Inverse Flocculation Patterns in *Saccharomyces cerevisiae* UOFS Y-2330

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ABSTRACT

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An interesting yeast strain was uncovered which showed an inverse flocculation pattern when cultivated in chemically defined and complex media. When inoculated in a defined medium with glucose as a sole carbon source, this strain immediately flocculated strongly and lost this ability before stationary phase was reached. In a complex malt medium containing glucose, this yeast strongly flocculated throughout the exponential and stationary growth phases. This inverse pattern may be ascribed to a switch in sensitivity of the yeast to flocculate in the presence of glucose as well as pH level, which may, in turn, influence the availability of calcium ions. In both media, matured cells produced protuberances or "wrinkles" upon flocculation as observed by electron and immunofluorescence microscopy. These protuberances may be involved in cell adhesion during the flocculation process.

Key words: Calcium, 3-hydroxyoxylipins, inverse flocculation, pH, *Sacch. cerevisiae,* sugars, zymolectin.

INTRODUCTION

In fermentation-based food and biotechnological processes, suspended cells are normally separated from the medium through flocculation before further processing. The processes for beer production are typical examples of this phenomenon.

In some yeasts (e.g. NewFlo phenotype), flocculation may develop only towards the early stationary phase of growth[11] through the binding of zymolectins with cell wall sugar receptors of neighbouring cell surfaces[6]. It has been demonstrated that these receptors are available throughout the growth of yeasts[10]. The presence of glucose has been found to inhibit the flocculation of yeast by binding to specific lectins[12]. The lectins are produced and inserted into the cell wall at an early stage of growth and are probably activated later during fermentation[13]. It has been reported that flocculation onset coincides with the termination of budding and glucose limitations with a concomitant increase in cell surface hydrophobicity[14]. Also, in the case of beer fermentation, flocculent yeasts undergo several morphological changes before flocculation occurs at the end of the process. These include for example, the deposition of chitin, an increase in the cell size, daughter cell retention and the occurrence of wrinkles on cell surfaces of matured cells[1]. The formation of wrinkles may resemble the hydrophobic oxylipin-associated "ghosting" phenomenon, that was reported previously in flocculent matured cells[8].

Moreover, it has also been reported that some yeasts (e.g. Flo1 phenotype) flocculate throughout the growth curve in all types of media[11]. In this case, glucose does not influence flocculation.

In this study, an inverse flocculation pattern is reported when *Saccharomyces cerevisiae* UOFS Y-2330 is grown in both complex and chemically defined glucose containing media. This phenomenon may shed additional light on yeast flocculation.

MATERIALS AND METHODS

Strain used

Saccharomyces cerevisiae UOFS Y-2330 was used throughout this study and is held at the University of the Free State (UOFS), South Africa.

Growth experiments

Saccharomyces cerevisiae UOFS Y-2330 was inoculated from YM[17] agar slants into 250 mL conical flasks containing 50 mL of glucose–YNB defined medium (12 g/L glucose, 6.7 g/L YNB) and incubated at 30°C while shaking (160 rpm) for 20 h until late exponential phase was reached. Appropriate volumes were then transferred to 500 mL side-arm conical flasks containing 100 mL of the same chemically defined medium to yield a final absorbency of 10 Klett units. The culture was incubated at 30°C while shaking (160 rpm) for 30 h. Growth was measured at regular intervals by measuring changes in optical density using a Klett Summerson colorimeter (red filter). The degree of flocculation (i.e. % Δ floc) was measured

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throughout growth by calculating the decrease in cell turbidity according to the method of Calleja and Johnson[4] by using the formula *Klett value at start of flocculation – Klett value after 5 min flocculation / Klett value at start of flocculation* while the pH was constantly monitored. This experiment was repeated when the yeast was grown for 22 h in complex media i.e. glucose-YM broth (12 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract) under similar growth conditions. All experiments were performed at least in duplicate. Materials from flocculating and non-flocculating cells grown on both complex and chemically defined medium were sampled for immediate immunofluorescence and electron microscopy analysis.

Immunofluorescence microscopy

Preparation and characterisation of antibody. Preparation and characterisation of antibodies raised against synthetic 3-HETE[2] has already been described earlier in detail[7]. Interestingly, the polyclonal antibodies recognised specifically all long-chain fatty acids carrying a hydroxyl group at C-3 position.

Microscopy. Immunofluorescence of fungal cells was performed as described[7]. In order to maintain aggregated cell floc structure, antibody, fluorescence and wash treatments were performed in 2 mL plastic tubes. Following adequate washing, the slides with fluorescing material were photographed using Kodak Gold Ultra 200 ASA film on a Zeiss Axioskop microscope equipped for epifluorescence with a 50 W high pressure mercury lamp. The stained cells were compared with appropriate controls as described[7]. All experiments were repeated at least in duplicate.

