Flocculation in ale brewing strains of *Saccharomyces cerevisiae*: re-evaluation of the role of cell surface charge and hydrophobicity

Ann Van Holle · Manuela D. Machado · Eduardo V. Soares

Abstract Flocculation is an eco-friendly process of cell separation, which has been traditionally exploited by the brewing industry. Cell surface charge (CSC), cell surface hydrophobicity (CSH) and the presence of active flocculins, during the growth of two (NCYC 1195 and NCYC 1214) ale brewing flocculent strains, belonging to the NewFlo phenotype, were examined. Ale strains, in exponential phase of growth, were not flocculent and did not present active flocculent lectins on the cell surface; in contrast, the same strains, in stationary phase of growth, were highly flocculent (>98%) and presented a hydrophobicity of approximately three to seven times higher than in exponential phase. No relationship between growth phase, flocculation and CSC was observed. For comparative purposes, a constitutively flocculent strain (S646-1B) and its isogenic non-flocculent strain (S646-8D) were also used. The treatment of ale brewing and S646-1B strains with pronase E originated a loss of flocculation and a strong reduction of CSH; S646-1B pronase E-treated cells displayed a similar CSH as the non-treated S646-8D cells. The treatment of the S646-8D strain with protease did not reduce CSH. In conclusion, the increase of CSH observed at the onset of flocculation of ale strains is a consequence of the presence of flocculins on the yeast cell surface and not the cause of yeast flocculation. CSH and CSC play a minor role in the auto-aggregation of the ale strains since the degree of flocculation is defined, primarily, by the presence of active flocculins on the yeast cell wall.

Keywords Brewing yeast · Cell–cell adhesion · Cell surface charge · Flocculation · Flo proteins · Hydrophobicity

Introduction

Flocculation can be defined as a nonsexual, reversible and multivalent process of aggregation of yeast cells into multicellular masses, called flocs, with the subsequent rapid sedimentation from the medium in which they are suspended (Stewart 2009). At industrial level, the auto-aggregation property of flocculent yeasts facilitates enormously the cell separation process. By this reason, flocculation can be seen as an effective, easy, fast and off-cost process of cell separation. Although yeast flocculation characteristics have been mainly employed in the brewing industry, this property can also be advantageously exploited in the making of other alcoholic beverages (wine, sparkling wine and cachaça), production of renewal fuels (bio-ethanol), in modern biotechnology (in the production of heterologous proteins) or used in environmental applications (bioremediation of heavy metals), as it was recently reviewed (Bauer et al. 2010; Soares 2011).

According to the lectin-like theory, formally proposed by Miki et al. (1982), flocculent cells display proteins in the cell wall, called “lectins” [or adhesins, flocculins (Verstrepen and

Klis 2006) and zymolectins (Speers et al. 1998)], that recognize and interact with carbohydrate residues of the α-mannans (receptors) of the neighbouring cells. Two main flocculation phenotypes were described in *Saccharomyces cerevisiae* cells: Flo1 and NewFlo phenotypes. Flocculation of yeast cells belonging to Flo1 phenotype is specifically inhibited by mannose and derivatives; on the other hand, NewFlo phenotype contains the majority of brewery ale strains, which flocculation is reversibly inhibited by mannose, maltose, glucose and sucrose but not by galactose (Stratford and Assinder 1991). Flo1 and NewFlo strains also display different sensitiveness to culture conditions, such as temperature (Soares et al. 1994), pH (Soares et al. 1994; Stratford 1996; Soares and Seynaeve 2000) and nutrients availability (Soares and Mota 1996).

The general architecture of the member proteins of the flocculin family, which in the case of *S. cerevisiae* includes the *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* genes (Dranginis et al. 2007) comprise three domains: an N-terminal, a central and a C-terminal (A, B and C domains, respectively). The C-domain carries a glycosylphosphatidylinositol anchoring site to covalently bind the Flo1p to β-glucans (de Groot et al. 2003). The middle part of Flo1p (domain B) contains many repeated sequences and a large number of serine and threonine residues, which O-glycosylation enables the Flo1 protein to adopt an extended conformation (Watari et al. 1994). The N-terminal region of Flo1 protein (domain A) contains the sugar recognition domain, which is important for flocculation definition of Flo1 and NewFlo phenotypes (Kobayashi et al. 1998). A detailed analysis of the adhesion domain (domain A) of the flocculin Flo5 revealed a bipartite organization constituted by a large β-sandwich domain (which is related with the PA14 domain of the anthrax-protective antigen); this domain takes an insertion, the Flo5 subdomain, which is composed by five short β-strands, stabilized by two disulphide bridges (Rigden et al. 2004; Veelders et al. 2010). It was also shown that calcium is directly involved in carbohydrate binding and not indirectly in the stabilization of the rod-like B domain (Veelders et al. 2010).

