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Flocculation in industrial strains of Saccharomyces cerevisiae: role of cell wall polysaccharides and lectin-like receptors

Running title: Role of sugars on the cell wall polysaccharides and lectin-like receptors

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Abstract:

Yeast flocculation is the reversible aggregation of yeast cells promoted by the interaction between lectin-like protein receptors with mannose side chains on adjacent cell walls. Flocculation is governed by several physiological factors, including the type of nutrient sugar available to yeast. We grew four industrial strains of *S.cerevisiae,* representing applications in the brewing, winemaking and bioethanol sectors, to late stationary phase and quantified the cellular content of mannans, glucans and lectin-like proteins on yeast cell surfaces. Results indicated that brewing and champagne strains showed moderate to high flocculation ability when grown with glucose, fructose, maltose or galactose, whereas winemaking and fuel alcohol strains only showed moderate flocculation when grown on maltose and galactose. All yeast strains studied were weakly flocculent when grown on mannose. With regard to lectin-like receptors, their number played a more important role in governing yeast flocculation than the mannan and glucan contents in yeast cell walls. We conclude that all the industrial strains of S. cerevisiae belonged to New-Flo type on the basis of their flocculation behavior observed when cultured on different sugars. Quantification of yeast cell wall polysaccharides and receptor sites indicate that mannan and glucan levels remain almost constant, irrespective of the strain under investigation. The main difference in flocculation characteristics in industrial yeast strains appears due to variations in concentrations of lectin-like cell surface receptors.

Our findings may benefit brewers, winemakers and other yeast-based technologies in design of media to prevent premature flocculation during fermentation.

*Deceased

Keywords: Carbon source, Flocculation, New-Flo phenotype, Lectin staining, Saccharomyces cerevisiae.

Introduction

Yeast flocculation is a type of asexual aggregation involving Ca^{2+}dependent interaction between lectins (also known as flocculins) and cell wall polysaccharides, notably mannans and glucans (*1,2*). Flocculins are lectins, i.e. proteins that bind to cell surface carbohydrates. Each yeast lectin is found to be specific for one or two monosaccharide haptens (eg. for mannose or glucose) and binds to haptenic oligosaccharides that contain these sugars in several different glycoside linkages (*3,4*). Lectins are sugar-binding proteins of non-immune origin, with no catalytic activity, which play a role in cell recognition (*5*). In yeast, these lectins are products of a family of genes known as Flo genes. *Saccharomyces cerevisiae* has five major flocculin – encoding genes (*FLO1, FLO5, FLO8, FLO9, FLO10* and *FLO11*). The genes *FLO1, FLO5, FLO9* and *FLO10* encrypt proteins related to cell-cell adhesion, while *FLO11* encodes a protein responsible for cellular adhesion to substrates, diploid pseudohyphae formation and haploid invasive growth (*6,7,8*).

The Flo1p, Flo5p and Flo11p, have been identified as "Flo1-type flocculins" whose activity is inhibited by mannose and not by glucose (*9*). "New-Flo type flocculins" on the other hand, are those strains whose activity is inhibited by both mannose and glucose as they have a variant of flocculin, Lg-Flo1. Studies by Liu et al. (2007) indicate that presence of truncated forms of *FLO1* (*FLO1NS, FLO1NL, FLO1S* and *FLO1M*) could also lead to phenotype conversion from

Flo1 to New-Flo type. Domain swap experiments have shown that sugar binding domains of Flo1p and Lg-Flo1 are externally exposed N-terminal domains (*10*).

Studies have shown that the N terminal part of a three-domain lectin protein is responsible for carbohydrate binding (*11*). The N terminal of the protein shows high affinity binding towards carbohydrate moieties, specifically to D-mannose, α-methyl-D-mannoside, various dimannoses, and mannans.

Three flocculation phenotypes are known in *S. cerevisiae*:

(i) Flo1 phenotype strains that are inhibited by mannose and derivatives
(ii) New-Flo type strains, which are inhibited by mannose, glucose, maltose and sucrose, but not by galactose, and
(iii) MI (mannose insensitive) strains, in which flocculation is insensitive to mannose (*12*).