Electron microscopy

Material for electron microscopy was chemically fixed (glutardialdehyde and osmiumtetroxide)[16]. Transmission electron micrographs were taken with a Philips CM 100 (The Netherlands) TEM.

pH adjustment during growth

The strain was cultivated in a chemically defined medium as described previously. When the yeast de-flocculated completely after 30 h at pH 2.2, the pH was raised (reversed) by titration with 1M NaOH while the degree of flocculation was measured as described before. All experiments were repeated at least in duplicate.

Flocculation and dependence on ions

In a separate experiment, $CaCl_2 \cdot 2H_2O$ was added to completely de-flocculated cells at pH 2.2 (see pH adjustment during growth section) at the following final concentrations: 0 mM, 0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM and 0.10 mM. The degree of flocculation was measured each time upon addition of $CaCl_2 \cdot 2H_2O$, while the pH 2.2 was maintained constant. All experiments were performed at least in duplicate.

Glucose determination

The residual glucose content of the supernatants was quantified at regular intervals over the growth cycle using a high-performance liquid chromatograph (HPLC) instrument (Waters, USA) equipped with a refractive index detector (Waters 2414, USA), and a Sugarpack 1 column (Waters), operating at 84°C with an eluent (deionised water) flow rate of 0.5 mL/min.

Measurement of flocculation using the sugar inhibition modified Helm's test

In order to determine the effect of glucose (0.5 M) on yeast flocculation, the modified Helm's test of D'Hautcourt and Smart[5] was used on two day old cultures grown in previously described media, respectively.

Chemicals used

All chemicals and solvents used were of highest purity and obtained from major retailers.

RESULTS AND DISCUSSION

When *Saccharomyces cerevisiae* UOFS Y-2330 was grown in complex glucose containing medium, this yeast reached stationary growth phase after about 14 hours (Fig. 1). The degree of flocculation (% Δ floc) also increased sharply towards late exponential phase (i.e. after 10 h), which is in accordance with literature for NewFlo phenotypes[6]. During this period, the pH decreased from pH 5.8 to pH 4.4 suggesting that hydrogen-ion concentration may play an important role in flocculation[3,9]. Also, the glucose concentration decreased dramatically after 8 h, which probably uplifted the competitive binding of this sugar with the available zymolectins, thereby increasing flocculation. This finding was confirmed with the modified Helm's sugar inhibition test where the addition of 0.5 M glucose to strongly flocculating cells grown in complex medium yield a percentage flocculation of only 13.1 ± 6.7%. Experiments were compared with Flo1 and NewFlo phenotype controls.

When the yeast was grown in defined medium at pH 5.5 (Fig. 2), the cells reached stationary phase after about 16 h at a rather low pH 2.2. Strikingly, the yeast started to flocculate (visual inspection) immediately upon inoculation into the fresh medium, which suggests limited inhibition by glucose as a substrate[6]. This was followed by a rapid increase in flocculation up to 8 h until a pH of 2.7 was reached. The latter phenomenon may be ascribed to the increase in cell density during exponential growth phase bringing the cells in closer contact to effect more efficient flocculation[15]. Moreover, the flocculation was followed by a rapid total de-flocculation of the cells as these entered the stationary growth phase, i.e. from 8 h to 12 h, with a concomitant decrease in pH from 2.7 to 2.4 after 12 h. The pH dropped after 8 h of growth (from pH 5.5 to pH 2.7) and then steadily decreased to a value of pH 2.2 after 30 h. This drop in pH may be attributed to the low buffer capacity of the chemically defined medium. In this case only a small amount of glucose was utilized in the first 8 h. This finding was supported by the modified Helm's sugar inhibition test, in which the addition of 0.5 M glucose to strongly flocculent cells grown in defined medium yield a percentage flocculation as high as 62.1 ± 10.1%. Experiments were compared with Flo1 and NewFlo phenotype controls.