Yeast flocculation is a cell surface property. Thus, it is not surprising that a considerable body of work has been dedicated to the impact of surface properties, namely cell surface charge (CSC) and cell surface hydrophobicity (CSH), on yeast flocculation. At physiological pH values, the yeast cell wall has a net negative charge (Jayatissa and Rose 1976; Beavan et al. 1979) mainly due to the phosphodiester groups and, in a minor extent, due to the participation of carboxylic groups (Amory and Rouxhet 1988; Mestdagh et al. 1990); as a consequence of the repulsion of the charges of the same sign, yeast cells remain dispersed, in suspension, at a distance of the order of 10 nm from each other (Dengis et al. 1995). It is conceivable that the reduction of global cell charge (non-specific electrostatic repulsion) should facilitate yeast cells approach; in this case, the macromolecules (lectins) present on yeast cell surface can easily overcome the negative barrier and the junction between lectins and carbohydrates (specific cell adhesion mechanism) is easier established. However, no clear relationship between yeast surface charge and the onset of flocculation was found (Amory et al. 1988; Dengis et al. 1995; Dengis and Rouxhet 1997).

Although an extensive research about the role of CSH on yeast flocculation has been undertaken, a controversy still exists. Some authors described no significant differences in hydrophobicity between flocculent and non-flocculent cells at stationary and exponential phases of growth, respectively (Dengis et al. 1995; Dengis and Rouxhet 1997). In contrast, there are several pieces of evidence that suggest a positive correlation between the increase of CSH and the onset of flocculation in brewing yeasts (Smit et al. 1992; Straver et al. 1993; Rhymes and Smart 2000; Jin et al. 2001; Speers et al. 2006). Nevertheless, the exact component of yeast cell wall responsible for the increase of hydrophobicity remains undetermined.

In the present work, the role of surface properties (CSC, CSH and the presence of active flocculins) on the flocculation of ale brewing yeast strains are re-evaluated using an integrated approach. Additionally, the role of CSH in the mechanism of cell–cell interaction is discussed.

Materials and methods

Strains, media and culture conditions

In this work, the flocculent ale brewing strains of *S. cerevisiae* National Collection of Yeast Culture (NCYC) 1195 and 1214 were used. The original strains were obtained from the NCYC, UK and are characterized as NewFlo phenotype (Stratford and Assinder 1991). In addition, two isogenic strains of *S. cerevisiae* S646-8D (*MAT*a/α, *HO/HO*, *trp1/trp1*) and S646-1B (*MAT*a/α, *HO/HO*, *FLO1/FLO1*, *ade1/ade1*) were also used. The strain S646-1B is a flocculent strain, belonging to Flo1 phenotype; the strain S646-8D is a non-flocculent strain (Stratford and Assinder 1991). The strains S646-8D and S646-1B were a kind gift from Dr. Malcolm Stratford. The strains were routinely maintained at 4°C on yeast extract peptone dextrose (YEPD) agar slants (10 g/l yeast extract, Difco-BD, USA), 20 g/l peptone (Difco-BD), 20 g/l glucose (Merck, Darmstadt, Germany) and 20 g/l agar (Merck).

The pre-cultures were prepared in 40 ml YEPD broth in 100 ml Erlenmeyer flasks. The cells were incubated at 25°C on an orbital shaker (Sanyo Gallenkamp IOC 400, West

Sussex, UK), at 150 rpm. After 48 h of growth, flocculent cells were harvested by centrifugation (2,000×g, 5 min) and washed twice with 30 mmol/l of ethylenediaminetetraacetic acid (EDTA) solution to ensure floc dispersion. Finally, cells were washed and suspended in deionised water. Cultures were prepared by inoculating 800 ml of YEPD broth, in 2-L Erlenmeyer flasks, with ~1×10^6 cells/ml from pre-cultures. Cells were grown at 25°C in an orbital shaker at 150 rpm. After growth, cells were harvested by centrifugation washed twice with 30 mmol/l EDTA solution (Merck), twice with deionised water and finally resuspended in deionised water.

Treatment with pronase E

Cells were harvested, washed and resuspended at 2×10^9 cells/ml in pre-warmed (at 30°C) phosphate buffer (50 mmol/l, at pH 7.5) with 20 mmol/l EDTA. Subsequently, cells were treated with 0.2 mg/ml of pronase E (protease from *Streptomyces griseus* type XIV, Sigma-Aldrich, St. Louis, MO, USA). After 60 min of cells incubation at 30°C, iced deionised water was added in order to stop the reaction. Then, cells were harvested by centrifugation (2,000×g, 5 min, 4°C), washed twice and resuspended in deionised water.

