REVIEW ARTICLE

Flocculation in *Saccharomyces cerevisiae*: a review

E.V. Soares

Keywords
aggregation, alcoholic beverages, bio-ethanol, brewing yeast, cell separation, *FLO* genes, flocculation.

Summary

The present work reviews and critically discusses the aspects that influence yeast flocculation, namely the chemical characteristics of the medium (pH and the presence of bivalent ions), fermentation conditions (oxygen, sugars, growth temperature and ethanol concentration) and the expression of specific genes such as *FLO1*, Lg-*FLO1*, *FLO5, FLO8, FLO9* and *FLO10*. In addition, the metabolic control of loss and onset of flocculation is reviewed and updated. Flocculation has been traditionally used in brewing production as an easy and off-cost cell-broth separation process. The advantages of using flocculent yeast strains in the production of other alcoholic beverages (wine, cachaça and sparkling wine), in the production of renewal fuels (bio-ethanol), in modern biotechnology (production of heterologous proteins) and in environmental applications (bioremediation of heavy metals) are highlighted. Finally, the possibility of aggregation of yeast cells in flocs, as an example of social behaviour (a communitarian strategy for long-time survival or a means of protection against negative environmental conditions), is discussed.

Introduction

Yeast flocculation can be defined as a nonsexual, homotypic (involving only one type of cell in the interactions), reversible (flocs can be reversible dispersed by the action of EDTA or specific sugars, like mannose) and multivalent process of aggregation of yeast cells into multicellular masses (composed by thousands or even millions of cells), called flocs, with the subsequent rapid sedimentation from the medium in which they are suspended (Fig. 1) (Stratford 1992b; Stewart 2009). The word floc derives from the Latin word *floccus*, which means a tuft of wool. The cells with the ability to form flocs are called flocculants and look like tufts of wool (Fig. 1a), while the cells that are not able to form flocs are usually known as powdery. *Saccharomyces cerevisiae* cells can be found aggregated in different ways, which should not be confused with flocculation, such as sexual aggregation, co-flocculation and chain formation. Sexual aggregation in haploid strains of *S. cerevisiae* of complementary mating types (α and a) can occur after exchange of pheromones a and α, respectively, which induces the appearance of complementary molecules (proteins) on surface of cells and facilitates the fusion of the haploid cells (Chen *et al.* 2007). Co-flocculation or mutual flocculation is a heterotypic aggregation process (while flocculation is homotypic) among two strains: one of them is nonflocculent and the other weakly flocculent; when mixed, in the presence of Ca^{2+}, the strains rapidly settle (Stewart 2009). Chain formation occurs because of a failure of the younger bud to separate from the mother cell; this results in an aggregate formation composed of 30–50 cells. These aggregates are covalently linked; thus, after mechanical dispersion, cells will not be able to re-aggregate (Stewart 2009). Other aggregation phenomena in *S. cerevisiae* include biofilm (Verstrepen *et al.* 2004) and pseudohyphae formation, which grow and invade the agar medium (Gimeno *et al.* 1992).

Saccharomyces sensu stricto complex had a long and profitable association with food, biotechnology and pharmaceutical industries; it is the micro-organism most industrially exploited by the man (Vaughan-Martini and

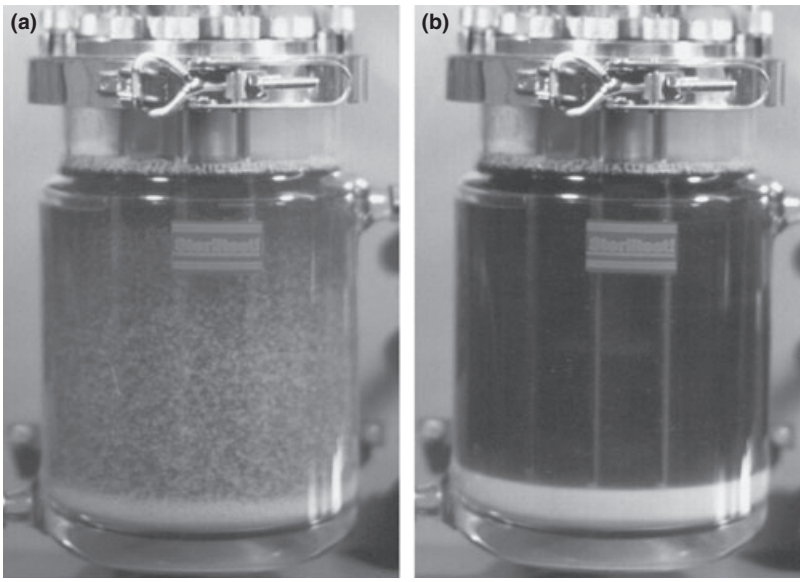

Figure 1 Flocculent ale-brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1195 cultured in yeast extract, peptone, dextrose medium, at 25°C, during 48 h. Culture after 30 s (a) or 1 min (b) of aeration and stirring stopped.

Martini 1998). In most of the industrial and environmental applications, after yeast growth and/or fulfilling its function, cells must be removed for further processing. The ability of some yeast strains to aggregate facilitates enormously cell separation (Fig. 1b). Thus, flocculation can be seen as an off-cost process of cell separation, which does not require energy input. As an auto-immobilization process, flocculent yeasts can be used in high density cell reactors, which increase the efficiency of the process (Bai *et al.* 2008). These advantages fit in the concept of 'White' Biotechnology (Paula and Birrer 2006) since flocculation increases the efficiency and reduces the energy consumption associated with cell separation.

Mechanism of flocculation

Cell wall charge and hydrophobicity

Saccharomyces cerevisiae cell wall is constituted by an inner layer, mainly composed by β-glucan and chitin, and a fibrilar outer layer constituted predominantly by α-mannan (highly glycosylated) associated with proteins (mannoproteins) (Klis *et al.* 2006). Flocculation is a surface characteristic. Heat-killed flocculent cells retain their flocculation ability (Machado *et al.* 2008), as well as cell walls prepared from flocculent cells (Sousa *et al.* 1992).

At physiological pH values, the yeast cell wall has net negative charge due to the ionization of carboxyl and phosphodiester groups of cell wall proteins and phosphomannans, respectively. The repulsion of charges of the same sign prevents cells from approaching sufficiently close and thus acts as an effective barrier to cell aggregation. As a consequence, cells remain dispersed in suspension at a distance of the order of 10 nm from each other (Dengis *et al.* 1995). The reduction of cell charge should facilitate cell–cell interactions and yeast flocculation. However, no clear relationship between yeast surface charge and the onset of flocculation was found (Dengis *et al.* 1995).

Conversely, a positive correlation between cell-surface hydrophobicity (CSH) and flocculation was found (Jin *et al.* 2001); CSH is partially responsible by the triggering of flocculation of brewing strains (Smit *et al.* 1992; Straver *et al.* 1993; Speers *et al.* 2006). Consistent with these results, other researchers described an increase of yeast surface hydrophobicity when Flo1, Flo5, Flo9, Flo10 and Flo11p are present in yeast cell wall (Verstrepen *et al.* 2001b; Govender *et al.* 2008; Mulders *et al.* 2009).

Lectin theory

Miki *et al.* (1982a) proposed that a specific lectin-like protein, only present on flocculent cells, recognizes and interacts with carbohydrate residues of α-mannans (receptors) of neighbouring cells (Fig. 2). Calcium ions enable the lectins to achieve their active conformation (Miki *et al.* 1982a; Stratford 1989) (Fig. 2a). While flocculation lectins are only present in flocculent cells, the receptors are present in flocculent and nonflocculent cells since the outer layer of *S. cerevisiae* cell wall is constituted by mannans. The analysis of the inhibitory action of sugars and the use of mannan synthesis mutants and Concanavalin A support that flocculation receptors are most likely the nonreducing termini of $\alpha(1 \rightarrow 3)$–linked mannan side branches, two or three mannopyranose residues in length (Stratford and Assinder 1991; Stratford 1992c). Adhesion forces of 121 ± 53 pN were measured between

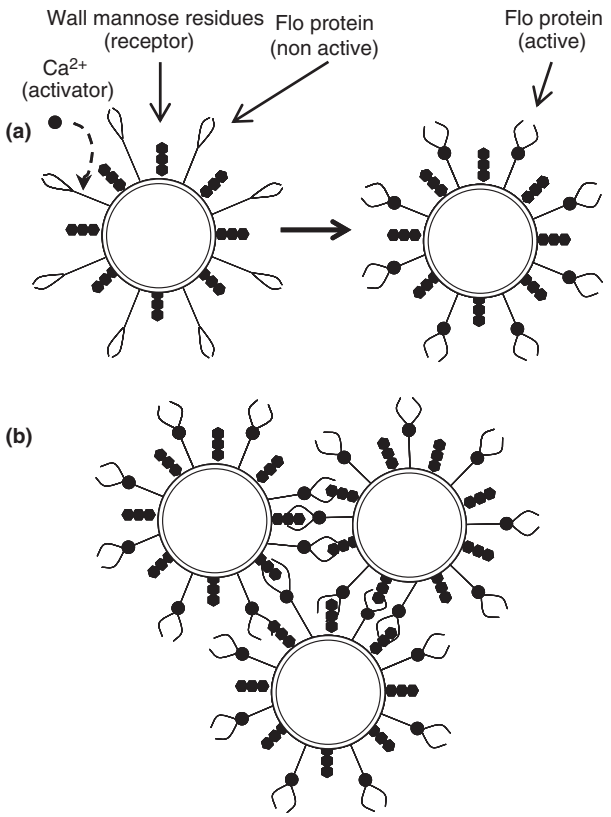

Figure 2 Lectin-like mechanism of *Saccharomyces cerevisiae* flocculation. (a) calcium ions enable the lectins to achieve their active conformation and (b) active flocculation lectins, only present in flocculent cells, stick out of the yeast cell wall and interact with mannose residues (receptors) on the neighbouring cell walls.

flocculation lectins and sugar residues (receptors) (Touhami *et al.* 2003). Most likely, besides specific (lectin-sugar) interactions, other nonspecific interactions, such as hydrogen bonds and hydrophobic interactions, should reinforce and stabilize the flocculent interactions (Dengis *et al.* 1995; Jin and Speers 2000; Jin *et al.* 2001).

