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Influence of Cell Surface and Nanomechanical Properties on the Flocculation Ability of Industrial *Saccharomyces cerevisiae* Strains

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Abstract

In the past few years, atomic force microscopy (AFM) has provided novel information on the ultrastructural and nanomechanical properties of yeast cell walls that play a major role in determining the flocculation characteristics of the yeasts. In this study, we used AFM to visualize at the nanoscale the cell surface topography and to determine cell wall nanomechanical properties (e.g. elasticity and adhesion) of different strains of *S. cerevisiae* employed for brewing, winemaking and fuel alcohol production. Cell surface topography was found to correlate with the flocculation behaviour of these strains during their late stationary phase, with the cell surface of flocculent cells being rougher than that of weakly flocculent cells. The elastic modulus of the yeast cell walls showed that weakly flocculent strains had a more rigid cell wall than highly flocculent strains. This difference in elasticity seemed to have an effect on the adhesive properties of the yeast cell walls, with weakly flocculent yeasts displaying lower adhesion energy than the highly flocculent strains. These findings seem to indicate that yeast cell surface nanomechanical properties play an important role in governing flocculation.

Keywords: atomic force microscopy, cell surface, flocculation, flocculins, mannans, nanomechanical properties, *Saccharomyces cerevisiae*

1. Introduction

The yeast cell wall is a complex carbohydrate membrane, which not only protects the yeast cells from adverse conditions but also helps maintain an optimum osmotic balance to ensure normal cellular activities. The yeast cell wall is composed of a microfibrillar matrix of β- glucans (β -1,3 and β -1,6 glucans) which gives it its mechanical and chemical resistance. The local mechanical properties of the cell wall are commonly investigated by utilizing non-functionalised and functionalised (e.g. decorated with specific biomolecules) AFM probes (Francois *et al.*, 2006; Dufrêne, Boonaert, Gerin, Asther & Rouxhet, 1999). Two of the main components of the yeast cell wall are β- glucans (~ 50-60%) and chitin (~ 1-3%). Additionally, the yeast cell wall is overlaid with highly glycosylated proteins, which are decorated by long chains of mannose residues and represent 40-50% of the cell wall mass (Kapteyn, Van Den Ende & Klis, 1999).

It is well-known that the yeast cell surface is decorated with proteins that play a pivotal role in adhesion, communication and microbial infection (Jendretzki, Wittland, Wilk, Straede & Heinisch, 2011). Proteins are thought to play an important role in cell wall molecular organization and remodelling due to the variety of organization patterns stemming from the different ways in which proteins attach to the polysaccharide moiety. Three main proteins' attachment classes can be distinguished. The first class comprises of the proteins that are bound non covalently to the β-1,3- glucan network (the SCWs family); the second category consists of proteins attached covalently through a remnant of the GPI anchor to β-1,6-glucans (the GPI-CWPs); and finally the third class is made of cell wall mannoproteins that are characterized by Protein Internal Repeat regions (PIR-CWPs or CCWs family) that are directly linked to β-1,3- glucans (Klis, Boorsma & de Groot , 2006). This outer layer is made of highly mannosylated proteins together with large polysaccahrides complex of 150 or more D-mannose units. Thus, the mannoprotein layer bears crucial biochemical and biotechnological properties, some of which are adhesion, aggregation and flocculation (Caridi, 2006; Verstrepen & Klis, 2006) as well as virulence (Francois

et al., 2013; de Groot, Ram & Klis , 2008).

According to the lectin-like theory, flocculation occurs because of the interaction between flocculins (specific flocculation proteins which are present only on flocculent cells) and the carbohydrate residues (receptors) of the cell walls of the neighbouring cells (Miki, Poon, James & Seligy, 1982). AFM plays an important role in the investigation of the mechanical properties of cells, as it allows manipulation at single-cell level, local determination of the viscoelasticity of cells and direct observation of the cell surface at nanometer resolution (Binnig, Quate & Gerber, 1986; Burnham & Colton, 1989; Mizes, Loh, Miller, Ahuja & Grabowski, 1991).