Evaluation of cell concentration

Cell concentration was monitored spectrophotometrically at 600 nm or by direct cell counting with a counting chamber, after appropriate dilution in 30 mmol/1 of EDTA solution to prevent cell aggregation. Calibration curves (number of cells or dry weight versus absorbance) were previously constructed for each strain.

Measurement of flocculation

Cells were removed by centrifugation, washed as described above and resuspended in deionised water at a final concentration ~2×10^9 cells/ml. Flocculation was monitored under standard conditions (50 mmol/l of citrate buffer, pH 4.0, containing 8 mmol/l of $CaCl_2$), using the microflocculation technique previously described (Soares and Vroman 2003).

Evaluation of cell surface charge

Cell surface charge was determined using the alcian blue retention assay (Rhymes and Smart 2001). Yeast cells were suspended in acetate buffer (20 mmol/l, pH 4.0, with 1 mmol/l Ca^{2+}), at 5×10^6 cells/ml, containing 15 μg/ml alcian blue 8 GX (Sigma-Aldrich, St. Louis, MO, USA). Then, cell suspensions were incubated for 5 min, at 25°C, on an orbital shaker (100 rpm), followed by centrifugation (500×*g*, 3 min). The amount of alcian blue 8 GX bound to yeast cells was calculated from the difference between the added dye and the amount of dye remained in the supernatant, measured by the absorbance at 607 nm. A calibration curve (dye concentration versus absorbance) was previously constructed.

Determination of cell surface hydrophobicity

Cell surface hydrophobicity was determined using microbial adhesion to hydrocarbon (MATH) test (Rosenberg 2006). Thus, cells were suspended in phosphate, urea, magnesium (PUM) buffer (pH 7.1), at 5×10^6 cells/ml. PUM buffer consisted on 19.7 g/l K_2HPO_4, 7.26 g/l KH_2PO_4, 0.2 g/l $MgSO_4\cdot7H_2O$ and 1.8 g/l urea. Cell suspensions (2.0 ml) were placed in standard glass test tubes, acid-washed, and *n*-hexadecane (0.4 ml) was added at each tube. Samples were vortex-mixed for 2 min and left to stand for 15 min, to ensure the complete separation of the two phases. Aqueous phase was carefully removed and the absorbance determined at 600 nm. The hydrophobicity (percent) was calculated according to the equation:

$$\text{Hydrophobicity}(\%) = (1 - A/A_0) \times 100 \quad (1)$$

where A_0 is the initial absorbance of the cell suspension and A the absorbance of the aqueous phase after mixing.

Detection of active flocculins

Active flocculation lectins on the yeast cell surface were detected using the fluorescent probe Avidin-fluorescein isothiocyanate (Avidin-FITC), as described before (Patelakis et al. 1998; Jin et al. 2001), with minor modifications. Yeast cells were washed, as described above, and resuspended at 5×10^6 cells/ml, in acetate buffer (20 mmol/l, pH 4.0), with 8 mmol/l Ca^{2+}. Avidin-FITC (Sigma-Aldrich, Steinheim, Germany) was added to a final concentration of 10 μg/ml. Yeast cells were incubated with the probe, at 25°C, for 60 min, in the dark. As a control, yeast cells were incubated with Ca^{2+} (8 mmol/l), the fluorescent probe and 1 mol/l glucose or mannose; in addition, yeast cells were also incubated with the probe, in acetate buffer, without Ca^{2+} or with 100 mmol/l EDTA.

Cells were examined using a Leica DLMB epifluorescence microscope (Leica Microsystems, Wetzlar GmbH, Germany) equipped with a HBO-100 mercury lamp and a filter set I3 (excitation filter BP 450-490, dichromatic mirror 510 and suppression filter LP 515) from Leica. Images were acquired with a Leica DC 300F camera (Leica Microsystems, Heerbrugg, Switzerland) and processed using Leica IM 50-Image manager software.

Reproducibility of the results

All experiments were repeated, independently, for each strain, two to four times. In each experiment, growth was evaluated in triplicate, flocculation and cell surface charge in quadruplicate and cell surface hydrophobicity sixfold. The data presented are the mean±standard deviation, with 95% confidence values.

Results

Two brewing flocculent strains (NCYC 1195 and 1214), belonging to the NewFlo phenotype, were used. The strains loss the flocculation in the exponential phase of growth; in the stationary phase (72 h), both strains were highly flocculent, i.e. >98% of the cells were flocculated (Fig. 1a, b). For comparative purposes, a laboratory strain belonging to the Flo1 phenotype (S646-1B) was also used; the strain S646-1B is highly flocculent (>97% of flocculated cells) during all growth phases (Fig. 1a, b).

