APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 718–728 Vol. 61, No. 2
0099-2240/95/$04.00+0

Mechanisms of Yeast Flocculation: Comparison of Top- and Bottom-Fermenting Strains

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Received 27 June 1994/Accepted 11 October 1994

The flocculation of two brewing yeast strains, top-fermenting strain *Saccharomyces cerevisiae* MUCL 38485 and bottom-fermenting strain *Saccharomyces carlsbergensis* MUCL 28285, has been investigated by means of a turbidimetric test. The two strains showed different electrical properties, a different hydrophobicity, and a different surface chemical composition. They flocculated according to completely different mechanisms; however, no correlation between the cell physicochemical properties and the onset of flocculation was found for either strain. Flocculation of the bottom strain was governed by a lectin-mediated mechanism. It was inhibited by mannose and some other sugars, required calcium specifically, occurred in a narrow pH range different from the isoelectric point, and was not influenced by ethanol. The onset of flocculation at the end of the exponential phase was controlled both by the appearance of "active" lectins at the cell surface and by the decrease in sugar concentration in the solution. Flocculation of the top strain was not inhibited by mannose, did not require the addition of calcium, and took place at the cell isoelectric point. Low concentrations of ethanol broadened the pH range in which the cells flocculated, and flocculation was favored by an increase of ionic strength. Adsorbed ethanol may induce flocculation by reducing the electrostatic repulsion between cells, by decreasing steric stabilization, and/or by allowing the protrusion of polymer chains into the liquid phase. The onset of flocculation was controlled by both a change of the cell surface and an increase in ethanol concentration. The only evidence for an adhesin-mediated mechanism was the specific requirement for a small amount of calcium.

Flocculation of yeast cells is usually observed at the end of beer fermentation and is of great importance in brewery (54). Its mechanism is not yet completely understood and is still the subject of much controversy (for reviews, see references 10, 11, 46, 55, and 56). It is a very complex process which depends on numerous factors: the yeast strain (genetics, physiological state, and metabolism), the composition of the culture medium, and the culture conditions (temperature, agitation, and aeration). The involvement of all of these parameters in the flocculation process makes it very difficult to compare different studies and may thus explain the many conflicting data found in the literature.

Cell flocculation can be controlled by specific (molecular recognition) and nonspecific (double-layer interactions, van der Waals forces, hydrophobic interactions, salt bridges, and steric repulsion) interactions. The most generally accepted mechanism is one mediated by lectins, which recognize mannan receptors on adjacent cells and require the presence of calcium (28, 33–35, 37, 57). van Hamersveld et al. (65) found that the net interaction energy of flocculated brewer's yeast was much higher than could be expected on the basis of nonspecific (van der Waals and electrostatic) interactions only. However, the involvement of a molecular recognition mechanism does not mean that nonspecific interactions are negligible: the specific binding can be revealed only if there is a nonspecific repulsion (5). Specific interactions take place at a very short distance (less than 1.5 nm) only, whereas nonspecific interactions can occur over a long distance (9).

The importance of physicochemical properties which are responsible for nonspecific interactions has been investigated for brewer's yeast. Van Haecht et al. (64) found a correlation between the isoelectric point of yeast cells and the N/P concentration ratio measured at the surface by X-ray photoelectron spectroscopy (XPS). Amory et al. (1) reported that the influence of the culture conditions on the tendency of the cells to flocculate was related to the variation of the surface electrical properties. In an extended study, Amory and Rouxhet (2) found that top-fermenting strains were systematically more hydrophobic and less negatively charged at pH 4 than bottom-fermenting strains. The onset of flocculation was found to coincide with an increase in surface hydrophobicity (27, 53, 62). Straver et al. (59) hypothesized that fimbriae were responsible for both specific interactions and hydrophobicity.

The aim of this work was to achieve a better understanding of the relative importance of different types of interactions in yeast cell flocculation. The flocculation behavior of a top-fermenting strain, which is not inhibited by sugars, and a bottom-fermenting strain, which is inhibited by sugars, is studied in detail by means of a turbidimetric test. The influences of pH, the concentration of sugars, cations, ethanol and other solvents, and the culture medium are investigated.

MATERIALS AND METHODS

Yeast strains. The brewing yeast strains *Saccharomyces cerevisiae* MUCL 38475 (top fermenting) and *Saccharomyces carlsbergensis* MUCL 28285 (bottom fermenting) have been kindly supplied by J.-P. Dufour (Laboratory of Brewing Science and Technology, Université Catholique de Louvain, Louvain-la-Neuve, Belgium).

Culture. The yeasts were stored in 25% (vol/vol) glycerol at −20°C. Cells were plated in a petri dish on solid medium (1% [wt/vol] yeast extract, 3% [wt/vol] glucose, 2.5% [wt/vol] agar) and incubated at 30°C for 3 days. The plates were stored at 4°C and repitched every 3 weeks. The culture broth contained 2% (wt/vol) yeast extract and 5% (wt/vol) glucose (pH of the medium unadjusted, about 6.5). The cells were cultivated in two steps as described by Kihn et al. (28). Two or three colonies from the solid medium plate were used as inoculum for the preculture. The stationary growth phase was reached after about 14 h for top strain MUCL 38475 (grown at 30°C) and after about 3 days for bottom strain MUCL 28285 (grown at 10°C). Cells of the exponential growth phase (hereafter called exponential cells) were harvested after 10 h and 2 days of culture for the top and the bottom strains, respectively; cells of the stationary growth phase

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(hereafter called stationary cells) were harvested after 24 h for the top strain and after 5 days, unless stated otherwise, for the bottom strain. The cells were harvested by centrifugation and washed twice with cold distilled water.

Microelectrophoresis. The electrophoretic mobility of the cells was determined with a Zetasizer III (Malvern Instruments, Malvern, England) and with a Laser Zee Meter, model 500 (Pen Kem, Bedford Hills, N.Y.). Cells of the last pellet were resuspended in 1 or 10 mM KNO_3 to a concentration of 5×10^6 cells per ml. The pH was adjusted with HNO_3 or KOH. For pH values below 6, the pH of the suspension did not change during the measurement; at higher pH values, the pH dropped as a function of time. The pH of the suspension was determined before and after the measurement, and the mean value was used for plotting the results. In the Malvern Zetasizer III, the measurements were performed at one of the stationary layers of the quartz capillary. For each pH value, at least three samples were injected into the capillary and measured for 10 s at an applied voltage of 150 V. In the Pen Kem Laser Zee 500, the measurements were performed at both the upper and lower stationary levels of the rectangular poly(methylmetacrylate) chamber; for each suspension, at least three measurements were performed at each level. The applied voltage was 90 V; a complete determination at a given pH took about 2 min.

Contact angle measurements. The hydrophobicity of the cells was estimated by measurement of the water contact angle on a lawn of cells (38). The cellular lawn was prepared by collecting 10 ml of a suspension of 2×10^8 cells per ml on a filter (4.5 cm in diameter) with a pore diameter of 0.45 μm (type HAWP filter; Millipore); this gave a lawn about 30 layers thick for the cells used. In order to standardize the moisture content, the filters with the cells were placed for 30 min in a petri dish containing 1% (wt/vol) agar and 10% (vol/vol) glycerol. The filters were mounted on a microscope slide with double-sided adhesive tape. Contact angles were measured as a function of drying time of the cellular lawns in air. The contact angles increased with drying time until a plateau was reached (after about 3 h); this was done for several independent cultures. The angles reported in this work are mean plateau values of the different independent measurements; the confidence intervals were calculated at the 95% confidence level.

Surface analysis by XPS. XPS provides a chemical analysis of the outermost layer (2 to 10 nm) of the analyzed solid; information about the principle of the technique and its application to microorganisms can be found in the literature (47, 48).

The surface analysis was performed on freeze-dried cells. One milliliter of a cell suspension (about 3×10^9 cells per ml) was deposited in a glass flask (with a diameter and a height of 2.5 cm each). The flasks were kept in liquid nitrogen for 15 min; they were then either placed immediately in a freeze-dryer (Lyovac GT4, Thermovac TM 22052; Leybold Heraeus) with the shelf temperature set at −50°C or stored in a freezer at −80°C until freeze-drying. In each freeze-drying operation, dry sorbitol powder was included as a control for surface contamination. The chamber was evacuated; when the pressure reached about 100 Pa, the shelf temperature was raised to −10°C in about 30 min, and the specimens were dehydrated under a vacuum of about 60 Pa. After one night, the shelf temperature was raised to 25°C, the chamber was connected to atmospheric pressure, and the flasks were stoppered and stored at room temperature in a desiccator containing silica gel.

The dried samples were homogenized with a spatula. A stainless-steel trough with an inner diameter of 4 mm was filled with the powder, which was gently compacted with a spatula. A polyacetal cylinder (Delrin), cleaned with isopropanol, was placed on the trough and pressed to obtain a smooth surface. The samples were mounted on a multispecimen carousel, which could accommodate up to 23 samples.

The XPS analyses were performed with an SSI X-Probe (SSX-100/206) photoelectron spectrometer from Fisons, interfaced with a Hewlett-Packard 9000/310 computer (monochromatized A1 K_α radiation; spot size, 1,000 μm; anode powered at 10 kV and about 17 mA; flood gun energy set at 6 eV). The constant pass energy in the hemispherical analyzer was 150 eV for survey analysis and 50 eV for individual peak analysis, giving full widths at half-maximum of the $Au_{4f7/2}$ peak of about 1.66 and 0.98 eV, respectively. Unless stated otherwise, the following sequences of spectra were recorded: for yeast cells, C_{1s}, O_{1s}, N_{1s}, P_{2p}, K_{2p}, and a survey spectrum; for sorbitol, C_{1s}, O_{1s}, and a survey spectrum.

