact flocculation, *FLO11* is a more dependable gene as it is more likely to not be silenced, and therefore be expressed (Vidgren et al., 2011). This is necessary as *FLO11* is a major cell adhesion molecule, with changes in the gene or the promoter resulting in significant changes in cell adhesion, which can lead to flocculation going array (Cullen et al., 2012). Other functions of *FLO11* include pseudohyphal growth, invasive growth and biofilm formation. These are important characteristics of this gene since both biofilm formation and pseudohyphal growth have been linked to increased adhesive ability of the yeast cells, which means increased ability to flocculate (Guo, 2000; Jin et al., 2003).

Flocculation is also influenced by factors such as strain type, and environmental factors like pH and temperature, which affect the cell to cell interactions and Flo protein activation. The cell to cell interactions include how often the cells are colliding, with an increase in collision promoting flocculation. The factors can influence activation as certain yeast strains prefer

different conditions, such as certain pHs causing flocculins to become inactive (Vestrepen et al., 2003). Flocculation of yeast can also be inhibited depending on if it is a FLO1 phenotype or a NewFloe phenotype. FLO1 phenotypes are inhibited by mannose, with the mannose taking the flocculins binding site, resulting in the cells no longer being able to bind together. NewFlo flocculation types are also inhibited mannose, along with glucose, sucrose and maltose. The gene of interest, the *FLO11* gene, has been found to be inhibited by mannose only, therefore being classified as a Flo-1 type flocculin (Bayly et al., 2005).

Due to *FLO11* being critical for cell adhesion as well as its location away from telomeres, it was chosen as the target gene in manipulating flocculation level. The goal of this project was to conduct a gene modification of a yeast strain through homologous recombination to observe a change in its flocculation level due to *FLO*11 expression. The strain chosen was BY4741, which is a non-flocculant laboratory strain. This strain is a non-flocculant due to a mutation of the *FLO8* gene, where it is unable to encode the transcriptional activators. Through a promoter swap, the *FLO*11 promoter was replaced with the pGAL1 and kanamycin resistance (KAN^R) gene (Figure 1). The promoter of the *GAL1* gene is used because it aids in the digestion of galactose, meaning when galactose is added the transcription of the gene is induced, which would then turn on *FLO11* (West et al., 1984). It was hypothesized through a promoter swap of *FLO11* promoter to pGAL1, there would be an increase in flocculation level due to overexpression of *FLO*11.

Materials and Methods

Experimental Design

To design this experiment, three factors were needed: a target gene, the wanted genetic change and a model strain. The chosen target gene was *FLO11* due to its proximity to the

telomere and it playing a large role in cell adhesion. To ensure that changes made to FLO11 were distinguishable, the laboratory strain BY4741 was chosen. Since it is a non-flocculant, any changes in flocculation in this model strain can be attributed to the modification of the *FLO11* gene. The modification of the *FLO11* gene was a promoter swap, swapping the FLO11 promoter for pGAL1.

PCR Reaction Setup

PCR was conducted with plasmid template pFA6-KanMX6-pGAL1 to make the amplicons needed for transformation (Longtine et al., 1998). These amplicons or DNA fragments contained pGAL1 and the KAN^R gene. Through homologous recombination, the amplicons were mixed into the yeast genome through homologous recombination, replacing the *FLO11* promoter. To achieve the recombination, primers were designed for the amplicons that were homologous to the *FLO11* gene. The forward primer contained 40 nucleotides and skipped 150 nucleotides upstream of the start codon. The F4 primer designed by Longtine was joined to this sequence to complete the forward primer. The reverse primer was identified by finding the first ATG of *FLO11*, and selecting the next 40 nucleotides, connecting the R2 primer designed by Longtine to complete the reverse primer.