The main aim of this study is to understand how cell surface roughness, cell adhesion force and cell surface elasticity, are correlated to flocculation, i.e. the reversible adhesion phenomenon that the cell undergoes during fermentation in the presence of calcium ions.

2. Materials and Methods

2.1 Yeast Strains

Four industrial strains of *S. cerevisiae* (provided by courtesy of Lallemand Inc. Montreal, Canada) were used: brewing yeast strain (LYCCI), champagne strain (LYCCII), wine strain (LYCCIII) and fuel alcohol strain (LYCCIV). The yeast strains were kept on Yeast Extract, Peptone, Glucose (YEPG) slopes at 4^{o}C (Nayyar, Walker, Canetta, Wardrop & Adya, 2014).

2.2 AFM Sample Preparation

The AFM samples were prepared by placing aliquots of 50µl (concentration of 1×10^{8}cells/ml) of yeast suspensions onto hydrophilic glass slides and allowed to air dry for about 3h at room temperature. AFM samples were used immediately soon after to preserve the original morphology of the yeasts under study. The glass slides were made hydrophilic by immersing them in aqueous 20% H_2SO_4 (sulphuric acid) for 24 h. The slides were then washed five times with ultrapure water and kept immersed in ultrapure water until use. The slides were air dried just before use. .

2.3 AFM Imaging of Yeast Surface Ultrastructure

A JPK Nanowizard I AFM (JPK, Berlin, Germany) was used. The yeast samples were imaged in contact mode (CM) by using Si_3N_4 (silicon nitride) triangular cantilevers (Veeco, Santa Barbara, CA, USA). The Si_3N_4 cantilever's spring constant was calibrated systemically using the thermal tune method (Song, Wu, Xu & Fu, 2015) and was found to be in the range of 0.01 - 0.02N/m. All the images (512×512 pixels) were captured at room temperature with a scanning speed of 0.5µm/sec and an applied force of 0.1nN. Since yeast cells can retain hydration water for several hours after having left the liquid environment (Canetta, Walker & Adya, 2009), the experiments were carried out in air at room temperature.

2.4 AFM Cytonanomechanics

After capturing the AFM image of each cell, AFM spectroscopy experiments were performed on the same cells in order to investigate their cytonanomechanics properties, such as cell elasticity and adhesion on the bud scar (B), cytoplasm (C) and edge (E) areas.

Fig. 1 shows a typical force spectroscopy curve. To probe only the elasticity (Young's modulus) of the cell wall without considering its adhesive properties (Arnoldi *et al.*, 2000; Yao *et al.*, 2002), the indentation speed was kept at 0.5µm/s with a maximal loading force of 1.5nN . The adhesive properties of the cell wall were probed during the retracting cycle (Fig. 1, points d-f and f-g) when the AFM probe was withdrawn from the cells surface at a constant speed of 0.5µm/s. The force *vs.* displacement curves obtained experimentally were converted into force-distance curves using the JPK Processing Software (JPK, Berlin, Germany). To access the elastic properties of the cells, the slope of each curve was interpolated using the Hertz model (Hertz, 1881):

$$F = \frac{2E\tan\alpha}{\pi\left(1-\vartheta^{2}\right)}\delta^{2} \tag{1}$$

where, F = loading force , E = Young's modulus, ϑ = Poisson's ratio, δ = indentation and α = half-cone angle. The assumption was made that the AFM probe used to perform the experiments was conical in shape with an opening angle of 35° (α) (Radmacher, Fritz & Hansma, 1995). A Poisson's ratio of 0.5 (incompressible material) was used.