Flo1 protein

The sequence of the open reading frame of *FLO1* gene predicts the presence of a protein of 1537 amino acids (Watari *et al.* 1994). Flo1 protein is a structural protein, located at the yeast cell surface, directly involved in the flocculation process (Bidard *et al.* 1995; Bony *et al.* 1997, 1998). Recently, it was also shown that Lg-Flo1p is the dominant flocculation protein in lager yeast strains; this protein is present in the yeast cell wall (Heine *et al.* 2009).

The amino acid sequence deduced from *FLO1* gene shows that Flo1 protein contains many repeated sequences, a large number of serine and threonine residues (which provide sites for O-glycosylation) and 14 potential N-glycosylation sites; the N- and C-terminal regions are more hydrophobic than the rest of the protein (Teunissen *et al.* 1993; Watari *et al.* 1994).

Cell wall proteins are synthesized at the endoplasmic reticulum, glycosylated intracellularly and transported to the cell surface by the secretory pathway. The glycosylphosphatidylinositol-proteins (GPI-proteins) are released from the plasma membrane by a processing step involving the GPI-anchor prior the attachment to the cell wall (Klis *et al.* 2006). In the case of Flo1p, this protein is anchored in the cell wall by a noncovalent stabilization (Bony *et al.* 1997); the hydrophobic C-terminal region of Flo1 protein seems to correspond to a GPI-anchor signal addition (Watari *et al.* 1994). Deletion of this hydrophobic region impairs the anchorage of protein to cell wall and results in the loss of flocculation (Bony *et al.* 1997).

The predicted secondary structure of Flo1 protein shows that this protein is almost composed of by β sheets and coils; the α-helix is only found at the N- and C-terminal regions (Watari *et al.* 1994). The O-glycosylation of serine and threonine residues enables the Flo1 protein to adopt an extended conformation, being the N-terminus exposed towards the cell surface (Teunissen *et al.* 1993; Watari *et al.* 1994). The deletion of the N-terminal region impairs the development of a flocculent phenotype (Bony *et al.* 1997). The N-terminal region of Flo1 protein contains the sugar recognition domain, which is important for flocculation definition of Flo1 and NewFlo phenotypes (Kobayashi *et al.* 1998).

In the middle part of *FLO1*, this gene is characterized by the presence of regions with high repeated sequences. A functional analysis revealed a linear correlation between the size of *FLO1* gene (which is determined by the number of repeated sequences) and the degree of flocculation (Bidard *et al.* 1995; Verstrepen *et al.* 2005). These repeats (repeated sequences) of *FLO1* gene seem to be highly instable and recombine at frequencies around 10^{-5} per generation; this results in loss or increase of repeated sequences with the consequent reduction or enlargement of the size of the gene, respectively (Rando and Verstrepen 2007). The changing of the number of repeats results in the modification of flocculation degree and sensitivity to stress conditions (Smukalla *et al.* 2008), as discussed in Flocculation: a social behaviour?. Additionally, it was suggested that the variation of the number of repeats can be the driving force behind the creation of novel Flo proteins; this results in an array of novel flocculation phenotypes, which enables yeast cells to adapt rapidly flocculation properties to a particular environment (Verstrepen *et al.* 2005; Verstrepen and Klis 2006; Dranginis *et al.* 2007). Surprisingly, it was proposed that the deletion of internal repeats of the *FLO1* gene is

responsible for the flocculation phenotype conversion from Flo1 to NewFlo (Liu *et al.* 2007).

Flocculation phenotypes

Taking into account the reversible inhibition of flocculation by sugars, salt, low pH value and proteases sensitiveness, two main flocculation phenotypes were distinguished: Flo1 and NewFlo phenotype (Stratford and Assinder 1991). Flo1 phenotype includes strains in which flocculation is specifically inhibited by mannose and derivatives. On the other hand, NewFlo phenotype contains the majority of brewery ale strains, which flocculation is reversible inhibited by mannose, maltose, glucose and sucrose but not by galactose. NewFlo phenotype strains are more sensitive to the inhibition by cations, low pH value and to the digestion by trypsin or proteinase K (Stratford and Assinder 1991). These phenotypes also display different sensitiveness to culture conditions, such as temperature (Soares *et al.* 1994), pH (Soares *et al.* 1994; Stratford 1996; Soares and Seynaeve 2000b) and nutrients availability (Soares and Mota 1996). The analysis of the N-terminal region of Flo1 protein, responsible to the Flo1 phenotype, has shown that the domain formed by tryptophan 228 and its neighbouring amino acid residues recognizes C-2 hydroxil group of mannose, but does not recognize the C-2 hydroxyl group of glucose. Similar analysis of the Lg-Flo1p, responsible to the NewFlo phenotype, revealed that the domain formed by leucine 228 and its neighbouring amino acid residues does not recognize C-2 hydroxyl group of mannose and glucose. On the other hand, threonine 202 most likely interacts with C-2 hydroxyl group of mannose and glucose allowing its recognition (Kobayashi *et al.* 1998).

Two other phenotypes have also been described: mannose insensitive (MI) phenotype, composed by strains in which flocculation is not inhibited by sugars, including mannose (Masy *et al.* 1992), and, a fourth phenotype, which flocculation only occurs in the presence of sufficiently high ethanol concentration (Dengis *et al.* 1995). The exact mechanism of aggregation of the strains belonging to these two phenotypes is far from being understood. However, a lectin mechanism, as was reported for Flo1 and NewFlo phenotype, seems to be excluded (Dengis *et al.* 1995).

Genetic basis of flocculation

Genes responsible for flocculation

Several dominant and recessive flocculation genes, as well as flocculation activators and suppressor genes, have been described. The genes that encode to flocculation lectins are called *FLO* genes. Among them, *FLO1* is the best known, which has been cloned and sequenced by different groups (Teunissen *et al.* 1993; Watari *et al.* 1994); *FLO1* is a dominant gene localized at 24 kb from the right end of chromosome I. Other *FLO* genes are *FLO2* and *FLO4*, which are alleles of *FLO1*. *FLO5*, *FLO9* and *FLO10* gene products are highly homologous to *FLO1*: 96, 94 and 58%, respectively (Teunissen and Steensma 1995). Four of the members of *FLO* gene family (*FLO1*, *FLO5*, *FLO9* and *FLO10*) are adjacent to their respective telomeres (Guo *et al.* 2000). The over expression of these four genes induces flocculation; nevertheless, the different *FLO* genes display different flocculation characteristics regarding the degree of flocculation and sugar sensitiveness (Govender *et al.* 2008; Mulders *et al.* 2009).

FLO1 is a structural gene for flocculation; it encodes a cell wall protein (Watari *et al.* 1994; Bony *et al.* 1997) and causes flocculation of a Flo1 phenotype (Kobayashi *et al.* 1998). In brewing lager yeasts, it was described that a new *FLO1* homologue, called Lg-*FLO1*, encodes to an adhesine responsible for the NewFlo phenotype (Kobayashi *et al.* 1998; Sato *et al.* 2002). In a lager-fermenting yeast, Lg-*FLO1* is located on chromosome VIII, at the same position as the *FLO5* gene of the laboratory strain of *S. cerevisiae* S288c (Ogata *et al.* 2008).

FLO8 gene encodes a transcriptional activator of *FLO1*, *FLO11* and *STA1* genes (Kobayashi *et al.* 1996, 1999). *FLO11* gene (also referred as *MUC1*) encodes a protein critical for pseudohyphal development and invasive growth (Lo and Dranginis 1998; Guo *et al.* 2000). It is also responsible for flocculation in *Saccharomyces diastaticus* but not in *S. cerevisiae* strains (Bayly *et al.* 2005). Flo11p enables yeast cells to adapt to nutritional stress conditions; in diploid cells, under nitrogen starvation, these yeast cells switch to pseudohyphal growth (Lo and Dranginis 1998), while in haploid cells, under glucose starvation, they may invade agar medium, a process called invasive growth (Cullen and Sprague 2000). Flo11 protein-dependent flocculation exhibits a Flo1 phenotype behaviour (Bayly *et al.* 2005).

In addition to the dominant genes, *flo3*, *flo6* and *flo7* genes have been described as recessive/semi-dominant genes (Teunissen and Steensma 1995). Many mutations [namely those involving regulatory genes (namely *TUP1* and *SSN6* genes), mitochondrial genes (*oli1* and *oxi2* genes) or genes involved in the cell wall biosynthesis (like *wal* and *abs* genes)] have been described to affect yeast flocculation; these mutations and their pleiotropic effects were reviewed by Teunissen and Steensma (1995).

