



Cell surface properties and flocculation behaviour of industrial strains of *Saccharomyces cerevisiae*

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ABSTRACT

Cellular adhesion properties of yeasts depend on the characteristics of the outer layer of the cell wall. In this study, the flocculation behaviour of four industrial strains of *Saccharomyces cerevisiae* used for production of beer, champagne, wine and fuel alcohol was evaluated; their flocculation abilities being, 42.5, 14.8, 13.8 and 11.6%, respectively. The brewing yeast strain was found to be the most flocculent. Very little flocculation was observed during the lag and logarithmic phases of growth (1-15%), while during the early and late stationary phases, different strains exhibited variable flocculation patterns. Cell surface hydrophobicity [assayed using hydrophobicity microsphere assay (HMA) and microbial adhesion to solvent (MATS)] and surface charge (assayed by Alcian Blue dye retention) played important roles in dictating flocculation behaviour in different yeast strains, as did the yeast growth phase. Percentage hydrophobicity index (HI) and % hydrophobicity of the four strains followed, respectively the same order, viz Beer (66.6, 21.5) > Champagne (33, 10.5) > fuel alcohol (22.4, 7.4) > wine (20.5, 2.7). Our findings provide new insight into yeast cell surface properties and how these relate to behavioural characteristics of yeasts employed in industrial fermentations.

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INTRODUCTION

The adhesion properties of microorganisms, which involve adhering of the microbe to other cells, tissues or solid substrates, have been the focus of wide ranging scientific and biotechnological interest (Verran and Whitehead, 2005; Verstrepen and Klis, 2006; Zhao and Bai, 2009; Kjeldsen 2000). Adhesion properties are known to play important roles in governing many essential aspects of the life cycles of microorganisms including sexual reproduction (Chen et al., 2007), cellular aggregation (e.g. flocculation), biofilm formation and invasion, and/or pathogenic behaviour (Reynolds and Fink 2001; Palmer et al., 2007; Ramage et al., 2009,

Maury J et al., 2005).

Yeast cells undergo Ca^{2+} -dependent, reversible, asexual aggregation known as flocculation. In *Saccharomyces cerevisiae*, floc formation is helpful in certain industrial fermentations such as brewing, as this aids in sedimentation of yeast cells at the bottom of cylindro-conical fermenter vessels at the end of the fermentation process (Bony et al., 1997; Stratford, 1989). In some cases, co-flocculation has been reported to occur by adhesion of flocculent and non-flocculent strains of *S. cerevisiae* and lactic acid bacteria (Miki et al., 1982a).

One important factor that governs the degree of flocculation is cell surface hydrophobicity which plays major roles in microbial adhesion phenomena. For example, an increase in flocculation ability is strongly

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correlated with an increase in cell wall surface hydrophobicity (Azeredo et al., 1997). Additional factors are involved, including electron donor/acceptor properties and zeta potential (White and Walker 2011). Techniques like microbial adhesion to solvent techniques (MATS), based on cell surface affinities for a monopolar and non-polar solvent, may be used to determine the electron donor or acceptor properties of yeast cells, whilst zeta potential can be quantified by measuring the electrophoretic mobility of cells (Vichi et al., 2010).

Yeast flocculation is also governed by genetic determinants. Five flocculin genes are expressed in *S. cerevisiae*, namely, FLO1, FLO5, FLO9 FLO10 and FLO11. Flo11p exhibits a variety of roles in yeast that helps cells change and adapt during nutritional deficiencies by switching to a pseudohyphal state, enabling cells to invade substrates in response to starvation of nitrogen and glucose (Dranginis et al., 2007, Saitoh et al., 2005).

It is important to study the cell wall properties of industrial yeast strains so as to have a better understanding of the phenomenon of flocculation. This study thus investigated the cell surface properties and flocculation behaviour of different strains of *S. cerevisiae* employed in brewing, winemaking and bioethanol industries. Cell surface parameters including cell surface hydrophobicity (CSH) and cell surface charge were investigated in these yeast strains at different (lag, logarithmic, early stationary and late stationary) phases of growth and our findings are pertinent to further understanding and potential manipulation of industrial fermentations involving *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strains

In this study, four industrial strains of *Saccharomyces cerevisiae* were used. The strains were provided by Lallemand Inc. Montreal, Canada. The strains (prefixed with LYCC: "Lallemand Yeast Culture Collection") were a brewing yeast strain, a champagne strain, wine strain and a fuel alcohol strain, each of which has a specific industrial application.