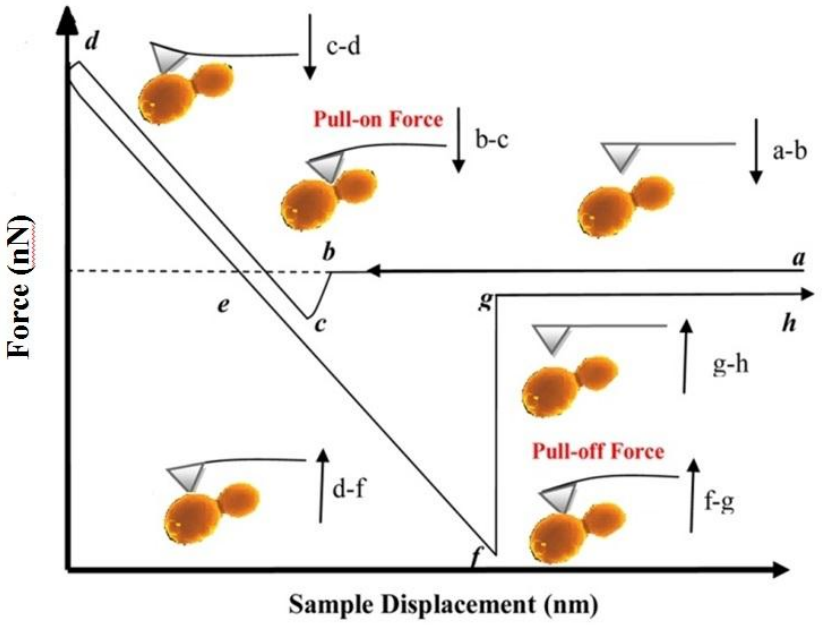

Figure 1. Schematic of the AFM force spectroscopy experiment. (a-b) - (c-d) represents the approaching cycle; (d-f) - (g-h) represents the retracting cycle. (a): The AFM probe approaches the cell surface, (b): The AFM probe jumps into contact with the cell surface due to Van der Walls forces, (c): The AFM probe indents the cell surface and during indentation the AFM cantilever on which the probe is micro-fabricated bends until the point (d) is reached, (e): The AFM probe is retracted from the cell surface and the latter stretches until point (f) is reached when the AFM probe and the cell surface detach from each other and the AFM probe-cell surface interaction force goes to zero (g).

2.5 Surface Roughness and Cell Thickness Analyses

Roughness analysis of the yeasts surface was carried out on raw (i.e. not subjected to any processing, such as flattening or filtering) AFM height images by measuring the root mean square roughness, R_{rms}, of each cell visible in the image using the JPK Processing software:

$$R_{rms} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{N}} \tag{2}$$

where x_i is the current height, $\bar{x}$ is the height of the mean data plane, and N is number of points within the selected area.

For each of the four strains, 35 cells were selected at random and the R_{rms} was evaluated at three different areas - budscar (B), cytoplasm (C) and edges (E) - on each cell using squares of 2.25 μm^2.

The cross sectional analysis was also performed over 35 cells using the JPK Processing software. Briefly, the height (peak-to-peak difference) and length of each cell were measured on the raw AFM height images. For each cell, the cross sectional analysis was repeated three times and statistics analysis was performed to get the average height and length of each individual cell.

2.8 Statistical Analysis

Statistical analysis was performed using IBM SPSS software (version 22). One-way ANOVA analysis was carried out to ascertain any change in the cell parameters (e.g. elasticity, adhesion force, cell surface roughness and cell thickness) with respect to yeast strains and type of sugar in the growth medium. Statistical significance was determined using Bonferroni and Tukey's tests. Correlation analysis was performed taking into consideration the Pearson's coefficient at two tailed level.

3. Results and Discussion

The nanostructural and nanomechanical properties of the four industrial yeast strains - (LYCCI - brewing),

(LYCCII - champagne), (LYCCIII - wine) and (LYCCIV - fuel alcohol) - were studied to determine a correlation between the morphology and cytomechanics of yeast cell surfaces and their level of flocculation.

3.1 Imaging Yeast Cells at High Resolution

The nanoscale morphological structures of the four industrial yeast strains were investigated by contact mode AFM imaging (Fig. 2). The AFM images of the four yeast strains showed the finest details of their surfaces and cellular shapes.