In addition to genetic determination, certain physical factors such as cell wall hydrophobicity, cell surface charge, cell surface topography, and cell age all contribute to the ability of yeast cells to flocculate (*13,14,15*). Nevertheless, all factors that determine yeast cell flocculation, especially in industrial strains, are unknown. For example, in brewing fermentation processes, repeated pitching of yeast leads to a loss in their flocculation ability, and this is difficult to predict (*16*).

We hypothesized that either a direct determination of yeast cellular mannose residues or cell wall associated lectins would provide more dynamic information regarding flocculation behaviour of industrial strains of *S. cerevisiae*. In addition, we discuss the roles that yeast sugar metabolic pathways, and sugar depletion pathways, play in expression of *FLO*-specific lectins on the cell surface. We selected four *S. cerevisiae* yeast strains used in different

fermentation applications to investigate if their flocculation behaviour when grown in different sugars was linked to lectin receptor density and the contents of cell wall glucans and mannans.

Materials and Methods

Yeast strains

In this study four industrial strains of *S. cerevisiae* were used. The strains were provided courtesy of Lallemand Inc. Montreal, Canada. The strains (prefixed with Lallemand Yeast Culture Collection (LYCC) designation) were: a lager brewing yeast strain, LYCCI, a champagne strain, LYCCII, a wine strain, LYCCIII, and a fuel alcohol strain, LYCCIV.

Growth media and culture conditions

Yeasts were routinely maintained at 4oC on YEPG agar slopes containing: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar.

Pre-cultures or seed cultures were prepared in 50 mL of YEPG in 100-mL Erlenmeyer flasks. Cells were incubated at 25°C on an orbital shaker (Infors HT Ecotron, Switzerland) at 170 rpm for 48 hours. Culture medium was prepared by inoculating YEPG at a starting cell density of 5×10^{6}cells/mL. The glucose in YEPG media was replaced with mannose, fructose, maltose, or galactose to investigate effects of sugar source on flocculation ability, glucan/mannan distribution on the cell wall and on the receptor density.

Flocculation assay

Flocculation abilities of the yeast strains were monitored using a method adapted from Bony *et al.* (1997) *(17)*. At defined periods of growth, yeast cells were harvested by centrifugation (4500×g for 5 min), washed and re-suspended in de-flocculation buffer (50 mM sodium acetate, 5 mM $CaCl_2$, pH 4.5) while the culture OD600 was adjusted at 2. The cells suspended in flocculation buffer containing calcium chloride ($CaCl_2$) were placed in test tubes of 15 mm diameter and 50 mm height was adjusted to give a final OD_{600} reading of 2. The tubes were sealed and kept on the shaking incubator at 140 rpm for 30 min. After agitation, 5 mL of the cell suspension was transferred to a new test tube and allowed to stand undisturbed for 6 min in a vertical position, after which, samples (1000 µL) were taken from just below the meniscus and the OD_{600} determined spectrophotometrically (Thermo Spectronic Genesys 10UV/10 UV Scanning Spectrophotometer 10-S). The percentage of flocculated cells was calculated by subtracting the fraction of cells remaining in suspension from the total cell count.

Mannan and glucan staining

We made use of the fluorescent lectins Concanavalin A- Alexa Fluor®-350 (Con A) and Pisum-sativum-agglutinate-fluorescein isothiocyanate (PSA-FITC) to determine contents of mannans and glucans, respectively, in yeast cells using a modification of the method of Heine *et al.* (2009).Yeast cells were harvested at late stationary phase of the growth curve by centrifugation at 5000 rpm for 5 min at 20°C.The pellet was then washed with PBS buffer (final concentration of 10 mM phosphate ions, 137 mM sodium chloride, and 2.7 mM potassium chloride) and then the cells were counted using a haemocytometer to a desired concentration of 3.15×10^6 cells/mL. Final concentration of the cells (1mL) was prepared in PBS and then 5 µL Pisum sativum-agglutinate FITC Conjugate was added and incubated in the dark for 25 min. The cells were centrifuged and the suspension was then transferred to 96 well plate and the fluorescence was read using a Modulus Microplate reader (Turners Biosystem). A small amount of sample

was placed on a clean slide and fluorescence observed using an inverted fluorescence microscope (Leica DMIRE2).The cells were then incubated with 25 µL Concanavalin A Alexa Fluor 350 nm for 10 min and then centrifuged and transferred to 96 well plate. The images were captured using a charged coupled EMCCD camera and analysed using Andor SOLIS for imaging X- 3043 software to visualise the distribution of the mannans and glucans on yeast cell walls.

