YEAST FLOCCULATION: A NEW METHOD FOR CHARACTERISING CELL SURFACE INTERACTIONS

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Flocculation is a property of yeast cells that has been traditionally used in industrial fermentations, especially in the brewing and wine industries. It is a cell wall interaction and an increase in flocculation is strongly correlated with an increase in cell surface hydrophobicity.

Methods to assay yeast cell surface hydrophobicity are only qualitative and while electrostatic interactions can play an important role, they are not always taken into account.

In this work, a simple and accurate method based on contact angle measurements is described to determine yeast cell surface hydrophobicity. With this method, it is possible to quantify yeast cell surface hydrophobicity by determining the free energy of interaction between cells and water molecules. Several yeast strains with different flocculation characteristics have been used to validate the method.

Key Words: Yeast, flocculation, contact angle, surface free energy, hydrophobic interactions.

NOTATION

ΔG_{iw}^{AB}	polar component of the free energy of interaction between yeast cells (i) and water (w) (mJ/m ²)
ΔG_{iw}^{AW}	apolar component of the free energy of interaction between yeast cells (i) and water (w) (mJ/m ²)
ΔG_{iw}^{tot}	free energy of interaction between yeast cells (i) and water (w) (mJ/m ²)
γ	surface tension (mJ/m ²)
γ^{AB}	polar component of surface tension (mJ/m ²)
γ^{AW}	apolar component of surface tension (mJ/m ²)
γ^+	electron acceptor parameter of the polar component of surface tension (mJ/m ²)
γ^-	electron donor parameter of the polar component of surface tension (mJ/m ²)

INTRODUCTION

Flocculation in yeasts is defined as the ability of cells to aggregate spontaneously and form flocs which sediment rapidly in culture medium^{1,2}. This property of yeast cells has been traditionally used by the brewing and wine industries and more recently in continuous ethanolic fermentation processes^{3,11}.

Yeast flocculation is described as a cell wall interaction, according to the model proposed by Miki *et al.*⁴. Several features of flocculation including the requirement for calcium, specific inhibition by sugars, interaction of non-flocculent cells with flocculent cells and culture conditions have been well identified^{5,8,9,10,13}. It is also clear that hydrophobic interactions play a crucial role in microbial adhesion phenomena⁶ and in *Saccharomyces* and *Kluyveromyces* strains an increase in flocculation is strongly correlated with an increase in cell wall surface hydrophobicity^{14,16,17}.

A variety of techniques are currently used to estimate the hydrophobicity of bacterial cell surfaces¹⁸. Some are based on the specific binding properties of bacteria, measuring the actual binding to substances that carry hydrophobic groups such as hexadecane, octyl sepharose and polystyrene. Others give an estimate of the overall surface properties, such as salting-out and contact angle measurements.

Adhesion to hexadecane is one of the most common methods used. As hexadecane is highly negatively charged, the electrical interactions between cells and hexadecane will influence the results¹. More recently Straver *et al.*¹⁴ proposed a new method based on adhesion to paramagnetic polystyrene-

coated latex beads. As both beads and yeast cells are reported to be negatively charged, this can result in electrostatic repulsion, influencing the results. Of all the methods mentioned above, only contact angle measurements yield quantitative data.

Contact angle measurements by the sessile drop technique, described by van Loodtsdrecht *et al.*¹⁹ is another technique that is commonly used to estimate yeast cell surface hydrophobicity. This method is based on the measurement of the contact angle formed between the surface and the tangent to the liquid drop in the contact point. To enable this measurement to be made, the microbial cells must be spread uniformly on a plane surface and a cell layer is usually built up on a membrane by filtration of a cell suspension. However, due to the high rate of liquid drop penetration into the cell layer, these measurements are very often difficult to perform⁵ and lack accuracy.

When considering cell–cell interactions in aqueous systems, it is also of interest to quantify hydrophobic interactions and the relative importance of polar and apolar interactions in aggregation mechanisms. This can be accomplished by measuring contact angles on a lawn of cells with liquids having different polarities and correlating values of contact angles with cell surface tension. This technique has been applied to clay minerals and gels^{22,23} but, no data are available, when interactions between microorganisms are considered.

In this work, a simple and accurate method based on contact angle measurements is presented to determine yeast cell surface hydrophobicity. It has been validated by correlating the values of contact angle with the flocculation ability of several yeast strains.

The importance of polar and apolar interactions between water molecules and cells in the flocculation mechanism is also quantified.

MATERIALS AND METHODS

Yeast strains and culture conditions

Baker's yeast was obtained from a local bakery. *Saccharomyces cerevisiae* NRRL Y265, used as a strain with constant flocculation capacity during all growth phases, was grown in a semi-synthetic medium, in a continuous bioreactor¹¹. Samples were collected from the bioreactor as required.

