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ORIGINAL ARTICLE

Flocculation onset in *Saccharomyces cerevisiae*: effect of ethanol, heat and osmotic stress

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Abstract

Aims: To examine the effect of different stress conditions on the onset of flocculation in an ale-brewing strain, *Saccharomyces cerevisiae* NCYC 1195.
Methods and Results: Flocculation was evaluated using the method of Soares, E.V. and Vroman, A. [*Journal of Applied Microbiology* (2003) 95, 325]; plasma membrane integrity was accessed using propidium iodide and the staining of the yeast cell wall was performed using calcofluor white M2R. Cells in exponential phase of growth were subjected to different stress conditions. The addition of 1%, 3% and 5% (v/v) ethanol, 1% and 3% (v/v) isopropanol or a brief heat shock (52°C, 5 min), did not induce an early flocculation phenotype when compared with control cells. The addition of 10% (v/v) ethanol, a continuous mild heat-stress (37°C) or an osmotic stress (0·5 or 1 mol l^{-1} of NaCl) did not induce a flocculent phenotype.
Conclusions: Flocculation seems not to be induced as a response to different chemical (ethanol and isopropanol) and physical (heat and osmotic) stress conditions. Conversely, osmotic and ethanol [10% (v/v)] stress, as well as a continuous mild heat shock (37°C), have a negative impact on the phenotype expression of flocculation.
Significance and Impact of the Study: The findings reported here contribute to the elucidation of the control of yeast flocculation. This information might be useful to the brewing industry, as the time when the onset of flocculation occurs can determine the fermentation performance and the beer quality, as well as in other biotechnological industries where flocculation can be used as a cell separation process.

Introduction

Yeast flocculation is of fundamental importance in the brewing industry. In addition, this property can be used in different biotechnological industries as an easy and cheap process of cell separation. Flocculation can be defined as 'the phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended' (Stewart and Russell 1981). In the brewing industry, ideally, flocculation should occur towards the end of fermentation and involves the interaction of specialized cell wall proteins called 'lectins' (Miki *et al.* 1982; Stratford 1992), 'adhesins' (Verstrepen *et al.* 2003; Verstrepen and Klis 2006), 'flocculins' (Verstrepen *et al.* 2003; Verstrepen and Klis 2006) or 'zymolectins' (Speers *et al.* 1998), present only on flocculent cells, with carbohydrates (receptors) on the neighbouring cell wall; calcium ions enable the adhesins to achieve their active conformation (Miki *et al.* 1982; Stratford 1992).

Taking into account the sensitivity to pH and sugar inhibition, two phenotypes have been described: Flo1 phenotype, which is mannose-sensitive only and NewFlo

phenotype, which is sensitive to several sugars like glucose, maltose and mannose (Stratford and Assinder 1991; Masy *et al.* 1992). The majority of brewery yeasts belong to NewFlo phenotype, which flocculate in the stationary phase of growth, while almost all Flo1 phenotype strains are constitutively flocculent (Stratford and Assinder 1991; Soares and Mota 1996). In addition to well-documented Flo1 and NewFlo phenotypes, other flocculation types have been described, namely mannose-insensitive flocculation (Masy *et al.* 1992) or strains whose flocculation only occurs in the presence of sufficiently high ethanol concentration (Dengis and Rouxhet 1997).

The effect of ethanol and other alcohols in yeast flocculation has been reported since 50 years. However, the influence of ethanol is strain-dependent: negative (Kamada and Murata 1984), positive or no-effect (Jin and Speers 2000), have been described. The mechanism through which ethanol exerts its influence, at surfacial level, is still unclear (Verstrepen *et al.* 2003), although it has been suggested that ethanol may act upon cell wall conformation and decreasing cell–cell electrostatic repulsion (Jin and Speers 2000); additionally, a slight increase of cell-surface hydrophobicity with the increase of ethanol concentration was reported (Jin *et al.* 2001).