The visualization of the presence of the active flocculation lectins on yeast cell surface was carried out using the fluorescent probe Avidin-FITC. The protein Avidin (labelled with fluorescein isothiocyanate) contains a mannose side chain, which functions as a binding site to flocculins. Active flocculation lectins are able to bind Avidin-FITC molecules, which results in the labelling of the yeast cells (Patelakis et al. 1998; Gouveia and Soares 2004; Speers et al. 2006). Brewing ale flocculent cells, in stationary phase of growth, and in the presence of Ca^{2+}, were able to fix Avidin-FITC probe, as it can be seen in Fig. 2a, c. In contrast, non-flocculent cells, in exponential phase of growth, were not labelled with Avidin-FITC probe (Fig. 2e). These results strongly suggest that flocculent lectins are not present (at least in an active conformation) during the exponential phase of growth of ale brewing yeast strains; similar results were found for the strain NCYC 1214 (data not shown). As control, cells (in stationary phase of growth) from the ale flocculent strains were incubated with the fluorescent probe in the absence of Ca^{2+} or with 100 mmol/l EDTA; in addition, cells were also incubated with Avidin-FITC, Ca^{2+} and 1 mol/l glucose or mannose. In all controls, cells were not labelled (data not shown). Without Ca^{2+} addition or in the presence of EDTA, the flocculins were not able to bind Avidin-FITC. In the presence of sugars, flocculent cells were not labelled because the presence of glucose or mannose competitively inhibited the flocculins to fix the fluorescent probe. These results are in agreement with the NewFlo phenotype described for these strains (Stratford and Assinder 1991).

The cell surface properties, namely cell surface charge (CSC) and cell surface hydrophobicity (CSH), were evaluated throughout the growth of the three strains. CSC was evaluated by the alcian blue assay. Alcian blue 8 GX is a dye with four positive charge groups enabling its

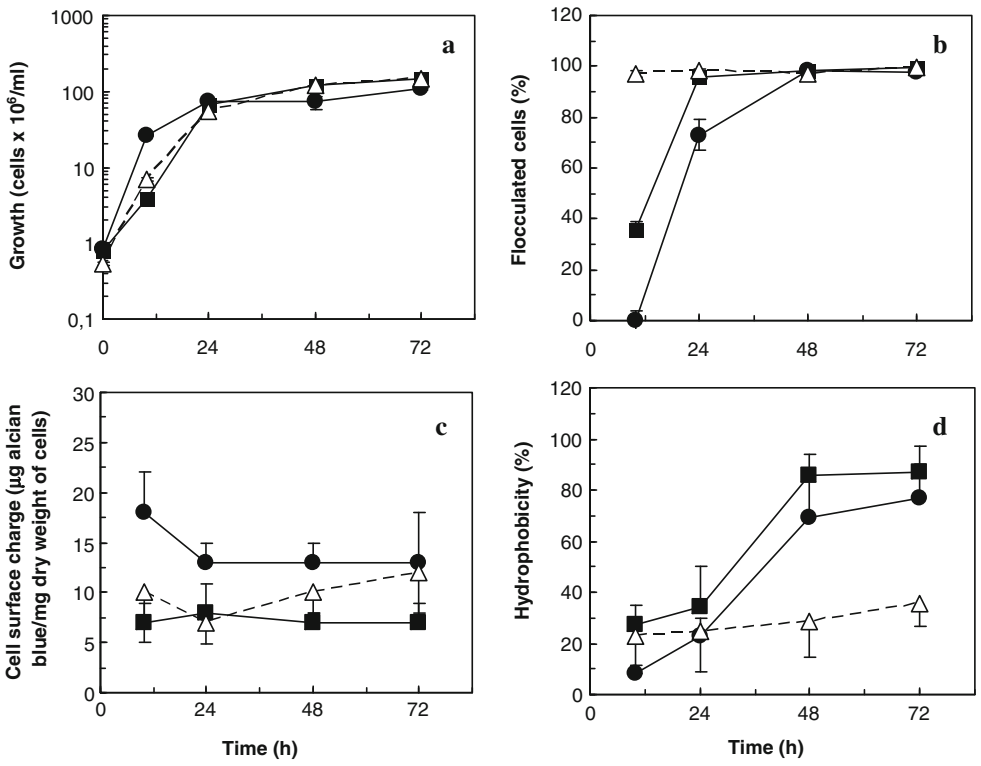

Fig. 1 Evolution of flocculation and cell surface properties during the growth of *S. cerevisiae* strains. The flocculent yeast strains are: (*filled circle*) NCYC 1195, (*filled square*) NCYC 1214 and (*empty triangle*) S646-1B. **a** Growth, **b** flocculation, **c** cell surface charge and (**d**) cell surface hydrophobicity. Mean and standard deviation are presented (with 95% confidence); where no *error bars* are shown, they are within the points