Medium and culture conditions

Yeasts were routinely maintained at 4°C on Yeast Extract Peptone Glucose (YEPG) slopes containing: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar.

Pre-cultures or seed cultures were prepared in 50 ml of YEPG broth in 100 ml Erlenmeyer flasks. Cells were incubated at 25°C on an orbital shaker (Infors HT

Ecotron, Switzerland) at 170 rpm for 48 h. Culture medium was prepared by inoculating medium at starting cell density of 5×10^6 cells/ml. Samples for testing were withdrawn in triplicate at 2, 8, 24 and 48 h interval, representing the lag, logarithmic, early and late stationary growth phases, respectively (see Appendix 1 in the online supplementary material).

Fermentation progress

Ethanol production

Ethanol production by yeast during fermentation was measured using a Fermento Flash (Funke Gerber, 3572, Germany) instrument. The ethanol concentration (expressed as v/v) and productivity (expressed as v/s) gave the ethanol producing efficiency of the four industrial yeast strains and also helped in choosing the correct time-points for determining cell surface characteristics in later experiments.

Carbon dioxide production

Carbon dioxide evolution during fermentation was measured using the ANKOM^{RF} Gas Production Measurement system, USA, which provides a measure of cumulative carbon dioxide gas produced in psi (versus) time graph. Fermentation performance of the yeast strains was therefore compared directly by the level of cumulative CO₂ production.

Flocculation assay

Flocculation abilities of the yeast strains were monitored using the modified Bony method (Bony et al., 1998). At defined times, yeast cells were harvested by centrifugation (4500×g for 5 min), washed and re-suspended in de-flocculation buffer (50 mM sodium acetate, pH 4.5, 5 mM EDTA buffer) and washed twice. The cells were then washed twice in double distilled water. Subsequently, cells were re-suspended in flocculation buffer (50 mM sodium acetate, 5 mM CaCl₂, pH 4.5) while the culture OD₆₀₀ was adjusted to 2. The cells suspended in flocculation buffer containing CaCl₂ were placed in test tubes (15 mm diameter, 50 mm height) at a final OD₆₀₀ of 2. The tubes were sealed and kept on the shaking incubator at 140 rpm for 30 min. After agitation, 5 ml of the cell suspension was transferred to a new test tube and allowed to stand undisturbed for 6 min in a vertical position, after which, samples (1000 µl) were taken from just below the meniscus and the OD₆₀₀ determined spectrophotometrically (Thermo Spectronic Genesys 10UV/10 UV Scanning Spectrophotometer

10-S, USA). The percentage of flocculated cells was calculated by subtracting the fraction of cells remaining in suspension from the total cell count.

Cell surface properties

Hydrophobicity assay 1

Cell surface hydrophobicity (CSH) was determined by hydrophobic microsphere assay (HMA) (Hazen and Hazen, 1992). Cells were harvested during the stationary growth phase and washed thrice with cold, sterile double distilled water. A final concentration of 4×10^6 cells were transferred to 2 ml cold HMA buffer (0.05 M NaPO₄, pH 7.2). Meanwhile, in a separate glass tube containing 6 μ l of bead suspension was added to 2 ml of cold HMA buffer, such that the final concentration of beads was 9.02×10^8 spheres/ml. In a polypropylene tube at room temperature, 100 μ l each of yeast cell and bead suspension was added and the tube left undisturbed for 2 min at 4°C. After incubation the tube was vortexed for 30 s and 20-30 μ l of the cell-bead suspension loaded on a haemocytometer. For statistical analysis, 100 cells were counted that had three or more than three beads attached to it. Determining the percentage of cells with 3 or more attached microspheres gave the Hydrophobicity Index.