The brewing yeast, *Saccharomyces uvarum* (carlsbergensis), was kindly supplied by UNICER. The non-flocculating variety was obtained from the propagation step and the flocculating variety was taken from the fermentation vessel at the end of the 3rd industrial generation.

Flocculation assay

The flocculation ability of the different yeast cells was measured using a modified Helm's sedimentation test as described by Soares *et al.*⁹

Preparation of the yeast substrata

Yeast cells were harvested by centrifugation, washed once with deionized water and then with increasing concentrations of ethanol (10%, 25%, 50%, 70%, 90%) and finally resuspended in pure ethanol to a concentration of 10^8 cells/ml. This procedure, using increasing ethanol concentrations, was used to avoid changes in cell wall structure due to dehydration.

This cell suspension was immersed in a sonication bath for 5 minutes in order to obtain better homogenization. Meanwhile, a solution of 20 g/litre of agar and 10% glycerol, was cast in glass petri dishes and cooled at room temperature. Finally, the agar was cut into small squares of 1.5×1.5 cm².

A small volume of the cell suspension (250 μ l) was spread over each agar square. After drying the first layer of cells, two more layers were added, completely covering the agar square.

Contact angle measurements

Contact angles were measured on the agar plates covered with cells. The measurements were carried out in a standard contact angle apparatus (Kruss-GmH, Hamburg).

The determinations of the contact angles were performed automatically, with the aid of an image analysis system (Kruss-GmH, Hamburg). The images were received by a video camera connected to a 486 DX4 100 MHz personal computer, with an automatic measuring system (G2/G40) installed.

All the measurements were made at room temperature and three different liquids were used: water, di-iodomethane and glycerol.

RESULTS AND DISCUSSION

Three yeast strains were used: a non-flocculating strain baker's yeast (*F*⁻), a flocculating yeast *Saccharomyces cerevisiae* NRRL Y265 (*F*⁺) and a brewer's yeast strain (*B*). The brewing yeast strain was obtained in different phases of the brewing process in order to have different flocculating characteristics: *B*⁻ and *B*⁺ denote the lowest and the highest flocculating ability, respectively.

The flocculation profile of this group of yeasts is presented in Figure 1.

Fig. 1 Sedimentation profiles of flocculating and non-flocculating *Saccharomyces cerevisiae* (*F*⁺, *F*⁻) and *Saccharomyces uvarum* (*B*⁺, *B*⁻).

TABLE I. Contact angles (and standard deviations) obtained with different yeast strains, using water, glycerol and di-iodomethane

Yeast strain	Contact angle (°)		
	water	glycerol	diiodomethane
<i>F</i> ⁻	13.7 ± 2.3	25.9 ± 3.3	61.9 ± 3.1
<i>F</i> ⁺	34.2 ± 2.2	36.9 ± 3.2	75.8 ± 2.5
<i>B</i> ⁻	11.6 ± 1.3	22.3 ± 3.4	60.7 ± 3.5
<i>B</i> ⁺	30.3 ± 3.1	37.5 ± 3.5	75.5 ± 1.0

The problems associated with liquid penetration in the cell layer were overcome, with the procedure above described for the measurement of contact angles. Scanning electron microscope observations made of dehydrated yeast cells, showed that the dehydration treatment to which yeast cells were submitted did not damage their structure.

The corresponding contact angles and the standard deviations for a set of 15 measurements (for each yeast sample with a different liquid) using water, glycerol and di-iodomethane are presented in Table I.

From these results it is apparent that:

- the strain with the highest flocculation ability clearly presents the highest contact angle of water. The non-flocculating strains have the lowest values. This is in agreement with published data¹⁶, describing the importance of cell wall hydrophobicity in flocculation.
- this technique seems to be very suitable for contact angle measurements, because there is a good degree of confidence in the data obtained.
- it is also worth noting the similar values of contact angles obtained for the flocculating strains with glycerol and di-iodomethane. In both cases, contact angles are significantly higher for the flocculating strains.

The contact angle values obtained for water, glycerol and di-iodomethane, in addition to testing the validity of the method, make possible the calculation of cell surface tension, which can be used as a measurement of cell surface hydrophobicity. It is known that a high surface tension means a small degree of hydrophobicity.