Yeast flocculation is under genetic control. Dominant and recessive genes and flocculation suppressor genes have been described (Teunissen and Steensma 1995; Verstrepen *et al.* 2003; Verstrepen and Klis 2006). Besides genetic control, environmental factors such as pH, calcium availability (Soares and Seynaeve 2000) and ionic strength (Jin and Speers 2000) deeply affect yeast flocculation; additionally, the increasing of cell-surface hydrophobicity (Straver *et al.* 1993; Jin *et al.* 2001) or the presence of 3-OH oxylipins (Strauss *et al.* 2005) have been positively correlated with the promotion of flocculation.

Brewing yeasts are usually exposed to different stress conditions during prefermentation (acid-washing, oxidative stress, cold shock and nutrient starvation), primary and secondary fermentations (osmotic stress, ethanol toxicity, pH, temperature fluctuations, and CO_2/hydrostatic pressure) or postfermentation (mechanical shear, cold shock and nutrient starvation) (Walker 1998). Yeast displays a rapid molecular response to sublethal injury. This response is characterized by synthesis of stress proteins, increasing the level of compatible solutes (trehalose and glycerol), modifications in the membrane lipid composition and plasma membrane ATPase activity and, in the case of oxidative stress, the production of antioxidant defences such as glutathione and superoxide dismutase (Rosa and Sá-Correia 1991; Estruch 2000; Hohmann 2002). Stress response is a transient reprogramming of cellular activities to ensure cell's survival and adaptation to adverse conditions (Estruch 2000). It was suggested that flocculation could be a response of yeast to stress (Iserentant 1996). In line with this possibility, flocculation can be seen as a mechanism of survival, as the cells at the outside of the floc can offer some protection, to external adverse environmental conditions, to the cells at the inside.

The aim of this work was to study the effect of different stress conditions (ethanol, heat and osmotic stress and the addition of an aliphatic alcohol which affect cell membrane) on the onset of flocculation of brewing yeast belonging to the NewFlo phenotype.

Materials and methods

Strain, media and culture conditions

In this work, the flocculent ale-brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1195 was used. The original strain was obtained from the NCYC, UK and is characterized as NewFlo phenotype (Stratford and Assinder 1991).

Precultures were prepared in 40 ml of YEPD broth (10 g of yeast extract l^{-1}, 20 g of peptone l^{-1}, 20 g of glucose l^{-1}) in 100-ml Erlenmeyer flasks. Cells were incubated at 25°C in an orbital shaker Sanyo Gallenkamp IOC 400 (West Sussex, UK) at 150 rev min^{-1}. After 48 h of growth, flocculent cells were harvested by centrifugation (2000 ***g***, 5 min) and washed twice with 30 mmol l^{-1} of ethylenediaminetetraacetic acid (EDTA) solution to ensure floc dispersion. Finally, cells were washed and suspended in deionized water.

Cultures in YEPD with 50 g of glucose l^{-1} were prepared by inoculating 800 ml of culture medium, in 2-l Erlenmeyer flasks, with about 1×10^6 cells ml^{-1} from precultures. Cells were grown at 25°C in an orbital shaker at 150 rev min^{-1}. After 13–14 h of growth, the cultures, in exponential respiro-fermentative phase of growth, were aseptically divided in three aliquots of 250 ml into 500-ml Erlenmeyer flasks. One of the aliquots was used as control and the others treated as described next. To determine the impact of osmotic and ethanol stress on flocculation, to the 250-ml aliquots of NaCl (at 0·5 or 1 mol l^{-1} final concentration) or ethanol [at 1 and 3% or 5% and 10% (v/v), final concentration], were added, according to the experiment. In both cases, cells were incubated at 25°C in an orbital shaker at 150 rev min^{-1}. For heat stress experiments, the 250-ml aliquots were subjected to a mild heat stress, by rapidly heating and incubation at 37°C, at 150 rev min^{-1}, or a brief heat shock induced by rapidly heating to 52°C, for 5 min, and then cooled and incubated at 25°C at 150 rev min^{-1}.