Regulation of *FLO* genes

FLO genes seem to be regulated by upstream genetic elements. The transcription of *FLO1* and *FLO11* genes is

activated by Flo8 protein (Kobayashi *et al.* 1996, 1999). The common used laboratory strain S288C is a nonflocculent strain due to a defect in *FLO8* gene (Liu *et al.* 1996); the replacement of the defective *FLO8* gene by an intact version, or the overexpression of *FLO8*, enables the expression of *FLO1* and *FLO11* genes with the consequent restoring of flocculation and pseudohyphal growth in diploids, respectively (Bester *et al.* 2006; Fichtner *et al.* 2007). It was proposed that Flo8p seems to inactivate the *TUP1-SSN6*-encoded cascade, which represses flocculation in certain strains (Kobayashi *et al.* 1996; Liu *et al.* 1996). In addition, it has been shown that the protein kinase A (PKA) pathway and the transcription factors Mss11p and Flo8p, as reported above, are the major regulators of the expression of *FLO1* gene (Bester *et al.* 2006; Fichtner *et al.* 2007). Besides *FLO1* and *FLO11* genes, *FLO5* gene also seems to be dependent of transcriptional regulation (Govender *et al.* 2008).

Another possibility of genetic regulation mechanism involves the complex proteins associated with Set1 (COMPASS) methylation complex. Mutants with a defective COMPASS showed a flocculent behaviour because of the increasing amounts of *FLO1*, *FLO5* and *FLO9* transcripts; flocculation was calcium dependent and inhibited by mannose. It was proposed that COMPASS was involved in silencing the expression of *FLO1*, *FLO5* and *FLO9* genes (Dietvorst and Brandt 2008). Recently, it was shown that in addition to COMPASS, the histone deacetylase (Hda1) and histone acetyltransferase (Gcn5) proteins are required to repress *FLO* genes in high-gravity fermentations (Dietvorst and Brandt 2010). Histone deacetylases (HDACs) are transcriptional repressors; they reduce acetylation of histones, creating regions of repressed chromatin. Other histone modification enzymes, like Gcn5, have also been shown to be involved in silencing. *FLO1* gene is expressed in the absence of Had1, whereas the expression of *FLO1* and *FLO9* genes is induced in the absence of Gcn5 (Dietvorst and Brandt 2010).

Expression of *FLO11* has been shown to be controlled by several major pathways, including cyclic adenosine monophosphate/protein kinase A (cAMP/PKA), the mitogen-activated protein kinase pathway, nutritional sensing pathways, quorum sensing pathways and cyclins (Verstrepen and Klis 2006; Dranginis *et al.* 2007). In addition to transcriptional regulation, *FLO11* expression also seems to be under control at posttranscriptional level (Strittmatter *et al.* 2006; Fischer *et al.* 2008). It has also been shown that *FLO10* and *FLO11* genes are regulated epigenetically. The epigenetic state of *FLO11* is heritable and silenced by Hda1p; when *FLO11* is expressed, diploid cells form pseudohyphal filaments; when *FLO11* is silenced, the cells grow as yeast form (Halme *et al.* 2004). *FLO10* gene is regulated by the transcription factors Sfl1p and Flo8p (like *FLO11* gene) and silenced by different set of HDACs: Hst1p and Hst2p (Halme *et al.* 2004).

Factors affecting flocculation

Flocculation is a complex phenomenon influenced by a multiplicity of factors. Besides genetic characteristics of the strains (*FLO* genes, suppressors and activators), different parameters affect yeast flocculation. The puzzle becomes even more complex since some of these effectors can act at more than one level: *FLO* genes expression, secretion of the lectins or interaction between the flocculation lectins and the receptors on yeast cell wall (Fig. 3).

Cations

Cations have a central role on *S. cerevisiae* flocculation. Among them, Ca^{2+} is generally recognized as the most

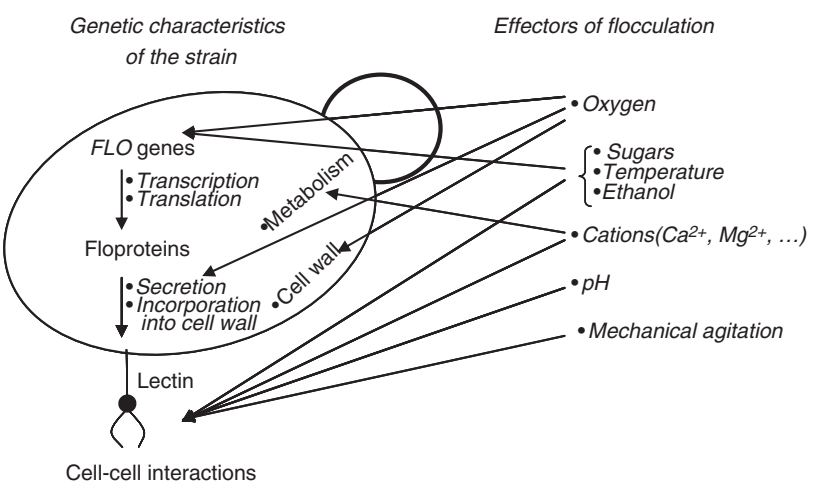

Figure 3 Diagrammatic representation of the multiplicity of factors that affect flocculation of *Saccharomyces cerevisiae*.

effective in the promotion of flocculation (Miki *et al.* 1982a; Stratford 1989). The amount required to induce the flocculation seems to be strain dependent; while for some strains, a trace amount of Ca^{2+} (10^{-5}–10^{-8} mol l^{-1}) (Taylor and Orton 1975) is enough to promote flocculation, other strains require a higher amount (5×10^{-4} mol l^{-1}) of Ca^{2+} (Soares and Seynaeve 2000b). It was shown that more important than the total Ca^{2+} concentration present in the media is the concentration of the available Ca^{2+} (i.e. free and labile Ca^{2+}) since this fraction is the only one that is able to induce the correct conformation of the lectins; the available Ca^{2+} concentration is influenced by the pH of the medium and the presence of complexing compounds in solution (Soares and Seynaeve 2000b).

Rb^{+}, Cs^{+}, Fe^{2+}, Co^{2+},Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+} and mainly Mg^{2+} and Mn^{2+} have also been described as flocculent inducers (Miki *et al.* 1982a; Nishihara *et al.* 1982; Sousa *et al.* 1992; Soares *et al.* 2002). On the contrary, cations like Ba^{2+}, Sr^{2+} and Pb^{2+} competitively inhibit yeast flocculation because of the similarity of their ionic ratio to Ca^{2+} (Nishihara *et al.* 1982; Gouveia and Soares 2004). Possibly, these cations compete for the same 'calcium site' of flocculation lectins, but are not able to induce the correct conformation of the lectins.

At low concentration, Na^{+} and K^{+} induce flocculation most likely because of the reduction of the electrostatic repulsive forces between the yeasts and/or stimulate the leakage of intracellular Ca^{2+} (Nishihara *et al.* 1982; Stratford 1989). At high concentrations, it seems that these ions provoke the distortion of the lectins and antagonize the calcium-induced flocculation (Stratford 1992b). Besides the surface action, the presence of cations in the culture medium (namely Mg^{2+} or the Ca^{2+}/K^{+} ratio), as micro-nutrients, seems to be essential for the expression of flocculation (Smit *et al.* 1992; Stratford 1992b).

pH of the medium

pH of the medium can have a deep effect on yeast flocculation. For several laboratory and industrial strains, flocculation occurs over a wide pH range (2·5–9·0), while many brewing strains (a sub-group of NewFlo phenotype) only flocculate within a narrow pH range (2·5–5·5) (Miki *et al.* 1982a; Dengis *et al.* 1995; Stratford 1996; Soares and Seynaeve 2000b). In both cases (strains with a wider or narrower pH range), the optimum pH value takes place between 3·0 and 5·0 according to the strain. Extreme pH values promote a reversible dispersion of the flocs. Probably, the modification of the pH value alters the ionization of the amino acids of the flocculation lectins with the consequent change of its conformation (Jin and Speers 2000; Jin *et al.* 2001).

Many ale strains do not flocculate in chemically defined media yeast nitrogen base (YNB) and also do not flocculate or flocculate poorly in rich media containing yeast extract, peptone, dextrose (YEPD); these strains are only flocculent in wort. Thus, it was proposed that these strains require the addition of nitrogen compounds (gelatine, peptones or yeast extract) in order to flocculate (Stewart and Russell 1981). More recent works have shown that the lack of flocculation observed could be explained by the narrow pH range of flocculation of these strains (Stratford 1996; Soares and Seynaeve 2000b) plus the limited available Ca^{2+}in solution (Soares and Seynaeve 2000b). In reality, YNB has a small buffer capacity, and consequently the pH falls rapidly to near 2·2 during yeast growth while in YEPD, the pH at the end of the growth is near 5·5–6·0; both pH values do not correspond to the pH range where these strains flocculate. The pH of the culture medium explains the lack of flocculation observed in YNB or in YEPD (Soares *et al.* 1994; Stratford 1996; Soares and Seynaeve 2000a; b). For these strains, flocculation in these culture media can be restored by correcting the pH of the media to an appropriate value and, for some of them, by simultaneous addition of calcium (Stratford 1996; Soares and Seynaeve 2000b).

Temperature

Temperature can act at different levels on yeast flocculation process (Fig. 3). The lowering of growth and fermentation temperature leads to a decrease in yeast metabolism and CO_2 production; as a consequence, there is a reduction of the turbulence, which favours yeast sedimentation. It was proposed that during beer fermentations, the agitation (shear force), caused by CO_2 production, exerts a major influence on the number of cells in suspension (Speers *et al.* 2006). Temperature can also affect yeast flocculation by acting on cell–cell interactions. A rise in temperature to 50–60°C, for a few minutes, promotes the reversible dispersion of the flocs (Taylor and Orton 1975) most likely because of denaturation of flocculation lectins. The incubation of the yeast strains at a supra-optimum temperatures (35–37°C) leads to a reduction (Soares *et al.* 1994) or impairment of yeast flocculation (Claro *et al.* 2007). A brief heat shock (52°C, 5 min) in a NewFlo strain, in exponential phase of growth, delayed the onset of flocculation (Claro *et al.* 2007), most likely by affecting direct or indirectly *FLO* genes expression.