These primers were then used to create the amplicons for PCR. In a 200 µl PCR tube, two microliters of DNA template (concentration 2ng/µl) was added with 28 µl distilled water and, 10 µl Forward Primer (concentration 4mM), 10 µl Reverse Primer (concentration 4mM) and 50 µl of 2x Master Mix. PCR tubes then went into the Thermal Cycler, and cycled according to the thermal profile for the PCR Program : 94^OC for 4 minutes, 20 cycles at 94^O C for 60 seconds, 55^O C for 60 seconds, then 72^O C for 2 minutes. Then the sample sat at 72^O C for 7 minutes. The PCR reaction was then stored in the freezer at 4^O C until further use.

Gel Electrophoresis/ Purification

Gel electrophoresis was run to confirm that DNA fragments were of the expected size following PCR. The DNA was prepared to be inserted into 1% Agarose gel by adding three microliters of the ampicon from PCR with 5 µL of water and 2 µL of 6X Loading Buffer. The solution was transferred and electrophoresis was run on a 1% agarose gel with 1XTAE. The gel was run at 100 volts until the blue dye reached two/thirds of the way through the gel.

To purify the DNA product from PCR, multiple washes were done, following the Monarch ® PCR and DNA Cleanup Protocol. To start, 50 µL of the DNA and 250 µL binding buffer were mixed and centrifuged for a minute and the flow-through was discarded. 200 µL of wash buffer was added and centrifuged again. This wash step was repeated twice. After the final wash step and the flowthrough was discarded, the solution was centrifuged again for 30 seconds to dry the tube. The DNA was then eluted twice with 20 µL of Elution buffer. The flow through solution was then spected using a Nanodrop to determine the concentrations of DNA. After concentrations were recorded an image of the agarose gel was taken.

Transformation

Yeast transformation was done to create a modified yeast strain, using the lab grown strain BY4741. The cells were transferred to a 1.5 ml conical tube and pelleted. The supernatant was discarded and cells were washed twice with 1 ml of sterile, distilled water. and then the cells were suspended by shaking. The cells were pelleted again for one minute in the centrifuge. Since two transformations were done, two samples were made. Both samples used 1 µg of purified PCR generated DNA. The amplicon DNA was prepared for transformation by mixing the samples with sterile water to reach a final volume of 34 µg. The samples were then vortexed and

suspended in 240 µl 50% PEG 3350, 36 µl 1M LiAcetate and 50 µl Carrier DNA (at 2.0mg/ml). and 34 µl PCR generated DNA (amplicon). The samples were incubated for 20 minutes at room temperature and then for 20 minutes at 42^{O}C in a water bath. The cells were then pelleted and resuspended in 1ml YPD and incubated for 2-3 hours at 20^{O}C. Then 1ml of the cell mixture was spread using glass onto a YPD and G418 coated petri dish and incubated at 30^{O}C for 2-4 days.

Flocculation Assay

A flocculation assay was performed to quantify the flocculation levels of the different yeast strains using a version of the Helm et al. 1953 protocol. For each yeast strain tested, 1.5ml of the yeast culture was centrifuge the samples for 1 minute at 18,000g in a microcentrifuge to remove it from the YPD media. The yeast pellet was resuspended in an EDTA wash using 1ml of 5mM EDTA (0.5mM) to remove calcium. This was done twice for a total of two washes. The yeast pellet was then resuspended in 1000 µL of deionized water to remove EDTA. The yeast pellet was resuspended in 1500 µL of the 50mM Citrate Buffer at pH 6. Each yeast sample, was split into two groups, a control and an experimental group. The control groups (A) would have no calcium added while the experimental group (B) added calcium. To each tube 700 µL of the yeast suspension was added To the un-flocculated control tubes (A), 300 µL of deionized water was added. To the experimental reaction tubes (B), 200 µL of deionized water and 100 µL of 0.5 $CaCl_2$ was added. The tubes sat for 5 minutes. Optical density (OD_{660}) of the samples were determined using a spectrophotometer. The absorbance reading was set at wavelength 660nm. The flocculation percentage was calculated using the formula below.

□□ = 100 * (□−□)/□

The assay was done three times for all experimental strains and once for control strains.