The binding energies were determined by setting the C—(C,H) component of the C_{1s} peak to 284.8 eV (40). The background was subtracted linearly. The ratios of atomic concentrations were calculated by using the sensitivity factors proposed by the manufacturer (mean free path varying according to the 0.7th power of the photoelectron kinetic energy; Scofield cross sections [49]; transmission function assumed to be constant). From the atomic concentration ratios, the mole fractions of the various elements, excluding hydrogen, could be computed. Decomposition of the peaks was done by using the SSI least-squares, best-fitting routine with a Gaussian/Lorentzian ratio of 85/15. In a first fitting, the initial binding energies, full width at half-maximum, and the intensity of the components were obtained without imposing constraints. For the subsequent fits, the full width at half-maximum of all components of a given peak was set equal to the value of the best-resolved component, rounded to 0.05 eV, and a new fitting was performed with this constraint. The peak components were attributed to chemical functions according to Rouxhet et al. (48).

Flocculation test. Flocculation was quantified by measuring the residual absorbance of a yeast suspension after agitation and subsequent undisturbed standing to allow floc settling. The test was based on the assay used by Kihn et al. (29). The compounds of interest ($CaCl_2$, $MgCl_2$, NaCl, ethanol or other organic compounds, and sugars) were introduced in test tubes (16 by 100 mm) as concentrated solutions; HCl, NaOH, or a buffer (sodium acetate-acetic acid or sodium citrate-citric acid) was added to adjust the pH. Distilled water (or culture medium) was added to a final volume of 5 ml. The yeast cells were then added (as a concentrated suspension of about 4×10^9 cells per ml) under whirl mixing (5 s); their final concentration in the test tubes was 10^8 cells per ml. The range of ionic strengths used in the flocculation tests included the ionic strength of the culture medium, estimated to be about 20 mM (based on the concentrations of calcium, magnesium, sodium, and potassium [34]), in agreement with a conductivity of 3 mS cm^{-1}. The tubes were agitated upside down at 25 rpm for 15 min and allowed to stand undisturbed for 15 min. Finally, the A_{660} was measured (Bausch and Lomb Spectronic 700 spectrometer).

Analysis of the culture medium. The total calcium concentration was measured by atomic absorption (Varian Techtron AA-5), using the standard addition method. The culture medium was centrifuged (15,000 rpm, 5 min), filtered under vacuum (0.22-μm pore size, type GSWP filter; Millipore), diluted by a factor of 2 in 1 M HCl, and filtered again (0.45-μm pore size, type HAWP filter; Millipore). Ten milliliters of the solution was transferred to a 20-ml volumetric flask, and $LaCl_3$ (spectral buffer) was added to a final concentration of 1%.

The free calcium activity was measured by potentiometry, using a membrane electrode specific for calcium ions (Philips IS 561-Ca^{2+}) and a double-junction calomel electrode (Philips RH 44/2-SD/1), with a Philips PW 9421 digital voltmeter. The electrodes were stored in 10^{-3} M $CaCl_2$. Preliminary tests were carried out at 5 and 21°C as it was feared that upon warming following transfer from 10°C to room temperature, the culture medium would be subject to a loss of CO_2 and thus to a change of pH and (free Ca^{2+})/(complexed Ca^{2+}) equilibria. The results obtained at the two temperatures were the same, and all of the following measurements were carried out at 21°C. Aqueous solutions of $CaCl_2$ were used to obtain a calibration curve of measured potential versus Ca^{2+} activity; concentrations of calcium were converted into activities by using the Debye-Hückel equation. The influence of ionic strength was tested by adding 25 mM, 100 mM, 1 M, or 2 M KCl or 100 mM NH_4Cl; similar curves were obtained in all cases. The calibration curve of potential versus activity with 25 mM KCl was used because this solution has an electrical conductivity close to that of the culture medium (3 mS/cm). Thus, a plot which covered a calcium activity range of 3×10^{-6} to 0.02 M with a slope of 29.1 ± 1.2 mV per log activity unit was obtained. The pH of the standard $CaCl_2$ solutions was not adjusted; it was found that the measured potentials were not influenced by pH in the range of interest (4.5 to 6.5). The reproducibility (defined as the standard deviation of measured potential of a 0.5 M solution, obtained when the electrode is transferred three times from a 0.5 to a 0.05 M solution) was 0.2 mV; the activity detection limit was 2×10^{-6} M.

The glucose concentration was determined according to the method of Dubois et al. (12); the results may contain a small contribution from reducing groups of other carbohydrates. All measurements were performed in triplicate.

The ethanol concentration was determined with a test kit (Boehringer Mannheim 123 960 [8]). Similar calibration curves were obtained with solutions in water and in culture medium, with or without pretreatment by $HClO_4$, which eliminates possible interfering proteins.

Surface tension measurements. The surface tensions of several organic compound-water mixtures were determined by the ring method (Interfacial Tensiometer K 8600; Krüss, Hamburg, Germany). Each value represents the mean of five measurements; confidence intervals are given at the 95% level.

Chemicals. Yeast extract was obtained from Difco (0127-01-7), and agar was from the Pasteur Institute (Pastagar A). All of the following products used were analytical grade, unless stated otherwise: HNO_3, KOH, HCl, NaOH, KNO_3, KCl, NH_4Cl, $CaCl_2$ dihydrate, $MgCl_2$ hexahydrate (99%), NaCl, EDTA-tetrasodium salt dihydrate (98%), D-(+)-glucose, D-(+)-mannose (99%), α-methyl-D-mannopyranoside (99%), *p*-nitrophenyl-α-D-mannoside, phenyl-α-D-mannoside, *n*-octyl-α-D-mannoside, D-(+)-galactose, D-(+)-maltose monohydrate, sorbitol, glycerol, methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, 1,2-propyleneglycol, 2,3-butanediol, acetone, sodium acetate (anhydrous), acetic acid, trisodium citrate dihydrate, and citric acid.

RESULTS

(i) Flocculation. Cells of the exponential and stationary growth phases of the two strains were submitted to the flocculation test in order to examine the influence of the following factors: calcium, magnesium, sodium, pH, organic solvents, sugars, and the culture medium.

Influence of calcium. The influence of calcium on the flocculation of cells of different ages at pH 4 (10 mM acetate buffer) is shown in Fig. 1A and B. For the stationary cells of the bottom strain, the addition of $CaCl_2$ (about 0.1 mM) was needed to produce flocculation; this was not appreciably influenced by the culture time, provided the latter was at least 3 days. On the other hand, the top strain flocculated without

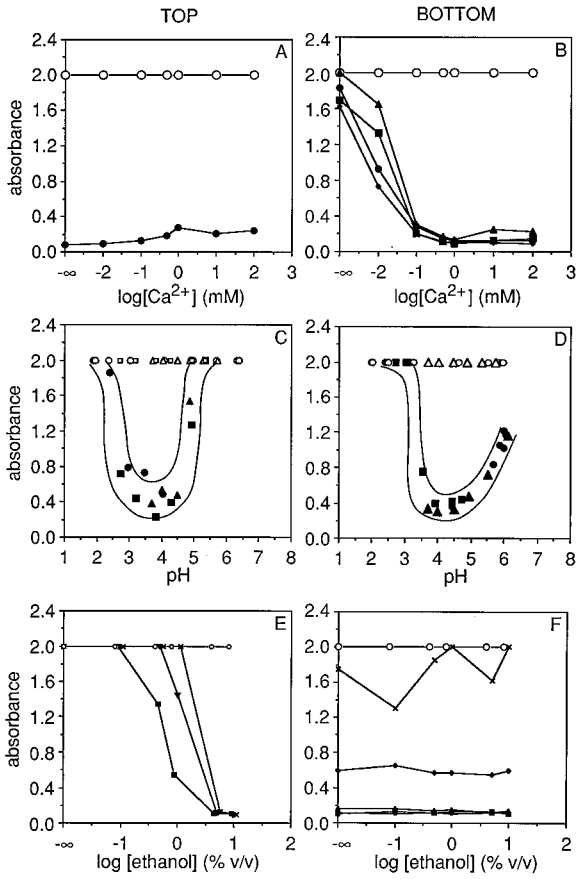

FIG. 1. Residual absorbance of yeast suspensions as a function of the solution composition. (Left) Top strain MUCL 38475; (right) bottom strain MUCL 28285. Open symbols, exponential cells; closed symbols, stationary cells. (A and B) Influence of $CaCl_2$ concentration (10 mM acetate buffer, pH 4). Cells of the bottom strain were harvested after 2 (○), 3 (▲), 4 (■), 6 (◆), and 8 (●) days. (C and D) Influence of pH; 0.5 mM (C) and 5 mM (D) $CaCl_2$ added. The pH was adjusted with HCl (○, ●), 10 mM acetate buffer (△, ▲), or 5 mM citrate buffer (□, ■). (E and F) Influence of ethanol concentration (10 mM acetate buffer, pH 5.2). $CaCl_2$ was added at 0 (×), 0.01 (◆), 0.1 (▲), 1 (▼), 5 (○, ●), or 10 (■) mM.

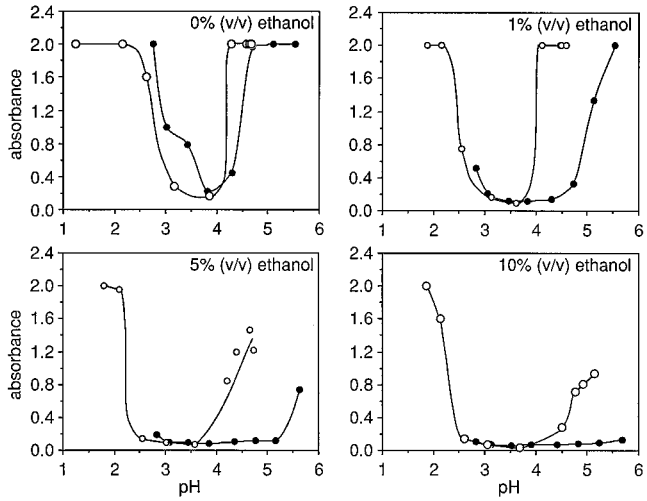

FIG. 2. Residual absorbance of suspensions of stationary cells of top strain MUCL 38475 as a function of pH at different ethanol concentrations: ●, 10 mM acetate buffer with 0.5 mM $CaCl_2$ added; ○, pH adjusted with HCl and no $CaCl_2$ added.

calcium addition. However, upon addition of 50 mM EDTA at pH 4 (results not shown), the flocs were completely redispersed. This indicates that while flocculation does not require addition of calcium, it involves calcium or other cations released by the cells. For the two strains, exponential cells did not flocculate upon $CaCl_2$ addition up to 100 mM.