Hydrophobicity assay 2

Hydrophobicity assay was also performed by microbial adhesion to hydrocarbons (MATS test; Bellon-Fontaine et al., 1996; Mortensen et al., 2005). This assay determines the hydrophobic nature and the Lewis acid-base (electron donor/acceptor) characteristics of yeast cell surfaces. In the MATS test, the affinity of microbial cells was compared to a pair of monopolar/apolar solvents of similar Lifshitz-van der Waals surface tension. The pair of solvents used was:

- Apolar solvent - hexadecane and the acidic monopolar solvent - chloroform,
- Apolar solvent - decane and the strongly basic monopolar solvent - ethyl acetate.

Since these solvents have different surface tensions, the affinity of yeast cells to the hexadecane-chloroform and decane-ethylacetate would reflect the electron donor and the electron acceptor property of the yeast cell surface. The hydrophobic nature was judged by the affinity to apolar solvents, specifically hexadecane.

The cell surface hydrophobicity was measured using the MATS test of Bellon-Fontaine et al., 1996 and Mortensen et al., 2005. Yeast cells from the stationary

growth phase were washed with 10 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer, 0.9% NaCl, pH 5.0 buffer and re-suspended to an optical density (OD) of 0.8 at 400 nm (A_0). Hexadecane + chloroform and decane + ethyl acetate constituted two electron donating and accepting pairs. A 0.4 ml of each of the solvent was added to four separate test tubes, each containing 2.4 ml cell suspension and vortex-mixed for 1 min. The mixture was allowed to stand for 15 min to ensure complete separation of the two phases. Once the distinct phases appeared, 1 ml of the sample was removed very carefully without disturbing the aqueous phase and OD measured at 400 nm (A). The percentage of bound cells was subsequently calculated by:

$$\% \text{ affinity} = 1 - \left(\frac{A}{A_0} \right) \times 100$$

Where;

A_0 = OD at 400 nm of the cell suspension before mixing,
A = absorbance after mixing.

Cell surface charge

Yeast cells were cultivated and centrifuged at 4500xg for 10 min and re-suspended in 0.02 M sodium acetate buffer (pH 4) at 5×10^7 cells/ml. The yeast cell suspension (1 ml) was then re-suspended in 1.8 ml of Alcian blue dye buffer solution (50 mg/L). Alcian blue is a phthalocyanine complex that has four charged sites in the molecule and is adsorbed by the negatively charged yeast cell surfaces. The suspension was incubated for 30 min at 25°C on the orbital shaker at 75 rpm, centrifuged and the free dye remaining in the supernatant was determined by OD at 615 nm (see Appendix 2 in the online supplementary material). The concentration of Alcian blue was determined by reference to an Alcian blue standard curve prepared from original dye/buffer solution. Thus Alcian blue retention was expressed as mg of Alcian blue per 5×10^7 cells/ml.

RESULTS AND DISCUSSION

The flocculation behaviour and cell surface properties of four different industrial strains of *S. cerevisiae* were studied. The aim of this study was to obtain greater insight into the fermentation performance of selected industrial strains and how their flocculation behaviour is affected by changes in cell surface properties which in turn are affected by changes in nutrient availability and physico-chemical conditions.