Results

PCR Verification

To verify that PCR had worked, gel electrophoresis was run to see if the samples were around the expected base pair length. The agarose gel after electrophoresis displayed that both samples A and B fell around 2 kilobases, as seen with the distinct band (Figure 2). This matches with the expected 2081 base pairs as seen with Longtine et al., 1998. The two samples were compared to the marker, with the NEB 1KB Ladder being used with the Catalog #N3232S. K1 and K2 are two independent reactions with the same sample and product, with the K2 sample being used for this lab. The primer sequences used were the YIR019C_SW forward and reverse (Table 2). To determine the concentration of the DNA, it first needed to be cleaned up and purified. This was done using the Monarch ® PCR and DNA Cleanup protocol. Using a Nanodrop to conduct spectrophotometric analysis, the DNA concentration to be 61.8 ng/µl and the total DNA yield was calculated to be 2.472 µg.

Transformation

To complete the promoter swap of the FLO11 gene to pGAL1, the yeast cells needed to go through yeast transformation to create a modified strain (Figure 3). Since there was no cell growth on the plates containing kanamycin, it meant that transformation was unsuccessful. The transformation was done again, this time changing the heatbath period from 40 minutes to 20 minutes, to decrease heat shock. This attempt also resulted in no growth, indicating transformation failed again. Due to limited time, a third attempt at transformation was not done.

Flocculation Assay

A Helms Assay was conducted to determine the percent flocculation of the yeast strains and to compare those values to those of the expected values that were registered by Lab Strains and Red Star (Table 1). Calcium was removed from the yeast cells using a EDTA wash to ensure that cells were unable to flocculate, as calcium is needed to form flocculins. Each strain was separated into two groups, an un-flocculated control and experimental group. No calcium was added to the control group and calcium was added to the experimental group to allow for flocculation. Each strain went through 3 repeats and the control strains went through a single trial. OD_{660} was measured to assess how much flocculation occurred and quantify the values of the yeast strains. To assess if the stains matched their expected flocculation, a percent error was calculated using the quantified values and the registered values.

Since both yeast transformations were unsuccessful, the focus switched to using other yeast strains in addition to ones already used, and performing the Helms Assay to quantify flocculation of each strain and comparing to their expected values to see if they were similar (Table 3). Flocculation percent varied amongst the stains in relation to their expected vs observed. The negative control, BY4741, had a percent flocculation of 0% and the positive control, WLP002, had a percent flocculation of 39%, which was the highest of the percent flocculation. Both controls, in comparison to the other strains, met expected flocculation. This is also seen with Premier Classique and Cote de Blanc, both of which were expected to have low flocculation and measured at 0.86% and 3.0%. The strains, WLP008 and WLP005 both did not match their expected flocculation. WLP008, which was expected to be low to medium, measured at 27.63% and WLP005, which was expected to be high, measured at 5.6%.

Discussion

Flocculation is a necessary process in the brewing industry, with it influencing both the flavor and alcohol content of different beers . This process is characterized by the clumping of yeast cells, where the cells adhere to one another forming flocs that separate from the medium they're in. The speed at which this flocculation occurs is categorized based on the yeast flocculation behavior, with strains usually falling into high, medium or low. High flocculating strains tend to flocculate the earliest, leading to less suspended yeast and a brighter beer. Medium flocculation strains tend to flocculate when sugars in the medium begin to run out and low flocculation strains lead to suspension of yeast cells even after the fermentation process has ended (Boulton et al., 2006). Knowing how a yeast strain is categorized can allow for selection of what outcome one may want, whether that be increased or decreased flocculation. Being able to alter a known strain's flocculation may be beneficial to those in the brewing industry.

The purpose of this project was to alter a yeast strain's flocculation level through a promoter swap. It was hypothesized that swapping the *FLO11* promoter to the GAL1 promoter would induce an overexpression of *FLO11*, meaning the flocculation level of the targeted yeast strain would increase. The *FLO11* gene was targeted for it being a major cell adhesion molecule and because it is not next to a telomere (Cullen et al., 2012, Halme et al., 2004). Research has found that the GAL1 aids in the digestion of galactose, meaning when galactose is added, the transcription of the gene is induced, which would then turn on *FLO11.* Since this project focused on a promoter swap, the primers designed for the promoter GAL1 followed the Longtine et al., protocol, with the forward primer having a skipped region that included the KAN and GAL.