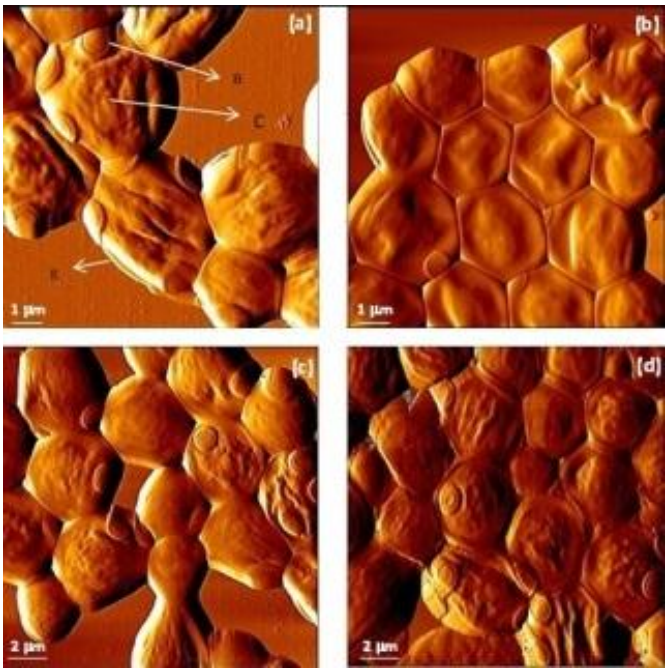

Figure 2. (a) AFM image of brewing yeast strain LYCCI (15 µm × 15 µm area; height scale 0 - 4.1µm). The bud (B), cytoplasm (C) and edge (E) areas on the yeast surface are indicated; (b) AFM image of champagne strain LYCCII, (15 µm × 15 µm area; height scale 0 - 3.1µm); (c) AFM image of wine strain LYCCIII (20 µm × 20 µm area; height scale 0 - 3.0µm); and (d) AFM image of fuel alcohol strain LYCCIV (20 µm × 20 µm area; height scale 0 - 2.7µm)

Cross sectional analysis on the AFM images showed that the brewing strain LYCCI were the largest cells (cell length = $2.10 \pm 0.05 \mu m$) followed by fuel alcohol strain LYCCIV (cell length = $1.1 \pm 0.1 \mu m$), champagne strain LYCCII (cell length = $0.9 \pm 0.1 \mu m$) and wine strain LYCCIII (cell length = $0.60 \pm 0.05 \mu m$).

Roughness analysis of the yeast cell surfaces displayed that the brewing strain LYCCI had the roughest cell wall (146 ± 8 *nm*), followed by the champagne strain LYCCII (95.1 ± 8.0 *nm*), fuel alcohol strain LYCCIV (73.5 ± 5.0 *nm*) and finally wine strain LYCCIII (35.7 ± 8.0 *nm*). The surface roughness and cell thickness of brewing, champagne and wine yeast cells seemed to be positively correlated ($r = 0.846$) to their flocculation ability (Nayyar et al. 2014) (Fig 3). In particular, it was observed that the rougher the cell surface and the thicker the cell, the higher the ability of the yeasts to flocculate. Interestingly, the fuel alcohol yeast strain showed a different behaviour compared to the wine yeast strain (Fig. 3); although it was rougher and thicker than the latter, its ability to flocculate was less good. The results for the brewing, champagne and wine yeast cells seem to indicate the presence on their surfaces of more sites for anchorage for the cells to hold on each other's surface; this could explain the formation of stable flocs in the fermenter and the increase in the flocculation ability for the strains (Ahimou, Touhami & Dufrene, 2003; Dague, Bitar, Ranchon, Durand & Yken, 2010). The low flocculation ability displayed by the fuel alcohol yeast strain compared to the wine strain despite being rougher and thicker than the wine yeasts could be caused by the fact that the fuel alcohol strain is less hydrophobic than the wine strain (Nayyar *et al.*, 2014).