Microscopy

The stained cells were observed using an inverted fluorescence microscope (Leica DM IRE2, Germany) and image analysis (camera: Charged coupled EMCCD iXon3, Andor, UK; software: Andor SOLIS for imaging X-3043). The fluorescence filters used were: Zeiss filter set 02 for Alexa Fluor®- 350 fluorescence (excitation G 365, BS 395, emission LP 420), Zeiss filter set 09 for FITC fluorescence (excitation BP 450-490, BS 510, emission LP 515).

Lectin-like receptor quantification

In general, the density of lectin like receptors present on the yeast cell surface was quantified using Avidin-FITC probe (Sigma) in the following modification of the method of Patelakis *et al.* (1998) (*18*). The probe and FITC conjugates were prepared at concentrations of 1500, 900, 540, 324, 192µg/mL in cCalcium-ethanol sodium acetate buffer (pH 4.0). 20 µL of the probe-FITC conjugates was added to 2980 µL and thoroughly mixed such that the final concentration of solution ranged from 10mg/mL down to 0.66mg/mL. The solution was then vortexed for 15 sec and measured using an excitation wavelength of 494±5 nm, and fluorescence read during 1 sec at 520±5 nm, using a Modulus Microplate reader (Turners Biosystem).

However, In order to quantify the presence of mannose receptors sites in specific (lectins) on the yeast cell surface, 137 the cells were harvested between 12-24 hours. The method of

analysis is based on spectrofluormetric measurements that generate the amount of free and bound probe. This concentration of free and bound probe on the yeast cell surface provides an estimate of the mannose binding receptors.

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

After measuring the yeast and buffer background fluorescence (free probe fluorescence intensity (B-D) and the bound probe fluorescence intensity (A-[C+(B-D)]), the actual amount of free and bound probe to the receptor was calculated in µg/mL. Further, free and bound probe concentrations were then analyzed according to the following Langmuir equation (*19*) in order to obtain the receptor density:

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

Where, P stands for the concentration of free probe, x is the concentration of bound probe, k is the proportionality constant and x' is the number of binding sites per molecule (number of

lectin sites). After deriving the equation by P, plotting 1/x versus 1/P will give a y-intercept of 1/x'.

Statistical Analysis

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

Results

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

Effect of sugars on flocculation

Fig (1A) shows the variation in flocculation ability when the strains were grown in media containing different sugars (i.e. maltose, glucose, mannose, galactose and fructose). The interactions mediated by Flo glycoproteins can be divided into two categories namely lectin-like (cell-to-cell adhesion) and sugar-insensitive (adhesion to abiotic surfaces) adhesion phenotypes (*4*). Furthermore, cell-cell adhesion phonotypes are divided into three sub types on the basis of their sensitivity towards sugars (*12, 20*). In our study, choice of yeast strain and sugar were observed to have significant effects on flocculation ability ($p \leq 0.001$). In terms of

strains, it was observed that a winemaking strain (LYCCIII), exhibited a range of flocculation from 2-24%. The strain almost lost flocculation when cultured on mannose and maltose in contrast to when the yeast cells were cultured in galactose, fructose and glucose where it showed high to moderate flocculation. A fuel alcohol strain, LYCCIV, exhibited consistently weak flocculation behaviour which ranged from 10-17%, when cultured on the five selected sugars. Unlike all the other strains, there was no effect on the flocculation when cultured on mannose. For a champagne yeast strain (LYCCII), this exhibited a range of 15- 29% flocculation ability. LYCCII yeast cells flocculated more when cultured on galactose and maltose, as the cells contained a high mannan content as well as mannose binding sites on the cell wall. Lastly, the brewing strain, LYCCI, flocculated highly on fructose (53%) and glucose (43%) and lost their flocculation ability when cultured on mannose (10%). Maltose and galactose had a moderate effect on the flocculation ability of the brewing strain.