Oxygen

A moderate aeration is beneficial for yeast flocculation while a strong aeration (Kida *et al.* 1989; Soares *et al.*

1991) or anaerobic conditions (Miki *et al.* 1982b; Soares *et al.* 1991; Straver *et al.* 1993) provoke the loss of flocculation. These observations are in agreement with the fact that cell wall mannoproteins are differently expressed under aerobic or anaerobic conditions (Abramova *et al.* 2001). It was proposed that the transition from aerobiosis to anaerobiosis, which occur in brewing conditions, may be associated with modifications in the expression of the genes that regulate or encode to flocculation lectins (Gibson *et al.* 2007).

Sugars

Sugars can affect yeast flocculation acting on cell–cell interactions, at surface level and on the regulation of *FLO* genes (Fig. 3). In the first case, sugars promote the reversible dispersion of flocs since they compete with the flocculation receptors (the sugars of yeast cell wall) for the flocculent lectins. Strain-sensitiveness to different sugars is the base of the distinction of Flo1 and NewFlo phenotypes. Furthermore, fermentable sugars, including those found in wort, induce the loss of flocculation in the early phase of growth (Soares *et al.* 2004) or in starved cells (Soares and Duarte 2002; Soares and Vroman 2003) most likely affecting the expression of *FLO* genes.

Ethanol

Ethanol seems to have a positive effect on yeast flocculation. However, the mechanism through which ethanol exerts a positive influence remains unclear. According to one of the possibilities, the adsorption of ethanol to yeast surface causes a reduction of the local dielectric constant and originates a decrease of cell–cell electrostatic repulsion (Dengis *et al.* 1995). It was also proposed that the presence of ethanol can allow the protrusion of polymer chains into the liquid phase, which carry binding sites for nonspecific (hydrogen bonds) or specific interactions (Dengis *et al.* 1995).

Lager strains seems to be less hydrophobic than ale strains, and NewFlo phenotype strains had higher CSH than Flo1 phenotype strains (Jin *et al.* 2001). A more marked effect of ethanol on the flocculation of yeasts with a strong surface hydrophobicity was observed. This effect was attributed to the fact that for these strains, organic compounds are better solvents than water (Dengis *et al.* 1995; Jin and Speers 2000). A slight increase of CSH with the increase of ethanol concentration in culture medium was also described (Jin *et al.* 2001).

Besides the surface action, ethanol seems to act on the expression of *FLO* genes. Flocculent cells when incubated in ethanol (4%, v/v), as sole carbon source, in growing or starvation conditions, never loss flocculation ability (Soares and Vroman 2003; Soares *et al.* 2004). However, high ethanol concentration (10%, v/v) impairs flocculation expression in a brewing ale strain most likely because of ethanol toxicity (Claro *et al.* 2007).

Genealogical and cultural age

In *S. cerevisiae*, after the cell division, the daughter cell leaves in the mother cell a birth scar. The number of bud scars presented on a yeast wall represents the cell genealogical age. In brewing practice, yeast cells are maintained and reused in many fermentation cycles (7–20 times). It was shown that virgin (daughter cells with genealogical age zero) and nonvirgin cells are both flocculent; irrespective of the brewing yeast classification (ale/lager), aged cells are more flocculent than the younger counter parts (Powell *et al.* 2003). Thus, the bottom part of the yeast crop could be composed by more aged and flocculent cells; this fact could explain the increase of flocculation with the brewing fermentation cycles (Powell *et al.* 2004).

In respect of the effect of growth phase (cultural age) on flocculation, two main types of yeast cells can be found: (i) cells that never lose its flocculation ability (constitutive flocculent strains) and thus are not affected by the cultural age and (ii) strains that flocculation change with growth phase (inductive flocculent strains – NewFlo phenotype). In the last group of strains (for more details, see Inductive flocculent strains), although aged cells could be more flocculent than virgin cells, the onset of flocculation observed towards the end of exponential phase of growth can hardly be explained by the difference of flocculation ability among the cells or by the increasing of the proportion of aged cells. Consistent with this possibility, it was shown that the distribution of the genealogical age of the cell population of an ale-brewing strain throughout the growth was basically identical; in all phases of growth, there were 44–54% of daughter cells (Soares and Mota 1996). Thus, it was suggested that daughter and parent (old) cells should be flocculent or nonflocculent depending of the growth phase rather than their genealogical age (Soares and Mota 1996). In NewFlo phenotype strains, the growth phase is the determinant factor in the definition of the flocculent ability of the cells and consequently in the flocculent state of the culture.

Cell density and mechanical agitation

It was shown that little or no flocculation occurs at cell concentrations below a minimum threshold concentration (Miki *et al.* 1982b). The presence of a low number of cells per millilitre limits the physical possibility of establishing a flocculent bond, and the rate of aggregation is practically zero (Soares and Mota 1997). At high cell

concentrations ($>4 \times 10^7$ cells ml^{-1}), the number of cells remaining in suspension is constant and independent of the initial cell concentration (Soares and Mota 1997).

In addition, a minimum threshold of agitation is required for flocculation to occur (Stratford 1992b). Mechanical agitation allows increasing the kinetic energy of cells to overcome the mutual repulsion among the cells; as a consequence, there is contact between the cells and flocculation-bond formation.

Metabolic control of flocculation

Constitutive flocculent strains

In a general way, the strains belonging to Flo1 phenotype are constitutively flocculent, being the flocculation expressed throughout the cell growth (Fig. 4a). During growth, the flocculation of these strains seems not to be affected by the variation of the physiological state of the cells and are immune to the presence of nutrients of the culture medium, namely carbon and nitrogen sources (Soares and Mota 1996; Soares and Vroman 2003). These strains can also flocculate over a wide range of pH values (Stratford and Assinder 1991; Soares *et al.* 1994). Together, these flocculation characteristics make Flo1 phenotype strains particularly suited to be used in bioreactors, in a continuous mode, without the risk of biomass washout, as the cells are self-immobilized in pellets (Domingues *et al.* 2000b).

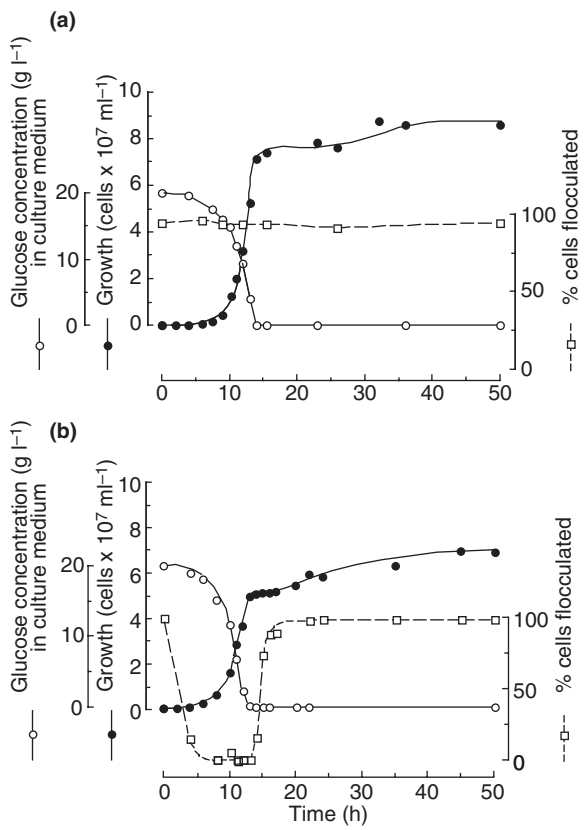

Figure 4 Flocculation of constitutive (Flo1 phenotype) and inductive (NewFlo phenotype) strains. (a) The flocculation of the strain of *Saccharomyces cerevisiae* NCYC 869 (Flo1 phenotype) is expressed during all grow phases, being insensitive to the presence of nutrients. (b) *S. cerevisiae* NCYC 1195 (NewFlo phenotype) shows the loss of flocculation in the early of growth and the onset of flocculation occurs at the end of exponential growth, when there is a shortage of nutrients (glucose reach the lowest concentration in the culture medium). Flocculation was determined under standardized conditions. Source: Soares and Mota (1996).

Inductive flocculent strains

The majority of brewer yeast strains belong to the NewFlo phenotype (Stratford and Assinder 1991) and possess cyclic flocculation ability (Soares and Mota 1996). The flocculent cells, when placed in fresh wort or laboratory culture medium, lose progressively the flocculation ability and become flocculent towards the end of logarithmic phase of growth (Fig. 4b).

Loss of flocculation

In the beginning of growth, the declining of flocculation of NewFlo phenotype strains can be attributed to a loss, blockage or inactivation of flocculation lectins or the receptors. However, it was shown that flocculation receptors are available in all stages of growth of these strains (Stratford 1993; Soares and Mota 1996); this means that flocculation cycle is dependent of the presence of active flocculation lectins (Stratford and Carter 1993).