The accepted approach for the calculation of the surface tension (or surface free energy) of solids is that of van Oss *et al.*^{20,21,22} The surface tension, γ , is assumed to be the sum of two components:

$$\gamma = \gamma^{LW} + \gamma^{AB} \quad (1)$$

where γ^{LW} is the Lifshitz-van der Waals component, comprising apolar interactions and γ^{AB} is the Lewis acid-base component, taking into account polar interactions. As the name of the last component indicates, these polar interactions involve two functionalities, one for the electron acceptor (Lewis acid) and the other for the electron donor (Lewis base). Thus, γ^{AB} is determined by two parameters:

$$\gamma^{AB} = 2\sqrt{\gamma^+ \gamma^-} \quad (2)$$

where γ^+ is the electron-acceptor (acid) parameter and γ^- is the electron-donor (base) parameter.

To determine the components of the surface tension of a solid, it is necessary to use at least three liquids with known components of surface tension²². One of the three liquids has to be apolar (in this case di-iodomethane).

The values obtained for the surface tension γ and its components for each yeast strain, calculated as described by van Oss *et al.*²² are presented in Table II.

The values of the surface tension are consistent with the flocculating ability of the yeast strains, with the lowest values of the surface tension pertaining to the flocculating strains. Another point to note is that the values of the parameter

TABLE II. Apolar and polar surface tension parameters for each yeast strain (mJ/m²)

Yeast strain	γ^{LW}	γ^+	γ^-	γ^{AB}	γ
F ⁻	27.5	4.4	53.0	30.5	58.0
F ⁺	19.7	6.1	43.7	32.7	52.4
B ⁻	28.2	4.7	51.8	30.2	58.4
B ⁺	19.9	5.4	48.8	32.5	52.4

comprising the apolar interactions γ^{LW} are lower for both flocculating strains than for the non flocculating ones and this parameter for the flocculating strains is smaller than the corresponding value for water (21.8 mJ/m²).

Considering the interaction between cells and water, the apolar component of the free energy of interaction ΔG_{in}^{LW} between the strain (i) and water (w) is given by:

$$\Delta G_{in}^{LW} = -2 \left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}} \right)^2 \quad (3)$$

in which γ_w^{LW} , γ_i^{LW} , denotes the apolar components of the surface tension for water and for the cell surface, respectively.

The ΔG_{in}^{AB} component is related to the individual surface tension components by:

$$\Delta G_{in}^{AB} = -4 \left[\sqrt{\gamma_i^+ \gamma_i^-} + \sqrt{\gamma_w^+ \gamma_w^-} - \sqrt{\gamma_i^+ \gamma_w^-} - \sqrt{\gamma_i^- \gamma_w^+} \right] \quad (4)$$

where γ_w^+ , γ_w^- and γ_i^+ , γ_i^- , denote the polar components of the surface tension for water and for the cell surface respectively.

The surface tension components for water are:

$$\gamma_w^{LW} = 21.8 \text{ mJ/m}^2 \text{ and } \gamma_w^{AB} = 51.2 \text{ mJ/m}^2 \\ (\text{with } \gamma_w^+ = \gamma_w^- = 25.5 \text{ mJ/m}^2).$$

The total free energy of interaction, ΔG_{in}^{tot} , is given by:

$$\Delta G_{in}^{tot} = \Delta G_{in}^{LW} + \Delta G_{in}^{AB} \quad (5)$$

The calculated values of the free energy of interaction between cells and water are given in Table III.

ΔG_{in}^{tot} is a measure of the hydrophobic interactions that can be established between cells and water molecules. Hydrophobic interactions comprise the LW attraction (which decay relatively slowly with distance and thus have a long range nature) and the short-range interactions (in particular, hydrogen bonds)²¹.

In the case of a negligible interaction LW, ΔG_{in}^{tot} expresses the degree to which the polar attraction of cells to water is greater (hydrophilicity) or smaller (hydrophobicity) than the polar attraction which water molecules have to each other²¹.

From the values in Table III, it can be observed that the resulting ΔG_{in}^{tot} is smaller for the flocculating cells than for the non-flocculating ones (Table II). This expresses the degree to which the interaction between flocculating cells and water is smaller and apparently this fact is responsible for a preferential interaction between cells.

TABLE III. Values of the free energy of interaction between cells and water (mJ/m²)

Yeast strain	ΔG_{in}^{LW}	ΔG_{in}^{AB}	ΔG_{in}^{tot}
F ⁻	-0.66	26.3	25.6
F ⁺	-0.11	16.1	16.0
B ⁻	-0.82	24.8	24.0
B ⁺	-0.09	21.1	21.0

CONCLUSIONS

From the above results, the following conclusions can be drawn:

- The technique presented here enables measurements of contact angle on microorganism cell surfaces with a good degree of confidence.
- The results obtained confirmed that the ability of yeast strains to flocculate is directly related to great cell surface hydrophobicity or to low surface tension.
- The preferential attraction between cells of flocculating strains, responsible for cell aggregation, can be explained by the smaller free energy of interaction between cells and water.

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