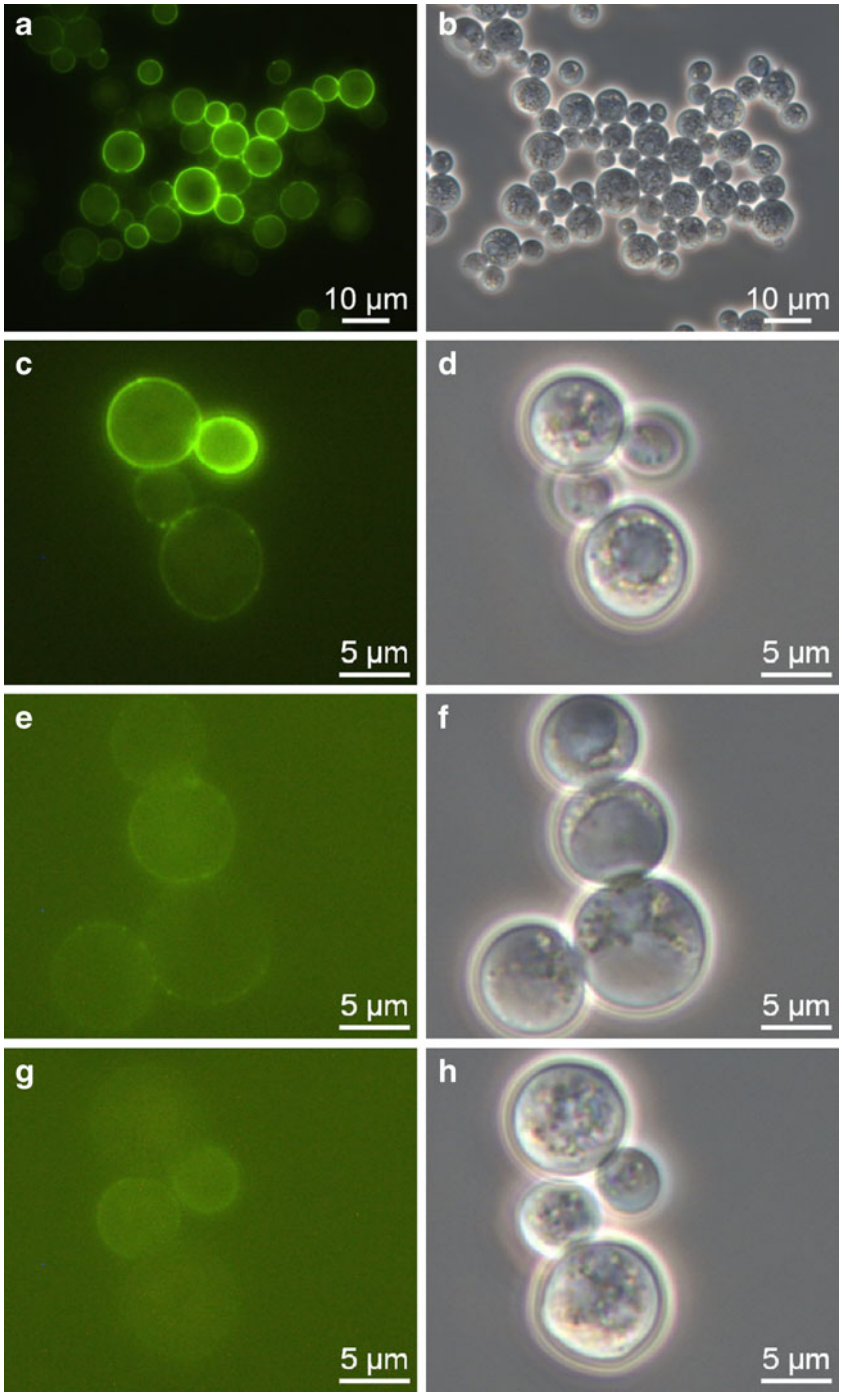

Fig. 2 Visualization of active flocculation lectins of the ale brewing strain of *S. cerevisiae* NCYC 1195 using Avidin-FITC. Cells were suspended in acetate buffer (20 mmol/l, pH 4.0), with 8 mmol/l Ca^{2+} and 10 μg/ml Avidin-FITC; cells were incubated at 25°C, during 60 min. Cells from a culture with 72 h (**a** and **c**) or 12 h (**e**). Cells from a culture with 72 h, treated with pronase E, during 1 h (**g**). Fluorescence micrographs (**a**, **c**, **e** and **g**); phase contrast micrographs of the same cells (**b**, **d**, **f** and **h**). Fluorescence images **e** and **g** were shot with 2.5× shutter time used in images **a** and **c**