Besides the surface level action described earlier, metabolizable sugars have a central role in the repression of flocculation (Soares and Duarte 2002). Flocculent cells incubated in 2% (w/v) of glucose, maltose or galactose, as sole nutrient (starved conditions), showed a progressive loss of flocculation, whereas other nutrients (nitrogen, sulphur sources or other minor nutrients) were unable to repress flocculation (Soares and Vroman 2003). Similarly, the growth of Newflo phenotype strains in media containing 2% (w/v) of glucose, fructose, galactose, maltose or sucrose induces the loss of flocculation in the early growth (Soares *et al.* 2004). Conversely, ethanol and glycerol (4%, v/v) did not induce the flocculation loss of starved or growing cells; these results indicate that the loss of flocculation is a process that requires energy and is influenced by the carbon source metabolism (Soares and Vroman 2003; Soares *et al.* 2004).

The loss of flocculation seems to be the result of two combined effects: the nonflocculent state of the new cells produced and the dismantling of the flocculation mechanism of the cells coming from the inoculum (Soares and

Mota 1996). The last effect requires *de novo* protein synthesis since the addition of cycloheximide to cells impairs the flocculation loss induced by glucose. Different proteases also seem to be involved as the presence of the protease inhibitor phenylmethylsulphonyl fluoride or the chelator EDTA (which inactivate metalloenzymes) prevented partially or totally, respectively, the flocculation loss induced by glucose (Soares and Vroman 2003; Soares *et al.* 2004).

Onset of flocculation

The onset of flocculation requires *de novo* protein synthesis; the addition of cycloheximide, which inhibits cytoplasmatic protein synthesis at the ribosomal level in eukaryotes, to cells in a nonflocculent state, impairs the onset of flocculation (Stratford and Carter 1993).

In a general way, the triggering of flocculation of NewFlo phenotype strains occurs at the end of exponential phase of growth (Fig. 4b), when a critical nutrient, like sugars (glucose, fructose or maltose) (Soares and Mota 1996; Sampermans *et al.* 2005), in ale strains, or nitrogen source (Smit *et al.* 1992), sterols or unsaturated fatty acids (Straver *et al.* 1993), in a lager strain, are almost depleted from the culture medium. The increase of the initial concentration of glucose of the culture medium provokes a delay in the expression of flocculation (Soares and Mota 1996). On the other hand, cells under catabolic repression when transferred to a medium with a lower sugar concentration have shown a rapid triggering of flocculation; this fact suggests a casual link between sugar limitation and the induction of flocculation (Sampermans *et al.* 2005).

In NewFlo phenotype strains, nutrient availability seems to control *FLO* genes expression (Verstrepen and Klis 2006). As it was described earlier, *FLO* genes can be regulated, at the transcriptional level, by the proteic complex Ssn6-Tup1 (Teunissen *et al.* 1995). Another possibility of flocculation regulation is associated with the so-called 'main glucose repression pathway'; once inside the cell, glucose is phosphorylated leading to inactivation of the central Snf1 protein kinase, which represses flocculation (Verstrepen and Klis 2006).

The flocculation expression is an energetic-dependent process, which requires the presence of an residual external energy source (Soares and Mota 1996), but not an external source of nitrogen. Nonflocculent cells, in exponential phase of growth, when placed in a culture medium without carbon source do not express flocculation; on the contrary, these cells when transferred to a complete medium except nitrogen source developed a flocculent phenotype (Sampermans *et al.* 2005).

The triggering of flocculation seems to be influenced by the carbon source metabolism. The presence of small amount of sugars or ethanol allows the onset of flocculation while the presence of glycerol impairs the expression of flocculation (Sampermans *et al.* 2005). Small amounts of ethanol have a positive effect on the triggering of yeast flocculation. The presence of 1% (v/v) of ethanol induces an early development of flocculation in cells growing in low amounts [0·2% (w/v)] of fermentable carbon source. The nutrients shortage combined with the presence of ethanol may be the signal nutrient that induces the onset of flocculation (Sampermans *et al.* 2005; Claro *et al.* 2007).

Industrial application of yeast flocculation

The solid–liquid separation process

Saccharomyces genus, especially *S. cerevisiae* and its relatives, included in the *Saccharomyces sensu stricto* complex (Vaughan-Martini and Martini 1998), is traditionally associated with the production of alcoholic beverages and bread (Fleet 2006); nowadays, *S. cerevisiae* is used in the production of bio-ethanol (more than 2×10^{10} l per year) [used as solvents for pharmaceutics or as a renewable energy source (Strehaiano *et al.* 2006)], food ingredients (Fleet 2006) and in environmental applications like industrial wastewater treatment (Wang and Chen 2006). The use of recombinant DNA technology allowed the expansion of industrial exploitation of *S. cerevisiae*, as a cell factory, for the production of heterologous products like human hormones and vaccines (Demain *et al.* 1998).

After yeast fulfilled its function, cells must be separated from the fermenting broth for further processing, in the so-called downstream processing. The problem of biomass separation is basically associated to its small size and density. In fact, yeast cells density is only slightly higher than the reaction liquors in which they are suspended. Conventional solid–liquid separation methods include sedimentation, filtration or centrifugation; these methods tend to be time consuming and/or expensive, which prohibit their use in many industries (Hatti-Kaul and Mattiasson 2001). The sedimentation process requires large settling tanks and extensive retention times to obtain a cell-free supernatant. Alternatively, cells can be removed by filtration using continuous rotary vacuum filters (Shuler and Kargi 2002); the expenditure associated with the equipment (capital investment of filter systems as well as the energy consumption for filter operation) increases the costs of the overall process and hinders their use in many industries, particularly those that produce low-cost/high-volume products. Separation of yeast cells from fermented medium can also be obtained by centrifugation; the two popular industrial centrifuges are disc stack and tubular bowl centrifuges (Shuler and Kargi

2002). Nevertheless, centrifugation can have a negative impact on yeast cells (see below The lack of flocculation in brewing yeast strains); in addition, this separation process is considered a relatively expensive method taking into account the costs of maintenance and power demand per quantity of micro-organisms removed (Hatti-Kaul and Mattiasson 2001). For example, the capital investments for centrifuges and the energy consumption for centrifuge operation hinder the use of this equipment in the production of bio-ethanol (Xu *et al.* 2005).

Another possibility is the precipitation of yeast cells by the addition of natural (chitosan) or synthetic (cationic or anionic) polymers (Weir *et al.* 1993; Kim *et al.* 2001; Wickramasinghe *et al.* 2010), inert powder (like nickel particles) (Weeks *et al.* 1983), lectin Concanavalin A (Stratford and Bond 1992), by the adhesion of fine magnetic particles followed by magnetic separation (Dauer and Dunlop 1991) or by co-flocculation with flocculent *S. cerevisiae* cells (Mortier and Soares 2007). Polyelectrolytes and polymeric particles provoke yeast settling by interfering with cell-surface charge; however, the addition of these compounds is not allowed in food industry because they are non-food-grade constituents.

In order to overcome the problem of solid–liquid separation processes associated with free cells, different processes of cell immobilization have been attempted namely: gel entrapment, adsorption to an inert support or entrapment within a porous matrix (Kourkoutas *et al.* 2004). Besides the cost associated with the immobilization process, differences in the metabolic activity among free cells and immobilized micro-organisms have been reported; these differences are probably due to the modification of the physical and chemical environment of immobilized cells or to a change in cell physiology induced by immobilization (Pilkington *et al.* 1998; Shen *et al.* 2003). The entrapment of yeast cells also inflicts mass transfer and diffusional limitations; these limitations cause gradients of oxygen, substrates and products in an extension which depends on the type of polymer used, porosity, texture and immobilized complex size (Strehaiano *et al.* 2006).

Yeast flocculation is a spontaneous process of auto-immobilization. In this context, it emerges as an easy, off-cost, fast and eco-friendly process of cell separation. Yeast flocculation also presents other advantages, namely: (i) the use of high density cell reactors, which leads to high volumetric productivity and shorter fermentation times (Teixeira *et al.* 1990); (ii) the use of different configurations of suspended biomass reactors without the risk of biomass washout (Domingues *et al.* 2000b); (iii) when used in a bioreactor operating in a continuous mode, it presents a lower risk of external contamination because of the high metabolic activity (Domingues *et al.* 2000a). Together, these advantages fit quite well in the concept of 'White' Biotechnology, which advises the optimization of the biotechnological processes with the reduction of wastes and energy consumption and contributes to a more sustainable future (Paula and Birrer 2006). A promising example of flocculation characteristics application is the production of renewable fuels (ethanol) (Zhao and Bai 2009). It is important to point out that yeast flocculation can have an importance beyond the cell separation process; this is the case of brewing industry, where the failure of yeasts to flocculate can adversely affect the beer flavour characteristics, as discussed later.

Brewing industry

Flocculation and beer production

The brewing process starts with the inoculation of yeast cells in the sweet wort (in beer terminology known as *pitching*). The yeast cells should grow, metabolize the sugars into alcohol (called *wort attenuation*) and impart the desired flavour to the beer. Subsequently, yeasts must be removed from the fermented wort by flocculation or by centrifugation, producing a 'green' or immature beer. For the brewer, two important aspects related with the yeast flocculation characteristics are considered: (i) flocculation degree, which affects yeast cell separation process efficiency; (ii) the time, during the fermentation cycle, that yeast culture flocculates (the onset of flocculation), which is the most important aspect (Stewart and Russell 1981). Flocculation allows an easy and fast remove of yeast cells for future re-use (*repitching*), usually after a washing step; in addition, flocculation reduces the cell concentration in the green beer for further flavour maturation and achievement of colloidal stability (a process called *conditioning*, *ageing* or *laggering*).