Unfortunately, both transformations were unsuccessful. This was indicated by the lack of colony growth on the petri dishes containing the G418 on them, which is essentially an antibiotic that works on yeast. Transformed cells are resistant to G418, meaning any cells not transformed are killed. Since transformation was unsuccessful, it means the KAN^R was not mixed into the yeast cells, which is why they were killed. Due to the transformations not working, the original hypothesis was not supported since there was a lack of data. Issues in the transformation protocol could have started with the primers, since primers can be a weak link as they are one of the first steps. Certain Flo genes are also homologous to one another, such as *FLO1, FLO5* and *FLO9*, which may have influenced the primers effectiveness as targeting *FLO11*. Other issues pertaining to the transformation may have been the heat shock period, with the second attempt reducing the heatbath from 40 minutes to 20 minutes, however, the second attempt was still unsuccessful.

Due to unsuccessful transformation, the focus for the Helms Assay was switched to quantifying the flocculation of yeast strains with established levels. These strains were assayed using a modified version of the Helms Assay and this was done to determine where they fell on a scale of low to very high flocculation. The results of the assay were compared to the registered flocculation levels, with there being some discrepancies. While most strains matched with their expected flocculation level, there were two strains that did not. This includes strain WLP005, which was expected to have high flocculation but appeared to have low flocculation. Strain WLP008 expected flocculation was low to medium and had a 27.63% flocculation, which could be argued to fall into the medium level, but appeared to be on the higher scale in comparison to all of the yeast strains flocculation measured. These differences could be attributed to procedural errors or how White Labs and Red Star registered the strains. An issue in the methods could be

how the samples were pipetted, such as accidentally causing the experimental group to always register a larger optimal density if the sample is always being taken from the bottom of a tube. This would mean the yeast cells could have settled, leading to a cloudier sample and therefore a larger OD_{660}. A way to fix this would be to shake the tube holding the yeast cells and then quickly pipette them into their separate tubes.

The flocculation of yeast is important, since it determines the rate of fermentation. Research has found that the improvement of fermentation is key to ensuring the best tasting beer while also being economically efficient. Since different factors such as pitching rate and magnesium and calcium concentrations can affect yeast fermentation performance, the flocculation levels of different strains need to be well established so modifications can be accurately measured when trying to improve flocculation (Verbelen et al., 2009). To create these modified strains, there needs to be a secure and effective transformation protocol that can ensure that transformation is successful. If transformation success can be improved, it could lead to more strains being modified and adapted to a brewers specific needs and wants.

Figures and Tables

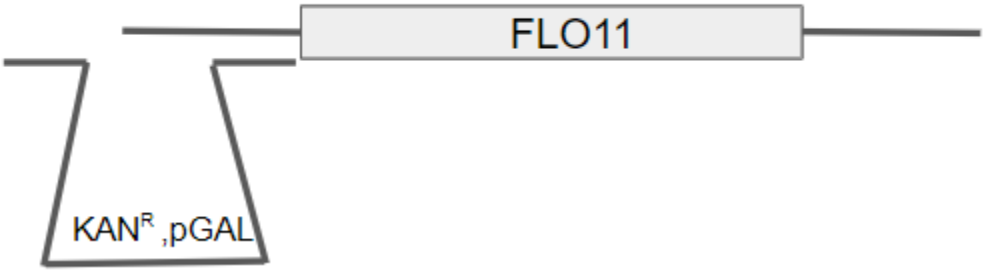

Figure 1. PCR Template. Using Longtine, MS. et. al., 1998 plasmid and PCR protocol to establish where the promoter swap needs to occur in relation to the gene of interest (GOI).