In order to investigate the impact, at the surfacial level, of the presence of 10% (v/v) ethanol and 0·5 or

1·0 mol l^{-1} of NaCl, control experiments were done, using high flocculent cells in nongrowing conditions. Thus, a 48-h culture was washed twice with 30 mmol l^{-1} of EDTA and then washed and suspended in deionized water at 1×10^7 cells ml^{-1}. Fifty-millilitre aliquots were aseptically transferred to 100-ml Erlenmeyer flasks and the cells placed in the presence of 10% (v/v) ethanol, 0·5 or 1·0 mol l^{-1} of NaCl. Cells were incubated during 24 h, at 25°C, in an orbital shaker at 150 rev min^{-1}. Flocculation was evaluated as described next.

Measurement of flocculation ability

At defined periods of growth time indicated in the figures, yeasts were harvested by centrifugation (2000 ***g***, 5 min), washed and suspended in ice-cold deionized water and stored at 4°C for a maximum of 72 h. Control experiments have showed that yeasts kept the same flocculation ability with this procedure. Before flocculation measurements, cells were washed twice in 30 mmol l^{-1} of EDTA solution. Subsequently, cells were washed twice and resuspended in deionized water at a final concentration near 2×10^9 cells ml^{-1}.

Flocculation was monitored under standard conditions (50 mmol l^{-1} of pH citrate buffer, pH 4·0, containing 8 mmol l^{-1} of $CaCl_2$), using the microflocculation technique previously described (Soares and Vroman 2003).

Growth

Growth was monitored spectrophotometrically at 600 nm or by direct cell counting with a counting chamber, after appropriate dilution in 30 mmol l^{-1} of EDTA solution to prevent cell aggregation. A calibration curve (number of cells versus absorbance) was previously constructed.

Glucose determination

Cells were harvested by centrifugation, at 2000 ***g***, for 5 min, and the supernatant was collected and immediately stored in the freezer until glucose determination. The glucose concentration in the culture medium was measured enzymatically with glucose oxidase–peroxidase method using the Adaltis glucose reagent kit (Adaltis, Bologna, Italy).

Assessment of plasma membrane integrity

Cells were concentrated by centrifugation, at 4500 ***g***, for 1 min, washed and resuspended in phosphate saline buffer (PBS) (50 mmol l^{-1}, pH 7·4), at 5×10^7 cells ml^{-1}. Propidium Iodide (PI) (Sigma, Steinheim, Germany) was added to a final concentration of 3 μg ml^{-1}. Cell suspension was incubated at room temperature, in the dark, for 20 min. Cells were examined using a Leica DLMB epifluorescence microscope (Leica Microsytems, 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 (Leica Microsystems, Heerbrugg, Switzerland). For each sample, at least 300 cells were scored in randomly selected fields.

Staining of the cell walls

Cells were harvested at the required phase of growth by centrifugation, at 4500 ***g***, for 1 min, washed twice with 30 mmol l^{-1} of EDTA solution, twice with water and resupended in water at 1×10^7 cells ml^{-1}. Calcofluor white M2R (Sigma) was added to a final concentration of 25 μmol l^{-1}. Cell suspensions were incubated at room temperature, in the dark, for 30 min. Cells were washed twice with deionized water and examined using an epifluorescence microscope equipped with a HBO-100 mercury lamp and a filter set A (excitation filter BP 340–380, dichromatic mirror 400 and suppression filter LP 425) from Leica. Images were acquired with a Leica DC 300F camera (Leica Microsystems) using a N plan ×100 objective; the images were processed using Leica IM 50-Image manager software.

Reproducibility of the results

All experiments were repeated, independently, three to five times. Although, absolute data were not comparable in the experiments performed on different days, the observed trends were fully consistent among the independent experiments and a typical example is shown. The data reported for growth and flocculation are the mean values and standard deviations, performed in triplicate and quadruplicate, respectively.