Typically, ale-brewing strains are classified as *S. cerevisiae* (Vaughan-Martini and Martini 1998) and rise to the surface of the fermenter, probably adsorbed to the rising carbon dioxide bubbles (top fermentation); yeasts are removed from the fermented wort and skimmed off. In contrast, lager strains, classified as *Saccharomyces pastorianus* (Vaughan-Martini and Martini 1998) and also referred as *Saccharomyces carlsbergensis* because of historical reasons (Stewart 2009), sediment in the bottom of the fermenter (bottom fermentation). Traditionally, lager fermentation is distinguished from ale fermentation by the bottom-cropping of yeast cells and lower fermentation temperatures. In the vertical cylindrico-conical fermentation vessels, yeasts with increased flocculation ability, independently of being ale or lager, tend to sediment in the cone of the fermenter, where they are cropped as concentrated slurry at the end of fermentation.

Premature yeast flocculation

A recurring problem observed in the brewing industry is the incomplete attenuation of the worth. This leads to 'hung' or 'stuck' fermentations, which can be attributed to a premature yeast flocculation (PYF); this fact affects beer quality since it results in sweeter beers with low alcohol contents. The most common causes underling PYF are as follows: (i) a successive selection of yeast cells with an increased flocculation ability; (ii) bacterial infection (Stratford 1992b) or (iii) by the action of wort components, more specifically high molecular weight polysaccharides rich in arabinose and xylose (Herrera and Axcell 1991). Recently, it was proposed that a malt barley factor, responsible for the induction of PYF, is a complex polysaccharide composed of a highly substituted glucurono-arabinoxylan-associated arabinogalactan protein with rhamnogalacturonan I (Koizumi *et al.* 2009). It was also postulated that the high molecular weight polysaccharides are generated by enzymatic degradation of barley husk because of fungal contamination during malting process (Van Nierop *et al.* 2004). These complex polysaccharides have a higher affinity for yeast flocculation lectins than sugars present in the medium; consequently, they trigger an early flocculation by acting as a bridge between cells (Koizumi *et al.* 2008).

After a number of consecutive fermentations (generations), a moderate flocculent strain can become progressively more flocculent. The increase of yeast flocculation ability has been attributed to a selection of more aged cells (see above – genealogical and cultural age) or to the accumulation of respiratory-deficient mutants with higher flocculation ability (Smart 2007).

The lack of flocculation in brewing yeast strains

Among many brewing properties, yeast flocculation is generally seen as the most instable property. A flocculent strain can gradually lose its aggregation characteristics and become powdery after successive generations. It was suggested that this behaviour can be attributed to genetic alterations, such as, chromosome deletion, Lg-*FLO1* C-terminal region deletion, partial deletion of the middle region of the Lg-*FLO1* gene or some defects in the post-transcriptional regulations (Sato *et al.* 2001). These genetic alterations of Lg-*FLO1* result in nonflocculent cells. It was proposed that nonflocculent cells can grow dominantly through successive cultivation. However, genetic instability of brewing yeasts seems to be strain dependent. Although some brewing yeasts are vulnerable to a genetic drift, other strains can remain stable over a serial repitchings. A study carried out by Powell and Diacetis (2007), which used ale and lager strains, did not show genomic variations or modifications of fermentations characteristics (flocculation and time required to achieve wort attenuation) over the course of 98 or 135 serial repitchings, respectively.

For the brewer, a delay or a failure of yeast flocculation can cause problems in beer clarification and in obtaining a bright sparkling beer. Moreover, off-flavours can often result during the ageing process because of yeast autolysis (Stewart and Russell 1981). Nowadays, the lack or insufficient flocculation can be minimized or overcome by using separation technologies. Among them, the use of disc stack centrifuge has become popular. However, the passage of yeast cells through the centrifuge exposes cells to mechanical and hydrodynamic shear stresses. It was shown that centrifuged cells exhibited lower viability and vitality. Centrifugation leads to a depletion of intracellular glycogen and trehalose yeast levels, induces the release of yeast cell wall mannan (an inducer of beer haze) and increases proteinase A activity, which results in a decrease of beer foam stability (Chlup *et al.* 2008). Thus, the use of centrifuges for cropping nonflocculent cells at the end of primary fermentation, besides the increase of operating costs, has a negative impact on yeast viability and vitality. As a consequence, a decrease of beer physical stability and quality can occur.

Wine, distilled beverage and bottle-fermented sparkling wine production

Yeast flocculation has also been exploited in the manufacturing of other alcoholic beverages. In wine production, grape fermentation can be carried out by the native flora present in the grapes or in the winery. As an alternative, the fermentation can be performed in more controlled conditions with a pure selected yeast strain (Pizarro *et al.* 2007). The ability to flocculate is one of the desirable characteristics that a wine strain should present (Pretorius 2000; Vilanova and Massneuf-Pomarede 2005). In wine production, several clarification strategies are usually performed such as sedimentation and filtration. Yeast flocculation facilitates enormously their removal from the fermented product in the tank or barrel, allows a rapid clarification and reduces the handling of wine. Moreover, yeast flocculation seems to be associated with the enhancement of ester production (Pretorius 2000).

Cachaça is a very popular alcoholic beverage in Brazil and is produced by batch fermentation of sugar cane, mainly by *S. cerevisiae*. Subsequently, the sugar cane wine is distilled in copper alembics. Like in brewing production, yeasts are reused in several fermentations batches; thus, it is not surprising that flocculent strains seem to be well suited for use, as a starter, in the production of high quality cachaça (Silva *et al.* 2009).

In the elaboration of sparkling wines, such as Champagne, performed by the so-called *Method Champenoise*,

after an initial fermentation generally placed in stainless steel tanks, a second fermentation occurs in the bottle itself. During this process, the bottles are inverted and cells sediment on the bottle neck. In this secondary fermentation, the use of flocculent cells is desirable since it facilitates enormously the skilled process of cell sediment removing from the bottle, without removing the wine (a process called *dégorgement*) (Valles *et al.* 2008).

In the production of the alcoholic beverages described earlier, the control of the onset of flocculation is of paramount importance. It is desirable that yeast cells remain in a nonflocculent state during the fermentation process, for a rapid fermentation rate, and at the end of sugar conversion an efficient sedimentation of the cells to simplify the problems of wine, cachaça or sparkling wine clarification (Dequin 2001; Pretorius and Bauer 2002); simultaneously, flocculation reduces the production costs (less energy consumed, becoming a 'greener' process) and can improve the quality of final products.

Production of bio-ethanol

Yeast flocculation seems to be well suited in the production of high volume products, like renewable fuels. Bio-ethanol, as a low value added product, is very sensitive to costs of production. The bio-ethanol industry uses a batch process, particularly in plants with small production capacity and cells are usually removed by centrifugation. The immobilization of yeast cells by gel entrapment seems not to be attractive (Bai *et al.* 2008). On the other hand, the use of flocculent cells, as a self-immobilization process, is a very promissory alternative since it reduces the expenses associated with the capital costs for centrifuge, energy consumption and centrifuges maintenance and makes the process more competitive (Zhao and Bai 2009; Sivakumar *et al.* 2010). It is estimated that the use of yeast flocculent cells allows an economy of 16% of processing costs and 10% of installation costs (Andrietta *et al.* 2008). Besides to facilitate cell-broth separation process, flocculent cells can be used in high density cell reactors, which improves ethanol productivity and reduces fermentation time.

At laboratory scale, several configurations of bioreactors, which use flocculent cells, have been developed for continuous ethanol production: bubble, columns, airlift reactors, packed and fluidized beds. The first ones are the most widely used (Domingues *et al.* 2000b; Bai *et al.* 2008). Most of the work has been carried out using constitutive flocculent strains in reactors operating in a continuous mode. In 2005, the operation of a fuel ethanol plant, which uses flocculent cells, with an annual capacity of production of 20 000 tons has been started; the plant consists of six bioreactors, each one working with a volume of 1000 m^3, arranged in a cascade mode (Bai *et al.* 2008).

Recently, two works described the production of bio-ethanol, in a batch mode, using a flocculent yeast strain. This process seems to be very promising due to the simplicity of process configuration, easy cell recycling and stability of operation. In one of them, ethanol was obtained from liquefied cassava medium using repeated-batch fermentations (Choi *et al.* 2009); the process allows an enhancement of volumetric ethanol productivity with a simple modification of the original batch fermenter. In another work, ethanol was produced under very high gravity medium conditions (Li *et al.* 2009). As in brewing industry, the use of a NewFlo phenotype yeast strains seems to be appropriate for successive batch ethanol fermentation. The strains are nonflocculent during the fermentation phase (avoiding diffusional limitations across the flocs) and develop flocculation towards the end of fermentation. In a flocculated state, cells are rapidly separated from the fermented broth and, simultaneously, flocculation can provide protection against ethanol stress, preventing them from loosing viability.

Production of heterologous proteins

Flocculent yeast strains have been constructed in which heterologous proteins were displayed on the yeast cell surface, such as lipase (Matsumoto *et al.* 2002), α-amylase or glucoamylase (Kondo *et al.* 2002; Seong *et al.* 2006). Yeasts displaying these enzymes on their cell surface can be used as whole-cell biocatalysts in several applications, namely in the production of bio-diesel (Matsumoto *et al.* 2002) or bio-ethanol (Kondo *et al.* 2002; Seong *et al.* 2006). Biodiesel can be produced from waste oil; flocculent cells displaying high lipase activity on their surface were used in transesterification reaction in a solvent-free system (Matsumoto *et al.* 2002). A critical step in bio-ethanol production from starchy materials is their hydrolysis because *S. cerevisiae* can not directly use starch. The use of a recombinant strain displaying amylolytic enzymes on their surface allows a single-step fermentation, wherein starchy materials are hydrolysed and subsequently converted to ethanol; at the end of fermentation, cells are recovered without centrifugation because of their sedimentation characteristics (Kondo *et al.* 2002; Seong *et al.* 2006). The production of an extracellular heterologous β-galactosidase, using recombinant flocculent cells, was also described (Domingues *et al.* 2005). The construction, through recombinant DNA technology, of flocculent yeasts in which heterologous proteins, like enzymes, vitamins or co-factors will be displayed or secreted, opens a new opportunity of exploiting the advantages of yeast flocculation.