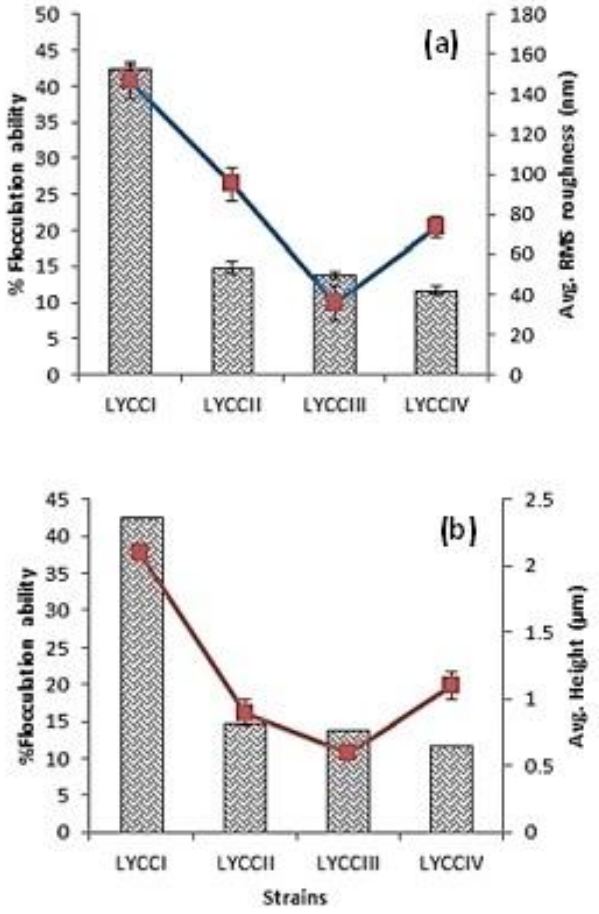

Figure 3. Relationship of (a) RMS roughness (nm) and (b) cell thickness (µm) with the % flocculation ability of the four *Saccharomyces cerevisiae* LYCCI, LYCCII, LYCCIII and LYCCIV yeast strains during their late stationary phase. The histograms represent the % flocculation ability, while the squares represent the RMS roughness in (a) and.the cell thickness in (b). Statistically significant direct relationship ($p \leq 0.001$) showed that for strains LYCCI, LYCCII and LYCIII the rougher the cell surface and the thicker the cell, the higher their flocculation ability. On the contrary, for the fuel alcohol strain LYCCIV displayed a lower flocculation ability than that of the wine strain despite being rougher and thicker than the latter

3.2 Effect of Elastic Modulus and Adhesive Force on the Flocculation Behaviour

Flocculation tests previously carried out by Nayyar *et al.* (Nayyar *et al.*, 2014) during all the phases of growth for the four industrial strains LYCCI, LYCCII, LYCIII and LYCCIV indicated that the brewing yeast strain LYCCI was highly flocculent throughout the fermentation including the late stationary phase (42.5%), followed by the champagne strain LYCCII (14.8%), wine strain LYCCIII (13.8%) and fuel alcohol strain LYCCIV (11.6%).

The Young's modulus, *Y*, adhesion force, F_{adh}, and adhesion energy, W_{adh}, of each of the four industrial strains at three different locations on the cell, bud scar (B), cytoplasm (C) and edge (E), was investigated by performing AFM force spectroscopy experiments.