Results

The effect of ethanol addition to the onset of flocculation was tested using cells at the middle of the exponential respiro-fermentative growth phase, when glucose concentration in the culture medium was about 40 g l^{-1}. The addition of 5% (v/v) ethanol induced a small reduction of the growth rate and did not trigger an early flocculation onset compared with the control (Fig. 1). However, a full flocculation development can be observed early, compared with the control; 7 h after the addition of 5% (v/v) ethanol more than 90% of cells were flocculated, while in the control 75% of the cells were flocculated (Fig. 1). Similar results to 5% (v/v) ethanol were observed with 1% or 3%

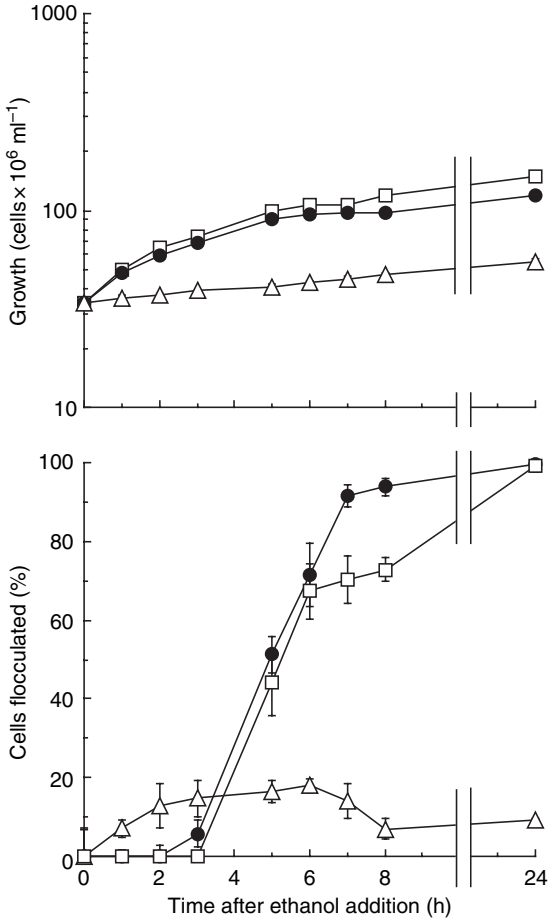

Figure 1 Effect of ethanol addition on growth and flocculation of an ale brewing strain of *Saccharomyces cerevisiae* NCYC 1195. Cells in exponential phase of growth, in YEPD broth (10 g of yeast extract l^{-1}, 20 g of peptone l^{-1}, 20 g of glucose l^{-1}), were subjected to ethanol stress, by the addition of an ethanol pulse at a final concentration of 5% (v/v) (circles) or 10% (triangles). Control (squares): nontreated cells. Flocculation was determined in standard conditions, in citrate buffer (pH 4·0, 50 mmol l^{-1}) containing Ca^{2+} (8 mmol l^{-1}) and agitated for 4 h, at 100 rev min^{-1}. The data reported for growth and flocculation are the mean values performed in triplicate and quadruplicate, respectively. Vertical error bars represent standard deviations. Where no error bars are shown, they are within the points.

