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Flocculence of *Saccharomyces cerevisiae* Cells Is Induced by Nutrient Limitation, with Cell Surface Hydrophobicity as a Major Determinant

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Initiation of flocculation ability of *Saccharomyces cerevisiae* MPY1 cells was observed at the moment the cells stop dividing because of nitrogen limitation. A shift in concentration of the limiting nutrient resulted in a corresponding shift in cell division and initiation of flocculence. Other limitations also led to initiation of flocculence, with magnesium limitation as the exception. Magnesium-limited *S. cerevisiae* cells did not flocculate at any stage of growth. Cell surface hydrophobicity was found to be strongly correlated with the ability of the yeast cells to flocculate. Hydrophobicity sharply increased at the end of the logarithmic growth phase, shortly before initiation of flocculation ability. Treatments of cells which resulted in a decrease in hydrophobicity also yielded a decrease in flocculation ability. Similarly, the presence of polycations increased both hydrophobicity and the ability to flocculate. Magnesium-limited cells were found to be strongly affected in cell surface hydrophobicity. A proteinaceous cell surface factor(s) was identified as a flocculin. This heat-stable component had a strong emulsifiying activity, and appears to be involved in both cell surface hydrophobicity and in flocculation ability of the yeast cells.

Flocculation of yeast (*Saccharomyces cerevisiae*) cells has been defined as "the phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended" (21). Yeast cells gain the ability to flocculate at the end of the fermentation process (2, 8, 12, 14), which makes this cell adhesion process of considerable interest in brewing industry (5, 23). However, the causal mechanism of initiation of flocculation ability is not known.

Data on determinants of flocculation are scattered and conflicting in the literature and concern different strains, treatments, and growth conditions. Initially, flocculation was reported to be a process based solely on ionic interactions, with Ca^{2+} ions acting as bridges between the yeast cells (13). A requirement for Ca^{2+} in flocculation of yeast cells is generally reported (see reference 5 for a review), but in some cases magnesium and manganese ions may act as substitutes (11, 22). Since flocs can be dispersed in the presence of sugars, most notably mannose (9, 11, 17), it was suggested that most likely a lectin-sugar interaction is involved in flocculation. Stratford and Keenan (24–26) provided evidence that agitation was required for initiation of flocculation. These results indicate that physicochemical cell surface interactions might also be involved in flocculation. Beavan and Belk (3) reported a correlation between flocculation and electrophoretic mobility of yeast cells under certain conditions, whereas others reported a correlation between hydrophobicity and flocculation for some yeast strains (1, 8). In most cases, however, the data are not conclusive and do not cover the possible role of specific interactions besides nonspecific adhesion. For instance, Kamada and Murata (8) found flocculation not to be cation dependent, in contrast to most reports, and Amory et al. (1) only used one method to measure hydrophobicity and showed the results from only two measuring points during growth of the yeast cells.

Taken together, the scattered results and the differences between reports dealing with various factors that affect flocculation of different yeast strains under different growth and test conditions hamper proper comparison of the results. The molecular basis of flocculation is still poorly understood, despite the importance of this process in industrial processes. The present study was initiated to determine characteristics of flocculation under standardized conditions and, subsequently, to identify yeast cell surface components involved in flocculation. Throughout this study, one strain and defined growth conditions were used. Nutrient limitation appeared to trigger an increase in cell surface hydrophobicity and, concomitantly, flocculation ability. The results strongly suggest that both Ca^{2+}-dependent sugar binding and physicochemical interactions, most notably cell surface hydrophobicity, are involved in the flocculation process of yeast cells. Furthermore, a proteinaceous surface compound with emulsifying activity was identified as a flocculin, involved in cell surface hydrophobicity and flocculation.