Cell-cell adhesion

The fermentation performance of the industrial yeasts

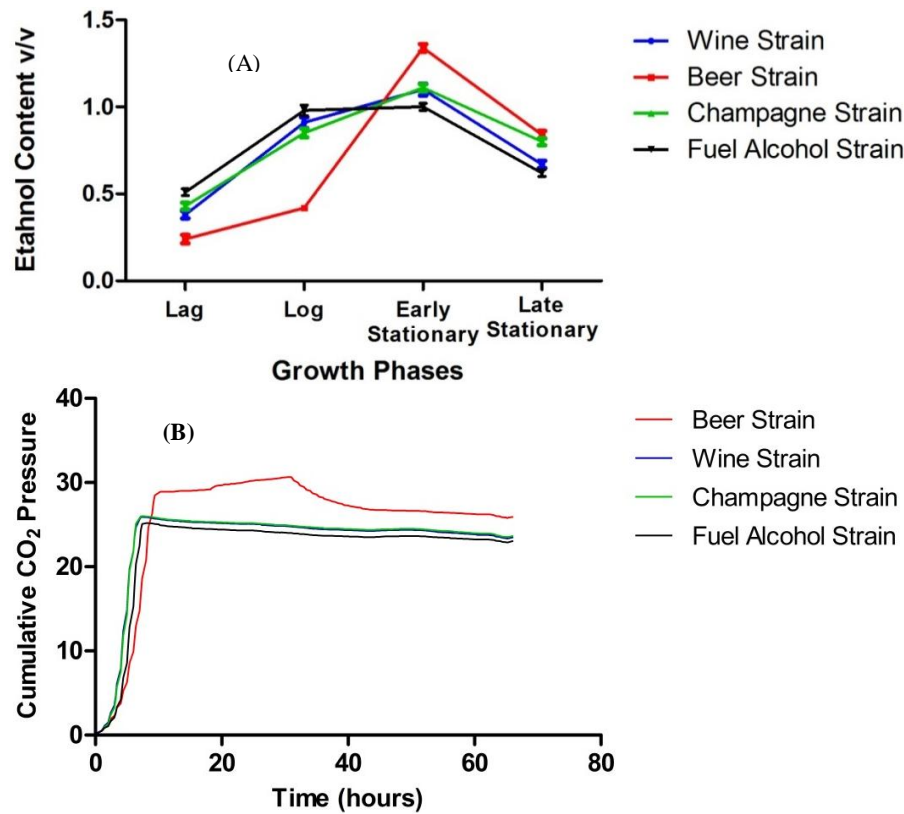


Figure 1. A: Ethanol production by four different strains of *Saccharomyces cerevisiae* during lag, log, early and late stationary phases of growth; **B:** Carbon dioxide production by four different strains of *S. cerevisiae* depicted in terms of cumulative CO₂ pressure measured in psi.

was initially evaluated in small-scale fermenters. The brewing yeast strain produced the highest amount of ethanol (v/v) followed by the champagne, wine and fuel alcohol strains. As shown in Figure 1, the CO₂ production kinetics indicated the transition of the yeast cells from one growth phase to another. For yeast kinetics during the four phases of growth for different strains (see Appendix 1 in the online supplementary material).

The activation and thus the expression of various flocculins resulted in the formation of macroscopic biofilms ranging in diameter from around 100 micrometres to several millimetres. Flocculation tests were carried out during all the phases of growth for the four industrial strains and it was found that all yeasts were flocculent either during the early or late stationary phase. The strains exhibited significant ($p < 0.05$) differences in their flocculation abilities during all the phases of growth (Figure 2). The brewing yeast strain was found to be highly flocculent throughout the fermentation including the late stationary phase (42.5%) when the flocculence character of the other strains diminished. The champagne yeast strain may also be

categorized as highly flocculent as it showed flocculence of about 28% during early stationary phase. The main reason for flocculation predominating during early and late stationary phase may be due to progressive crenellation and wrinkling of the cell wall during aging. This increases the potential surface area of contact compared with that of smooth younger cells and therefore promotes cell-cell adhesion (Barker and Smart, 1996).

Cell surface hydrophobicity

Cell surface hydrophobicity was determined by two different and independent techniques, HMA and MATS. While the first method, HMA, semi quantitatively explores the hydrophobic nature of the yeast cell surface, the MATS test in addition provides information on the electron donating and electron accepting nature of the yeast cell wall.

HMA employs latex microspheres with a diameter of $0.845 \pm 0.001 \mu\text{m}$. About 100 cells for each of the four strains were counted and percentage hydrophobicity was