If the pH of the suspensions was set at 5.2 (which is the pH of the growth medium in the stationary growth phase of the top and bottom strain cultures) with 10 mM acetate buffer, the results for the bottom strain (not shown) were identical to those at pH 4. For the top strain, however, no flocculation occurred over the whole range of $CaCl_2$ concentrations (up to 100 mM).

Similar results were obtained when the pH was adjusted to 4 or 5.2 with HCl instead of buffer (results not shown).

Influence of pH. The influence of pH on flocculation is presented in Fig. 1C and D. $CaCl_2$ was added to a final concentration of 0.5 mM for the top strain and 5 mM for the bottom strain. For the two strains, the exponential cells did not flocculate in the pH range investigated. Stationary cells flocculated only in a narrow pH range: from pH 3 to 4.5 for the top strain and from pH 3.5 to 6 for the bottom strain. For the top strain, no flocculation occurred at pH 5.2, which is the pH of the growth medium in the stationary growth phase. For both strains the pH range of flocculation was not strongly affected by the nature of the electrolyte used to set the pH: HCl, 10 mM acetate buffer, or 5 mM citrate buffer.

Influence of ethanol. The influence of ethanol on flocculation of the two strains at pH 5.2 (10 mM acetate buffer) is shown in Fig. 1E and F. Cells of the exponential growth phase did not flocculate upon ethanol addition. For the stationary cells, the results differed from one strain to another. Flocculation of the bottom strain was not influenced by ethanol regardless of the calcium concentration (Fig. 1F).

For the top strain, flocculation was induced at 5% (vol/vol) ethanol when no calcium was added and at a slightly lower ethanol concentration when calcium was added (Fig. 1E). If the pH was adjusted to 5.2 with HCl instead of buffer, thereby maintaining a low ionic strength, flocculation in 10% (vol/vol) ethanol was only observed when $CaCl_2$ was added at 1 mM or more (results not shown). This points to the importance of ionic strength for flocculation of the top strain at pH 5.2 in the presence of ethanol.

Combined effect of ethanol and pH. The combined effect of ethanol and pH on the flocculation of stationary cells of the top strain in acetate buffer is shown in Fig. 2 (closed symbols). Upon addition of ethanol, the pH range in which flocculation occurred was broadened. When no $CaCl_2$ was added, the pH profiles obtained with 0 and 5% (vol/vol) ethanol (results not shown) were the same as those shown in Fig. 2 (closed symbols). At ethanol concentrations of 5 or 10% (vol/vol), the pH interval in which flocculation occurred included the pH of the culture medium in the stationary phase (5.2). This points to the requirement of a sufficient concentration of ethanol for flocculation of the top strain to occur during fermentation. At pH 5.2 in the presence of 5% (vol/vol) ethanol and with no addition of $CaCl_2$, total deflocculation was observed upon addition of 5 mM EDTA, demonstrating the specific role of $CaCl_2$ or other cations.

If the pH was adjusted with HCl and no calcium was added (Fig. 2, open symbols), the extension of the range of flocculation towards a higher pH was less important than that with the acetate buffer, and no flocculation occurred at pH 5.2 in 5%

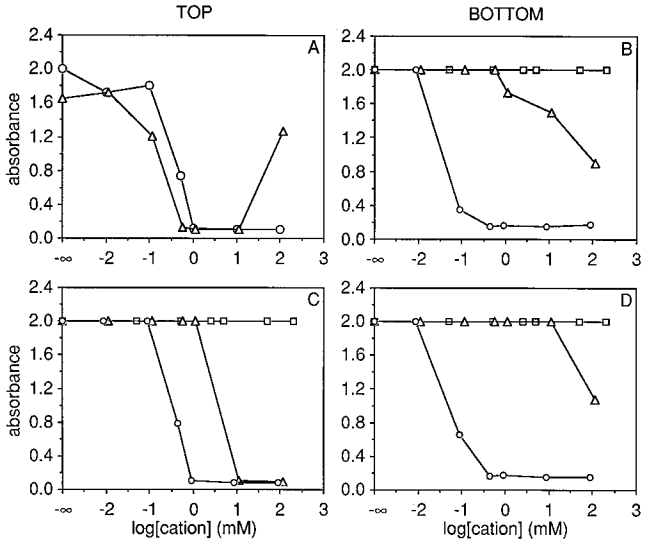

FIG. 3. Residual absorbance of suspensions of stationary cells as a function of the concentration of added cation (○, calcium; △, magnesium; □, sodium). (Left) Top strain MUCL 38475; (right) bottom strain MUCL 28285. In left panels, 5% (vol/vol) ethanol was added. (A and B) pH was adjusted to about 5 with HCl; (C) pH 5.2, with 10 mM acetate buffer and 10 mM EDTA; and (D) pH 5.2, with 10 mM acetate buffer.

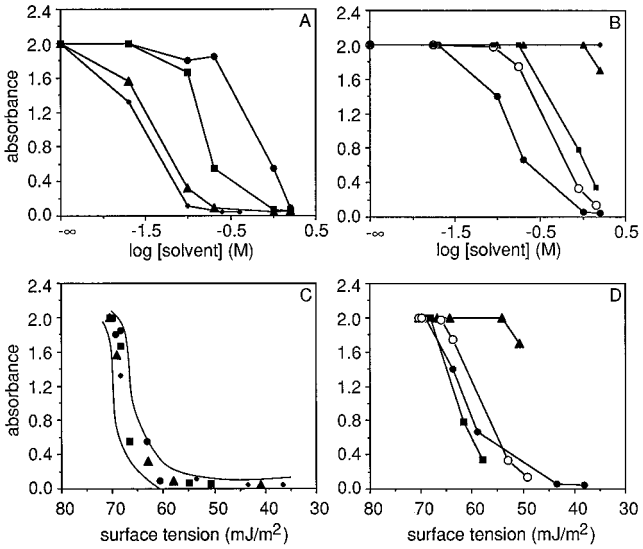

FIG. 4. Residual absorbance of suspensions of stationary cells of top strain MUCL 38475, in 10 mM acetate buffer (pH 5.2) with 5 mM $CaCl_2$ added, as a function of the concentration of organic compounds in aqueous solutions (A and B) or the surface tension of mixtures of pure water and the organic compounds at the concentration given in panels A and B (C and D). (A and C) ●, methanol; ■, ethanol; ▲, *n*-propanol; ◆, *n*-butanol. (B and D) ●, isopropanol; ■, 1,2-propyleneglycol; ▲, 2,3-butanediol; ◆, glycerol; ○, acetone.

(vol/vol) ethanol. This confirms the influence of ionic strength on flocculation of the top strain.

Influence of other ions. The influence of other ions (magnesium and sodium) on the flocculation of stationary cells of the two strains was investigated in buffered (Fig. 3C and D) and nonbuffered (Fig. 3A and B) systems at pH 5 to 5.2. For the top strain (Fig. 3A and C), ethanol was added at a concentration of 5% (vol/vol). When the pH was adjusted with HCl (Fig. 3A), both $CaCl_2$ and $MgCl_2$ induced flocculation at a concentration of about 0.5 to 1 mM; the flocs redispersed partially in 100 mM $MgCl_2$. For sodium, the results were not reproducible. In the buffered system (Fig. 3C), 10 mM EDTA was added in order to avoid flocculation in ethanol in the absence of additional salt. Flocculation was provoked by the addition of 1 mM $CaCl_2$ or 10 mM $MgCl_2$, but not by NaCl up to 200 mM.

For the bottom strain, the influences of calcium, magnesium, and sodium ions on flocculation were similar in the buffered (Fig. 3D) and the nonbuffered (Fig. 3B) systems: $CaCl_2$ induced complete flocculation at a concentration of about 0.5 mM, in agreement with the results shown in Fig. 1B, whereas only partial flocculation occurred in 100 mM $MgCl_2$ and no flocculation was observed with NaCl concentrations up to 200 mM.

Effect of different organic solvents. The effects of different organic solvents as inducers of flocculation of stationary cells of the top strain at pH 5.2 (10 mM acetate buffer; 5 mM $CaCl_2$ added) are shown in Fig. 4A and B. The efficiency of monofunctional aliphatic alcohols in inducing flocculation increased in the following order: methanol, ethanol, *n*-propanol, and *n*-butanol. Isopropanol was less effective than *n*-propanol and as effective as ethanol. Alcohols with two hydroxyl (1,2-propyleneglycol and 2,3-butanediol) or three hydroxyl (glycerol) functions had little or no effect; 1,2-propyleneglycol was as effective as methanol. The effect of acetone was comparable to that of methanol.

In order to compare more closely the efficiency of the organic compounds in inducing flocculation, the concentration required to obtain a residual absorbance of 1 in the flocculation test is given in Table 1, together with the dielectric constant and the surface tension of the pure compounds. There seems to be a correlation between the efficiency of the organic compound and its dielectric constant for the series methanol, ethanol, *n*-propanol, and *n*-butanol; however, no correlation is found if all of the compounds used are considered. Similarly, no correlation with the surface tension of the pure organic compound is found.

