New Insights on the brewer's yeast flocculation

Luís Miguel Santos Sousa

Supervisor: Doctor Ir. Niels Gerard Adriaan Kuijpers

Co-supervisor: Professor Doctor Isabel Maria de Sá Correia Leite de Almeida

Abstract

Yeast flocculation is a highly important process in the brewing industry. Due to its importance, in depth studies regarding its regulation and influencing parameters is emergent. Moreover, the prediction of the flocculation capacity of lager yeast remains a difficult task, yet important for a better standardization of brewer's yeast strains. In the present work, a transcriptomic analysis of the most important flocculation related genes was performed. A new flocculation method was created, with which a new parameter regarding the flocculation characteristics of cells was resolved. Flocculation capacity was tested in different wort compositions, and interesting new insights regarding flocculation regulation and efficiency were sought to be solved.

Keywords: flocculation; lager yeast; *FLO* genes; transcription; measurement

Introduction

Flocculation is described as the asexual, reversible, calcium-dependent and homotypic process by which yeast cells aggregate into clumps composed of thousands/millions of cells that quickly sediment from the bulk medium where they are suspended (Soares 2011). In the brewing context, the *curriculum vitae* of a yeast strain must include not only the ability to flocculate, but as well the perfect timing to do so. Specifically, at the end of beer fermentation, when all fermentable sugars are converted into ethanol and CO_2, yeast flocculation should occur, allowing for its sedimentation to the bottom of the fermenter (in the case of bottom-fermenting yeast) or its flotation to the surface of the fermentation vessel (top-fermenting yeast) (Van Mulders et al. 2010). The capacity for yeast cells to flocculate at the end of the fermentation imposes a considerable advantage to the process, since it represents a cost-free and effective way to separate yeast cells from the fermented beer, enabling its re-pitching in subsequent fermentations (Verstrepen et al. 2003). The mechanisms by which yeast cells flocculate and how the process is initiated is not completely understood. However, the more consented hypothesis takes as assumption the lectin-like theory of flocculation (Miki et al. 1982). According to this theory, specific proteins present on the yeast cell wall named lectins (or flocculins) interact with oligosaccharide receptors that decorate the cell wall of an adjacent cell, in the presence of calcium (Bony et al. 1997). The oligosaccharide receptors consist of mannan side-branches, with the length of two to three mannose residues. These are present in flocculent or non-flocculent cells, contrarily to flocculins, which are only expressed by flocculent strains. The way that yeast flocculates is highly strain–dependent, with the genetic background as a major influence on that characteristic (Vidgren and Londesborough 2011). Flocculins are encoded by the sub telomeric located *FLO* genes, which include *FLO1*, *FLO5*, *FLO9* and *FLO10* genes, all encoding for different cell wall flocculins (Vidgren and Londesborough 2011). *FLO8* gene also belongs to this gene family, but it encodes for a transcriptional activator, which activates *FLO1* transcription (Kobayashi et al. 1996). There are three known different flocculation phenotypes: (i) mannose sensitive or Flo1 phenotype, in which aggregation is inhibited only by mannose residues in solution; (ii) the NewFlo phenotype, in which glucose, sucrose, maltose, maltotriose and mannose are able to inhibit the aggregation process (Malcolm Stratford and Assinder 1991; Sato et al. 2001) and (iii) a flocculation phenotype in which aggregation is not inhibited by any sugar in solution (Masy et al. 2010). NewFlo phenotype is directly correlated with the expression of the *Lg-FLO1* gene, which product encodes for a flocculin with broader sugar sensitiveness (Kobayashi et al. 1998). Lager yeast is reported to present this gene in its genome, which makes it suitable for the flocculation onset at the end of beer fermentation, when all sugars in wort are almost depleted, enabling the flocculins to bind neighbour cell walls, instead of the free sugars in wort (M Stratford and Assinder 1991).

Besides representing a biological phenomenon, yeast flocculation comprises a multi-disciplinary thematic, in