MATERIALS AND METHODS

Yeast strain and culture conditions. *S. cerevisiae* MPY1 used in these studies is a bottom-fermentation brewery isolate. The yeast cells were maintained at 4°C on wort agar slopes. The standard medium used contains (per liter of deionized water): maltose, 80.0 g; KH_2PO_4, 3.0 g; $MgCl_2 \cdot 6H_2O$, 0.6 g; K_2SO_4, 0.4 g; L-alanine, 0.107 g; L-arginine, 0.126 g; L-asparagine, 0.083 g; L-isoleucine, 0.078 g; DL-leucine, 0.158 g; L-lysine, 0.103 g; L-proline, 0.369 g; L-serine, 0.069 g; L-threonine, 0.063 g; L-tyrosine, 0.100 g; L-valine, 0.150 g; Ca^{2+}-pantothenate, 1.0 mg; myoinositol, 25.0 mg; nicotinic acid, 0.2 mg; biotin, 0.05 mg; EDTA, 30 mg; $ZnCl_2$, 2.2 mg; $CaCl_2 \cdot 2H_2O$, 18 mg; $MnCl_2 \cdot 2H_2O$, 1.0

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mg; $FeCl_2 \cdot 4H_2O$, 4.3 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg; $CuCl_2 \cdot 2H_2O$, 0.4 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; H_3BO_3, 1.0 mg; and KI, 1.0 mg. In carbon-limited medium, the amount of maltose in the medium was reduced to 16.0 g/liter. Zn^{2+}-limited medium contains 44 μg of $ZnCl_2$ per liter instead of 2.2 mg, and Mg^{2+}-limited medium contains 6 μg of $MgCl_2 \cdot 6H_2O$ per liter instead of 0.6 mg.

Yeast cells were cultivated at 28°C in 100-ml cotton-plugged Erlenmeyer flasks containing 50 ml of standard medium (180 rpm). Yeast cells for isolation of flocculins were grown in 2-liter Erlenmeyer flasks containing 1.0 liter of standard medium. Growth was measured by the A_{620} value (after proper dilutions) with a Novaspec II spectrophotometer (Pharmacia-LKB, Uppsala, Sweden), by direct cell counting with a hemacytometer, and by dry weight determination of cells. Direct cell countings were done in the presence of 10 mM EDTA, which prevents aggregation of the cells. For dry weight determinations, three culture samples of 1.0 ml each were filtered over a 0.45-μm-pore-size membrane filter (Sartorius GmbH, Göttingen, Germany) and washed three times with deionized water before drying at 80°C for 24 h.

Flocculation assay. The flocculation assay is essentially based on the assay described by Miki et al. (11), with a number of modifications. Briefly, after determination of the A_{620} value of the culture, yeast cells were harvested by centrifugation, washed, and resuspended in a 50 mM Na acetate–0.1% $CaCl_2$ buffer (pH 4.5) (flocculation buffer) to a final A_{620} value of 2.5. After 30 min of acclimatization at room temperature, 650 μl of cell suspension was added to a 1.0-ml cuvette. With this sample volume, the light beam of a Novaspec II spectrophotometer monitors the optical density slightly below the surface of the cell suspension. The cell suspension was whirlmixed for 20 s with a Vortex Genie apparatus at maximum speed; this was followed by five inversions of the cuvette. Immediately thereafter, the settling profiles were spectrophotometrically determined (see Fig. 1). The maximal decrease in optical density per minute was chosen as a measure of flocculation ability of the cells. Very strong flocculation resulting in a decrease in optical density higher than 0.5/min could not be measured accurately and is represented as >0.5. The influence of adding salts and monosaccharides was tested after these compounds were added to the flocculation buffer just before whirlmixing. The variability of this test is about 10%.

Treatments of yeast cells. Yeast cells were harvested by centrifugation at an A_{620} value of 5.0 and suspended in 10 mM Tris-HCl buffer (pH 7.5). Proteinase K and pronase (both from Sigma) were added to the cells in a final concentration of 0.1 and 1.0 mg $\cdot$ ml^{-1}, respectively, and then incubated for 60 min at 28°C under gentle agitation. Subsequently, the yeast cells were harvested by centrifugation, washed three times with deionized water, and resuspended in flocculation buffer to a final A_{620} value of 2.5. Controls were incubated for 60 min in Tris-HCl buffer only.

Yeast cells were sheared for 30 min in 50 mM Na-acetate buffer (pH 4.5) with a Sorvall Omnimixer (DuPont Instruments, Newton, Conn.) at a setting of 6.0. Subsequently, the cells were harvested, washed, and resuspended in flocculation buffer.