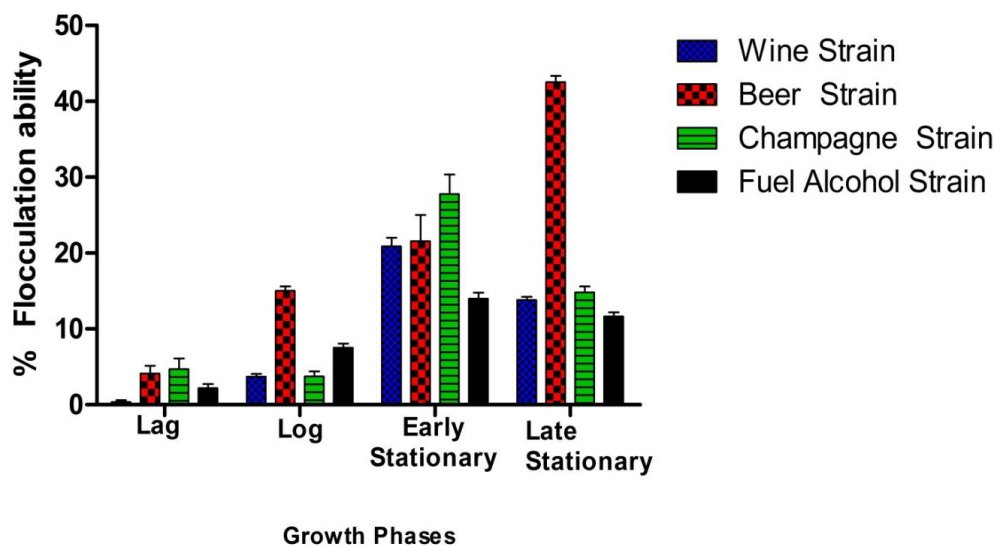


Figure 2. Percentage flocculation ability of different strains of *Saccharomyces cerevisiae* during different phases of growth. Champagne strain (Green) showed high flocculation during early stationary phase, while comparatively less flocculation was seen during late stationary phase. The same held true for wine strain (blue) and fuel alcohol strain (black). In contrast, beer strain (red) became more flocculent during late stationary phase. Significant difference ($p \leq 0.05$) was observed between the flocculation ability of all the strains during different phases of growth curve.

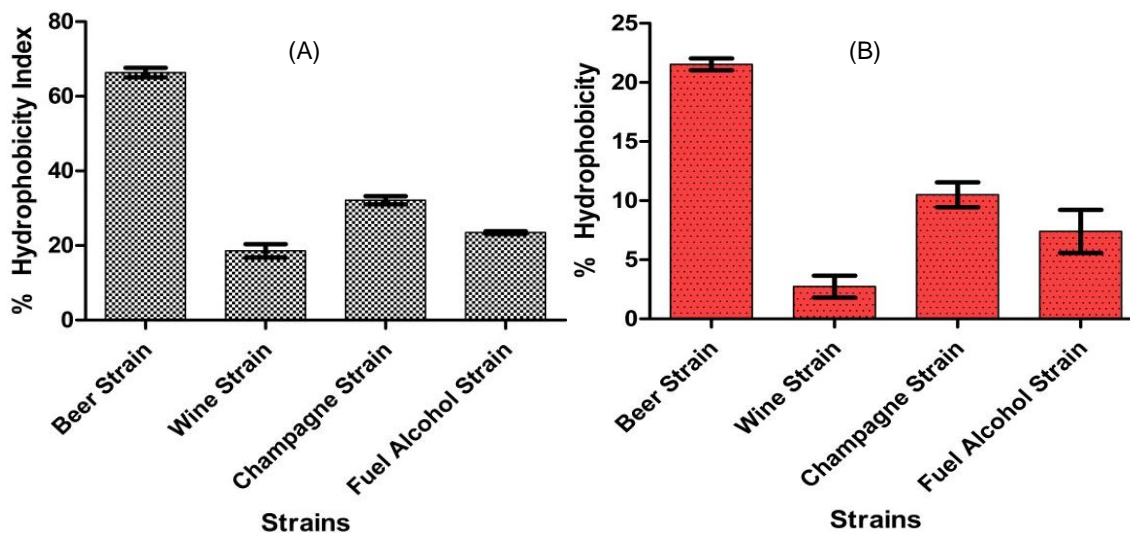


Figure 3. A: Percentage Hydrophobicity Index (HI) of different strains of *Saccharomyces cerevisiae* by HMA test. Significant differences ($p \leq 0.01$) were observed amongst the strains. Beer strain exhibited highest %HI. Due to high CSH, more latex beads were observed to be attached to the cell surface ; **B:** Percent Hydrophobicity of different strains of *S. cerevisiae* by MATS test. Significant differences ($p \leq 0.01$) were observed between different strains. HMA and MATS tests showed similar pattern of results (compare Figures 3A and 3B).

calculated for those cells having ≥ 3 beads attached. The reason for using $0.845 \pm 0.001 \mu\text{m}$ microspheres was the ease of homogeneous suspensions as opposed to smaller microspheres ($< 0.845 \mu\text{m}$ dia). Figure 3 shows

that there were significant ($p < 0.05$) differences in the hydrophobicity indices of the 4 industrial yeasts, with the brewing strain exhibiting the highest hydrophobicity index (65%). These observations were validated using MATS