which chemical and physical aspects represent great importance. Physiological factors as pH, temperature, ethanol, cations and nutrients availability are reported to impact yeast cell flocculation, by direct implications on the physical aspects of the aggregation process or by impacting gene expression and regulation (Soares 2011; Vidgren and Londesborough 2011; Stewart 2018). Those factors might have implications in the physical state of yeast cell walls, impacting their charge or hydrophobicity (Smit et al. 1992; Jin et al. 2001). Cell surface hydrophobicity (CSH) is reported to impact cell flocculation and known to increase as the population of cells attains the stationary phase of growth (Speers et al. 2006). An increase in CSH might enhance the hydrophobic interactions between neighbour cells, facilitating aggregation (Gregory 1993). Apparently, CSH increases as the concentration of flocculins in the cell walls increases (Van Mulders et al. 2009). Together, lectin-binding and hydrophobic interactions are the mechanisms that permit yeast cells to aggregate in bigger clumps. However, to know the forces and mechanisms by which cell aggregate remains incomplete for a deep understanding and prediction of cells flocculence. Understanding colloid kinetics might be helpful, especially in the prediction collision and association rates. In 1977, van de Van and Mason (van de Ven and Mason 1977) modified a mathematical expression for the orthokinetic aggregation mechanism of yeast cells, accounting for perfect spheres in laminar flow:

$$\frac{N_t}{N_0} = e^{-\left(\frac{4\,\alpha_0\,\dot{\gamma}\,\varphi_0}{\pi}\right)t}$$

where N_t is the concentration of particles/flocs at time t, N_0 is the initial concentration of particles, α_0 corresponds to the orthokinetic capture coefficient, φ_0 corresponds to the initial volume fraction of particles and $\dot{\gamma}$ is the shear rate. In the above-mentioned equation, the capture coefficient takes a clear importance to the expression, since it comprises all the forces involved in cell collisions plus the probability of colliding cells/flocs forming a doublet.

By analysing the collision rates and forces of interaction, one might think that increased forces would enhance the flocculence and sedimentation of cell flocs. However, yeast flocs are regarded as fractal objects (Meakin 1987). Fractal object is defined as structure which presents self-similarity, so that its macroscopic form and structure is a mirror of the small clusters that associated together to form it. The consequence of this structure formed from clusters of clusters is that, as its size increases, its density decreases. In this way, if the mass of the object is plotted against its size (in a logarithmic scale), the result is a linear slope, which is called the *fractal dimension* – the smaller the slope, the less dense is the macroscopic aggregate (Elimelech 1995). High interaction forces between cells might be detrimental for flocs sedimentation, since it can lead to the formation of a less compact clump, which will might not completely settle to the bottom of the fermenter (Stratford 1992).

Research in flocculation genes and expression and regulation is still a matter of great debate. Moreover, most of the studies performed had as model organism *S. cerevisiae* strains, which genomic background is rather simple than the hybrid yeast *S. pastorianus*(Gibson and Liti 2015). The difficulty in solving DNA sequences for the sub telomeric located FLO genes and the added complexity of *S. pastorianus* hydride genomes makes this topic even more complex, in the lager brewing context. However, the availability of high-quality genome sequences of Heineken yeast strains opens the possibility for an in-depth study of FLO genes expression and regulation.

In this work, three lager yeast strains were tested for their flocculation phenotype in different fermentation setups and medias. Its flocculation was followed by an innovative flocculation measurement method, which permitted to follow their flocculation profile throughout the fermentation time, in a user-friendly and online manner. To try to answer some questions regarding the *FLO* genes expression and flocculation genes regulators, a transcriptomic analysis was performed for all the three tested strains. Gene transcripts were analysed in four different stages of the fermentations, and in two different wort compositions. Together, some new insights on gene transcription and regulation and wort media impact on flocculation onset and strength were achieved, and a flocculation profile of each tested strain was possible to record.

Materials and methods

Yeast strains and media

Lager brewing yeast strains of *Saccharomyces pastorianus* species used in this study include A, B and C strains (HEINEKEN's property).

Heineken standard wort: full malt wort with an extract content of 16.8 °P, produced at the Heineken brewery of Zoeterwoude, The Netherlands, directly collected from the coolers at the brewery. The collected wort was afterwards supplemented with 0.6mg.L^{-1} of $ZnSO_4.7H_2O$ (Merk KGaA, Germany) and 1mL.L^{-1} of antifoam (Snapsil antifoam FD10, Brunschwig Chemie). It was then autoclaved for 15 min at 121°C.

50% Adjunct wort: full malt wort mixed with sucrose solution, with a final mass/mass ratio of 1:1 and a final extract content of 16.8 °P. The prepared Adjunct wort was afterwards supplemented with 0.6mg.L^{-1} of $ZnSO_4.7H_2O$ (Merk KGaA, Germany), 1mL.L^{-1} of antifoam (Snapsil antifoam FD10, Brunschwig Chemie) and 20mg.L^{-1} of $CaCl_2$ (Merck KGaA, Germany), in order to achieve a final concentration of Ca^{2+} in the final wort superior to 30mg.L^{-1}. It was then autoclaved for 15 min at 121°C.