Determination of physicochemical cell surface characteristics. Cell surface hydrophobicity of yeast cells was determined by three methods: (i) interaction of yeast cells with hexadecane; (ii) adhesion of yeast cells to polystyrene; and (iii) contact angle determinations of water droplets on a layer of yeast cells. Determination of the interaction of yeast cells with hexadecane was done essentially as described previously for bacteria (18). Two volumes of yeast cells, suspended in flocculation buffer to an A_{620} value of 2.5, were whirlmixed for 20 s with 1 volume of hexadecane (Sigma). After 30 min at room temperature, the interaction was determined as the amount of emulsification of the hexadecane in the water phase.

Adhesion of yeast cells to polystyrene was measured as follows: yeast cells were harvested and suspended in 50 mM Na-acetate buffer (pH 4.5) to an A_{620} value of 2.5. Ten milliliters of this suspension was transferred into a standard petri plate (Greiner) and incubated for 2 h at room temperature. Subsequently, the supernatant fluid was removed and the plate was rinsed five times under a gentle stream of deionized water. Adhesion of the cells to the petri dish was then monitored with a light microscope.

The contact angle of water on a layer of yeast cells was determined essentially by the method of Van Loosdrecht et al. (27). Briefly, 10.0 ml of yeast culture was harvested, and the cells were suspended in phosphate-buffered saline (PBS; NaCl, 8.5 g/liter; KH_2PO_4, 0.272 g/liter; $Na_2HPO_4 \cdot 2H_2O$, 1,424 g/liter; pH 7.2) and collected on a 0.20 μm-pore-size Sartorius membrane filter. The number of cells used yielded a film of cells with a thickness of approximately 50 cell layers, as judged from the cell size. With chitosan pretreatment, a 100-fold-diluted PBS was used, which prevents dissociation of chitosan from the cell surface. The PBS concentration itself did not affect the contact angle, as tested for untreated controls in which PBS was used in different concentrations up to 10 times the standard concentration. Filters were mounted on glass slides and air dried in the presence of silica gel for at least 3 h. No change in contact angle occurred after 3 h of drying, which is in accordance with results from Busscher et al. (4). Contact angles were measured directly by using a microscope with a goniometric eye piece (Smit Science Control Systems, Leiden, The Netherlands). Each reported contact angle is the mean of at least 10 independent measurements.

Electrophoretic mobility, as a measure for the negative surface charge of the yeast cells, was determined as described by Van Loosdrecht et al. (28), using a Doppler velocimeter with a ZetaSizer (Malvern Instruments, Malvern, England). Flocculation buffer without $CaCl_2$ (50 mM Na acetate buffer, pH 4.5) was used for mobility studies.

Isolation of flocculins. *S. cerevisiae* cells were grown in 1.0 liter of standard medium to an A_{620} of 5.0 to 6.0, harvested by centrifugation, and suspended in 50 ml of 10 mM Tris-HCl buffer (pH 7.5). After shearing in an Sorvall Omnimixer for 15 min at a setting of 6.0, the cells were removed by centrifugation at 9,000 × *g* for 10 min, and the resulting supernatant fluid was used as the cell surface-derived preparation. This preparation was further purified by ultrafiltration by the use of membrane filters with nominal cutoffs of 10, 100, and 300 kDa (Amicon Co., Danvers, Mass.). Flocculin fractions were heat treated by incubation of the samples at 100°C for 15 min. Enzymatic digestion was done with 0.1 mg of proteinase K $\cdot$ ml^{-1} for 30 h at 28°C. The prolonged incubation time with protease was done to inactivate the enzyme itself as well.

RESULTS

Flocculation characteristics of *S. cerevisiae* MPY1. Flocculation could be quickly and conveniently quantified by the decrease in turbidity of a yeast cell suspension in flocculation buffer with time, which reflects the cell settling profiles

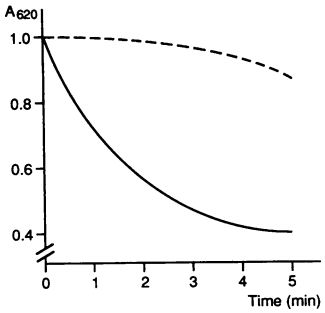

FIG. 1. Flocculation of *S. cerevisiae* MPY1 cells in the presence of 7 mM $CaCl_2$ (——) and 1 mM EDTA (– – –) as quantified by measuring the decrease in A_{620} of a cell suspension. The decrease in the presence of EDTA observed after 3 to 4 min is due to settling of (nonflocculated) cells.