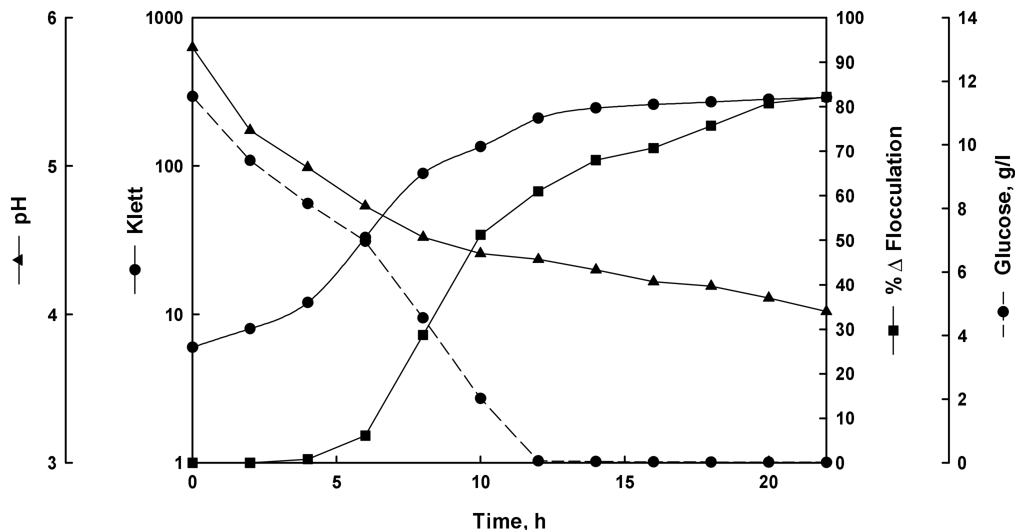

Fig. 1. Changes in growth (Klett-units), degree of flocculation, pH and glucose utilisation over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in complex medium. This experiment was repeated in duplicate and produced similar patterns.

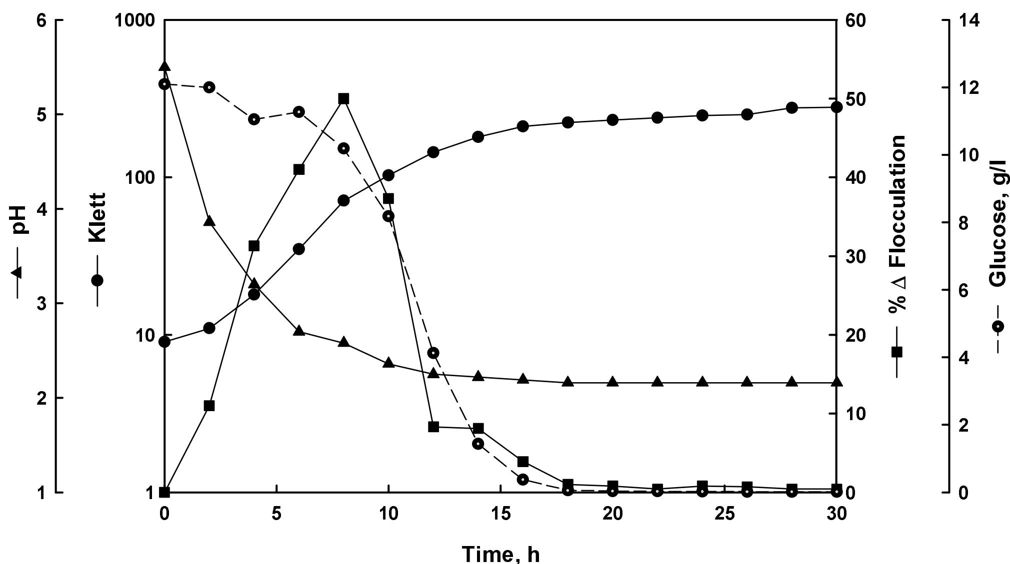

Fig. 2. Changes in growth (Klett-units), degree of flocculation, pH and glucose utilisation over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in chemically defined medium. This experiment was performed in triplicate and produced similar patterns.

To determine whether flocculation was dependent on pH, it was decided to steadily increase the pH after 30 h of growth by titrating with 1 M NaOH and follow the changes in the degree of flocculation. Surprisingly, the degree of flocculation was almost perfectly reversed, with the slopes depicting the rate of de-flocculation and flocculation staying more or less the same (Fig. 3). In this case, the flocculation was switched on over a rather narrow pH range stretching from 2.5 to 2.6. From pH 3.8 to pH 10.7, the cells flocculated maximally and again de-flocculated rapidly at a narrow pH range of 10.7 to 11.0. The flocculation/de-flocculation phenomenon may be ascribed to the sensitivity of the zymolectin–cell wall sugar bonds to pH.

To shed more light on the mechanism of de-flocculation occurring at low pH, the calcium level was raised by addition of different concentrations of $CaCl_2 \cdot 2H_2O$ to the de-flocculated culture while maintaining the pH 2.2 (Fig. 4). Calcium was chosen since it has been reported that these divalent cations play a significant role in flocculation, probably by influencing the zymolectin conformation.[6] Consequently, the degree of flocculation increased significantly with each addition of calcium ions suggesting that these may not be available for flocculation at low pH 2.2. This phenomenon is at present under investigation.