Bioremediation of heavy metals

Heavy metal pollution, mainly due to industrialization, is a cause of concern particularly in developed countries. Conventional technologies, such as precipitation, ion exchange and reverse osmosis are not efficient or often economically prohibitive for the treatment of large volumes of wastewaters containing relative low metal concentrations. Several types of biomass, including yeast cells, have been proposed as the base of a new technology. *Saccharomyces cerevisiae* yeast cells constitute a good alternative, mainly because of its availability (can be obtained in high amount at low-cost as a by-product of fermentation industries) and capacity to accumulate a broad variety of heavy metals under a wide range of external conditions (Wang and Chen 2006).

Flocculent yeast cells are able to flocculate in a single or multi-element solutions containing Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+} or Cr^{3+} (Soares *et al.* 2002; Machado *et al.* 2008). Among the most common heavy metals, Pb^{2+} inhibits competitively yeast flocculation; however, as it was reported above (Cations), small amounts of Ca^{2+} strongly alleviate Pb^{2+} inhibitory effect (Gouveia and Soares 2004). This means that the presence of a small amount of calcium, in the effluent, is enough to revert the Pb^{2+} inhibition; these results evidence that flocculation of yeast cells occurs in the great majority of industrial effluents. Flocculent yeast cells have a higher ability of metal accumulation than nonflocculent ones; most likely, heavy metals occupy lectin Ca^{2+}-binding sites with the consequent increase of metals removal (Soares *et al.* 2002). Recently, it was shown that heat-inactivated flocculent yeast cells display a higher metal accumulation than live cells and retain their flocculent characteristics (Machado *et al.* 2009). Flocculent cells of *S. cerevisiae* seem to be a promissory type of biomass for the bioremediation of wastewaters containing heavy metals, as they combine the ability to remove efficiently a wide variety of metals from synthetic (Machado *et al.* 2008, 2010a) and real effluents containing multi-elements (Machado *et al.* 2010a,b,c) with a fast separation of biomass after effluent treatment (Machado *et al.* 2008); flocculation overcomes the need of cell immobilization or the use of a solid–liquid separation technique, which is a critical issue when we are looking for an effective and low-cost technology.

Flocculation: a social behaviour?

A floc, when suspended in a liquid culture medium, presents a negative gradient of nutrients from the periphery to the centre; therefore, cells in the centre of the floc can suffer from nutrients limitations and accumulation of waste products. Although undoubtedly beneficial to man, for their own cells, what is the advantage of flocculation?

Strains in which flocculation is regulated, like the typical brewing yeasts belonging to the NewFlo phenotype, when placed in the presence of abundant nutrients, like sugars, lose their flocculation ability. Under these conditions, these cells are preferentially under the form of free-living unicellular organisms. On the other hand, nutrients limitations, probably combined with the presence of ethanol, induce the onset of flocculation. It means that in the presence of nutrients shortage, cells aggregated in flocs do not have anything to lose, under a nutritional point of view. In addition, cells can cooperate within a multicellular structure (floc) when the environment becomes exhausted of nutrients. The cooperation may be the basis of the strength of the cell community. In an evolutionary and selective adaptation point of view, yeast cells with the ability to form flocs are in advantage; the autolysis of some cells of the centre of the floc will originate compounds (proteins, carbohydrates and vitamins), which can support the survival of the other cells of the floc. In agreement with this perspective, it was proposed that yeast cells commit altruistic suicide (apoptosis – programmed cell death) in order to provide nutrients for the others, probably younger and healthy cells (Herker *et al.* 2004). Thus, yeast flocculation can be a form of making possible a long-term survival of a cellular community of yeast cells in an unfavourable environment due to limited nutrients supply.

Brewing yeasts are usually exposed to several negative conditions such as cold-shock, nutrient starvation, osmotic stress and ethanol toxicity (Gibson *et al.* 2007). Consequently, flocculation can act as a communitarian mechanism of survival: the external cells from the floc structure can protect the inside cells against a harmful environment by physical shielding. In agreement with this possibility, it was shown that dispersed cells of a flocculent yeast treated with ultraviolet (UV) light suffered a dramatic loss of viability, while the same yeasts in an aggregated form were more resistant (Stratford 1992a). In the same line, it was shown that a floc cell population increased ethanol tolerance with the increase of floc size up to 300 μm (Lei *et al.* 2007). The possibility that flocculation can be a response to stress seems to be strain dependent. It was shown that flocculation of an ale-brewing strain was not induced as a response to different chemical [1, 3 or 5% (v/v) ethanol, 1 and 3% (v/v) isopropanol] or physical [a brief heat shock (52°C, 5 min)] stress conditions (Claro *et al.* 2007). Conversely, it was described that flocculation of a laboratory strain protects the *FLO1* expressing cells from several stresses not only because of shielding effect reported above but also because of altered gene expression in cells imbedded in flocs (Smukalla *et al.* 2008). Cells with *FLO1* expression are two times more resistant to 10% ethanol and

one hundred times more resistant to oxidative stress than the corresponding nonflocculent strain. Stress resistance raises with the increasing number of tandem repeats in the *FLO1* gene and consequently with the degree of flocculation (Smukalla *et al.* 2008). Thus, it was proposed that yeast cells can rapidly adapt the adhesion properties (Verstrepen *et al.* 2005; Verstrepen and Klis 2006; Dranginis *et al.* 2007) and stress resistance to new environmental conditions by the variation of the repeated sequences. Therefore, it was suggested that *FLO1* gene could be a green beard gene (a gene for altruism) that drives cooperation in yeast cells (Smukalla *et al.* 2008).

Most likely, microbial sociality is more common that it is supposed; the domestication of the strains can lead to the loss of social behaviours (Queller 2008). Although yeasts are classically defined as unicellular fungi, a floc can be seen as a community of cells and flocculation as an example of social behaviour: a strategy for a long-time survival or a mechanism of protection to harmful environments.

Conclusions

Yeast flocculation, as a particular case, of yeast aggregation, is a complex, fascinating and useful phenomenon. The ability of yeast cells to form flocs facilitates enormously the downstream processing. As a natural way of yeast self-immobilization, the use of flocculent strains opens the possibility of exploiting different fermentation configurations and novel fermentation designs. However, these useful potentialities remain largely unexploited.

The use of genetic engineering builds the possibility of obtaining strains in which the expression of flocculation can occur under controlled conditions by placing the *FLO* genes under the control of a suitable promoter (Verstrepen *et al.* 2001a; Cunha *et al.* 2006; Govender *et al.* 2010). However, nowadays, the use and/or release of genetic modified (GM) yeasts by the industry require several guaranties, which are under many complex laws and guidelines. In addition, the negative public perception of GM yeasts strongly impairs its use. Thus, the potential application of these strains seems, at the present, limited to other industries besides the food industry, like the bio-ethanol production. The use of NewFlo phenotype strains, because of its cyclic flocculation, emerges as an alternative, particularly those that only flocculate in a narrow pH window. These cells can grow outside the favourable flocculation pH and when desired the switching of pH leads to a rapid triggering of flocculation (Soares and Seynaeve 2000b). In brewing or other alcoholic beverages production, even though the modification of fermentation parameters, like sugar concentration or pH value can only occur to a limited degree, the usefulness of these strains remains in other industrial processes where the fermented broth is not the bio-product.

The new era opened by functional genomics, in which new tools like transcriptome, proteome and metabolome analysis are used, enlarges the possibility of obtaining a solid knowledge about yeast flocculation control at a global and molecular level. The improvement of the knowledge of *FLO* genes regulation, namely the elucidation of the upstream sensors and the signalling pathway(s) involved in the regulation of flocculation, generates the opportunity of manipulating this useful characteristic. This opens the possibility of expanding the usefulness of flocculation to other biotechnological industries besides bio-ethanol and alcoholic beverage production.

References

Abramova, N., Sertil, O., Mehta, S. and Lowry, C.V. (2001) Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. *J Bacteriol* **183**, 2881–2887.

Andrietta, S.R., Steckelberg, C. and Andrietta, M.D.S. (2008) Study of flocculent yeast performance in tower reactors for bioethanol production in a continuous fermentation process with no cell recycling. *Bioresour Technol* **99**, 3002–3008.

Bai, F.W., Anderson, W.A. and Moo-Young, M. (2008) Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol Adv* **26**, 89–105.

Bayly, J.C., Douglas, L.M., Pretorius, I.S., Bauer, F.F. and Dranginis, A.M. (2005) Characteristics of Flo11-dependent flocculation in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **5**, 1151–1156.

Bester, M.C., Pretorius, I.S. and Bauer, F.F. (2006) The regulation of *Saccharomyces cerevisiae FLO* gene expression and Ca^{2+}-dependent flocculation by Flo8p and Mss11p. *Curr Genet* **49**, 375–383.

Bidard, F., Bony, M., Blondin, B., Dequin, S. and Barre, P. (1995) The *Saccharomyces cerevisiae FLO1* flocculation gene encodes for a cell surface protein. *Yeast* **11**, 809–822.