In order to relate flocculation to the properties of the liquid phase, surface tensions were determined for mixtures of pure

TABLE 1. Critical concentration[a] of organic solvents inducing flocculation of top yeast MUCL 38475, dielectric constant (ε) and surface tension (γ) of pure compounds, and surface tension (γ) of mixtures with water corresponding to the critical concentration

Solvent	Critical concn (M)	ε (25°C) of pure solvent[b]	γ of pure solvent (mJ/m^2)[c]	γ of mixtures at critical concn (mJ/m^2)
Glycerol	>2.0	42.5	63.4	(69.0 ± 0.7)[d]
2,3-Butanediol	>2.0		30.0 ± 0.9[e]	(47.8 ± 0.7)[d]
1,2-Propyleneglycol	0.84	32.0 (20°C)	35.4 ± 0.8[e]	62.3 ± 0.3
Acetone	0.75	20.7	23.2	56.0 ± 0.5
Methanol	0.65	32.6	22.6	65.8 ± 0.5
Isopropanol	0.20	18.3	20.9	59.7 ± 0.7
Ethanol	0.16	24.3	22.3	66.5 ± 0.5
n-Propanol	0.05	20.1	23.7	66.9 ± 0.6
n-Butanol	0.04	17.1	23.7	60.9 ± 2.4
Water		78.5	72.8	

[a] Concentration giving a residual absorbance of 1 in the flocculation test performed in 10 mM acetate buffer at pH 5.2, with 5 mM $CaCl_2$ added (determined by interpolation).
[b] From reference 67.
[c] From reference 41.
[d] Surface tension of the highest concentration tested (2 M).
[e] Measured in this work.

water with the organic compounds, and the results were used to plot Fig. 4C and D. It appears that with the exception of 2,3-butanediol, the organic solvents all have the same effect if the surface tension of their mixture with water is taken into consideration. This is also shown in Table 1, which lists the surface tensions of the mixtures at the critical concentration. Flocculation was induced if the surface tension dropped below a value near 65 mJ/m^2 for the series methanol, ethanol, *n*-propanol, and *n*-butanol. For isopropanol, acetone, and 1,2-propyleneglycol, the critical value appeared to be slightly lower, i.e., about 60 mJ/m^2. Glycerol did not induce flocculation, as the surface tension of all glycerol-water mixtures tested remained higher than 65 mJ/m^2. The effect of 2,3-butanediol does not follow the rule observed for the other compounds; in this case, no flocculation occurred even though the surface tensions of the mixtures used were far below 60 mJ/m^2.

The effect of *n*-butanol on flocculation of stationary cells of the bottom strain was tested in the presence and in the absence of 5 mM $CaCl_2$ (10 mM acetate buffer, pH 5.2). The addition of *n*-butanol up to a concentration of 400 mM had no effect on flocculation of this strain (results not shown).

Influence of sugars. The influence of sugars on flocculation of stationary cells at pH 4 (10 mM acetate buffer) was tested. The addition of up to 500 mM glucose, mannose, CH_3-mannose, or galactose did not affect flocculation of the top strain (0.5 mM $CaCl_2$ added [results not shown]). For the bottom strain (5 mM $CaCl_2$ added), on the contrary, inhibition of flocculation occurred after the addition of 50 to 500 mM CH_3-mannose, mannose, maltose, or glucose but not galactose. This confirmed results previously published by Masy et al. (33) and Kihn et al. (28) for the same strain.

The influence of sugars and derivatives with a hydrophobic substituent was also tested on stationary cells of the bottom strain at pH 5.2 (10 mM acetate buffer; 5 mM $CaCl_2$ added). The inhibition of flocculation by 500 mM glucose and mannose, and the lack of inhibition by galactose, was again observed. Inhibition was found with phenyl-α-D-mannoside (about 10 mM) and *p*-nitrophenyl-α-D-mannoside (about 10 mM) and not with *n*-octyl-α-D-glucopyranoside (results not shown). None of these compounds was found to inhibit flocculation of the top strain at pH 5.2 (10 mM acetate buffer; 5 mM $CaCl_2$ added) in the presence of ethanol.

Influence of culture medium composition. The direct influence of the culture medium composition on flocculation was investigated by submitting exponential and stationary cells to a flocculation test in which, instead of using water, the cells were resuspended in fresh medium, in culture medium separated from a culture in the exponential growth phase, or in medium separated from a culture in the stationary growth phase. The results are shown in Fig. 5A and B for the top and the bottom strains, respectively. For the two strains, exponential cells did not flocculate regardless of the medium used, and stationary cells did not flocculate when resuspended in fresh medium. Stationary cells of the top strain flocculated partially in medium separated from an exponential culture and completely in medium separated from a stationary culture. Stationary cells of the bottom strain flocculated only in medium separated from a stationary culture (3 days of culture or more). Thus, besides the cell physiological state, the "actual" medium composition was important for flocculation.

For the two strains, the activity of free Ca^{2+} and the concentrations of calcium, ethanol, and glucose were determined in fresh medium and in media separated from cultures at different growth phases. The results are presented in Table 2. In the fresh medium, a total calcium concentration of 350 μM was found; the free Ca^{2+} activity was about 68 μM. In the exponential phase, little calcium was consumed; in the stationary phase, the total calcium concentration decreased to about 220 μM and the free Ca^{2+} activity dropped to 30 and 15 μM for the top and bottom strains, respectively. The sugar concentration in the culture medium of the two strains dropped to about 32 g/liter in the exponential growth phase and about 3 g/liter in the stationary phase. Ethanol was formed at concentrations of about 1.3 to 1.9 and 2.3 to 3.9% (vol/vol) in the exponential and stationary phases, respectively.

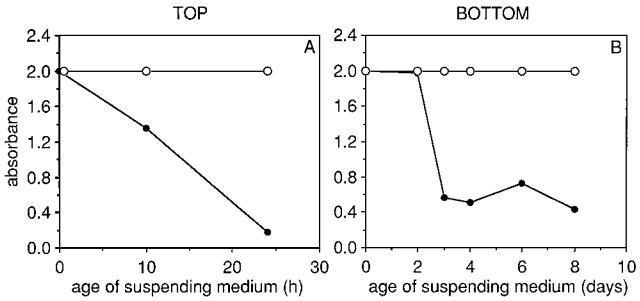

FIG. 5. Residual absorbance of yeast suspensions: exponential (○) and stationary (●) cells, resuspended in media separated from cultures of different ages and in fresh medium (0 on the abscissa scale). (A) Top strain MUCL 38475; (B) bottom strain MUCL 28285.

To test whether flocculation of the top strain was caused by the appearance of ethanol (shown above to be a possible inducer of flocculation) in the medium or by the disappearance of an inhibitor present in the fresh medium, the flocculation test was repeated with the three media, adding ethanol to a final concentration of 1, 5, or 10% (vol/vol). Figure 6A presents the absorbance of the cell suspension against the added ethanol concentration; in Fig. 6B, the same results are plotted against the total ethanol concentration (added ethanol plus ethanol already present in the culture medium [Table 2]). The results show that stationary cells flocculated in the fresh medium provided the ethanol concentration was at least 3% (vol/vol) regardless of the freshness of the medium; thus, no inhibitor was present in the fresh culture medium. The exponential cells did not flocculate in any of the media supplemented with ethanol up to 10% (vol/vol).

(ii) Surface properties. Electrophoretic mobilities were measured as a function of pH for stationary cells of the top strain and the bottom strain in 1 and 10 mM KNO_3. The two

TABLE 2. Activity of free Ca^{2+} and concentrations of total calcium, sugars, and ethanol in fresh culture medium and in supernatants of cultures of yeasts, harvested at different times[a]

Strain	Culture stage	Activity of free Ca^{2+} (μM)	Concn of:		
			Total Ca^{2+} (μM)	Glucose (mM)	Ethanol (%, vol/vol)
None	Fresh medium	68	350	287	
Top strain MUCL 38475	10 h	51	325	190 ± 35	1.9 ± 0.1
	24 h	30	225	29 ± 12	3.9 ± 0.1
Bottom strain MUCL 28285	2 days	53	313	178 ± 29	1.3 ± 0.1
	3–8 days	15	200	35 ± 12	2.3 ± 0.2

[a] Each value is the mean of at least two independent experiments.

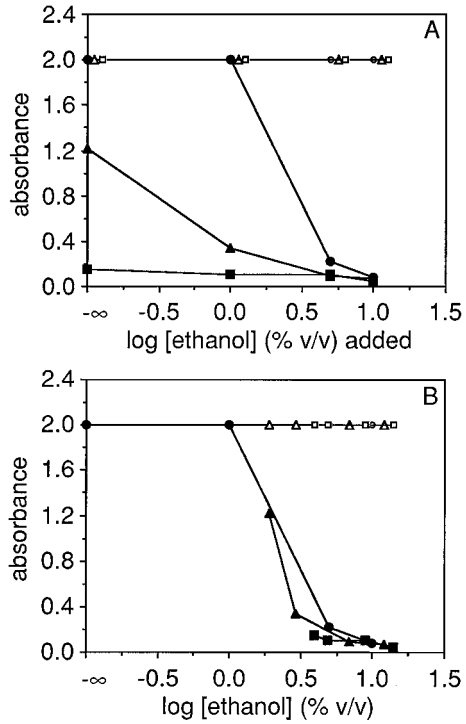

FIG. 6. Residual absorbance of suspensions of top strain MUCL 38475 as a function of the concentration of added (A) and total (B) ethanol exponential (open symbols) and stationary (closed symbols) cells resuspended in fresh culture medium (○, ●), culture medium separated from the exponential growth phase (△, ▲), and culture medium separated from the stationary growth phase (□, ■).

instruments gave identical results; those obtained with the Malvern Zetasizer III are presented in Fig. 7. The isoelectric point was near 2 for the bottom strain and about 4 for the top strain. At pH 5.2, the pH of the culture medium in the stationary phase, the two strains had comparable electrophoretic mobilities, i.e., about -1.1×10^{-8} and -0.5×10^{-8} m^2 V^{-1} s^{-1} in 1 and 10 mM KNO_3, respectively. However, at pH 7.5 and low ionic strength (1 mM KNO_3), the electrophoretic mobilities were -2.0×10^{-8} and -1.3×10^{-8} m^2 V^{-1} s^{-1} for the top and bottom strains, respectively.

The electrophoretic mobility can be converted into zeta potential by the Smoluchowski equation: zeta potential (mV) = 12.9 × electrophoretic mobility (10^{-8} m^2 V^{-1} s^{-1}) at 25°C (23); this equation is valid when the particle radius is much larger than the Debye length. At pH 5.2 and ionic strength 10 mM (Fig. 7C and D), the zeta potential covers a range of −5 to −12 mV. Zeta potentials of yeasts in the literature are mostly higher than those measured here: values in the range of −7 to −56 mV at ionic strengths of 10 to 30 mM were reported (4, 6, 7, 17, 20, 65).