Fermentation setups

Fermentations settings: The controlled fermentations were carried out at the Pilot Brewery facilities, which belong to the Global Innovation and Research of the Heineken Supply Chain department, located in Zoeterwoude, The Netherlands. At the Pilot Brewery, bioreactors were used for the controlled fermentations. The bioreactors, with a working volume of 7L, allowed the online measurement, monitoring and control (if specified) of temperature, aeration, rotation speed, pressure and

pH. Additionally, the optical density probe allowed for the online monitoring and measurement of the total cell density during the ongoing fermentation. Cleaning and sterilization of the bioreactor are done in place (CIP and SIP).

All the different fermentation setups were performed in duplicate. The applied temperature profile consisted of pitching at 9°C and controlled linear temperature rise to 16°C in 72h and kept at 16°C until the end of the fermentation. Pitching was done with full volume of the 240 mL propagation flask in 6.50 L of fermentation medium in the bioreactor. pH was not adjusted during the fermentation, unless stated otherwise. Offline samples were taken two times per day (unless stated otherwise), for analysis of fermentation parameters as apparent extract, ethanol content, pH, cell count and viability – performed at Heineken Pilot Brewery facilities. Upon this general parameters, additional analysis of the fermenting wort (2 times per day, unless stated otherwise) included: glucose, fructose, sucrose, maltose, maltotriose, acetaldehyde, dimethyl sulphide (DMS), acetone, ethylformiate, ethylacetate, methanol, ethylpropionate, propanol, isobutanol, isoamylacetate, amyl alcohols, ethylcapronate, total higher alcohols, diacetyl and 2,3-Pentanedione. All these analyses were performed by the Heineken Quality Assurance Laboratory (QAL).

Fermentations for transcriptomic and cell surface hydrophobicity analysis (F19F, F19G, F19H and F19I fermentation series): the three tested strains were cultivated in duplo, each one in full malt wort and 50% Adjunct wort compositions. During the ongoing fermentations, 4 samples at different stages of cell population growth – (by order) exponential phase, deceleration phase and two at the stationary phase – were performed, in order to see the evolution on genes expression and CSH throughout the fermentation time. Flocculation parameters were also analysed throughout those fermentations.

Fermentations for volume impact analysis (F19J series): On this experiment, no samples were taken during the ongoing fermentations, in order to evaluate the impact of sampling volume on the OD profile and flocculation profile of the strains.

Fermentations for pH impact analysis (F19K1 series): These fermentations were carried out in 50% Adjunct wort with pH controlled with automated addition of 2 M NaOH, in order to evaluate the impact of a higher pH on the OD profile and flocculation profile of the used strains.

Fermentations for increased nitrogen content impact analysis (F19K2 series): These fermentations were carried out in 50% Adjunct wort with increased nitrogen content, in order to evaluate the impact of a higher initial nitrogen value in the OD and flocculation profile of the yeast strains. Fully prepared Adjunct wort was supplemented with 0.3 $g.L^{-1}$ of $(NH_4)_2HPO_4$ (VWR chemicals, Belgium)

RNA sequencing analysis

Performed samples were aimed to obtain a total of 240mg of biomass per sample. The samples for RNA-seq analysis were immediately quenched in liquid nitrogen to avoid impact of sampling on the transcriptome. The RNA isolation and sequencing procedures were performed at BaseClear (Leiden, The Netherlands) and RNA was sequenced using the Illumina HiSeq sequencing platform (Illumina, San Diego, CA). Paired-end sequence reads were generated using the Illumina NovaSeq 6000. FASTQ read sequence files were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool, version 0.11.5. RNA sequences were statistically analysed by the service provider, including quality controls and reads quantification, which were mapped against the reference genome (StrainA_v4). Effect of media and sampling times on the reads count was performed for all the three strains, as well as the differences between strains.

Results

Flocculation measurement method development and proof of concept – non-volume changed fermentations

During the fermentations, an OD probe was used to monitor the cell density inside the fermenter, and by manipulation of the fermenter settings, two flocculation parameters were prone to be analysed: flocculation strength and flocculation behaviour. Flocculation strength accounts for the strength of binding between the cells, and flocculation behaviour accounts for the floc morphology – higher values are correlated with less dense flocs, and the lower the value, the higher is the flocs density. Flocculation strength parameter is obtained by the calculation of the $Floc_{bind}$ value, and the flocculation behaviour parameter is obtained by calculation of the $Floc_{behav}$ value. Each of the values was calculated during the fermentations, at specific times – RS intervals – at which the fermenter settings were manipulated to do so.