TABLE 1. Influence of presence of various monosaccharides on flocculation of *S. cerevisiae* MPY1 cells[a]

Sugar added	Concn (mM) required to abolish flocculation
α-D-Mannose	25
α-Methyl-mannopyranoside	25
D-Glucose	100
D-Trehalose	100
Maltose	150
2-Deoxy-D-glucose	150
α-Methyl-glucopyranoside	200
D-Lyxose	250
α-L-Rhamnose	>250
D-Xylose	>250
D-Ribose	>250
D-Galactose	>250

[a] Monosaccharides were added to the cells in the flocculation buffer just before whirlmixing.

(Fig. 1). In the absence of flocculation, the turbidity drops slowly, due to settling of the cells after several minutes. Agitation of the yeast cells (by whirlmixing) was found to be essential for initiation of flocculation.

Flocculation occurred in the presence of Ca^{2+} ions, whereas no flocculation was observed in the absence of Ca^{2+} or in the presence of EDTA or EGTA (Fig. 1). Mg^{2+}, Sr^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, K^+, and Na^+ were not able to replace the Ca^{2+} ions, indicating that Ca^{2+} is specifically required for flocculation of MPY1 cells. Flocculation was found to be strongly dependent on the pH of the flocculation buffer, with an optimum at pH 4.5 (Fig. 2). No flocculation was observed above pH 6.0 or at pH 3.0. Flocculation is also highly sensitive to the presence of certain saccharides during flocculation (Table 1). Especially, mannose and α-methyl-mannopyranoside were found to be very effective inhibitors of flocculation at concentrations as low as 25 mM.

Nutrient limitation and initiation of flocculation. It is not known at which moment during growth flocculation ability is initiated, nor what is the causal factor(s) in this process. Therefore, we examined initiation of flocculation ability in a defined medium, which enabled us to accurately vary the composition of the growth medium. Spontaneous flocculation was not observed in the standard growth medium used, unless extra $CaCl_2$ was added to the medium. This indicates that although the cells do not flocculate in the medium itself, which is apparently due to low Ca^{2+} conditions, they do become flocculent. To test whether a positive correlation exists between flocculence and growth limitation of yeast cells, the flocculation ability of MPY1 cells was determined during growth in standard defined medium, with appropriate changes in nutrient limitation.

During growth in batch culture, a positive correlation was observed between the dry weight of the cells and the optical density. However, these parameters and the number of cells per ml did not correlate at an A_{620} of more than 3.5. This result can be clearly visualized when the number of cells during culture growth is plotted against the optical density in a so-called *n/A* plot (Fig. 3; see also reference 10). At an A_{620} of 3.5, the cell number in standard medium reached its maximal value, whereas the optical density subsequently increased. These results indicate that already at an A_{620} of 3.5, the cells are growth limited. Subsequently, the A_{620} and the dry weight of the cells still increases, possibly due to an increase in cell volume. Significantly, the stop in cell division coincides with the initiation of flocculation ability as shown in Fig. 3.

By addition of various components to standard medium, it was found that the nitrogen source was the growth-limiting factor. Addition of either glutamate or an extra amount of the standard mixture of amino acids (the N source of the medium) resulted in an upward shift of the maximum number of yeast cells, the ultimate dry weight, and the ultimate optical density. Moreover, a decrease of the amount of

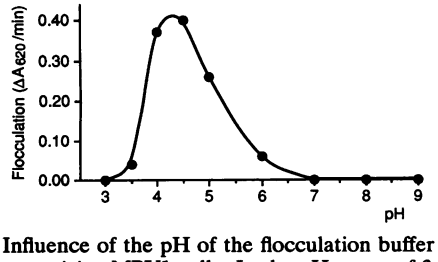

FIG. 2. Influence of the pH of the flocculation buffer on flocculation of *S. cerevisiae* MPY1 cells. In the pH range of 3.0 to 6.0, a 50 mM Na acetate–1% $CaCl_2$ buffer was used, and a 10 mM Tris-HCl–1% $CaCl_2$ buffer was used, and a 10 mM Tris-HCl–1% $CaCl_2$ buffer was used for pH 7.0 to 9.0.