adsorption onto negative charged surfaces; the amount of adsorbed dye can be related with the charge of yeast wall (Jin et al. 2001; Rhymes and Smart 2001). The value of CSC is strain dependent. At pH 4.0, the strain NCYC 1195 displayed a more negative cell surface charge, i.e. a higher amount of alcian blue 8 GX was adsorbed by the yeast cells; the strain NCYC 1214 showed the lower negative cell charge (Fig. 1c). For all strains, CSC varied during the growth even for the constitutively flocculent strain (S646-1B). A reduction of the repulsion of yeast cells, due to the lowering of the CSC, should facilitate yeast flocculation. However, the analysis of the CSC of the three strains showed that no relationship could be found among the cell charge and the flocculation ability during the growth (Fig. 1a–c).

CSH was evaluated by the MATH assay, which is based on the microbial adhesion to liquid hydrocarbons (Rosenberg 2006); a high affinity to nonpolar solvents (such as n-hexadecane) indicates a high CSH and vice versa. The cells of the constitutively flocculent strain (S646-1B) were less

hydrophobic than the ale brewing strains. For S646-1B strain, the CSH (~30%) and the flocculation remained approximately constant during the growth (Fig. 1a, b, d). In contrast, the hydrophobicity of ale brewing cells (NCYC 1195 and 1214) were low, in exponential phase of growth, in a non-flocculent state (10% and 27% for the strain NCYC 1195 and NCYC 1214, respectively) and increased enormously in the stationary phase of growth (72% and 87% for the strain NCYC 1195 and NCYC 1214, respectively); a positive correlation between the increase of cell hydrophobicity and flocculation was found (Fig. 1a, b, d). However, two questions remain still unsolved: what is the cell wall component responsible for the increase of CSH? Is the flocculation a consequence of the increase of hydrophobicity? Or, in contrast, is the increase of CHS a consequence of the presence of lectins on yeast cell surface?

In order to test if flocculation lectins themselves are the wall components responsible by CSH, flocculent ale brewing strains were treated with a protease (pronase E). In addition, the flocculent strain S646-1B and the isogenic (except for the marker genes *ade1* and *trp1* and the gene *FLO1*) non-flocculent strain S646-8D were also treated with pronase E. After 1 h of protease treatment, all the flocculent strains lost the flocculation ability (<12% of flocculated cells) (Fig. 3a). Most likely, protease E treatment degraded the proteins on yeast surface and flocculins were also removed. Consistently with these results, cells treated with pronase E were not able to fix the fluorescent probe Avidin-FITC and, consequently, remained unlabelled (Fig. 2g). The action of protease E had a marginal impact on CSC, since no clear modification of CSC values occurred after the treatment with the protease (Fig. 3b). In contrast, pronase E treatment provoked a deep reduction of hydrophobicity: 76%, 92% and 96% for the strains NCYC 1195, 1214 and S646-1B, respectively; interestingly, no effect on hydrophobicity was observed in the non-flocculent isogenic strain S646-8D (Fig. 3c).