Effect of sugars on mannan and glucan content in the cell wall

Fluorometric analysis of the selected industrial yeast strains was performed by applying fluorescent lectins ConA-Alexa Fluor and PSA-FITC to measure the levels of mannans and glucans, respectively, on the cell wall (Fig 1 (B) (C)). The strains were grown in media containing five different sugars (glucose, mannose, maltose, galactose or fructose) and harvested at the early stationary growth phase.

The protocol followed was modified from Heine et al. (2009) and showed good fluorescence when 5 µg PSA-FITC per 3.15×10^6 cells/mL was used for 25 min followed by incubation with 25 µg ConA-Alexa Fluor for 10 min. PSA-FITC application was done first in order to

mask the effect of excess cell wall-associated glucan (Fig 2). From the spectrofluormetric findings, it was observed that the type of sugar nutrient employed governed the extent of distribution of glucans on the yeast cell wall ($p \leq 0.001$), while no such effect was observed for

mannans ($p \geq 0.05$). The overall distribution of mannan remained same for all the strains when grown in different sugars. Interestingly, glucose and galactose had similar effects on cell wall glucan distribution as compared to maltose, mannose and fructose. The overall distribution pattern of mannan and glucan helped in understanding the flocculation pattern of these strains grown in different sugars as these are the binding sites for the lectin-like protein receptors (*2*).

Effect of sugars on the density of lectin like receptors

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

Fluorescence due to binding of the avidin-FITC complex to lectin sites provided an indication of the number of lectin sites available on the cell surface for the attachment to the neighboring mannan residues. The bound and free probe concentrations were analysed according to the Langmuir relationship (*19*) to obtain the receptor density, and data revealed significant relationship ($p \leq 0.001$) in the four strains when grown on different sugars (Fig 1D). This implies that the number of lectin-like receptors on the cell surface differs depending on the type of sugar available in the growth medium. In general, the brewing yeast strain LYCCI exhibited the maximum number of receptors on the cell surface, except when grown on galactose, followed by the champagne strain LYCII. Sugars in the medium may affect the transcription of FLO genes (*4*). The protein products for these genes (flocculins) could either be Flo1 or New-Flo type. Flo1 type strains have flocculins that are only mannose sensitive while New-Flo has a broader sugar range for sensitivity. Thus, despite having high receptor numbers on the yeasts when cultured on mannose sugars, they exhibited weak flocculation ability.

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

Yeast flocculation is generally considered to result from interactions between protein components on one cell surface and carbohydrate components on an adjacent cell surface. Thus, it is important to ascertain the effect of sugars on the cell wall polysaccharides as well as the protein or the lectin sites. Figure 3 shows the relationship amongst the mannan-glucan content (RFU), flocculation ability and the receptor density in yeast cells cultured with different sugars. When the data was analysed on the basis of sugars, taking each independent parameter namely (mannan content, glucan content and receptor density) vs. % flocculation ability, it was observed that sugars like maltose, fructose and galactose gave a negative correlation when plotted for mannan content (r^2 = -0.013,-0.066,-0.914, respectively) while glucose and mannan gave a positive correlation (r^2= 0.785,0.663, respectively). Significantly less important appears to be the role of cell wall glucans in yeast flocculation. We quantified the amount of glucans in cells cultured on different sugars and their role in yeast flocculation.

It was observed that glucan content vs. flocculation ability gave negative correlation for all the sugars except for maltose, which gave a positive correlation (r^2= 0.802). Finally, when the receptor density was plotted against flocculation ability, we observed a positive correlation (r^2= 0.872, 0.613, 0.938, 0.708, 0.748) for glucose, mannose, maltose, fructose and galactose, respectively.

Discussion

We examined the effect of sugars commonly found in industrial fermentation media employed for brewing, winemaking, fuel alcohol production processes (mainly glucose, fructose, maltose

or sucrose), as well as the other carbohydrates like galactose and mannose, on yeast flocculation. Flocculation was determined in washed cells, in standard conditions making it possible to correlate flocculation with the presence of lectin-like cell wall receptors.