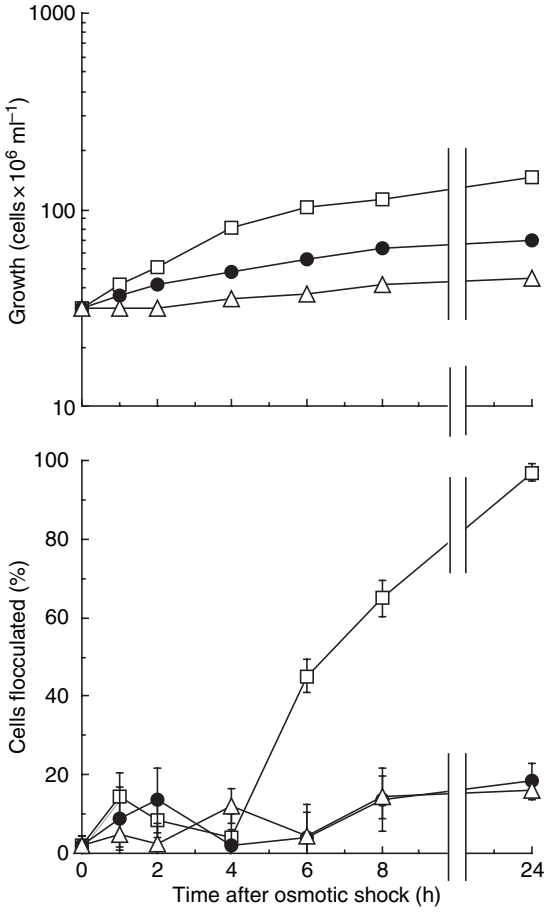

Figure 2 Effect of osmotic stress on growth and flocculation of the strain *Saccharomyces cerevisiae* NCYC 1195. Cells in exponential phase of growth, in YEPD broth (10 g of yeast extract l^{-1}, 20 g of peptone l^{-1}, 20 g of glucose l^{-1}), were subjected to an osmotic stress, by the addition of NaCl at a final concentration of 0·5 mol l^{-1} (circles) or 1 mol l^{-1} (triangles). Control (squares): nontreated cells. The data reported for growth and flocculation are the mean values performed in triplicate and quadruplicate, respectively. Vertical error bars represent standard deviations. Where no error bars are shown, they are within the points.

(v/v) ethanol (data not shown). Increasing the ethanol pulse to 10% (v/v) induced lethality, accessed by PI, of 10% and 40% after 8 and 24 h, respectively, and did not allow the triggering of flocculation (Fig. 1). The possibility of ethanol to affect the cell surface was ruled out as no diminishing of flocculation occurred in control experiments, where flocculent cells, in nongrowing conditions (suspended in water) were exposed during 24 h to 10% (v/v) ethanol (data not shown).

The effect of isopropanol, which could produce a stress signal, was also tested. The addition of 1% and 3% (v/v) isopropanol to exponentially growing cells did not induce an early flocculation phenotype, when compared with the control cells, as was described with 5% (v/v) ethanol (data not shown).

The osmotic stress produced by 1 mol l^{-1} of NaCl caused cessation of cell growth (Fig. 2) and triggered morphological changes (Fig. 3a). After 2 h of growth was arrested, the cells resumed growth with a reduced rate (Fig. 2). While control cells exhibit a uniform faint fluorescence on their surface, after being stained with calcofluor white (Fig. 3b), cells shifted to hyperosmotic medium (1 mol l^{-1} of NaCl) showed clearly visible fluorescent patches after 1 h of incubation (Fig. 3a). In