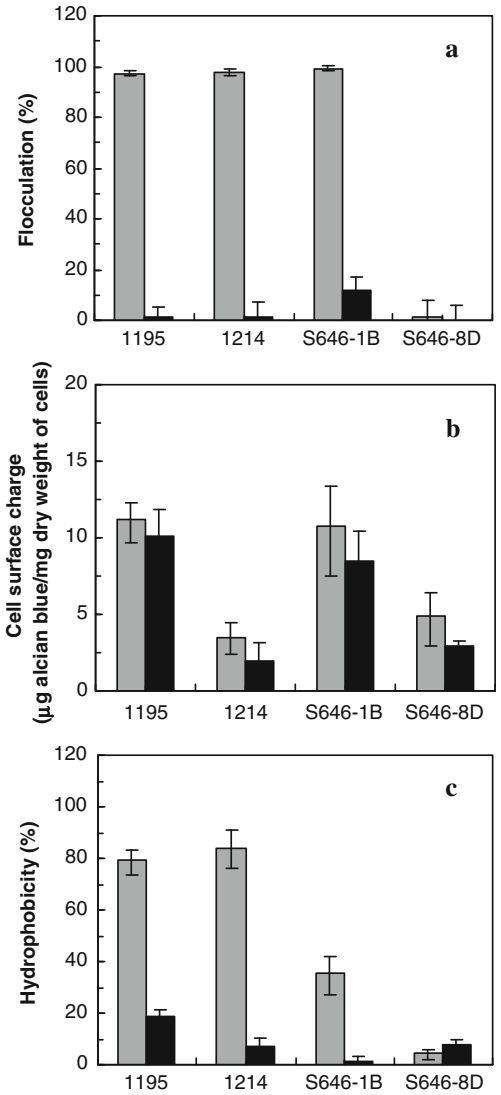

Fig. 3 Effect of the pronase E treatment on the surface properties of *S. cerevisiae* cells. The strains studied were: the flocculent ale brewing NCYC 1195 and NCYC 1214; in addition, the flocculent strain S646-1B and the isogenic non-flocculent strain S646-8D were also used. **a** Flocculation; **b** cell surface charge and **c** hydrophobicity. Before (*grey bar*) or after (*black bar*) pronase E treatment during 1 h. Mean and standard deviation are presented (with 95% confidence)

Discussion

In the present work, the role of the surface properties on the flocculation of two ale brewing strains belonging to the NewFlo phenotype was investigated. For comparative purposes, a constitutively flocculent strain belonging to the Flo1 phenotype and its isogenic non-flocculent strain S646-8D were also used.

The ale brewing strains displayed a typical NewFlo phenotype flocculation behaviour: loss of flocculation during the exponential phase of growth and recovering of flocculation toward the end of exponential phase of growth. The constitutively flocculent strain remained flocculent during all phases of growth. The cell charge of brewing and the constitutively flocculent strains varied during the growth; however, no clear relationship among the CSC and the onset (or loss) of flocculation of the brewing strains was observed. These results are in agreement with other authors, which attribute a minor role of CSC on the onset of flocculation (Smit et al. 1992; Dengis et al. 1995; Dengis and Rouxhet 1997). Although it is described that ale (top) brewing fermentation strains are less negative charged than lager (bottom) strains (Amory and Rouxhet 1988; Mestdagh et al. 1990), no clear difference on the CSC was observed in the three strains used.

The hydrophobicity of the ale brewing strains was low when the strains are in a non-flocculent state and a huge increase of CSH occurred when the cells become floccu-

lent. These data are in agreement with other works in which a positive correlation among the increase of hydrophobicity and the onset of flocculation was found (Smit et al. 1992; Straver et al. 1993). On the other hand, the presence of active lectins on yeast surface could only be observed in flocculent cells, in Ca^{2+} solution. The absence of Ca^{2+} or the presence of 1 mol/l glucose or mannose also inhibited the cells to fix Avidin-FITC; this fact is compatible with the Ca dependence and sugar-sensitive flocculation of the brewing ale strains under study. During the exponential phase of growth, the ale brewing yeast strains NCYC 1195 and NCYC 1214 cells were not able to fix the fluorescent prove Avidin-FITC. Together, these facts prompted us to investigate if the variation of the CSH during the growth of ale brewing yeast strains is dependent or not of the presence of active lectins in yeast surface.

The treatment of flocculent cells with pronase E provoked the hydrolysis of peptidic bonds of surface proteins; this treatment did not affect the cell surface charge of the strains studied. These results can be explained by the fact that phosphates play a determinant role in the development of CSC, being minor the importance of carboxylic groups (Amory and Rouxhet 1988). In contrast, the treatment with pronase E, during 1 h, provoked a complete loss of flocculation; additionally, no active flocculation lectins can be visualized in the pronase E-treated cells and a loss of CSH occurred in the three flocculent strains under study. Together, these results strongly suggest that the percentage of CSH of yeast cells is mainly due to the presence of flocculation lectins on yeast cell surface. Consistent with this possibility, after the treatment with the protease, the isogenic strains S646-8D (non-flocculent) and S646-1B (flocculent) displayed similar flocculation and CSH. In addition, the comparison of the percentage of the hydrophobicity of the non-treated isogenic strains shows that the flocculent strain (S646-1B) is more hydrophobic than the non-flocculent one (S646-8D). Since the strains are isogenic (except for the marker genes *ade1* and *trp1* and the gene *FLO1*), the difference in CSH can only be attributed to the presence of flocculins in the S646-1B strain. These data strongly suggest that the concomitant increase of CSH and the onset of flocculation, in ale brewing yeast cells, can now be explained by the expression of flocculins on yeast cells surface. This possibility is in agreement with the fact that N- and C-terminal regions of Flo1 protein are more hydrophobic than the rest of the protein (Watari et al. 1994); N-terminal region corresponds to the domain A of Flo proteins, which is responsible by the recognition and adhesion to receptors (Kobayashi et al. 1998; Veelders et al. 2010).