In order to get a good comparison between different fermentations, several tests were performed with a constant working volume. This was achieved by not taking samples during the fermentations. In this way, the position of the probe relative to the surface of the culture remained constant. Strains B and C were cultivated in Full malt wort and the OD was measured over time.

Two important characteristics can be taken from the flocculation strength plots (fig. 1): 1 – the onset of flocculation, time at which the $Floc_{bind}$ values sharply increase during the fermentations, and which correspond to the first inflexion point in the plots; 2 – the maximum flocculation strength, time at which the curves show a stable maximum value, which permits the comparison of the binding strength between the cells of the different strains. The data shows for both strains C and B a similar flocculation onset time at 75h of fermentation. Furthermore, the maximum flocculation strength value is also similar between the two strains.

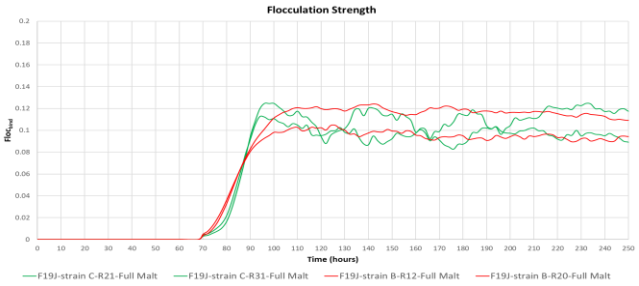

Figure 1 – Flocculation strength for the two tested strains B and C in Full malt wort composition. Fermentations carried out without volume change (F19J series). Each duplo fermentation is represented with the same colour, but in two separate lines (duplos). $Floc_{bind}$ (y axis) are averaged. Green lines account for strain C and red lines for strain B.

The second analysed parameter, linked to floc morphology, is shown on figure 2 for both C and B strains. An increase in the values is observed at onset of flocculation for both strains. This increase reaches a plateau between 120-130 hours of fermentation, and the $Floc_{behav}$ values progressively decrease afterwards, until the end of the fermentation time. The connection of the obtained values to floc morphology points to more loose flocs formed by strain C cells in comparison to strain B cells. Towards the end of the fermentation, both strains cell flocs become progressively more compact.

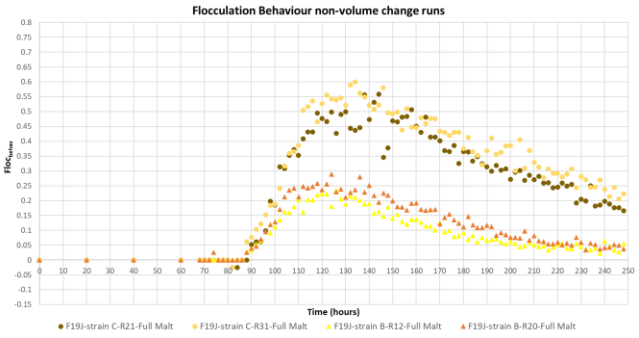

Figure 2 – Flocculation Behaviour graph for the non-volume change runs (F19J series). In the graph are represented strains B (triangles) and C (spheres) in Full malt wort. Each point of the graph represents the calculated $Floc_{behav}$ (y axis) value.

Different media compositions impact on flocculation

Adjunct wort vs Full malt wort: The use of Adjunct wort formulations in breweries is already a practice worldwide. However, a deeper understanding of its impact on the flocculation characteristics of the different strains is emergent. Better prediction of the flocculation onset and characteristics might be achieved by its investigation. To do so, A, B and C strains were tested in two different wort types – full malt wort and 50% Adjunct wort. The flocculation characteristics of the three strains were followed during such experiments.

Strain A, regarded as the low flocculent strain, was settled as the negative control of all the experiments. The values for the $Floc_{bind}$ value on this strain never surpassed 0.02, being that this was set as the threshold flocculation value, below which no flocculation is taking place.

Both strains B and C presented higher flocculation strength in 50% Adjunct wort than in full malt wort compositions. For both wort types, strain B always presents a slightly lower flocculation strength than strain C. The flocculation onset of strain C in 50% Adjunct wort happens 30h later than in full malt wort, pointing for a retardation effect of adjunct wort formulation in this strain flocculation onset. Strain B starts to flocculate earlier (65h) in 50% Adjunct wort, in comparison to Full malt wort (75h). Strikingly, an opposite behaviour is presented by C and B strains: strain C cells start to flocculate earlier in Full malt wort than in 50% Adjunct wort, while B strain cells flocculate earlier in 50% Adjunct wort than in Full malt wort.