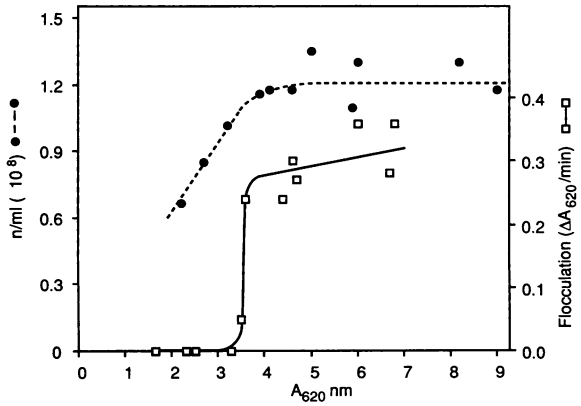

FIG. 3. Relationship between the optical density (A_{620}), the cell number, and the ability to flocculate of *S. cerevisiae* MPY1 cells growing in standard medium. Note that the moment at which the cells become flocculent coincides with cell division stop.

TABLE 2. Influence of the initial concentration of amino acids in, and addition of glutamate to, the growth medium on the final optical density, final cell number, and final dry weight of *S. cerevisiae* MPY1 cells and optical density at which the cells become flocculent

Content of amino acids[a] (%)	Additional combined nitrogen[a] (%)	Final A_{620}	Final dry wt (g/liter)	Final cell no. (10^8/ml)	Initiation of flocculation (A_{620})
50	None	6.0	6.3	0.70	1.9
80	None	7.7	7.4	0.75	2.9
100	None	9.5	7.8	1.20	3.5
120	None	11.0	8.6	1.45	3.7
140	None	11.6	9.1	1.50	4.1
100	20 (glutamate)	11.8	8.0	1.70	4.0

[a] Percentage of the amount present in standard medium.

amino acid mixture added to the medium resulted in a downward shift of cell number, optical density, and dry weight reached at the stationary phase of growth (Table 2). Flocculation ability of the yeast cells was determined during growth, and in all these cases, initiation of flocculation was shifted accordingly (Table 2).

The positive correlation between a stop in cell division and initiation of flocculation ability was not found only for N limitation, but for other nutrient limitations as well. Changing the medium composition in such a way that the limiting factor was not the N source but another nutrient, e.g., the carbon source or Zn^{2+}, again yielded a positive correlation between the moment that the cells stop dividing and the initiation of flocculation ability. These results led us to hypothesize that flocculation ability is induced at the moment cell division stops because of limitation for a nutrient.

One limitation tested, namely, Mg^{2+} limitation, differed from the other limitations mentioned above in that it resulted in yeast cells which were unable to flocculate during any stage of growth (Table 3).

Physicochemical surface characteristics of yeast cells and flocculation. We analyzed whether cell surface hydrophobicity and surface charge are important in the flocculation process of *S. cerevisiae* MPY1 cells. Hydrophobicity and electrophoretic mobility of yeast cells were determined during growth in standard medium, during growth under Mg^{2+} limitation, and after various treatments of the yeast cells. Flocculent yeast cells appeared to show a high contact angle with water (Fig. 4; Table 3) and to adhere strongly to polystyrene (data not shown). When the interaction between flocculent yeast cells and hexadecane was studied by light microscopy, the yeast cells appeared to form a monolayer of cells around each hexadecane droplet, resulting in formation of an emulsion of hexadecane in water (data not shown). These results demonstrate that flocculent yeast cells are highly hydrophobic.