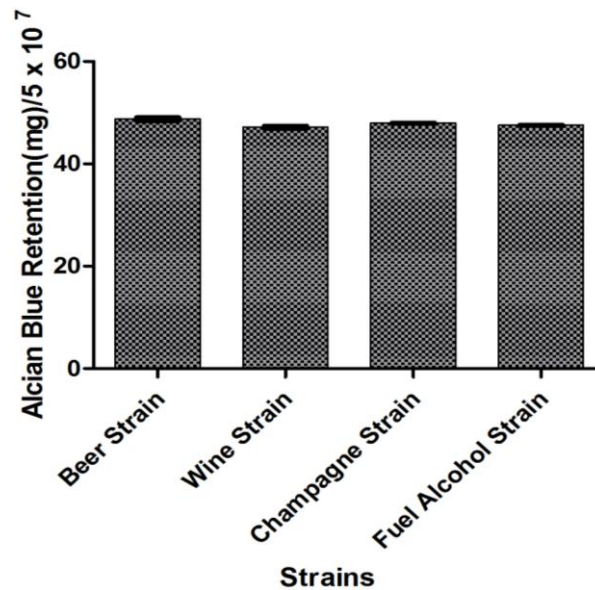


Figure 4. Alcian Blue retention ability of different strains of *Saccharomyces cerevisiae*. Significant differences ($p \leq 0.05$) were observed for Alcian blue retention for beer strain compared to Champagne, wine and fuel alcohol strains.

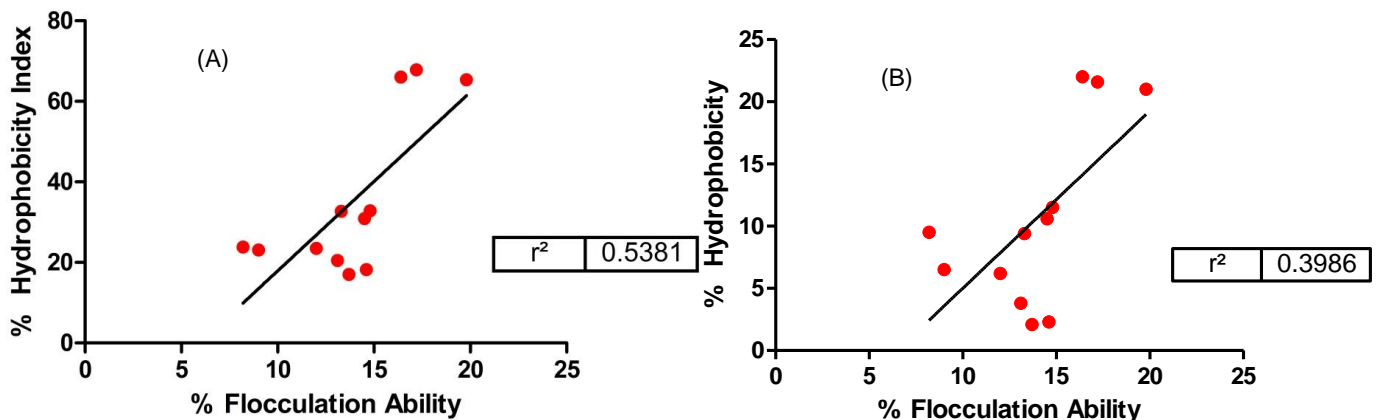


Figure 5. A: Correlation between hydrophobicity index and flocculation ability of *Saccharomyces cerevisiae*; **B:** Correlation between percentage hydrophobicity and flocculation ability of *S. cerevisiae*.

test, indicating that the brewing strain was the most hydrophobic (21.5%) toward hexadecane (apolar solvent), followed by the champagne strain (10.5%), fuel alcohol strain (7.4%) and wine strain (2.7%). In addition, the strains also showed high electron donor capacity (percentage affinity to chloroform minus percentage affinity to hexadecane). The brewing strain showed highest electron donating capacity (68.8%) while champagne strain showed the lowest (44.1%). Our studies show a direct correlation between increased CSH and initiation of flocculence during fermentation (Figures 5A and B). A high level of CSH facilitates higher cell-cell

contact in an aqueous medium resulting in more specific lectin-carbohydrate interaction (Jin and Speers, 1998).