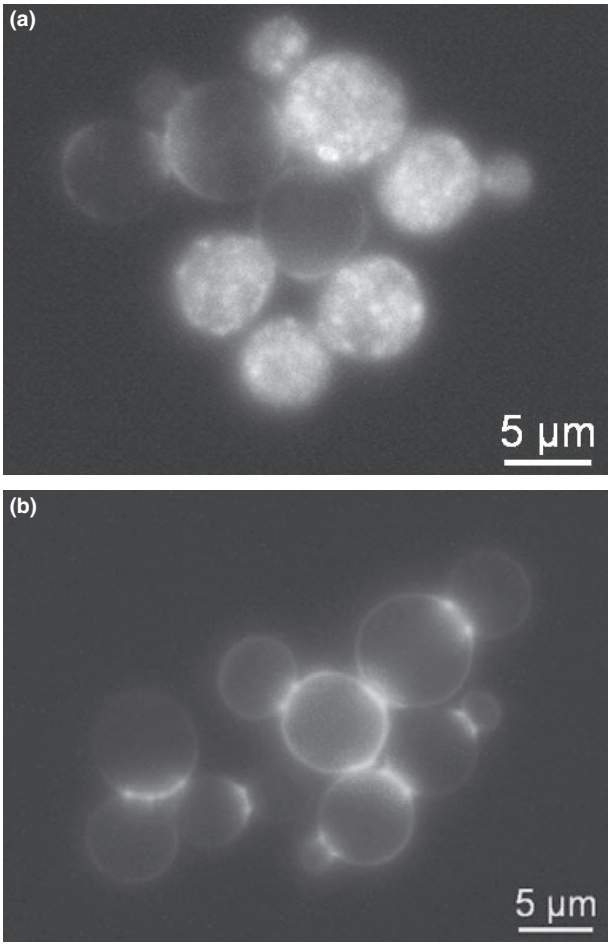

Figure 3 Fluorescent labelling, using calcofluor white M2R, of the cell wall of the strain *Saccharomyces cerevisiae* NCYC 1195. Cells in exponential phase of growth were incubated for 1 h in YEPD broth (10 g of yeast extract l^{-1}, 20 g of peptone l^{-1}, 20 g of glucose l^{-1}) with 1 mol l^{-1} of NaCl (a). Control: nontreated cells with the same time of growth (b).

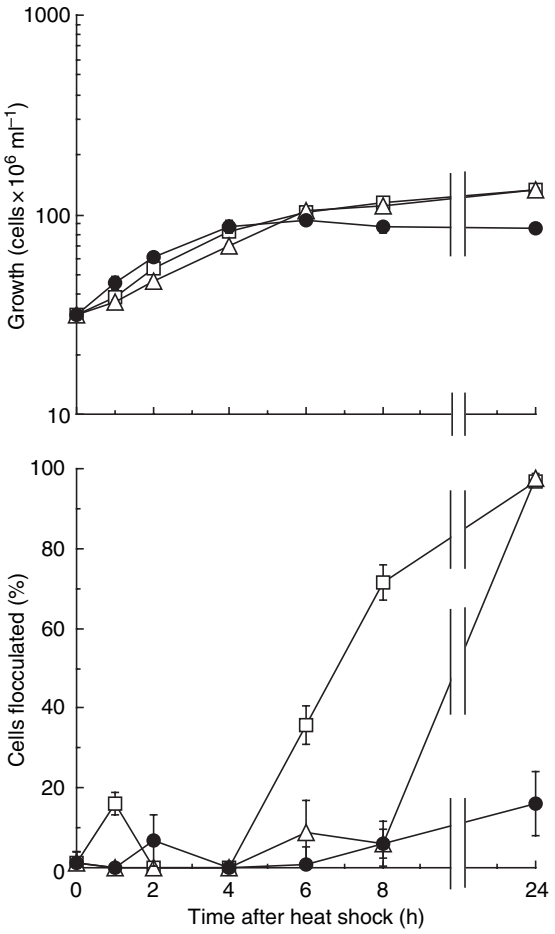

Figure 4 Effect of heat stress on growth and flocculation of the strain *Saccharomyces cerevisiae* NCYC 1195. Cells in exponential phase of growth, in YEPD broth (10 g of yeast extract l^{-1}, 20 g of peptone l^{-1}, 20 g of glucose l^{-1}), were subjected to mild heat stress, by rapid heat and incubation at 37°C (circles), or heat shock induced by rapid heat at 52°C, for 5 min, and then cooled and incubated at 25°C (triangles). Control (squares): nontreated cells. The data reported for growth and flocculation are the mean values performed in triplicate and quadruplicate, respectively. Vertical error bars represent standard deviations. Where no error bars are shown, they are within the points.

0·5 mol l^{-1} of NaCl, the osmotic shock induced a smaller reduction of cell growth rate, compared with 1 mol l^{-1} of NaCl (Fig. 2) and no patches were observed. Nevertheless, the incubation of cells in 0·5 or 1 mol l^{-1} of NaCl had a negative impact on yeast flocculation, as the onset of flocculation did not occur during a 24-h period, after the osmotic shock (Fig. 2). Control experiments were done, exposing flocculent cells, in no growing conditions to 0·5 and 1·0 mol l^{-1} of NaCl, during 24 h; no modification of flocculation occurred (data not shown).