Treatment of flocculent yeast cells with proteolytic enzymes resulted in a strong decrease in hydrophobicity as judged from strongly decreased values of contact angles and from the abolishment of emulsifying activity of the yeast cells (Table 3). Such treatment also abolished flocculation ability of the cells (Table 3). Addition of either monosaccharides (e.g., mannose) in concentrations up to 1,000 mM or EDTA (25 mM), which both inhibit flocculation, did not affect the cell surface hydrophobicity of yeast cells as judged from hexadecane interactions (data not shown). Also, changes in the pH of the buffer in which the interaction with hexadecane is determined, in the range of pH 2.0 to 10.0, had no significant effect on hydrophobicity of the yeast cells.

Hydrophobicity of the yeast cells was found to increase rapidly at the end of the logarithmic phase during growth in standard medium (Fig. 4). Significantly, this increase in hydrophobicity shortly precedes the moment at which the cells stop dividing and become flocculent (Fig. 3). In contrast, growth of the yeast cells in Mg^{2+}-limited medium resulted, in addition to absence of flocculence, in strongly reduced cell surface hydrophobicity as judged from the low contact angle between water and Mg^{2+}-limited cells, in poor adhesion ability to polystyrene, and in lack of emulsifying activity (Table 3).

No significant differences in the electrophoretic mobility of yeast cells were observed under all conditions tested (data not shown). This indicates that the negative surface charge of the cells is most likely not significantly affected during growth and by the various treatments used.

TABLE 3. Influence of culture conditions, shearing, and treatment with protease or chitosan on hydrophobicity and flocculation ability of *S. cerevisiae* MPY1 cells[a]

Growth medium[b]	Optical density (A_{620})	Treatment	Hydrophobicity		Flocculation ability (ΔA_{620}/min)
			Contact angle (degrees)	Hexadecane	
Std	2.3	None	53 ± 2	+++	0
Std	2.3	Chitosan[d]	65 ± 4	+++	0.28
Std	5.9	None	68 ± 3	+++	0.36
Std	5.0	Chitosan	78 ± 2	+++	>0.5[c]
Std	5.0	Shearing	32	±	0.03
Std	5.0	Protease	<10	−	0
Mg^{2+} limited	3.0	None	33 ± 2	±	0
Mg^{2+} limited	3.0	Chitosan	44 ± 2	+++	0.25

[a] *S. cerevisiae* cells were harvested by centrifugation and suspended in either 50 mM sodium acetate buffer (pH 4.5) (shearing) or 10 mM Tris-HCl buffer (pH 7.5) (protease treatment). For details about treatments, see Materials and Methods.

[b] Std, standard medium. Mg^{2+}-limited medium contains 1% of the $MgCl_2$ concentration of standard medium. The turbidity of 3.0 represents cells harvested at the end of the growth phase.

[c] Flocculation could even be observed in the absence of whirlmixing.

[d] Chitosan was used in a final concentration of 1 μg/ml.

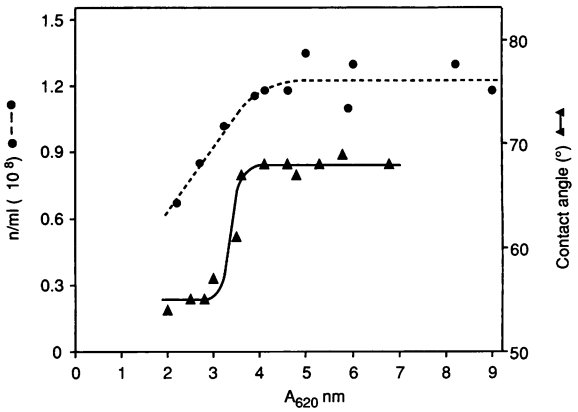

FIG. 4. Relationship between the optical density (A_{620}), cell number, and cell surface hydrophobicity. The number of yeast cells (●) and the contact angle (▲) are plotted against the A_{620} of the culture. Note that the increase in hydrophobicity precedes the cell division stop as well as initiation of flocculation ability (Fig. 3).