Cell surface charge

Many forces are involved in cell-cell interactions that determine the extent of attachment/adhesion of cells and also the extent of floc formation. After testing all the strains using the Alcian blue retention test, it was observed that all the strains were negatively charged during their late stationary phase. Figure 4 shows that the

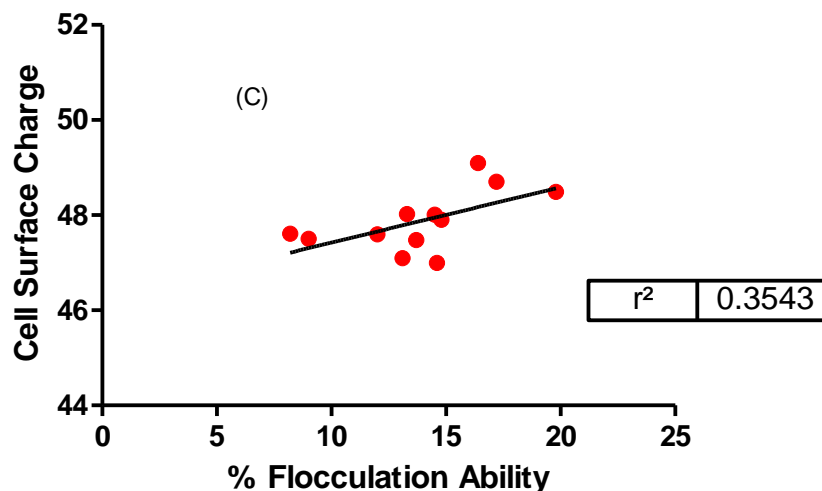


Figure 5. C: Correlation between cell surface charge and flocculation ability of *Saccharomyces cerevisiae*. Significant difference ($p \leq 0.05$) in the above figures indicates that to some extent CSH and CSC are directly correlated with flocculation ability. These observations were made for cells in their late stationary phase.

brewing yeast strain was significantly ($p < 0.05$) highly negatively charged compared to the Champagne, wine and fuel alcohol strains. The presence of carboxylic and phosphodiester groups are responsible for the negative character of yeast (Jin and Speers, 1998). Cells in aqueous environments are subjected to many forces that influence cell-cell and cell-water interactions. Due to highly negative charges on the cell surface, electrostatic forces of repulsion keep cells about 10 nm from one another (Dengis et al., 1995). Such forces act as a barrier to flocculation. When yeast cells age, the zymolectin biosynthesis is initiated and the CSH increases, thus hydrophobic forces come into play. These forces consist of long range van der Waals attractions and the short range interactions, particularly hydrogen bonding (van Oss and Giesse, 1995).

The present study is limited in the sense that it provides information only on how the flocculation behaviour of the studied industrial yeast strains could be controlled by cell surface properties, such as hydrophobicity and surface charge. There are several other parameters, such as presence or absence of dominant flocculation genes (FLO genes), mannan and glucan distribution levels, presence of lectin like receptors and environmental parameters of the fermenter that also play an important role in governing the flocculation behaviour of these strains. Nevertheless, our present findings indicate that cell surface properties play important roles in determining the extent of flocculation in industrial strains of *S. cerevisiae* (Figure 5). This phenomenon is important in industrial fermentation processes and deeper understanding of it may lead to practical approaches (e.g. manipulation of media or physical conditions) to alter

CSH and CSC, thereby providing some degree of control over the timing and extent of yeast flocculation.