For the exponential cells, the mobility curves obtained in 1 mM KNO_3 were identical to those of the stationary cells (results not shown).

Water contact angle measurements gave the following results: 73 ± 17 (six independent experiments) and 77 ± 12 (nine independent experiments) for exponential and stationary cells, respectively, of the top strain; 21 (two independent experiments) and 31 ± 15 (four experiments) for exponential and stationary cells, respectively, of the bottom strain. The top strain was thus more hydrophobic than the bottom strain. For the two strains, the differences observed between the exponential and stationary growth phases were not significant.

The cell surface chemical composition obtained by XPS is given in Table 3. Mean atom fractions (excluding hydrogen) were calculated for samples originating from independent cultures. According to a Student's *t* test (95% confidence level), the top strain did not show any significant difference between the exponential and the stationary phases. For the bottom strain, a significant decrease in surface phosphate and potassium concentrations was observed between the exponential and the stationary phases.

When stationary cells of the top and bottom strains were compared, some significant differences were found (Table 3). The cell surface of the top strain contained more hydrocarbonlike carbon [C—(C,H)], less oxygen, and more nitrogen than the cell surface of the bottom strain; for the minor elements (P and K) and the minor components of the major peaks (less than 5% mole fraction), no significant differences were detected.

DISCUSSION

Physicochemical properties. (i) Surface composition and electrical properties. Differences in electrical properties between the two strains were found: the top strain had a higher isoelectric point and its electrophoretic mobility seemed to be strongly dependent on pH as opposed to the bottom strain. Amory and Rouxhet (2) found systematically less negative electrophoretic mobilities below pH 5 for top-fermenting strains compared with bottom-fermenting strains (all cultured at 30°C). They related the more negative electrophoretic mobility of bottom strains at pH 4 to a higher surface concentration of phosphate, measured by XPS. In the present study, the difference between the surface phosphate concentrations of the top and bottom strains is not significant.

Apart from phosphate groups in phosphomannans, the electric charge at yeast cell surfaces may arise from carboxylate and protonated amino groups in proteins (25). Unfortunately, the concentrations of carboxylate and protonated amino groups at the cell surface of the strains could not be determined by XPS, as their contribution to the total signals of C_{1s}, O_{1s}, and N_{1s} was very small. Furthermore, in the N_{1s} peak, protonated amine cannot be distinguished from ammonium ions, which do not contribute to the surface charge but act as counterions for negative surface groups.

There is still much controversy about the relative importance of carboxylate and phosphate groups in flocculation (26, 31, 32, 36). Eddy and Rudin (16) and Beavan et al. (3) attributed the mobility at pH 4 to phosphate and the difference in mobility between pH 7 and pH 4 to carboxylate groups; Bowen and Cooke (6) adopted a similar approach. However, this interpretation does not take into account the nature of the cell wall-solution interface. Deprotonation or protonation of surface groups changes the surface electric potential, which in turn influences the apparent equilibrium constants of acid-base reactions at the surface. On that basis, Amory and Rouxhet (2) suggested that the contribution of carboxylate groups to the surface charge was small compared with the contribution of phosphate groups. Moreover, the surface is not ion impenetrable and should be regarded as a three-dimensional gel-like structure (7, 43). Finally, it is not a homogeneous system, neither chemically, as proteins are distributed in the polysaccharide matrix, nor morphologically, as the surface is not necessarily smooth.

No difference in electrical properties was found between nonflocculent and flocculent cells of the two strains. For the bottom strain a significant decrease in surface phosphate concentration, and a concomitant decrease of potassium as coun-

TABLE 3. Surface chemical composition of exponential and stationary cells of top-fermenting yeast strain MUCL 38475 and bottom-fermenting yeast strain MUCL 28285 determined by XPS: average atom fraction, excluding hydrogen; confidence intervals (95%); and expression of the surface composition in terms of model molecular constituents

Yeast strain	Growth phase	Avg atom fraction (%)											Model compounds (wt %)			
		Total C	C—(C,H)	C—(O,N)	C=O	O=C-OH	Total O	C—OH	O=C	Total N	N—C	N^+	P	K	Proteins	Polysaccharides	Hydrocarbons
MUCL 38475	Exponential[a]	70.5 ± 3.0	38.7 ± 9.5	24.1 ± 5.6	6.5 ± 1.4	1.2 ± 0.2	26.3 ± 3.7	21.6 ± 6.0	4.7 ± 3.2	2.6 ± 1.1	2.4 ± 0.8	0.3 ± 0.2	0.28 ± 0.22	0.33 ± 0.15	14.3 ± 5.1	52.9 ± 6.0	32.8 ± 6.5
	Stationary[b]	70.4 ± 1.2	36.3 ± 3.2	25.9 ± 1.8	6.6 ± 0.5	1.5 ± 0.2	27.4 ± 1.4	24.3 ± 2.0	3.1 ± 1.0	2.0 ± 0.3	1.8 ± 0.2	0.2 ± 0.1	0.15 ± 0.09[c]	0.15 ± 0.07[c]	10.5 ± 1.3	56.9 ± 3.2	32.7 ± 2.7
MUCL 28285	Exponential[d]	67.8 ± 1.8	30.7 ± 2.1	28.9 ± 1.0	7.1 ± 0.7	1.1 ± 0.3	30.3 ± 1.5	27.7 ± 1.0	2.6 ± 1.0	1.2 ± 0.3	1.1 ± 0.2	0.1 ± 0.1	0.28 ± 0.06	0.45 ± 0.31	6.7 ± 1.1	65.2 ± 3.2	28.1 ± 3.8
	Stationary[e]	67.6 ± 2.4	29.2 ± 5.0	30.0 ± 2.6	7.2 ± 0.7	1.2 ± 0.3	30.9 ± 2.5	28.9 ± 2.8	1.9 ± 0.7	1.2 ± 0.3	1.1 ± 0.3	0.1 ± 0.1	0.17 ± 0.05	0.16 ± 0.08	6.7 ± 1.5	66.1 ± 5.9	27.2 ± 5.0
Significant difference, MUCL 38475 vs MUCL 28285[g]		+	+	–	NS[f]	+	–	–	+	+	+	NS	NS	NS	+	–	+

[a] Three samples originating from independent cultures.
[b] Thirteen samples originating from independent cultures.
[c] For samples in which the concentration was below the detection limit (0.1%), the value was set equal to half of the detection limit.
[d] Five samples originating from independent cultures.
[e] Fourteen samples originating from independent cultures: six were harvested after 5 days, five were harvested after 6 days, and three were harvested after 8 days.
[f] NS, not significant.
[g] For stationary cells.

terion, was found with culture time, but this was not reflected in the electrophoretic mobility. Thus, no relationship was found between electrical properties and the onset of flocculation.

(ii) Surface composition and hydrophobicity. The concentration of hydrocarbonlike carbon is much higher than could be expected from the biochemical composition of the whole yeast cell wall (18), and thus the question of whether this was an artefact due to sample preparation was raised. A systematic study of the effect of sample handling on XPS results, however, showed that only a minor part of hydrocarbonlike moieties could be due to artefacts (48).

Using the ratios O/C and N/C, given by XPS, and using average elemental compositions of model constituents (21), the cell surface can be modeled roughly in terms of "polysaccharides," "proteins," and "hydrocarbonlike compounds" (48); the results are shown in Table 3. The concentrations of proteins and hydrocarbon moieties are significantly higher for the top strain than for the bottom strain. This may be related to higher water contact angle measurements revealing a greater hydrophobicity for the top strain. The hydrophobicity of the yeast cell surface has already been positively correlated both with the presence of proteins in the cell wall (27, 53) and with the protein surface concentration (1).

The difference in hydrophobicity may explain the difference in behavior of these strains at the end of fermentation. The flocs of the top strain associate with CO_2 bubbles and rise to the top of the fermented wort, whereas aggregates of the bottom strain settle down. This is supported by the relationship between flotation and hydrophobicity observed for *S. cerevisiae* (63).

No significant differences in hydrophobicity or surface concentrations of proteins, polysaccharides, or hydrocarbons were found between nonflocculent and flocculent cells. Thus, hydrophobicity does not seem to be correlated with the onset of flocculation. This is in contrast to the recent observation of a small increase of the water contact angle at the moment of initiation of flocculation (62).

Flocculation behavior and flocculation mechanisms. (i) Bottom-fermenting strain MUCL 28285: factors controlling flocculation. Flocculation of the bottom strain showed the following characteristics: occurrence in the stationary phase only, specific requirement for Ca^{2+}, inhibition at low pH, and inhibition by mannose, glucose, or maltose but not by galactose. This behavior corresponds with the mechanism proposed by Miki et al. (35), in which flocculation is mediated by a lectin which binds with mannose residues on adjacent cells and requires calcium to adopt its active conformation. Bottom strain MUCL 28285 thus belongs to the NewFlo phenotype according to the classification of Stratford and Assinder (57) or to the GMS group according to the classification of Masy et al. (33). Stratford observed this phenotype predominantly for ale strains; MUCL 28285 used in this work is a lager strain.