The flocculation behaviour parameter presented similar results on these fermentation runs, as for the non-volume changed runs, but with a higher duplo-to-duplo variation and less data quality. Strain C presented the higher values for the $Floc_{behav}$ value, with the same trend as presented in figure 2, for full malt wort composition. However, much lower values were obtained in Adjunct wort, pointing to the fact that the 50% Adjunct wort composition has some impact on the floc morphology of strain C cells, namely by increasing its density. For strain B, results for both wort types are unclear, but overall lower $Floc_{behav}$ values are observed. As for the non-volume changed runs (fig. 2), strain B presents a more compact structure of its cell flocs than strain C, in both wort types.

Sugars consumption and ethanol production was also analysed. For all A, B and C strains, a complete consumption of all sugars in wort is not achieved in 50% Adjunct wort. Maltose and Maltotriose are not fully consumed in this wort type, for all the tested strains. However, strain C presents the higher consumption of both sugars, with the lower concentration of carbohydrates in wort at the end of the fermentation. Consequently, it also presents the higher concentration of ethanol at the end of the fermentation, for the 50% Adjunct wort formulation. In full malt wort, all three strains fully consume sugars present, and ethanol production profiles are similar for the three strains.

The pH was also analysed during the fermentations. For the three trains, pH achieves lower values for the 50% Adjunct wort formulation, than for the full malt wort type. This fact might be due to the lower buffering capacity of 50% Adjunct wort composition.

Adjunct wort pH- controlled fermentations: The goal of this set of fermentations was to know whether a change in the pH value throughout the fermentation would impart differences in the flocculation phenotype showed by the strains. Since pH in Adjunct wort declines to lower values than for the full malt wort composition, pH was controlled in 50% Adjunct wort formulation, in order to achieve similar profiles as for the full malt wort fermentations.

The increased pH value of the fermentation media incremented the flocculation strength values of both B and C strains, without changing the flocculation onset times in the fermentation – in relation to a normal Adjunct wort fermentation. For the B strain, the increased pH values imparted interesting differences on the maximum flocculation strength of this strain. The maximum $Floc_{bind}$ values were higher in pH-controlled adjunct wort fermentations, in comparison with the normal adjunct wort

formulations. For strain C, similar results were obtained, with higher $Floc_{bind}$ values in pH-controlled fermentations, but for just one of the duplo fermentations. In the previous section, results showed higher flocculation strength values in Adjunct wort in comparison to Full malt wort. In the present section, results show that flocculation strength values are even more incremented in relation to the two previous wort formulations, by increasing the pH value in Adjunct wort. This observation loses validity for the C strain due to low quality of the duplos.

Differences on the flocculation behaviour parameter were also observed, but with low data quality. For B strain, the increase in pH incremented the $Floc_{behav}$ values dramatically, which points to the interpretation of much less dense flocs being formed in higher pH. Results for this parameter in strain C are not conclusive, since the quality of the data is highly questionable, and further fermentations should be performed.

Adjunct-wort fermentations with higher initial nitrogen content: As influence of the 50% dilution of Full malt wort with a sucrose solution, to make a 50% adjunct wort, a 50% decrease in the Total Nitrogen and Free Amino Nitrogen is observed in the Adjunct wort configurations. In this way, the goal of this fermentation sets was to evaluate the impact of nitrogen supplementation of normal Adjunct wort with Di-ammonium phosphate, which functions as an ammonia nitrogen source for the growing yeast. The results showed profound effects on the flocculation strength parameter of B and C strains.

Two important changes occurred in the flocculation strength of C and B strains, while the wort was supplemented with a nitrogen source: 1) the $Floc_{bind}$ values significantly reduced with the supplementation of DHAP for both strains and 2) the onset of flocculation was significantly delayed for B strain, with the supplementation of nitrogen. B strain cultivated in Adjunct wort with additional nitrogen content showed the actual flocculation onset at around 112h of fermentation, 37h delayed in relation to the normal Adjunct wort fermentation. Not only the flocculation onset is later, but also the increase to the maximum flocculation strength is slower, and much lower values for this parameter are achieved – around 0.1, contrarily to normal Adjunct wort, which achieves 0.16. In the case of strain C, the flocculation onset is not significantly but a significant decrease in the maximum flocculation extent is observed, with a variation of around 0.16 in normal Adjunct wort, to 0.11 in Adjunct wort supplemented with nitrogen. Regarding the flocculation behaviour for both strains, no significant differences were found between the normal Adjunct wort configurations and Adjunct wort supplemented with nitrogen.