The effect of a continuous mild heat stress (by exposing the cells at 37°C) and a brief heat shock (by exposing the yeast cells at 52°C for 5 min, followed by a rapid cooling and incubation at 25°C) on the triggering of flocculation of exponentially growing cells was also tested. The continuous exposure of yeast cells to a mild heat stress did not stimulate the triggering of flocculation, while a brief heat shock followed by incubation at 25°C delayed the onset of flocculation, compared with the control cells (Fig. 4).

Discussion

In the present work, we examined the effect of different chemical (ethanol and isopropanol) and physical (heat and osmotic) stresses on flocculation. The imposition of

those stress conditions can influence yeast flocculation at different levels: at a genetic level, influencing *FLO* gene expression; at a surface level, affecting the presence of active Flo proteins or acting upon cell–cell interactions. It is well described in the literature that cell–cell interactions could be deeply affected by the chemical composition of the solution, namely pH (Soares and Seynaeve 2000) and ionic strength (Jin and Speers 2000). In the present work, flocculation was always determined in washed cells, under defined and standard buffer conditions, in order to eliminate the effect of the environmental conditions where the cells were suspended on cell–cell interactions. Thus, it is possible to correlate the flocculation capacity with the presence of active surface zymolectins on yeast cell wall, as it is described that flocculation receptors are present in all phases of growth (Soares and Mota 1996).

It is generally accepted that the onset of flocculation, in brewing yeasts of NewFlo phenotype, is triggered at the end of respiro-fermentative growth phase, which coincides with the carbon source exhaustion and the attainment of the higher ethanol concentration (Soares and Mota 1996; Sampermans *et al.* 2005). Thus, it was proposed that flocculation could be considered a stress reaction, as a response of a shortage of nutrients or a defense mechanism against high ethanol concentrations (Iserentant 1996). The results presented in this work regarding the addition of 1%, 3% or 5% (v/v) ethanol to exponential growing cells in the presence of high (40 g l^{-1}) glucose concentration did not support this possibility, as an early flocculation phenotype was not induced, when compared with control cells. However, the presence of a moderate ethanol concentration [1%, 3% or 5% (v/v)] had a positive effect on the kinetics of flocculation development, which is in agreement with previous results (Soares and Vroman 2003; Soares *et al.* 2004; Sampermans *et al.* 2005) where a positive effect of ethanol on the flocculation was found. Increasing the ethanol concentration to 10% (v/v) induced a toxic effect, which can be responsible for the absence of flocculation in these cells. PI allows the evaluation of the effect of the ethanol stress on yeast plasma membrane integrity; cells with injured plasma membranes (nonviable cells) incorporate PI, which stains double-stranded nucleic acids (Haugland 2005). This loss of plasma membrane integrity in cells exposed to 10% (v/v) ethanol is in agreement with the data reported in the literature in which the main target of ethanol toxic effects are the lipids and proteins of plasma membrane and organelles (mitochondria); during ethanol stress, membrane fluidity is modified, decreasing their structural integrity (Birch and Walker 2000).

It has been shown that some *Saccharomyces cerevisiae* strains are able to form pseudohyphae as a physiological response to starvation (carbon and nitrogen) and stressful environmental conditions such as the addition of aliphatic alcohols (*n*-propanol and isopropanol) at 2% (v/v) (Zaragoza and Gancedo 2000). Aliphatic alcohols are nonmetabolizable compounds that affect the lipid bilayer organization of the cell membrane (Zaragoza and Gancedo 2000). In the present work, the addition of 1% and 3% (v/v) isopropanol to exponentially growing cells did not induce an early flocculation phenotype, when compared with the control cells.

A rapid increase in the osmolarity of the growth medium, by the addition of NaCl (0·5 or 1 mol l^{-1}) to exponentially growing cells, caused a reduction in growth rate (0·5 mol l^{-1} of NaCl) and 2 h of arrested growth (1 mol l^{-1} of NaCl). Arrested growth and change in the permeability of plasma membrane are reported in the literature as examples of immediate yeast cell responses to an increasing osmolarity of the growth medium (Hohmann 2002). The exposure of yeast cells to a hyperosmotic stress provokes a loss of cytoplasmatic water and several mechanisms are induced to counteract cell dehydratation and protect cellular structures. The sensors of external osmolarity are the plasma membrane proteins Sln1p and Sho1p, which transmit the signal to MAP kinase cascade; this cascade when activated, culminates in the phosphorylation and activation of Hog1p (high osmolarity glycerol pathway – HOG pathway). The HOG pathway is involved in the regulation of the majority of the genes induced by osmotic stress (Estruch 2000; Hohmann 2002).