An increase of yeast surface hydrophobicity when the glycoproteins Flo1p, Flo5p, Flo9p and Flo10p are present in yeast cell walls (Verstrepen et al. 2001; Govender et al. 2008; Mulders et al. 2009; Govender et al. 2010) was described. Similarly, the expression of *FLO11* also confers hydrophobicity to yeast cells (Guo et al. 2000; Reynolds and Fink 2001; Fidalgo et al. 2006; Mortensen et al. 2007; Govender et al. 2008; Mulders et al. 2009). Nevertheless, contradictory results regarding the flocculation behaviour of *FLO11* transformants have been presented. Guo et al. (2000) described that upon overexpression of *FLO11*, the cultures flocculated. However, the aggregates are calcium independent and are not inhibited by the presence of sugars (glucose, mannose, maltose or sucrose); these facts suggest a cell–cell interaction mechanism different than those observed in the brewing yeast cells. On the other hand, Bayly et al. (2005) reported Ca dependence and mannose inhibition of Flo11p-dependent flocculation, while other authors did not observe a flocculent phenotype in strains overexpressing *FLO11* gene (Verstrepen and Klis 2006; Govender et al. 2008; Mulders et al. 2009; Govender et al. 2010). These data are consistent with our possibility: although the presence of Flop on yeast cell wall seems to be important on the determination of the percentage of cell hydrophobicity, the degree of yeast flocculation is not related with the level of hydrophobicity. Cells did not need to be highly hydrophobic for being flocculent. In the present work, it was shown that brewing and constitutively flocculent strains displayed a similar flocculation degree (>97%) and a different percentage of hydrophobicity; the brewing strains were ~2.5–3 times more hydrophobic than the strain S646-1B. On the other hand, CSH alone seems not to be sufficient to provide calcium-dependent and sugar-sensitive flocculation because strains expressing *FLO11* presented a high CSH but did not yield a flocculent phenotype (Mulders et al. 2009; Govender et al. 2010).

Since flocculation receptors are present in all phases of growth of *S. cerevisiae* cells (Stratford 1993; Soares and Mota 1996; Mortier and Soares 2007), the onset of flocculation seems to be only dependent of the presence of active lectins on yeast surface. Thus, the mechanism of flocculation of the brewing yeasts belonging to the NewFlo phenotype can be simplified: cells are flocculent or non-flocculent depending on the presence of flocculins. In agreement with this possibility, the presence of active flocculins in flocculent cells was visualized (Fig. 2a, c), while the non-flocculent cells remained unlabelled (Fig. 2e). Teunissen et al. (1995) proposed that *FLO* genes can be regulated at the transcriptional level; most likely, the expression of flocculation, in NewFlo phenotype strains, seems to be controlled by the availability of the nutrients (Sampermans et al. 2005; Verstrepen and Klis 2006). In this context, flocculation can be seen as an example of a social behaviour: a communitarian strategy for a long-time survival in the absence of nutrients (Soares 2011).

Although non-specific forces, such as hydrogen bonds, hydrophobic interactions or electrostatic forces can be involved in the yeast flocculation (Dengis et al. 1995; Jin and Speers 2000; Hsu et al. 2001; Jin et al. 2001), the specific (lectin–sugars) interactions seem to govern the flocculent state of the brewing yeast cells. Using atomic force microscopy, Touhami et al. (2003) described adhesion forces of 121±53 pN between flocculation lectins and sugar residues (receptors). Even though CSH can be important in the adhesion process of yeast cells to the abiotic surfaces (like plastics or agar) and its consequent colonization, CSH seems to play a marginal role in the cell–cell interaction process associated with the self-flocculation of brewing yeasts.

In conclusion, the correlation between the increase of CSH and flocculation of ale brewing yeast cells is most likely due to the presence of active flocculins on yeast cell surface. This conclusion is supported by the following reasons: (1) flocculation only occurred when active flocculation lectins were observed on the yeast cell surface; (2) the hydrolysis of peptidic bonds of the surface proteins led to a reduction of hydrophobicity; (3) the treatment of the non-flocculent strain with pronase E did not reduce CSH; (4) the percentage of hydrophobicity was not related with the level of flocculation. Together, these facts strongly indicate that the cell hydrophobicity plays a minor role in the auto-aggregation of the ale brewing yeast cells; in these cells, the degree of flocculation is primarily defined by the presence (or absence) of active flocculins on yeast cell wall rather than the decrease of cell surface charge or the increase of cell surface hydrophobicity.

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