Strain B experienced an increase in maltose and maltotriose consumption, as an effect of nitrogen supplementation. The final values for maltose concentrations are 12.5 g/L in the nitrogen supplemented Adjunct wort, much lower than the above 20g/L values in normal Adjunct wort. In the same way, the final values for maltotriose concentrations are 6g/L in nitrogen supplemented Adjunct wort, lower than the 7.5 g/L concentration in the normal Adjunct wort configuration. Subsequently, the values for the final total fermentable sugars differ from 2 g/L in nitrogen supplemented Adjunct wort, to almost 3 g/L in normal Adjunct wort. Strain C presented similar results to strain B for sugars consumption, but the differences from Adjunct wort to nitrogen supplemented wort are not as dramatic as for strain B.

As result from the different sugar consumption patterns and extents at the different wort compositions, different alcohol production and pH trends are also observable. In fact, the decrease in pH for the nitrogen supplemented wort is higher, probably result of a higher sugar consumption and so higher fermentation rate. The pH values in normal Adjunct wort never dropped below 3.5 value, whereas for nitrogen supplemented wort, a 3.3 value was achieved. Regarding the alcohol production, no great difference is observed from nitrogen supplemented wort to the normal Adjunct wort, for strain C. However, more alcohol is produced by B strain, in the nitrogen supplemented Adjunct wort configuration.

Transcriptomic analysis

In order to know the influence of different fermentation conditions on the FLO genes expression, RNA-seq analysis was performed. The collection of RNA samples was done at 4 different time points per strain (A, B and C), in each wort type (Full malt and 50% Adjunct wort). Sample times were chosen in such way that samples were representing the same growth stages for each strain. The four samples for RNA-seq analysis were performed at the end of the exponential phase, deceleration phase and twice on the stationary phase of growth.

Gene expression levels: The analysis of the different transcripts and their quantity showed differences on the expression level, throughout the different sample times, strains and media composition. Some of the transcripts identified include most of the known FLO genes: genes X, Y, Z, W and gene O (O') were identified, showing differences in expression for the tested strains, media and sample times. Among these different genes, paralogs for each gene were also identified. However, due to their relative higher expression levels, three transcripts were categorized as the potential effectors of the observed flocculation differences among the strains, all of them paralogs of Y gene. The paralogs found were nominated Y, Y1 and Y2, all located at chromosomes R1, R2 and R3, respectively. The higher transcript levels are found for the Y1 paralog. Both strains C and A show high transcript levels, in comparison with the B strain. However, for all the three strains, a variation in the mRNA quantity of this paralog is found throughout the four samples, and differences are also found among different wort compositions. For strain C in Adjunct wort, an increase from the first to the third sample was observed, declining at the fourth sample time. A different pattern is found for the same strain in Full malt wort, in which the maximum transcript level is found at the second sample time, and with overall lower expression values. Strain A shows similar trends, with increase in expression from the first to

the third sample in Adjunct wort, and only from the first to the second sample in Full malt wort. Strain B shows the lower expression values, and the variations in expression with the time of sampling are very subtle for both wort compositions.

The second more expressed paralog was Y2. For strain C, this gene showed a similar expression trend to Y1, in both wort types. Y2 paralog was barely expressed by A and B strains, in both wort compositions. Finally, Y paralog, the least expressed one, shows similar trends to all the other paralogs for C strain, and its absent for A strain. Strain B shows similar expression trends of this paralog to C strain.

Gene structure of Y1 homologue: In order to see potential DNA level differences among the Y1 homologues on the different strains, a whole genome comparison of this gene and protein level predictions were performed, using the reference genomes of each strain, which were already previously sequenced. The analysis of the gene in three different strains show evident differences in terms of size, with Y1 homologue on strain C genome assembly presenting a longer gene than for the other two strains, A and B. Regarding the protein level prediction, it was predicted that the PA14 domain, which is present on the N-terminal domain of all known yeast flocculins (Brückner and Mösch 2012), is absent on the Y1 homologues of A and B strains, but present on C strain Y1 gene (fig. 3).