In order to determine if hydrophobic oxylipins were associated with the cell walls of flocculating cells, as previously reported by us[8], we applied specific 3-hydroxyoxylipin antibodies in an immunofluorescence microscopic investigation. We found that these 3-hydroxyoxylipins were present in both flocculent cells (i.e. after 8 h) and

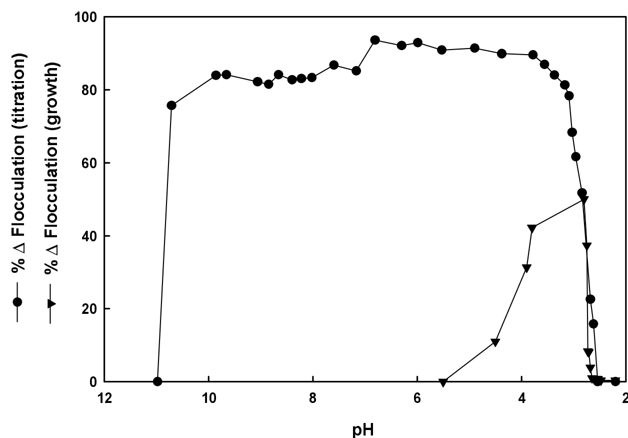

Fig. 3. Changes in the degree of flocculation over concomitant changes in pH from 5.5 to 2.2 when *Saccharomyces cerevisiae* UOFS Y-2330 was grown in chemically defined medium (read left to right on X-axis) followed by changes in the degree of flocculation when de-flocculated cells were titrated with 1 M NaOH, pH change from 2.2 to 11.0 (read right to left on X-axis). This experiment was performed in triplicate and produced similar patterns.

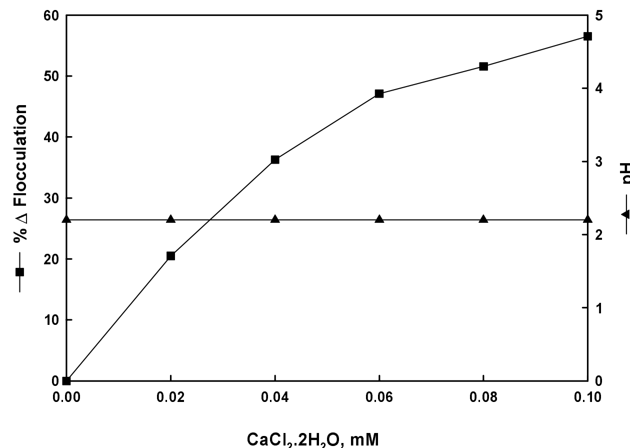

Fig. 4. Changes in the degree of flocculation in the presence of different concentrations of $CaCl_2 \cdot 2H_2O$, while the pH was kept at about 2.2. This experiment was performed in duplicate and produced similar patterns.

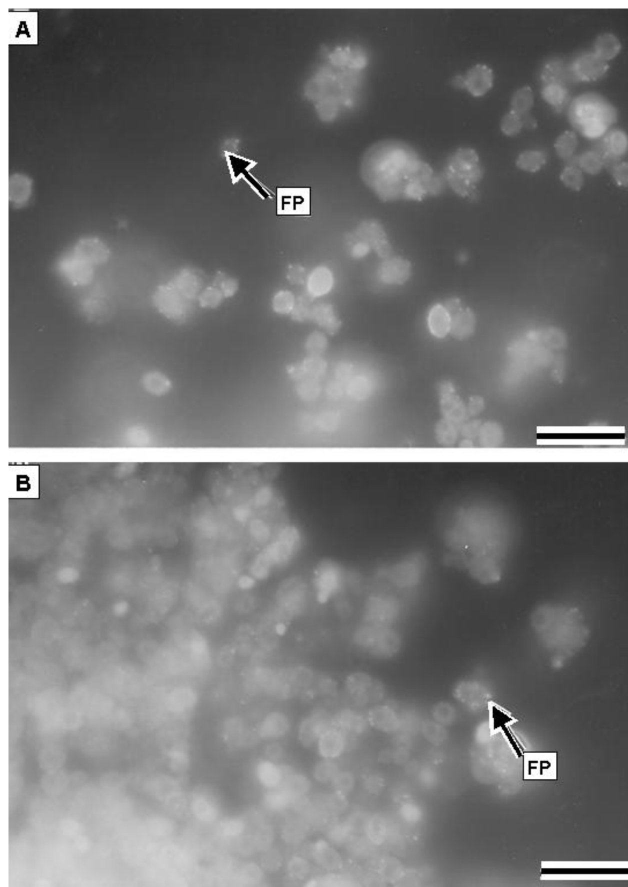

Fig. 5. Oxylipin specific immunofluorescence micrograph of flocculating cells when grown in chemically defined medium. Similar results were obtained for flocculating cells produced from complex medium. Fluorescing mature cells with oxylipin specific fluorescing protuberances on cell surfaces (FP). Scale bar represents 12 μm.