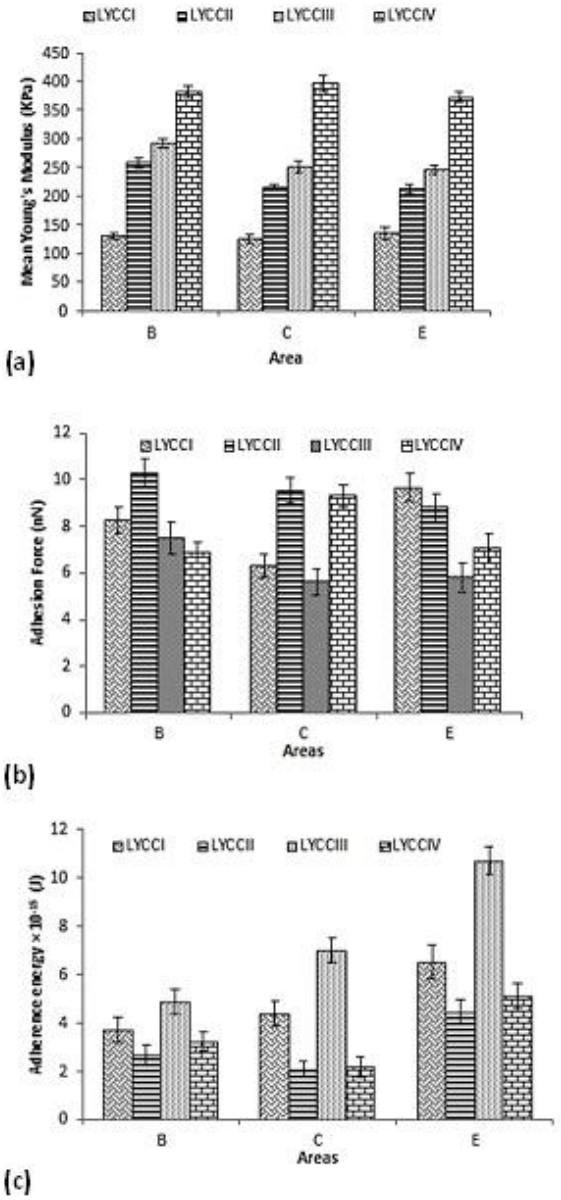

Figure 4. (a) Young's (elastic) modulus, (b) adhesion force and (c) adhesion energy for each of the four strains was measured at three different locations on the cell surface, i.e. on the bud scar (B), the cytoplasm (C) and the edge (E) of the cell

The results for the elastic modulus (Fig. 4a) were the same across the cell surface and showed that the brewing yeasts LYCCI were the softest ones ($Y = 389 \pm 7\ kPa$) and the fuel alcohol yeasts LYCCIV ($Y = 1152 \pm 11\ kPa$) the stiffest ones. These findings further confirmed the pattern observed in the flocculation essay and the surface roughness and cross sectional analyses, i.e. there is a positive correlation between the elasticity of the cell wall, the surface roughness and thickness of the cell, and its flocculation ability. In particular, these results seem to indicate that a high level of flocculation is achieved when the yeast cells have a rough surface and a soft cell wall because this morphological-mechanical synergy could allow for a higher mobility of GPI-anchored proteins (Verstrepen & Klis 2006; Zhang *et al.*, 2013).

To investigate the adhesive force that comes into play when the cell surfaces of two or more yeast come in contact, the adhesion force between the cell membrane and the AFM probe was measured. The hypothesis is that the higher the adhesion force of the cell membrane, the higher the stability of the flocs in the fermenter. Conversely to the elastic modulus, the values of adhesion force, F_{adh}, of the cell wall to the AFM probe was cell area dependent (Fig. 4b) with the differences at the bud scar, cytoplasm and edge of individual cells statistically

significant ($p \leq 0.001$). The brewing strain LYCCI was found to be most adhesive at the edges of the cells (F_{adh} = 10.0 ± 0.6 *nN*) followed by the champagne strain LYCCII ($F_{adh} = 9.0 \pm 0.6$ *nN*). Conversely, the fuel alcohol strain LYCCIV ($F_{adh} = 7.00 \pm 0.63$ *nN*) and wine strain LYCCIII ($F_{adh} = 6.00 \pm 0.65$ *nN*) displayed the least adhesive properties. These findings agreed with the values obtained for the elastic modulus because the LYCCI strain is the softest and also the most adhesive as expected. Similarly, the LYCCIV strain is the stiffest and also the least adhesive.

To gain a deeper insight into the relation between the adhesive properties of the strains and their flocculation abilities, the adhesion energy, W_{adh}, of their membranes was also studied. Adhesion energy describes the amount of work required for the cell to detach from a surface (in our case the AFM probe):