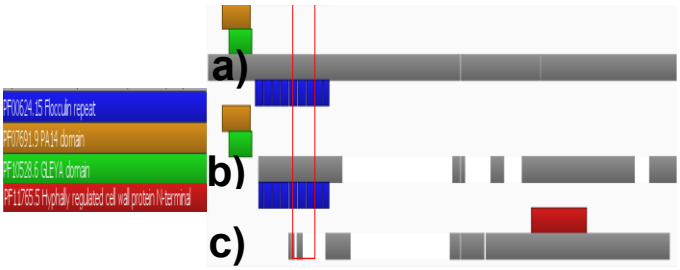

Figure 3 – Protein level comparison of the Y1 homologue for each of the studied strains C (fig. a)), A (fig. b)) and B (fig. c)), with the sequence of each strain's homologue represented in grey. The coloured blocks represent known protein domains being them flocculin repeats (blue), PA14 domain (yellow), GLEYA domain (green) and the hyphally regulated cell wall protein N-terminal (red).

Discussion

The present work had as aims to have new insights regarding the complex flocculation phenomena. Flocculation is highly dependent on the flocculins, proteins encoded by the sub telomeric regions-located FLO genes. The testing and development of a new flocculation measurement was crucial, to enable the correct comparison of flocculation phenotypes among the strain A, B and C. This method not only sought to give online results, by which better correlations between fermentation conditions and different yeast phenotypes could be achieved, but also showed to unravel a new parameter, regarding the cell binding characteristics of different yeast strains. Since strain C is reported to show some incoherence in respect to the flocculation times, different fermentation conditions and wort compositions were tested. A correct transcriptomic comparison is enabled by RNA-seq during different fermentations, for the above-mentioned strains.

In previous investigations (D'Hautcourt and Smart 1999; Speers et al. 2006; Claro et al. 2007), higher ethanol concentrations are related with increased flocculation efficiency. In Adjunct wort fermentations, strain C produces more ethanol than in Full malt wort fermentations, correlating with the previous findings said before. For both B and C strains cultivated in Adjunct wort, maltose and maltotriose are not fully consumed. Several are the studies that correlate sugars presence to flocculation inhibition (Soares et al. 2004), even more for brewing yeast strains, which normally start to flocculate at the end of the fermentation, when all the sugars in wort are depleted (Verstrepen et al. 2003). However, results for flocculation strength show that the flocculation is higher in Adjunct wort, where sugars are still present in solution, than in Full malt wort, where all sugars in wort are totally consumed. Besides this, it should be considered that different flocculins have different binding affinities towards different types of sugars (Van Mulders et al. 2009; Willaert 2018). It could be that the flocculins expressed by C and B strains do not have a broad specificity towards maltose and maltotriose, enabling flocculation even with some of these sugars still present in the wort.

The pH controlled Adjunct wort fermentations showed, for B strain, an increase in cell binding strength and higher fluffiness of the flocs. For the C strain, the great duplo to duplo variation disables a clear comparison, and the fermentations should be repeated. The fact that a higher cell binding strength is followed by an increase in fluffiness totally correlates with the relocation phenomena. However, it could be asked why the cell binding increases in response to the increase of 0.5 value in pH. Previous research (Dengis et al. 1995) showed that lager yeast shows an optimal pH range-value for flocculation between 4-4.5. However, at the time of this paper, no structure of the flocculins was solved. Veelders et al. (Veelders et al. 2010) showed, for the first time, the sub-atomic structure if the N-terminal part of the Flo5 protein. At the sugar binding site, two aspartic acid residues are highly preserved among N-terminal domains of different flocculins. These two residues are known to be responsible for calcium binding. With the increase in pH, these residues might be much less protonated, and more able to bind calcium, a preponderant requirement for flocculation to take place (Stratford 1989). Moreover, the isoelectric point of the aspartic acid amino acid is between 3.9-4, below which the sidechains of the residue are highly protonated, and less able to bind other ions (like calcium). However, it should be noted that some characteristics of the amino acids, as the isoelectric point, may be different, when it they are in a protein context (Urry et al. 1993).

The supplementation of Adjunct wort with nitrogen showed severe implications on the flocculation strength of the cells. Both B and C strains show lower flocculation strength values, in comparison with normal adjunct wort, and the flocculation onset of B is delayed in response to a higher initial nitrogen content. The nitrogen was already reported as being correlated with the flocculation onset in