cells that lost this ability (i.e. after 20 h) when cultivated in chemically defined medium. In flocculating and deflocculating cells, the oxylipins differentiated into localised fluorescing protuberances (Fig. 5) on the cell wall. 3-Hydroxyoxylipins were also present between the flocculating cells suggesting a possible role in flocculation. Similar results were obtained when this yeast was grown in complex medium. These results are consistent with those reported previously by Kock and co-workers[8].

Consequently, these results prompted us to further investigate the fluorescing protuberances, which appeared similar to the wrinkles as described by Barker and Smart[1]. For this purpose, transmission electron microscopy (TEM) was performed on osmium tetroxide fixed yeast samples (see Materials and Methods). Again "ghost-like" osmiophilic protuberances were observed (Fig. 6) similar to that reported by Kock et al[8]. These protuberances extend randomly across any region of the cell wall (Fig. 6A, 6B) and may contain both oxylipins as well as activated lectins necessary for binding to the cell walls of adjacent cells.

CONCLUSIONS

We uncovered in this study an interesting yeast which showed an inverse flocculation pattern when cultivated in complex and chemically defined media. Seemingly, an apparent switch in sensitivity towards glucose and flocculation inhibition occurred. Can this be attributed to the presence of different sugar receptors on the cell walls of this yeast when grown in two different media? Is this yeast the possible ancestor of the Flo1 and NewFlo phenotype lines? This phenomenon remains the subject of further research.

In the chemically defined medium, a drop in pH was experienced over the growth cycle probably due to the low buffer capacity of this medium. The decrease in pH to below 2.5, probably caused the reversible breaking of the zymolectin–cell wall sugar bonds thereby affecting deflocculation. Upon increasing the pH, these bonds were

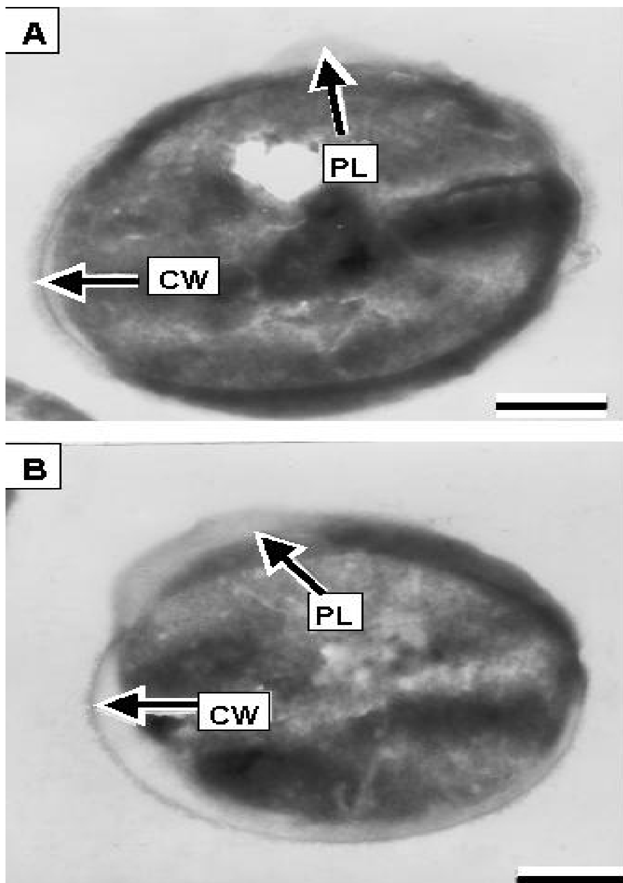

Fig. 6. TEM micrographs of *Saccharomyces cerevisiae* UOFS Y-2330. A,B: Cells showing characteristic cell walls (CW) and extensively protruding layers (PL). Scale bar represents 1 μm.

restored, thereby resulting in renewed flocculation of the cells. This may be related to the availability of divalent ions, such as calcium, that affect flocculation. Further studies on the physiological, genetic as well as colloidal aspects of flocculation using this model strain may lead to important new insights into this fascinating phenomenon.

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