$$W_{adh} = F_{adh} \cdot x_{def} \tag{3}$$

where x_{def} accounts for how the amount of deformation undergone by the cell membrane before complete detachment from the AFM probe. Hence, W_{adh} provided information on the deformability of the cell membrane. Same as for the elastic modulus, the adhesion energy was area-independent, i.e. for each yeast strain the adhesion energy pattern was the same in the bud scar, cytoplasm and edge areas (Fig. 4c). However, the differences in the adhesion energy were more statistically significant at the cytoplasm and edge areas ($p \leq 0.001$) of the cells of the strains, and less significant on the bud scar region of the cell ($p \geq 0.05$). This was expected due to the rigidity and low adhesion of bud scars compared to the cytoplasm and edge areas of the yeasts. The results showed that wine making strain LYCCIII had highest adhesion energy ($W_{adh} = (22.5 \pm 0.6) \times 10^{-15}$ *J*) and champagne strain LYCCII presented the lowest adhesion energy ($W_{adh} = (9.1 \pm 0.5) \times 10^{-15}$ *J*). Comparing the findings for F_{adh} and W_{adh} highlighted that the champagne yeast cells LYCCII had a high F_{adh} but the lowest W_{adh}, while the wine yeasts LYCCIII had a low F_{adh} but the highest W_{adh}. Eq. (3) indicates that these differences between adhesion force and energy are due to the cell wall of LYCCII deforming less than that of LYCIII. Since the elasticity of the cell membrane of LYCCII is higher (i.e. softer) than that of LYCCIII, the lower deformation presented by LYCCII could indicate a more liquid-like behaviour of its cell membrane compared to LYCCIII.

3.3 Correlation between the Flocculation Ability and the Different Nano Mechanical Properties of Cells

The surface roughness, nanomechanical and flocculent properties of the four strains are summarised in Table 1.

Table 1. Summary of the average surface roughness, nanomechanical and flocculation properties of the four strains. *E* represents the Young's modulus (kPa), F_{adh} the adhesion force (nN), and W_{adh} the adhesion energy (10^{-15} J), respectively

Strain	R_{rms} (nm)	Y (kPa)	F_{adh} (nN)	W_{adh} (10^{-15} J)	Flocculation (%)
LYCCI	146.0 ± 8.0	126.0 ± 7.0	8.0 ± 0.6	4.7 ± 0.7	42.5 ± 9.5
LYCCII	95.1± 8.0	251.0 ± 7.0	10.0 ± 0.6	3.1 ± 0.5	14.8 ± 3.4
LYCCIII	35.7± 8.0	303.0 ± 9.0	6.3 ± 0.6	7.4 ± 0.6	13.8 ± 3.3
LYCCIV	73.5± 5.0	374.0 ± 11.0	7.1 ± 0.7	3.5 ± 0.5	11.6 ± 2.9

A pattern in the relationship between flocculence, surface roughness and nanomechanics of the cell wall could be observed. In particular, our findings showed that is LYCC1 (brewing strain) is the most flocculent and presents a high level of adhesion. Moreover, LYCCI was the softest yeast and seemed to deform more than others, and its surface was the smoothest. Conversely, LYCCIV (fuel alcohol strain) displayed the lowest flocculation ability and was found to be the most rigid of the four yeast strains with a relatively rough surface. Additionally, LYCCIV cell membrane showed a relative low level of adhesion and deformability. The results appeared to indicate a negative correlation between the flocculent and elastic properties of the cell: the softer (low elastic modulus) and more deformable (high adhesion energy) the cell membrane, the more flocculent the strain. This seemed to suggest that the flexibility of the cell wall was an important parameter to enhance the flocculation ability of the yeast strain during fermentation. To verify this hypothesis, the surface roughness, elastic modulus, adhesion force and adhesion energy of the cells were plotted as functions of the flocculation ability of the strains (Fig. 5). As expected, a statistically significant ($p \leq 0.001$) and strong negative correlation ($r = -0.825$) between the flocculation ability and the elasticity was found (Fig. 5a). Conversely, the flocculation ability of the yeasts and their surface roughness displayed a statistically significant ($p \leq 0.001$) and strong positive correlation ($r = 0.846$). These findings could indicate that the flocs in the liquid medium are held by the better and stable anchorage that rough surfaces provide.