The hypothesis of a lectinlike mechanism for this strain is further supported by the fact that the onset of flocculation in the stationary growth phase is not accompanied by a change of surface properties as revealed by XPS analysis, electrophoretic mobility measurement, or contact angle measurement. Flocculation is thus due to more subtle modifications. Nagarajan and Umesh-Kumar (39) reported the appearance of an antigen with lectinlike function during the transition of the yeast cells from the nonflocculent to the flocculent state. Some authors suggested that lectins and/or receptors are not present at the surface in the exponential growth phase or are already present but not accessible or operational (51, 55, 58). Recently, cell surface proteins involved in flocculation were isolated from *S.*

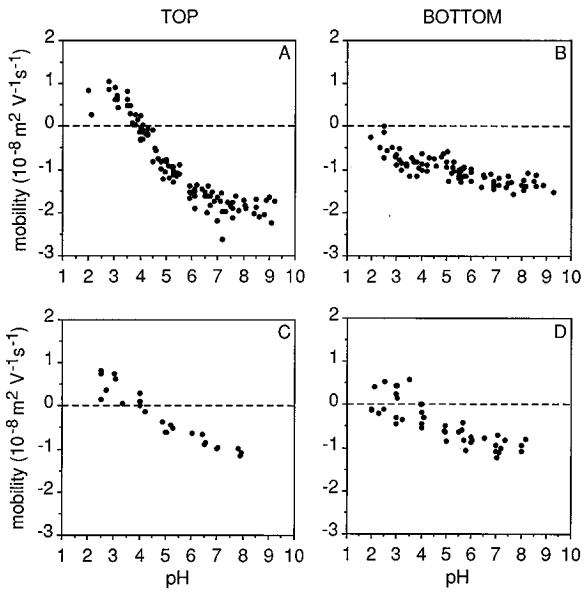

FIG. 7. Electrophoretic mobility as a function of pH for top strain MUCL 38475 (A and C) and bottom strain MUCL 28285 (B and D). Measurements were performed in 1 mM (A and B) and 10 mM (C and D) KNO_3.

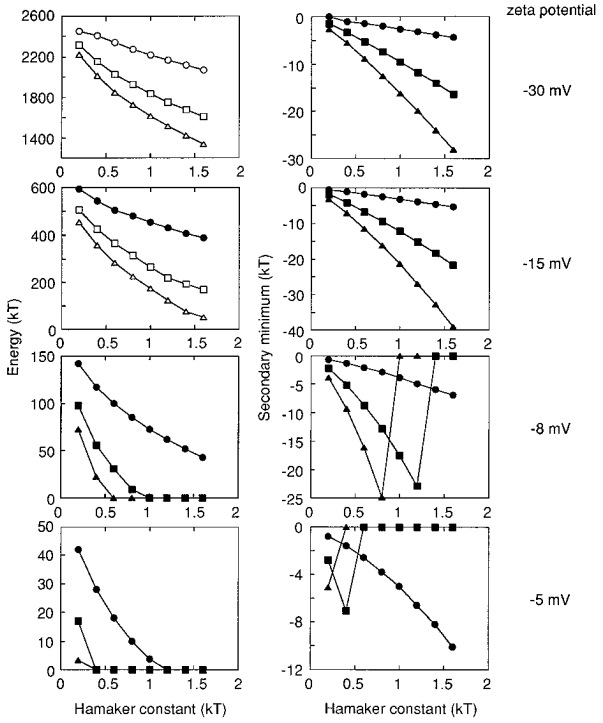

FIG. 8. Data computed for the interaction energy between yeast cells according to DLVO theory (radius, 3 μm; dielectric constant, 78.5) as a function of the Hamaker constant at different zeta potentials and different ionic strengths (●, ○, 1 mM; ■, □, 10 mM; ▲, △, 25 mM): energy barrier (closed symbols) or energy at 1-nm distance (open symbols) and depth of the secondary minimum.

cerevisiae by two independent groups (50, 61); these proteins showed lectinlike activity as they were mannose specific and required calcium. Straver et al. (60) also isolated another protein involved in flocculation but without lectinlike activity; the authors suggested that this flocculin might act as a receptor for surface lectins.

Masy et al. (34) reported a change, during the culture, of the calcium activity required to provoke flocculation in water, called calcium threshold. It was found that, at the onset of flocculation, the calcium threshold measured in flocculation tests was close to the calcium activity in the fermented broth. The above results show that the variation of the calcium threshold (100 mM to 10 μM [Fig. 1B]), a characteristic of the cells, is much stronger than the variation of calcium activity in the fermentation broth (53 to 15 μM [Table 2]). The drop of the calcium threshold might be explained by the appearance of an operational lectin in the stationary growth phase.

However, Fig. 5 shows that flocculation requires not only a certain physiological state of the cells but also an adequate composition of the surrounding aqueous solution. The key factor is not the calcium concentration or activity but the sugar concentration. In the culture medium the glucose concentration dropped from 180 mM in the exponential growth phase to 35 mM in the stationary growth phase (Table 2), while the flocculation tests showed that the inhibitory concentration was around 100 mM.

Mechanism of flocculation. In order to achieve a better appreciation of the interplay between nonspecific and specific interfacial interactions, the DLVO (Derjaguin-Landau-Verwey-Overbeek) theory has been used to calculate curves of the interaction energy versus the separation distance between the cell surfaces (65). The same approach was adopted here, using a cell radius of 3 μm and a dielectric constant of 78.5 (pure water); the Hamaker constant for the cell-water-cell interface was varied from 0.2 to 1.6 kT (range of Hamaker constants across water between vesicles coated with different polysaccharide-protein mixtures as reported by Nir [42]), the zeta potential was varied from −5 to −30 mV, and the ionic strength was varied from 1 to 25 mM. Figure 8 presents the height of the energy barrier and the depth of the secondary minimum for different combinations of the parameters. The DLVO theory supposes that the interacting particles are smooth spheres; in the case of a rough surface, it may refer to the approach between two protuberances, and the results should be modified in proportion to the radius of curvature.

Figure 8 shows that due to the uncertainties in the zeta potential (range, −5 to −12 mV [Fig. 7]) and the Hamaker constant, the possibility that the energy barrier is low enough to allow flocculation in the primary minimum cannot be ruled out. The potential barrier is indeed negligible for a range of situations when the zeta potential is equal to −5 or −8 mV. The results presented in Fig. 8 indicate that flocculation may also occur in the secondary minimum of the interaction energy curve. The distance of the secondary minimum with respect to the surface provides a convenient evaluation of the minimal distance from which the cells can easily approach each other (45). Table 4 gives this distance for the range of parameters selected in Fig. 8. It is interesting that while the height of the energy barrier and the depth of the secondary minimum depend strongly on the physical parameters used, the distance of the secondary minimum with respect to the surface varies only a little as a function of these parameters in the range of ionic strengths (10 to 25 mM), which are relevant to the culture medium and to the solutions used for flocculation tests.

Flocculation of the bottom strain cannot be explained only by the action of London-van der Waals forces and double-layer repulsion (DLVO theory). According to the latter model, both exponential and stationary cells would be expected to behave in the same way because they have similar surface chemical

TABLE 4. Distance of the secondary minimum from the cell surface, as derived from the net attraction energy curve between two cells, according to the DLVO theory for different values of zeta potential, ionic strength, and Hamaker constant

Hamaker constant (kT)	Distance of secondary minimum from surface (nm) at given zeta potential and ionic strength											
	−30 mV			−15 mV			−8 mV			−5 mV		
	1 mM	10 mM	25 mM	1 mM	10 mM	25 mM	1 mM	10 mM	25 mM	1 mM	10 mM	25 mM
0.2		28	18	90	24	14	75	19	10	65	14	7
0.4	90	27	15	85	21	12	65	15	8	50	10	
0.6	90	25	14	75	19	11	60	13	7	50		
0.8	90	24	14	70	18	10	55	11	5	40		
1.0	90	22	13	70	17	9	50	10		40		
1.2	90	22	13	70	16	9	50	8		29		
1.4	85	21	12	70	15	8	50			28		
1.6	80	21	12	60	15	8	45			23		

compositions and zeta potentials. Furthermore, the observation of the effect of specific interactions is not compatible with flocculation in the primary minimum and requires a repulsion, either electrostatic or steric (5, 28).

The following model can then be considered. Electrostatic repulsion could keep the cell surfaces at a distance of the order of 10 nm from each other. When the cells are in the stationary growth phase, lectins and/or macromolecular chains carrying a receptor can protrude out of the surface over that distance and bind with each other, thus provoking flocculation. This scheme remains valid if the cell surface is not smooth and if the cells are bound through fimbriae (59), noting that the radius of curvature of the surfaces in contact is then much smaller. The lack of influence of the ionic strength on flocculation and the fact that the optimum pH for flocculation is appreciably higher than the cell isoelectric point indicate that the repulsion is not electrostatic in nature. Steric repulsion is thus probable, as suggested in the case of bacterial adhesion (45). Considering the hydrophilic and polymeric nature of this yeast surface, it is expected that polymers (polysaccharides) are solvated and protrude from the surface.

(ii) Top-fermenting strain MUCL 38475: factors controlling flocculation. The top strain behaves like the bottom strain in the sense that its flocculation requires both a certain physiological state and an adequate solution composition (Fig. 5). However, other characteristics show that the process involved is of quite a different nature: flocculation is not inhibited by sugars, it is induced by ethanol and other organic solvents, it takes place at the cell isoelectric point, and it does not require the addition of calcium.

The cells of the stationary phase were not inhibited by any of the sugars tested, in contrast to many brewing yeast strains which are at least partially inhibited by mannose (55). Eddy (13, 15) found one top and two bottom strains that were not inhibited by mannose or maltose. Kihn et al. (28) and Masy and coworkers (33) reported a few strains which were not inhibited by mannose and other sugars; they proposed a third class, besides the two classes Flo1 and New Flo defined by Stratford and Assinder (57), which was named MI (mannose insensitive). The top strain thus belongs to this class.

Flocculation is induced by low concentrations of ethanol (Fig. 1E and 2). In the flocculation test, induction was observed between 1 and 5% (vol/vol); in the culture medium the ethanol concentration increased from 2% in the exponential phase to 4% (vol/vol) in the stationary phase. The ethanol concentration is the characteristic which makes the composition of the test solution or of the culture medium adequate for flocculation to occur. This is demonstrated by Fig. 6, which shows that stationary cells flocculate in fresh culture medium provided ethanol is added at a final concentration of about 5% (vol/vol). A promoting effect of ethanol and other alcohols was reported by several authors (1, 14, 15, 36, 44). Eddy (14, 15) described the particular behavior of yeasts that were induced for flocculation by low concentrations of certain alcohols; they were at least partly deflocculated by mannose or maltose. On the other hand, Kamada and Murata (27) reported an inhibition of flocculation of a set of top and bottom yeasts by ethanol.