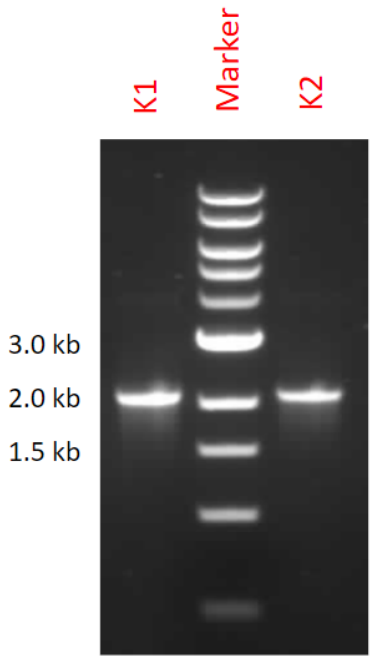

Figure 2. Results of PCR Amplification with Gel Electrophoresis. K1: Kan: GAL1p::*FLO11*-sample B, K2: Kan: GAL1p::*FLO11*-sample A. The label marker between the two samples is the NEB 1KB Ladder.

Table 1. Primer Sequences Used. The primers designed target the promoter of FLO11 and replace it with template plasmids containing, pGAL1 and KAN^R, pulled from Longtine, 1998.

Target	Name	Primer sequence	Objective	PCR	Template plasmid
FLO11	YIR019C_SWA_FOR	ATCAGTTATT ATCCCTCGTC ATGTTGTGGT TCTAATTAAAGAATTCGAGCTCGTTTAAAC	Promoter swap	Yes	pFA6a-kanMX6-PGAL1
FLO11	YIR019C_SW_REV	GAAGCGAAAGGACCAAATAAGCGAG TAGAAATGGTCTTTGCATTTTGAGATC CGGGTTTT	Promoter swap	Yes	pFA6a-kanMX6-PGAL1

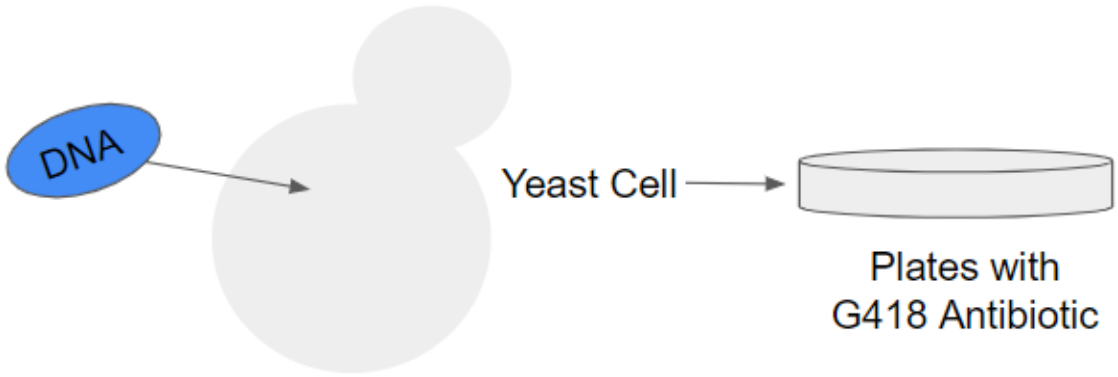

Figure 3. Transformation of Yeast Cells. DNA was mixed into the yeast cells and plated onto plates with G418 antibiotics.

Table 2. Yeast Strains Chosen. Yeast strains sourced from White Labs (White Lab, 2021).

Strain Name	Flocculation Level	Change/Purpose
BY4741	None	Promoter swap to GAL1
WLP008	Low to medium	Control
WLP004	Medium to high	Control
WLP002	Very high	Control

Yeast Strain	Average % Flocculation	Expected Flocculation
BY4741	0	None
Premier Cote du Blanc	3.00	Low
Premier Classique	0.86	Low
WLP008 *	27.63	Low-Medium
Premier Blanc	5.11	Low-Medium
WLP005 *	5.61	High
WLP002	39.62	Very High

Table 3. Average Percent Flocculation. Percent flocculation following Helms Assay and how it compared to expected flocculation. A (*) indicates that the flocculation of the strain did not meet expected flocculation level. Expected flocculation levels were determined by Whitelabs and Redstar.

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