TABLE 4. Influence of growth conditions and treatments on emulsifying and flocculation-stimulating activity of cell surface-derived preparations of *S. cerevisiae* MPY1

Growth medium[a]	Treatment of cell surface-derived prepn	Flocculation-stimulating activity[c]	Emulsifying activity
Standard	None	+	+++
Standard	Heat[b]	+	+++
Standard	Protease[b]	−	−
Mg^{2+} limited	None	−	−

[a] Cell surface-derived preparations were obtained from yeast cells grown in standard medium and Mg^{2+}-limited medium and harvested at the moment cell division stopped.

[b] See Materials and Methods.

[c] Stimulation of flocculation was tested by addition of cell surface preparations to flocculent yeast cells grown in standard medium and harvested at an A_{620} of 4.0.

Recently, Rosenberg and coworkers (7) reported that adhesion of microorganisms to polystyrene could be enhanced in the presence of polycations, such as chitosan and poly-L-lysine. We tested the effect of treatment of yeast cells with these polycations and compared these treated cells with control cells for hydrophobicity, cell surface charge, and flocculation ability. Low-molecular-weight poly-L-lysine was used, since high-molecular-weight poly-L-lysine was found to cause lysis of yeast cells (6). Addition of chitosan (1 μg/ml) or poly-L-lysine (1 mg/ml) significantly increased the hydrophobicity of the cells (Table 3), whereas this treatment did not significantly affect electrophoretic mobility (data not shown). Polycation-treated yeast cells were not only more hydrophobic, but also showed a stronger ability to flocculate. Chitosan treatment resulted even in spontaneous flocculation in the absence of whirlmixing. Moreover, Mg^{2+}-limited yeast cells which normally do not flocculate and exhibit a strongly reduced cell surface hydrophobicity were partially restored for these characteristics after treatment with polycations (Table 3). Furthermore, nonflocculent cells harvested at low A_{620} values and treated with chitosan showed an increase in contact angle to a value comparable with that of flocculent cells (65 and 68 degrees, respectively) and concomitantly became flocculent (Table 3). Taken together, these results clearly show that hydrophobicity of yeast cells is positively correlated with their ability to flocculate. In all cases, flocculation appeared to be Ca^{2+} dependent and mannose sensitive.

Isolation and preliminary characterization of yeast flocculin. A flocculin was experimentally defined as a surface component of yeast cells able to inhibit or enhance (depending on it being monovalent or multivalent) flocculation of *S. cerevisiae* cells when added during the flocculation assay. After treatment of the yeast cells by shearing forces, we found that the cell surface hydrophobicity as well as their ability to flocculate was strongly reduced (Table 3). Cells did not loose viability after this treatment. The resulting surface-derived preparation, obtained after this treatment, was tested for the presence of flocculins. Addition of this preparation to untreated cells in the flocculation assay resulted in an enhancement of flocculation in a Ca^{2+}-dependent and mannose-sensitive way, indicating that this fraction indeed contained one or more flocculins (Table 4). The flocculin preparation could even induce flocculation when added to (nonflocculent) Mg^{2+}-limited cells (data not shown). Furthermore, this fraction had a high emulsifying activity, similar to intact MPY1 cells (data not shown). A cell surface-derived preparation isolated from cells grown under Mg^{2+}-limiting conditions neither stimulated flocculation nor contained significant emulsifying activity (Table 4).

Treatment with proteinase K abolished both the flocculation-stimulating activity and the emulsifying activity completely, whereas heat treatment did not significantly affect its activity (Table 4). Ultrafiltration of the flocculin preparation revealed the molecular mass of the flocculation-stimulating and emulsifying component to be higher than 300 kDa. However, after heat treatment of the preparation, nearly all activity passed through a 100-kDa-cutoff membrane but was retained by a 10-kDa-cutoff membrane, indicating that the >300-kDa flocculin fraction is a complex which can be reduced in size by heat treatment without loss of activity. Taken together, these results strongly indicate that a hydrophobic cell surface compound is involved in yeast flocculation.

DISCUSSION

Characterization of flocculation mechanism of *S. cerevisiae* MPY1. Flocculation of MPY1 cells (i) requires agitation, (ii) requires calcium, (iii) is highly sensitive to mannose and mannose derivatives, and (iv) is pH dependent. These results indicate that a Ca^{2+}-dependent lectin-sugar interaction is involved in flocculation of MPY1 cells.