As we know, the generally accepted mechanism of yeast flocculation is lectin mediated adhesion of adjacent yeast cells to form large cell clusters. Lectins (flocculins) are required for flocculation to occur, as their presence on one cell binds to mannose residues in the cell wall of adjacent cells and so link the yeast cells into clusters that contain thousands of cells. (*20,21,22,23*).

Maximum flocculation ability was observed for the strains when they were in the stationary phase of growth curve due to sugar depletion. We found that all the industrial yeast strains under the study belonged to the New-Flo type as flocculation behaviour in these strains was inhibited by mannose, glucose, maltose and fructose. This suggests that cell surface proteins were able to bind to a wider range of sugars, except galactose. Homologues of the *FLO1* gene known as *Lg-FLO1*, *FLONL* and *FLONS* are believed to encode for flocculin proteins conferring the New-Flo phenotype (*10,24*).

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

flocculate. Our results indicate a higher number of lectin like receptors compared to the work of Patelakis *et al.(18),* which focused more on the role of fermentation time on the density of lectin like receptors in a laboratory strain of *S. cerevisiae*. Our study was based on industrial strains of *S. cerevisiae*, that revealed approximately 100 fold more number of lectin like receptors on the yeast cell surface.

Strain-sensitivity to different sugars is the basis of the distinction of Flo1 and New-Flo phenotypes. Furthermore, fermentable sugars, including those found in brewer's wort, induce the loss of flocculation in the early lag and logarithmic phases of growth (*25*) or in starved cells (*26,27*) most likely affecting the expression of *FLO* genes. Although in the current study we did not isolate and amplify *FLO* genes, other studies have reported such findings in *S. cerevisiae* with various genes including *FLO1, FLO5* and *FLO9* (*6,7,8,10*). These studies, and the work reported here, highlight the diversity and strain dependency of flocculation gene expression in *S. cerevisiae*.

Reversible inhibition of flocculation by specific sugars such as mannose, maltose, glucose or fructose, which leads to dispersal of the flocs and eventually leading to loss of flocculation ability, by competitive inhibition with sugars of the yeast cell wall for lectin like receptors, has been described previously (*2,20,28*). Emergence of these receptors may be directly related to the expression levels of *FLO genes*. Sugars like glucose, fructose and galactose are responsible for expression of *FLO genes* though the Ras/ cAMP / PKA pathway (*4,29*). In this pathway, activation of PKA, TpK2 kinases leads to inactivation of Sfl1 (a suppressor of flocculation) and activates the positive regulation gene *FLO8 (30,31)*. The *FLO8* gene product acts as a transcriptional factor for other *FLO genes* mainly, *FLO1* gene that is responsible for the formation of the lectin like receptors on the yeast cell surface (*32,33*). In addition to the interconnection of the sugar metabolic pathways with the expression of flocculation genes, Amory *et al.* (1988) have identified other physiological factors in the medium that influence the emergence of receptors on the yeast cell surface (eg. pH, temperature, cell age etc.). Activation of the FLO genes is not only governed by presence of glucose but is also triggered by carbohydrate depletion, which may explain the role of the glucose repression pathway, which represses FLO11 as long as glucose is present in the medium. Thus, these pathways cannot be considered as single, independent entities, but rather as integrated systems working

together to control cellular adhesion (*30,34,35*). Therefore, when cultured on glucose, fructose, galactose and maltose (to some extent) cells exhibit sufficient receptors on their cell surfaces, which in turn govern the extent of flocculation (*10,36*).

Although the industrial strains of all *S. cerevisiae* strains selected for this investigation appear to belong to the NewFlo type, it remains to be seen if other strains employed in brewing, winemaking and bioethanol fermentations exhibit similar NewFlo flocculation phenotypes. Our findings may benefit brewers, winemakers and other yeast-based technologies in design of fermentation media comprising sugars that would not induce premature flocculation during the early stages fermentation.

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Conflict of Interest

This statement is to certify that all Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. We warrant that the article has not received prior publication and is not under consideration for publication elsewhere. On behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission and declare a “NO conflict of interest”.

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