As flocculation is a superficial phenomenon where the composition, structure and architecture of cell wall play a critical role, the effect of osmotic stress on yeast cell wall was investigated. Our results are in agreement with previous observations that the osmotic shock caused by 1 mol l^{-1} of KCl, induces a change in the cell wall organization, with formation of fluorescent patches, probably as a consequence of the shrinkage of the cytoplasm and redistribution of periplasmic and/or matrix cell wall material (Slaninová *et al.* 2000). This modification of cell wall organization could affect the presence of active Flo proteins, explaining the negative impact of 0·5 and or 1 mol l^{-1} of NaCl on yeast flocculation. Thus, osmotic stress could act influencing *FLO* gene expression and/or Flo protein secretion or activation. However, in nongrowing conditions, 0·5 and/or 1 mol l^{-1} of NaCl, did not affect yeast flocculation, which suggests a more complex effect of the osmotic stress in yeast cell metabolism, rather than a mere surfacial action.

Temperature is one of the most important factors affecting growth and metabolism of yeasts. The exposition of yeast cells to supra-optimum temperature increases protein aggregation and denaturation, changing the structure of membranes and affecting different cellular processes (Estruch 2000). The flocculation reduction in cells

growing at 37°C is in agreement with previous observations were it was found that flocculation is a thermosensitive event in *Saccharomyces cerevisiae* (Williams *et al.* 1992; Soares *et al.* 1994). Probably, a continuous mild heat stress (37°C) acts directly on the mitochondrial activity and indirectly on the cell membrane structure, affecting the secretion of zymolectins, with the consequent reduction of flocculation. Interestingly, the triggering of flocculation in *Kluyveromyces marxianus* was described as a consequence of the appearance of a protein of 37 kDa in the yeast cell wall, upon an upshift of the growth temperature from 26 to 40°C (Fernandes *et al.* 1993). When shifted to higher temperatures, yeast cells respond by inducing the synthesis of heat-shock proteins (hsps). Two distinct regulatory elements are involved in the induction of genes by heat shock: stress response elements (STRE) and the heat-shock elements (HSE) (Estruch 2000). However, in the present work, the brief heat shock (52°C, 5 min) did not induce an early flocculation phenotype, when compared with the control cells; on the contrary, the heat stress delayed the onset of flocculation.

Many modifications induced in yeast cells exposed to heat stress are identical to those caused by ethanol; in addition, the protective responses induced by heat shock and ethanol show a high degree of similarity (Birch and Walker 2000; Estruch 2000; Alexandre *et al.* 2001). It is known that STRE are activated by various stress conditions such as nitrogen starvation, osmotic and oxidative stress, low external pH, weak organic acids, heat shock and ethanol (Ruis and Schuller 1995). Thus, it has been proposed that high ethanol concentrations may induce the *FLO* genes through the numerous stress-responsive heat-shock elements, which are found in the promoter region of these genes (Verstrepen *et al.* 2003). The results found in the present study do not support this possibility, at least for the ale-brewing yeast strain used. However, it is important to bear in mind that regulation of *FLO1* expression, which seems to occur at the transcriptional level, is dependent on the genetic background of the strains (Verstrepen *et al.* 2003; Verstrepen and Klis 2006).

In conclusion, the results presented in this paper show that for the ale-brewing yeast strain used, flocculation seems not to be induced as a response to a specific stress: chemical (ethanol and isopropanol) or physical (heat and osmotic) stress. Conversely, ethanol [10% (v/v)] and osmotic stress or a continuous mild heat shock (37°C) had a negative impact on the phenotypic expression of flocculation.

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