Mechanism of flocculation. Mill (36) related the flocculation behavior to the dielectric constant of suspensions containing methanol, ethanol, isopropanol, acetone, and dioxane in high solvent concentration (more than 30% [vol/vol]); this was explained by an increase in the strength of hydrogen bonds and a decrease in ionization of salt bonds. Fletcher (19) studied the influence of low concentrations of methanol, ethanol, *n*-propanol, and *n*-butanol on bacterial attachment to surfaces. Whether the support was hydrophilic or hydrophobic, the least attachment was observed when the liquid surface tension was in the range of 64 to 69 mJ/m^2; however, this minimum was not very pronounced.

Figure 4 shows that, except for 2,3-butanediol, all of the solvents examined behave in the same way provided that they are compared on the basis of the decrease of the liquid surface tension: they all provoke flocculation as long as the surface tension is lowered below 65 mJ/m^2. The influence of the organic solvents thus acts undoubtedly through adsorption at the cell-water interface. This might be interpreted by one of two approaches: the balance of surface energies approach (neglecting electrical interactions) or the DLVO approach (see above). According to the balance of surface energies approach, the free energy of cell-cell adhesion, ΔG_{CC}, would be given by: $\Delta G_{CC} = \gamma_{CC} - 2\,\gamma_{CL}$, where γ_{CL} and γ_{CC} are the interfacial tensions of the cell-liquid and cell-cell interfaces, respectively. Spontaneous flocculation occurs if ΔG_{CC} is negative. Decreasing γ_{CL} by adsorption of organic compounds would be unfavorable to adhesion, and thus this model has to be rejected. As a matter of fact, electrical interactions cannot be neglected in the flocculation mechanism of the top strain. This is indicated by the fact that the optimum pH of flocculation is close to the cell isoelectric point and by the influence of ionic strength when the flocculation takes place above the isoelectric point in the presence of ethanol.

Adsorption of organic molecules can have several consequences in relation to the DLVO model. It will disrupt the water structure near the surface and change the dielectric constant locally (24), and it may influence the adsorption of counterions (22) and modify the zeta potential (66). If the yeast is considered a hydrophobic colloid, the influence of ethanol and other organic solvents may thus be attributed to

their adsorption at the cell surface, leading to a lowered local dielectric constant and to decreased cell-cell electrical repulsion. Unfortunately, the effect of the solvents on the electrical properties of the yeast surface could not be investigated by electrophoretic mobility measurements because, in the presence of solvents, the cells flocculated and sedimented in a few seconds.

Let us now discuss the possible nature of the difference between stationary and exponential cells. The former flocculate at the isoelectric point, at a higher pH in the presence of ethanol, and at a sufficient ionic strength, while the latter do not flocculate under the same conditions. The fact that the exponential cells do not flocculate at the isoelectric point indicates the existence of a steric stabilization by macromolecular chains protruding from the surface into the solution. The stationary cells could then differ from the exponential cells by a lower efficiency of the steric stabilization, due to a smaller chain length, a lower concentration, or a different structure of these polymers, without noticeable difference of surface elemental composition, zeta potential, and water contact angle.

Table 4 shows that cell surfaces can approach one another at a distance of about 10 nm without encountering a net repulsion. The organic solvents could exert their influence by reducing cell-cell electrical repulsion. They could also decrease steric stabilization by being poorer solvents or, on the contrary, by allowing the protrusion of polymer chains carrying binding sites for nonspecific (hydrogen bonding) or specific interactions. The latter hypothesis seems more probable: since these cells have a hydrophobic surface, organic compounds will be better solvents than water.

Although no inhibition by sugars was observed and no addition of calcium was required for flocculation, the implication of specific interactions in the flocculation of the top strain cannot be ruled out. Deflocculation by EDTA points to a specific role for calcium or another divalent cation. In the presence of EDTA, calcium was about 1,000 times more effective in inducing flocculation than magnesium (10 times more magnesium needed to restore flocculation; complexing constant of EDTA for calcium of 100 times greater than that for magnesium [52]). In the nonbuffered system without EDTA, calcium and magnesium appeared to be equally efficient. This apparent contradiction was also observed by Kuriyama et al. (30). Accordingly, the role of calcium may be twofold: on the one hand, trace amounts are required to activate a protein; on the other hand, calcium contributes to reduce electrostatic repulsion between the cells. The second role can be equally well played by magnesium. The redispersion of the cells at high magnesium concentration in the flocculation test (Fig. 3A) may be due to competition of magnesium for the calcium binding site.

The transition of the nonflocculent to the flocculent character of the top strain at the end of the exponential growth phase may thus be attributed to changes which decrease steric stabilization and/or to an adhesin which is only operational in the presence of a certain concentration of ethanol. In the latter case, the adhesin is not a lectin as reported for most brewing strains (57).

Conclusion. The top and the bottom strains show different electrical properties, a different hydrophobicity, and a different surface chemical composition. They are found to flocculate according to totally different mechanisms; however, no correlation between the cell surface physicochemical properties and the onset of flocculation is found for either strain.

Flocculation of the bottom strain (MUCL 28285) is governed by a lectin-mediated mechanism. It is inhibited by mannose and some other sugars, requires calcium specifically, flocculates in a narrow pH range different from the isoelectric point, and is not influenced by ethanol. The strain can be classified in the NewFlo group as defined by Stratford and Assinder (57) or in the GMS group as defined by Masy et al. (33). The onset of flocculation for the bottom strain at the end of the exponential phase is controlled by the conjunction of two effects: the appearance of "active" lectins at the cell surface on the one hand, and the decrease of sugar concentrations in the solution on the other hand. Calculations of the cell-cell interaction energy by the DLVO theory do not establish firmly the existence of an energy barrier due to electrostatic repulsion between the cells. However, the observation that no flocculation occurs near the isoelectric point and that flocculation is independent with respect to ionic strength rather point to the effect of steric repulsion. Cells of the stationary phase probably possess protruding polymers carrying lectins and/or receptor sites that can bridge two cell surfaces across a repulsion barrier.

The top strain (MUCL 38475) does not flocculate according to the above mechanism. Its flocculation is not inhibited by mannose, does not require the addition of calcium, and takes place at the cell isoelectric point. The transition of the nonflocculent to the flocculent character at the end of the exponential phase is controlled by the conjunction of two effects: a change of the cell surface on the one hand and an increase of ethanol concentration on the other hand. Low concentrations of ethanol broaden the range of pH in which the cells flocculate, and under these conditions, flocculation is favored by an increase of ionic strength. Other alcohols have the same effect as ethanol when the comparison is made at constant solution surface tension. Adsorbed organic solvents may induce flocculation by reducing the electrostatic repulsion between cells, by decreasing steric stabilization, and/or by allowing the protrusion of polymer chains into the liquid phase. The only evidence for an adhesin-mediated mechanism is the specific requirement of a small amount of calcium.

ACKNOWLEDGMENTS

P.G.R. is member of the Research Center for Advanced Materials. We thank J.-P. Dufour and G. Derdelinckx for providing yeast strains and freeze-dryer facilities.

The support of the National Fund for Scientific Research (P.B.D.) and of the Department of Education and Scientific Research (Concerted Action Physical Chemistry of Interfaces and Biotechnology) is gratefully acknowledged.

REFERENCES

1. **Amory, D. E., J.-P. Dufour, and P. G. Rouxhet.** 1988. Flocculence of brewery yeasts and their surface properties: chemical composition, electrostatic charge and hydrophobicity. J. Inst. Brew. **94:**79–84.
2. **Amory, D. E., and P. G. Rouxhet.** 1988. Surface properties of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*: chemical composition, electrostatic charge and hydrophobicity. Biochim. Biophys. Acta **938:**61–70.
3. **Beavan, M. J., D. M. Belk, G. G. Stewart, and A. H. Rose.** 1979. Changes in electrophoretic mobility and lytic enzyme activity associated with development of flocculating ability in *Saccharomyces cerevisiae*. Can. J. Microbiol. **25:**888–895.
4. **Beezer, A. E., R. J. Miles, and S. R. Sivayogan.** 1983. Study of the interaction of *Saccharomyces cerevisiae* with glucose by particle microelectrophoresis. Biochim. Biophys. Acta **763:**251–257.
5. **Bell, G. I.** 1988. Models of cell adhesion involving specific binding, p. 227–258. *In* P. Bongrand (ed.), Physical basis of cell-cell adhesion. CRC Press Inc., Boca Raton, Fla.
6. **Bowen, W. R., and R. J. Cooke.** 1989. Studies of *Saccharomyces cerevisiae* during fermentation—an in vivo electrokinetic investigation. Biotechnol. Bioeng. **33:**706–715.
7. **Bowen, W. R., H. A. M. Sabuni, and T. J. Ventham.** 1992. Studies of the cell-wall properties of *Saccharomyces cerevisiae* during fermentation. Biotechnol. Bioeng. **40:**1309–1318.
8. **Bücher, T., and H. Redetzki.** 1951. Eine spezifische photometrische Bestimmung von Äthylalkohol auf fermentotovem wege. Klin. Wochenschr. **29:**615.