Flocculence is initiated by nutrient limitation. In the defined standard medium used, the component which first becomes limited is the nitrogen source. At the moment the cells stop dividing, because of this limitation, the ability to flocculate increases rapidly (Fig. 3). An increase or decrease of the initial amount of nitrogen in the medium results in a shift in the moment at which the cells stop dividing and concomitantly become flocculent (Table 2). This correlation between growth limitation of the cells and initiation of ability to flocculate was also observed for other nutrient limitations, with Mg^{2+} limitation being an exception (Table 3). Addition of $MgCl_2$ during the flocculation assay or addition of extra $CaCl_2$ during growth did not restore the ability of Mg^{2+}-limited cells to flocculate (data not shown), indicating that Mg^{2+} is an essential nutrient required for synthesis of a flocculin or involved in a more general feature, e.g., membrane stability (see also references 15 and 16). Previous reports from our laboratory on attachment of *Rhizobium* bacteria have also shown that growth limitation acts as a trigger for initiation of cell adhesion phenomena (10, 19, 20). Thus, limitation-induced flocculation ability might illustrate a more general feature of microorganisms.

Future research will focus on initiation of flocculation during fermentation in wort. This is necessary to address the question whether flocculation in wort is primarily initiated by the same mechanism and/or by flocculation-promoting compounds released during fermentation, as proposed in a number of reports (see reference 5 for a review).

Positive correlation between cell surface hydrophobicity and flocculence. MPY1 cells grown in standard medium were hydrophobic. Several observations support the hypothesis that cell surface hydrophobicity is a major determinant in flocculation of yeast cells: (i) hydrophobicity of MPY1 cells significantly increases shortly before initiation of flocculation (Fig. 4); (ii) growth of MPY1 cells under Mg^{2+}-limiting conditions resulted in a concomitant decrease in hydrophobicity and flocculence (Table 3); (iii) treatment of nonflocculent cells, either harvested at low optical densities or grown under Mg^{2+}-limiting conditions, with polycations renders these cells both more hydrophobic as well as flocculent

(Table 3); (iv) treatment of flocculent cells with proteases or with shearing forces resulted in a strong decrease both in cell surface hydrophobicity and in ability to flocculate (Table 3).

Electrophoretic mobility of MPY1 cells did not change significantly under all conditions tested (Table 3). Apparently, cell surface charge is not correlated with any of the differences found in flocculence of MPY1 cells. However, it is important to recognize the possible role of (nonspecific) electrostatic repulsion in flocculation, since without this repulsion, selective cell-cell adhesion cannot function.

It is important to notice that monosaccharides and Ca^{2+} as well as variations in pH had no effect on cell surface hydrophobicity. Besides the profound effect of hydrophobicity on flocculation ability, involvement of a Ca^{2+}-dependent lectin-sugar binding is essential for flocculation. The strong correlation between flocculence and cell surface hydrophobicity indicates that the regulation of flocculation might be controlled by the expression of this surface characteristic.

Identification of a hydrophobic surface protein as a flocculin. Shearing of MPY1 cells resulted in poorly flocculent cells with reduced cell surface hydrophobicity (Table 3). The cell surface-derived fraction obtained after such a treatment possessed both emulsifying and flocculation-stimulating activity, indicating that this fraction contains a flocculin. Both the emulsifying as well as the flocculation-stimulating activity were found to be protease sensitive (Table 4). This corroborates the results from protease treatment on whole yeast cells (Table 3). Furthermore, a cell surface-derived preparation from Mg^{2+}-limited cells showed neither flocculation-stimulating nor emulsifying activity (Table 4). Both emulsifying activity and flocculation-stimulating activity of the cell surface-derived preparation were found in a high-molecular-weight complex, which after heat treatment could be degraded into smaller fragments. These results strongly indicate that a heat-stable, proteinaceous cell surface component is involved in cell surface hydrophobicity and flocculation ability of *S. cerevisiae* MPY1 cells, adding weight to the hypothesis that cell surface hydrophobicity is a major factor in flocculation. Future research will focus on purification, characterization, and regulation of this emulsifier, the putative Ca^{2+}-dependent lectin and its receptor.

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