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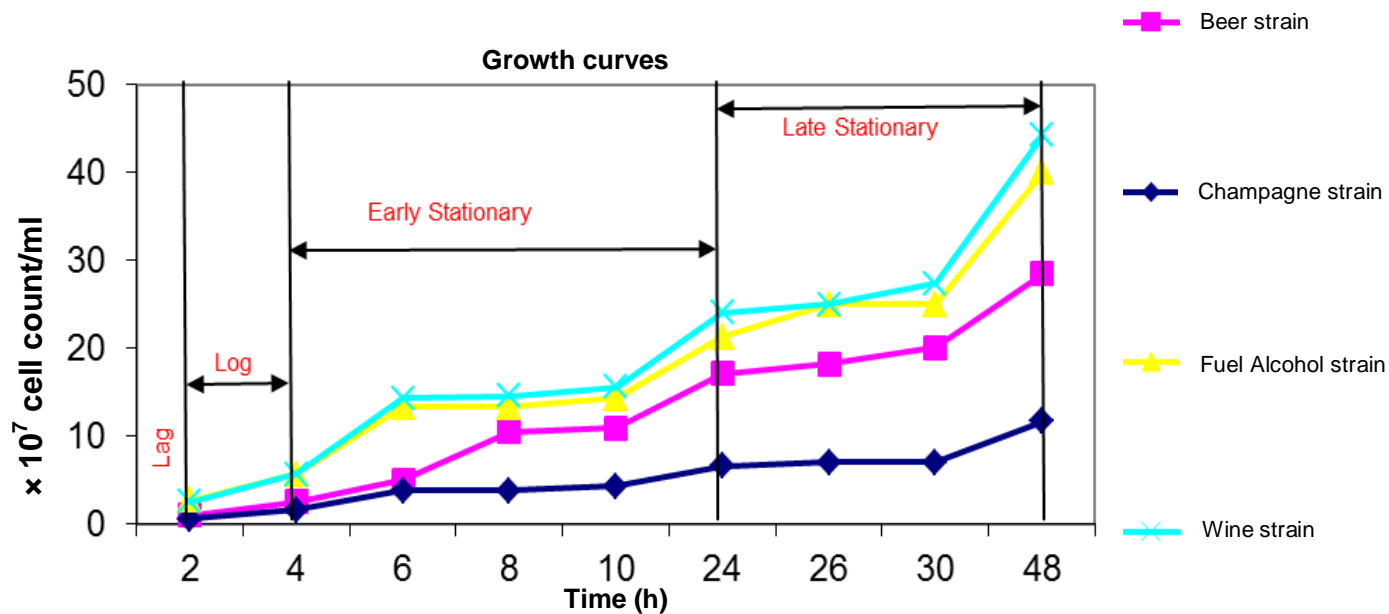
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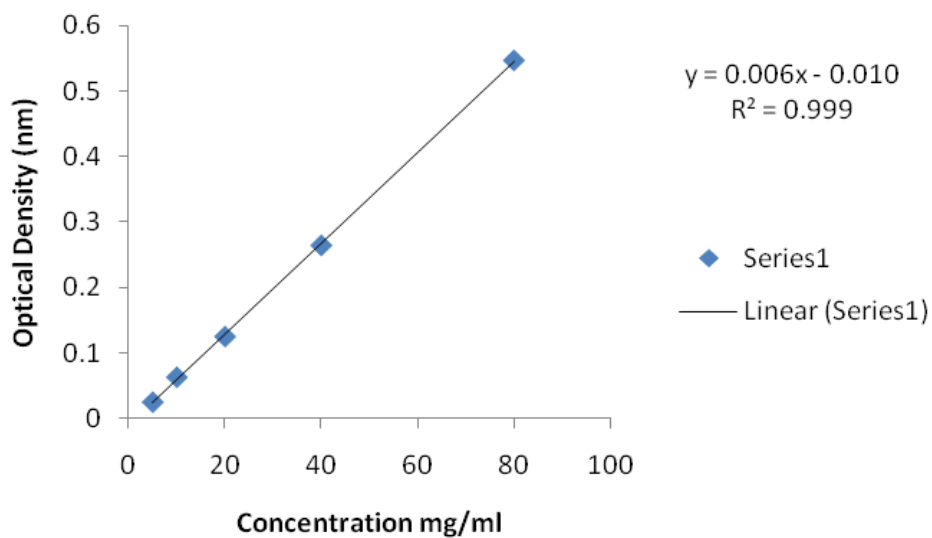
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APPENDIX



Appendix 1. Growth curve showing time-points of different growth phases. From this data, time points of 2 h (lag), 8 h (logarithm), 24 h (early stationary) and 48 h (late stationary phase) were selected.



Appendix 2. Calibration curve obtained by plotting the optical density (OD) and concentration (mg/ml) Alcian blue dye stock.