9. **Busscher, H. J., M. M. Cowan, and H. van der Mei.** 1992. On the relative importance of specific and non-specific approaches to oral microbial adhesion. FEMS Microbiol. Rev. **88:**199–210.
10. **Calleja, G. B.** 1989. Cell aggregation, p. 165–238. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 2. Yeast and environment. Academic Press Ltd., London.
11. **Calleja, G. B.** 1994. Hooks, loops, and the shallow minimum: mechanistic aspects of yeast flocculation. Colloids Surf. B **2:**133–149.
12. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
13. **Eddy, A. A.** 1955. Flocculation characteristics of yeasts. I. Comparative survey of various strains of *Saccharomyces cerevisiae*. J. Inst. Brew. **61:**307–312.
14. **Eddy, A. A.** 1955. Flocculation characteristics of yeasts. III. General role of flocculating agents and special characteristics of a yeast flocculated by alcohol. J. Inst. Brew. **61:**318–320.
15. **Eddy, A. A.** 1958. Composite nature of the flocculation process of top and bottom strains of *Saccharomyces*. J. Inst. Brew. **64:**143–151.
16. **Eddy, A. A., and A. D. Rudin.** 1958. The structure of the yeast cell wall. I. Identification of charged groups at the surface. Proc. R. Soc. London B Biol. Sci. **149:**419–432.
17. **Fisher, D. J.** 1975. Flocculation—some observations on the surface charges of yeast cells. J. Inst. Brew. **81:**107–110.
18. **Fleet, G. H.** 1991. Cell walls, p. 199–277. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 4. Yeast organelles, Academic Press Ltd., London.
19. **Fletcher, M.** 1983. The effects of methanol, ethanol, propanol and butanol on bacterial attachment to surfaces. J. Gen. Microbiol. **129:**633–641.
20. **Gage, R. A., A. R. P. Theuvenet, and G. W. F. H. Borst-Pauwels.** 1986. Effect of plasmolysis upon monovalent cation uptake, 9-aminoacridine binding and the zeta potential of yeast cells. Biochim. Biophys. Acta **854:**77–83.
21. **Gerin, P. A., Y. Dufrêne, M.-N. Bellon-Fontaine, M. Asther, and P. G. Rouxhet.** 1993. Surface properties of the conidiospores of *Phanerochaete chrysosporium* and their relevance to pellet formation. J. Bacteriol. **175:**5135–5144.
22. **Hesleitner, P., N. Kallay, and E. Matijevic.** 1991. Adsorption at solid/liquid interfaces. 6. The effect of methanol and ethanol on the ionic equilibria at the hematite/water interface. Langmuir **7:**178–184.
23. **Hiemenz, P. C.** 1986. Principles of colloid and surface chemistry. Marcel Dekker, New York.
24. **Israelachvili, J.** 1992. Interactions involving polar molecules, p. 48–66. *In* J. Israelachvili (ed.), Intermolecular and surface forces, 2nd ed. Academic Press Ltd., London.
25. **James, A. M.** 1991. Charge properties of microbial cell surfaces, p. 221–262. *In* N. Mozes, P. S. Handley, H. J. Busscher, and P. G. Rouxhet (ed.), Microbial cell surface analysis: structural and physicochemical methods. VCH Publishers, New York.
26. **Jayatissa, P. M., and A. H. Rose.** 1976. Role of wall phosphomannan in flocculation of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **96:**165–174.
27. **Kamada, K., and M. Murata.** 1984. On the mechanism of brewer's yeast flocculation. Agric. Biol. Chem. **48:**2423–2433.
28. **Kihn, J.-K., C. L. Masy, and M. M. Mestdagh.** 1988. Yeast flocculation: competition between nonspecific repulsion and specific bonding in cell adhesion. Can. J. Microbiol. **34:**773–778.
29. **Kihn, J.-K., C. L. Masy, M. M. Mestdagh, and P. G. Rouxhet.** 1988. Yeast flocculation: factors affecting the measurement of flocculence. Can. J. Microbiol. **34:**779–781.
30. **Kuriyama, H., I. Umeda, and H. Kobayashi.** 1991. Role of cations in the flocculation of *Saccharomyces cerevisiae* and discrimination of the corresponding proteins. Can. J. Microbiol. **37:**397–403.
31. **Lyons, T. P., and J. S. Hough.** 1970. Flocculation of brewers' yeast. J. Inst. Brew. **76:**564–571.
32. **Lyons, T. P., and J. S. Hough.** 1971. Further evidence for the cross-bridging hypothesis for flocculation of brewer's yeast. J. Inst. Brew. **77:**300–305.
33. **Masy, C. L., A. Henquinet, and M. M. Mestdagh.** 1992. Flocculation of *Saccharomyces cerevisiae*: inhibition by sugars. Can. J. Microbiol. **38:**1298–1306.
34. **Masy, C. L., M. Kockerols, and M. M. Mestdagh.** 1991. Calcium activity versus "calcium threshold" as the key factor in the induction of yeast flocculation in simulated industrial fermentations. Can. J. Microbiol. **37:**295–303.
35. **Miki, B. L. A., N. H. Poon, A. P. James, and V. L. Seligy.** 1982. Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. J. Bacteriol. **150:**878–889.
36. **Mill, P. J.** 1964. The nature of the interactions between flocculent cells in the flocculation of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **35:**61–68.
37. **Moradas-Ferreira, P., P. A. Fernandes, and M. J. Costa.** 1994. Yeast flocculation—the role of cell wall proteins. Colloids Surf. B **2:**159–164.
38. **Mozes, N., and P. G. Rouxhet.** 1987. Methods for measuring hydrophobicity of microorganisms. J. Microbiol. Methods **6:**99–112.
39. **Nagarajan, L., and S. Umesh-Kumar.** 1990. Antigenic studies on flocculating brewer's yeast *Saccharomyces cerevisiae* NCYC 227. J. Gen. Microbiol. **136:**1747–1751.
40. **National Institute of Standards and Technology.** 1989. User's guide to the NIST X-ray photoelectron spectroscopy database version 1.0. National Institute of Standards and Technology, Gaithersburg, Md.
41. **Nelson, R. D.** 1988. Dispersing powders in liquids. Elsevier Science Publishing Co. Inc., New York.
42. **Nir, S.** 1976. Van der Waals interactions between surfaces of biological interest. Prog. Surf. Sci. **8:**1–58.
43. **Ohshima, H., and T. Kondo.** 1991. On the electrophoretic mobility of biological cells. Biophys. Chem. **39:**191–198.
44. **Patel, G. B., and W. M. Ingledew.** 1975. A flocculation test for moderately flocculent brewing yeast. J. Inst. Brew. **81:**123–126.
45. **Rijnaarts, H. H. M., W. Norde, J. Lyklema, and A. J. B. Zehnder.** DLVO and steric contributions to bacterial deposition in different ionic strength environments. Submitted for publication.
46. **Rose, A. H.** 1984. Physiology of cell aggregation: flocculation by *Saccharomyces cerevisiae* as a model system, p. 323–335. *In* K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag, Berlin.
47. **Rouxhet, P. G., and M. J. Genet.** 1991. Chemical composition of the microbial cell surface by X-ray photoelectron spectroscopy, p. 173–220. *In* N. Mozes, P. S. Handley, H. J. Busscher, and P. G. Rouxhet (ed.), Microbial cell surface analysis—structural and physicochemical methods. VCH Publishers, New York.
48. **Rouxhet, P. G., N. Mozes, P. B. Dengis, Y. F. Dufrêne, P. A. Gerin, and M. J. Genet.** 1994. Application of X-ray photoelectron spectroscopy to microorganisms. Colloids Surf. B **2:**347–369.
49. **Scofield, J. H.** 1976. Hartree-Slater subshell photoionization cross-sections at 1254 and 1487 eV. J. Electron Spectrosc. Relat. Phenom. **8:**129–137.
50. **Shankar, C. S., and S. Umesh-Kumar.** 1994. A surface lectin associated with flocculation in brewing strains of *Saccharomyces cerevisiae*. Microbiology **140:**1097–1101.
51. **Sharon, N., and H. Lis.** 1993. Carbohydrates in cell recognition. Sci. Am. **268:**74–81.
52. **Skoog, D. A., and D. M. West (ed.).** 1982. Fundamentals of analytical chemistry, 4th ed. Saunders College Publishing, Philadelphia.
53. **Smit, G., M. H. Straver, B. J. J. Lugtenberg, and J. W. Kijne.** 1992. Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. Appl. Environ. Microbiol. **58:**3709–3714.
54. **Stewart, G. G.** 1975. Yeast flocculation—practical implications and experimental findings. Brew. Dig. **50:**42–62.
55. **Stratford, M.** 1992. Yeast flocculation: a new perspective. Adv. Microb. Physiol. **33:**1–71.
56. **Stratford, M.** 1994. Genetic aspects of yeast flocculation: in particular, the role of *FLO* genes in the flocculation of *Saccharomyces cerevisiae*. Colloids Surf. B **2:**151–158.
57. **Stratford, M., and S. Assinder.** 1991. Yeast flocculation: Flo1 and NewFlo phenotypes and receptor structure. Yeast **7:**559–574.
58. **Stratford, M., and A. T. Carter.** 1993. Yeast flocculation: lectin synthesis and activation. Yeast **9:**371–378.
59. **Straver, M. H., G. Smit, and J. W. Kijne.** 1994. Induced cell surface hydrophobicity influences flocculation of brewer's yeast in a flocculation assay. Colloids Surf. B **2:**173–180.
60. **Straver, M. H., G. Smit, and J. W. Kijne.** 1994. Purification and partial characterization of a flocculin from brewer's yeast. Appl. Environ. Microbiol. **60:**2754–2758.
61. **Straver, M. H., G. Smit, and J. W. Kijne.** Isolation and partial characterization of a mannose-specific agglutinin from brewer's yeast involved in flocculation. Yeast, in press.
62. **Straver, M. H., P. C. van der Aar, G. Smit, and J. W. Kijne.** 1993. Determinants of flocculence of brewer's yeast during fermentation in wort. Yeast **9:**527–532.
63. **Tybussek, R., F. Linz, K. Schügerl, N. Mozes, A. J. Léonard, and P. G. Rouxhet.** 1994. Comparison of the continuous flotation performances of *Saccharomyces cerevisiae* LBG H620 and DSM 2155 strains. Appl. Microbiol. Biotechnol. **41:**13–22.
64. **Van Haecht, J. L., C. Defosse, B. Van den Bogaert, and P. G. Rouxhet.** 1982. Surface properties of yeast cells: chemical composition by XPS and isoelectric point. Colloids Surf. **4:**343–358.
65. **van Hamersveld, E. H., M. C. M. van Loosdrecht, and K. C. A. M. Luyben.** 1994. How important is the physicochemical interaction in the flocculation of yeast cells? Colloids Surf. B **2:**165–171.
66. **Vincent, B.** 1992. Dispersion stability in mixed solvent (aqueous/organic) media. Adv. Colloid Interface Sci. **42:**279–302.
67. **Weast, R. C. (ed.).** 1985. Handbook of chemistry and physics. CRC Press Inc., Boca Raton, Fla.