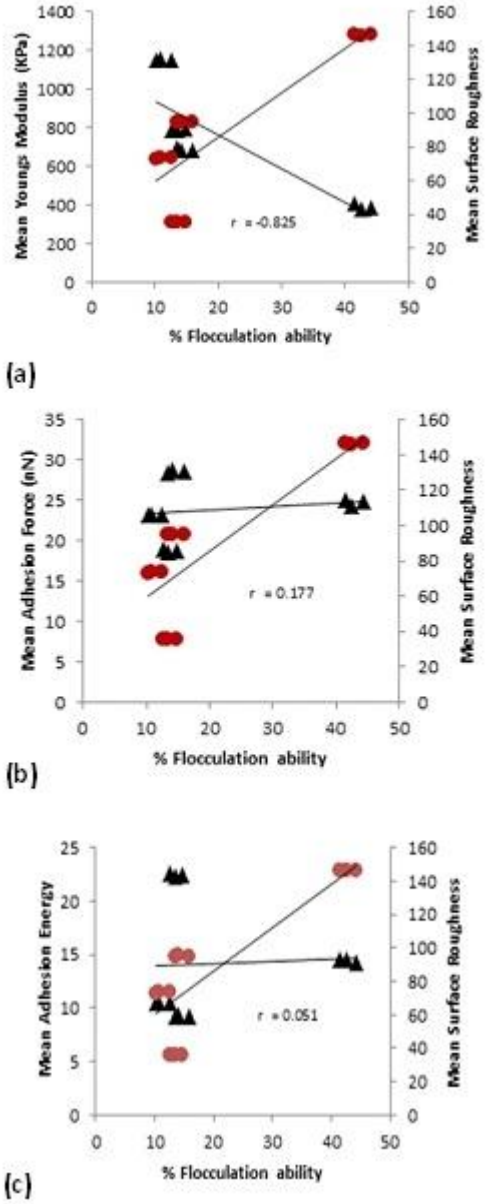

Figure 5. Correlation studies between the percentage (%) flocculation ability, the surface roughness and nanomechanical properties of the four industrial yeast strains LYCCI (brewing yeast), LYCCII (champagne yeast), LYCCIII (wine yeast), and LYCCIV (fuel alcohol yeast). The circles represent the relationship between the surface roughness of the yeasts and their % flocculation ability. This correlation is positive ($r= 0.846$). The triangles represent the Young's modulus, adhesive force and adhesive energy of the four industrial strains. (a) Statistically significant ($p \leq 0.001$) negative correlation ($r = -0.825$) between % flocculation ability and the Young's (elastic) modulus of the cells. (b) and (c) Positive correlation between % flocculation ability and the adhesion force ($r = 0.177$) and the % flocculation ability and the adhesion energy ($r = 0.051$) of the yeasts. In both cases the correlation is statistically insignificant ($p \geq 0.05$).

Interestingly, a statistically insignificant ($p \geq 0.05$) and weak positive correlation between the flocculation ability of the strains and the adhesion force ($r = 0.177$) and adhesion energy ($r = 0.051$) of their cell walls was observed, suggesting that the adhesion force between the yeast surfaces and the deformability of their cell walls played a less important role in governing the flocculation ability compared to the elasticity and roughness of the cell membrane. These findings are important in food technology because of their potential critical impact on fermentation performance. In fact, knowing the physical biomarkers (i.e. surface roughness and cytonanomechanical properties) of highly flocculating *S. Cerevisiae* yeast strains can be used not only to reliably

characterise yeast strains but also for engineering them in order to achieve the best yeast management.

4. Conclusions

Morphological changes in the yeast cell surface for different strains, at nanoscopic level were observed by immobilizing the cells on hydrophilic slides by air drying for as long as 5h (Canetta, Adya & Walker, 2006a, 2006b; Canetta *et al.*, 2009).The topological changes were quantified in terms of changes in roughness, which was found to be maximum for the brewing strain LYCCI, which was also found to be highly flocculent. The dramatic change in roughness may have some consequence on the adherence capacity of yeast to material surface (Gallardo-Moreno, González-Martín,Perez-Giraldo, Bruque & Gomez-Garcıa, 2004; Mercier-Bonin, Ouazzani, Schmitz & Lorthois, 2004). Also it is clear from our data that the elasticity of the cell wall, as determined by force-indentation curves, is directly related to the flocculation patterns for the strains, as it was seen that strains with higher mean Young's modulus, adherence energy and adhesion force exhibited higher flocculation ability. Altogether these changes help us understand the rigidness of the cell wall which could help us relate to the reasons contributing to different flocculation patterns for the strains.

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