S. cerevisiae strains (Sampermans et al. 2005). Coincidently, the shortage in nitrogen leads to earlier flocculation onsets, which agrees with what was observed for the B strain. Strain B lacks a correlation for its flocculation onset and the amount of sugars in solution. For the nitrogen supplemented fermentation, B strain consumes more sugars, but flocculates much less than in normal Adjunct wort, in which leaves more sugars in solution. Same can be said about the ethanol concentrations. B strain produces more ethanol if it has more nitrogen at the beginning of the fermentation, but the flocculation strength is lower. For the C strain, no correlation is found between nitrogen concentration and flocculation onset. In fact, this strain flocculates later in normal Adjunct wort, but no shift was found in nitrogen supplemented Adjunct wort. Conversely, it seems from the analysis of sugars in all fermentations that C strain starts to flocculate, as soon as the sugars concentrations in wort drop below 4g/100mL. An interesting correlation between state of growth and flocculation onset is found for both strains. For B strain, the flocculation onset in Full malt wort and normal Adjunct wort happens to coincide with the beginning of the deceleration phase of the cell population. However, when Adjunct wort is supplemented with nitrogen, the flocculation onset is delayed to the end of the same phase. For C strain, the same difference was observed by comparing the flocculation onset of this strain in Full malt (earlier onset) and Adjunct wort (later onset). Finally, for both strains, the pH values dropped slightly more than for normal Adjunct wort fermentations. From the pH-controlled fermentations, it was seen that an increase of 0.5 induced a higher flocculation efficiency. In the same way, it might be the case that those slightly lower pH values imparted an extreme decrease in the flocculation efficiency in nitrogen supplemented Adjunct wort, when compared with normal Adjunct wort fermentations.

The results from RNA-seq analysis showed interesting transcript patterns. The most transcribed genes included gene Y and its paralogs, namely, Y1 and Y3. Among these three, Y1 showed the higher transcript levels, and differences in its expression throughout the fermentation time are observable for all the three tested strains. C strain showed for this gene a good correlation between level of transcription and onset of flocculation. In Adjunct wort, on which the higher transcript levels of Y1 are achieved, an increase from the 1st to the 3rd samples are observed. Interestingly, this increase in expression until the third sample time is coincident with the onset of flocculation for this strain in this wort composition. The 3rd sample was performed at 123 hours of fermentation, and the onset of flocculation was around 105 hours of fermentation time. Coincidently, the same can be said for Full malt wort results. C strain started to flocculate at around 75 hours of fermentation in Full malt wort, and the maximum transcript level for Y1 gene is observed at the 2nd sample, which was performed at 92 hours of fermentation. In both wort compositions, the transcript levels decline after the flocculation onset was achieved. Besides not so evident, B strain also reveals a similar pattern, and the achievement of the flocculation onset is coincident with the higher transcript levels, which decline afterwards. Strain A also presented high transcription of the mentioned gene, but its flocculation levels are almost null, in comparison with C and B strains. However, the protein level comparison revealed that Y1 homologue on this strain is lacking for the PA14 domain, which contains the binding site of the sugar residues (Brückner and Mösch 2012). Without this domain, no binding can take place, and so the protein cannot be functional. B strain revealed the same result for the protein prediction, besides its flocculation phenotype. However, it should be noted that other FLO genes revealed expression for this strain, revealing that other gene or genes besides Y1 are responsible for the flocculation phenotype showed by this strain.

Besides the difference in transcription levels, the data coming from the RNA-seq analysis fails in confidence. The RNA samples showed a good quality, meaning that the sample and subsequent treatment were successful. However, the DNA sequencing assemblies are not 100% reliable for the chromosome regions in which the analysis was performed. The FLO genes show zero coverage for some parts of their DNA sequence, meaning that assembly mistakes are present, which on their way make it difficult to draw solid conclusions about the transcript expression data. In fact, two great obstacles are found when analysing FLO genes in *Saccharomyces pastorianus* strains. First, FLO genes are presented in sub-telomeric regions, which by itself makes it difficult for reliable sequencing data, due to the high repetitive regions present (Van Mulders et al. 2010). Secondly, the fact that *S. pastorianus* is a hybrid of *S. cerevisiae* and *S. eubayanus* species makes it even harder for correct assembly procedure of their genomes, due to the high rates of aneuploidies present on this yeast species (Nakao et al. 2009).

The objective of following the relevant flocculation genes in the *Saccharomyces pastorianus* tested strains was revealed to be incomplete. Y gene and its paralogs were classified as the most relevant, due to their relative high expression, in relation to all the other gene transcripts. However, the lack of confidence on the reference genome doesn't enable to answer clearly to the raised questions. To answer them, upgrades regarding genome sequencing techniques must be done, since the highly repetitive zones of *Saccharomyces pastorianus* genome are still out of reach. The flocculation measurement method, however, revealed potential in solving the question of how to characterize a flocculation phenotype of a strain. Besides being an online method, also permits the evaluation of two characteristics of yeast cells flocculation: binding strength and floc morphology. Further improvements must be performed, but all points for a new perspective regarding this so important parameter, which will might improve the prediction of flocculation capacity of certain yeast strains.

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