Bony, M., Thines-Sempoux, D., Barre, P. and Blondin, B. (1997) Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *J Bacteriol* **179**, 4929–4936.

Bony, M., Barre, P. and Blondin, B. (1998) Distribution of the flocculation protein, flop, at the cell surface during yeast growth: the availability of flop determines the flocculation level. *Yeast* **14**, 25–35.

Chen, E.H., Grote, E., Mohler, W. and Vignery, A. (2007) Cell–cell fusion. *FEBS Lett* **581**, 2181–2193.

Chlup, P.H., Bernard, D. and Stewart, G.G. (2008) Disc stack centrifuge operating parameters and their impact on yeast physiology. *J Inst Brew* **114**, 45–61.

Choi, G.W., Kang, H.W. and Moon, S.K. (2009) Repeated-batch fermentation using flocculent hybrid, *Saccharomyces*

cerevisiae CHFY0321 for efficient production of bioethanol. *Appl Microbiol Biotechnol* **84**, 261–269.

Claro, F.B., Rijsbrack, K. and Soares, E.V. (2007) Flocculation onset in *Saccharomyces cerevisiae*: effect of ethanol, heat and osmotic stress. *J Appl Microbiol* **102**, 693–700.

Cullen, P.J. and Sprague, G.F. (2000) Glucose depletion causes haploid invasive growth in yeast. *Proc Natl Acad Sci USA* **97**, 13619–13624.

Cunha, A.F., Missawa, S.K., Gomes, L.H., Reis, S.F. and Pereira, G.A.G. (2006) Control by sugar of *Saccharomyces cerevisiae* flocculation for industrial ethanol production. *FEMS Yeast Res* **6**, 280–287.

Dauer, R.R. and Dunlop, E.H. (1991) High-gradient magnetic separation of yeast. *Biotechnol Bioeng* **37**, 1021–1028.

Demain, A.L., Phaff, H.J. and Kurtzman, C.P. (1998) The industrial and agricultural significance of yeasts. In *The Yeasts: A Taxonomic Study* ed. Kurtzman, C.P. and Fell, J.W. pp. 13–19. Amsterdam: Elsevier Science B.V.

Dengis, P.B., Nélissen, L.R. and Rouxhet, P.G. (1995) Mechanisms of yeast flocculation: comparison of top- and bottom-fermenting strains. *Appl Environ Microbiol* **61**, 718–728.

Dequin, S. (2001) The potential of genetic engineering for improving brewing, wine-making and baking yeasts. *Appl Microbiol Biotechnol* **56**, 577–588.

Dietvorst, J. and Brandt, A. (2008) Flocculation in *Saccharomyces cerevisiae* is repressed by the COMPASS methylation complex during high-gravity fermentation. *Yeast* **25**, 891–901.

Dietvorst, J. and Brandt, A. (2010) Histone modifying proteins Gcn5 and Hda1 affect flocculation in *Saccharomyces cerevisiae* during high-gravity fermentation. *Curr Genet* **56**, 75–85.

Domingues, L., Lima, N. and Teixeira, J.A. (2000a) Contamination of a high-cell-density continuous bioreactor. *Biotechnol Bioeng* **68**, 584–587.

Domingues, L., Vicente, A.A., Lima, N. and Teixeira, J.A. (2000b) Applications of yeast flocculation in biotechnological processes. *Biotechnol Bioprocess Eng* **5**, 288–305.

Domingues, L., Lima, N. and Teixeira, J.A. (2005) *Aspergillus niger* β-galactosidase production by yeast in a continuous high cell density reactor. *Process Biochem* **40**, 1151–1154.

Dranginis, A.M., Rauceo, J.M., Coronado, J.E. and Lipke, P.N. (2007) A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. *Microbiol Mol Biol Rev* **71**, 282–294.

Fichtner, L., Schulze, F. and Braus, G.H. (2007) Differential Flo8p-dependent regulation of *FLO1* and *FLO11* for cell–cell and cell–substrate adherence of *S. cerevisiae* S288c. *Mol Microbiol* **66**, 1276–1289.

Fischer, C., Valerius, O., Rupprecht, H., Dumkow, M., Krappmann, S. and Braus, G.H. (2008) Posttranscriptional regulation of *FLO11* upon amino acid starvation in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **8**, 225–236.

Fleet, G.H. (2006) The commercial and community significanceof yeasts in food and beverage production. In *Yeasts in Food and Beverages* ed. Querol, A. and Fleet, G.H. pp. 1–12. Heidelberg: Springer.

Gibson, B.R., Lawrence, S.J., Leclaire, J.P.R., Powell, C.D. and Smart, K.A. (2007) Yeast responses to stresses associated with industrial brewery handling. *FEMS Microbiol Rev* **31**, 535–569.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A. and Fink, G.R. (1992) Unipolar cell divisions in the yeast *Saccharomyces cerevisiae* lead to filamentous growth: regulation by starvation and Ras. *Cell* **68**, 1077–1090.

Gouveia, C. and Soares, E.V. (2004) Pb^{2+} inhibits competitively flocculation of *Saccharomyces cerevisiae*. *J Inst Brew* **110**, 141–145.

Govender, P., Domingo, J.L., Bester, M.C., Pretorius, I.S. and Bauer, F.F. (2008) Controlled expression of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **74**, 6041–6052.

Govender, P., Bester, M. and Bauer, F.F. (2010) *FLO* gene-dependent phenotypes in industrial wine yeast strains. *Appl Microbiol Biotechnol* **86**, 931–945.

Guo, B., Styles, C.A., Feng, Q. and Fink, G. (2000) A *Saccharomyces* gene family involved in invasive growth, cell–cell adhesion, and mating. *Proc Natl Acad Sci USA* **97**, 12158–12163.

Halme, A., Bumgarner, S., Styles, C. and Fink, G.R. (2004) Genetic and epigenetic regulation of the *FLO* gene family generates cell–surface variation in yeast. *Cell* **116**, 405–415.

Hatti-Kaul, R. and Mattiasson, B. (2001) Downstream processing in biotechnology. In *Basic Biotechnology* ed. Ratledge, C. and Kristiansen, B. pp. 187–211. Cambridge: Cambridge University Press.

Heine, F., Stahl, F., Strauber, H., Wiacek, C., Benndorf, D., Repenning, C., Schmidt, F., Scheper, T. *et al.* (2009) Prediction of flocculation ability of brewing yeast inoculates by flow cytometry, proteome analysis, and mRNA profiling. *Cytometry A* **75A**, 140–147.

Herker, E., Jungwirth, H., Lehmann, K.A., Maldener, C., Fröhlich, K.U., Wissing, S., Büttner, S., Fehr, M. *et al.* (2004) Chronological aging leads to apoptosis in yeast. *J Cell Biol* **164**, 501–507.

Herrera, V.E. and Axcell, B.C. (1991) Induction of premature yeast flocculation by a polyssacharide fraction isolated from malt husk. *J Inst Brew* **97**, 359–366.

Jin, Y. and Speers, R.A. (2000) Effect of environmental conditions on the flocculation of *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **58**, 108–116.

Jin, Y., Ritcey, L.L. and Speers, R.A. (2001) Effect of cell surface hydrophobicity, charge, and zymolectin density on the flocculation of *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59**, 1–9.

Kida, K., Yamadaki, M., Asno, S., Nakata, T. and Sonoda, Y. (1989) The effect of aeration on stability of continuous ethanol fermentation by a flocculating yeast. *J Ferment Bioeng* **68**, 107–111.

Kim, J.S., Akeprathumchai, S. and Wickramasinghe, S.R. (2001) Flocculation to enhance microfiltration. *J Membr Sci* **182**, 161–172.

Klis, F.M., Boorsma, A. and De Groot, P.W.J. (2006) Cell wall construction in *Saccharomyces cerevisiae*. *Yeast* **23**, 185–202.

Kobayashi, O., Suda, H., Ohtani, T. and Sone, H. (1996) Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Mol Gen Genet* **251**, 707–715.

Kobayashi, O., Hayashi, N., Kuroki, R. and Sone, H. (1998) Region of Flo1 proteins responsible for sugar recognition. *J Bacteriol* **180**, 6503–6510.

Kobayashi, O., Yoshimoto, H. and Sone, H. (1999) Analysis of the genes activated by the *FLO8* gene in *Saccharomyces cerevisiae*. *Curr Genet* **36**, 256–261.

Koizumi, H., Kato, Y. and Ogawa, T. (2008) Barley malt polysaccharides inducing premature yeast flocculation and their possible mechanism. *J Am Soc Brew Chem* **66**, 137–142.

Koizumi, H., Kato, Y. and Ogawa, T. (2009) Structural features of barley malt polysaccharides inducing premature yeast flocculation. *J Am Soc Brew Chem* **67**, 129–134.

Kondo, A., Shigechi, H., Abe, M., Uyama, K., Matsumoto, T., Takahashi, S., Ueda, M., Tanaka, A. *et al.* (2002) High-level ethanol production from starch by a flocculent *Saccharomyces cerevisiae* strain displaying cell–surface glucoamylase. *Appl Microbiol Biotechnol* **58**, 291–296.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R. and Koutinas, A.A. (2004) Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol* **21**, 377–397.

Lei, J., Zhao, X., Ge, X. and Bai, F. (2007) Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distribution. *J Biotechnol* **131**, 270–275.

Li, F., Zhao, X.Q., Ge, X.M. and Bai, F.W. (2009) An innovative consecutive batch fermentation process for very high gravity ethanol fermentation with self-flocculating yeast. *Appl Microbiol Biotechnol* **84**, 1079–1086.

Liu, H.P., Styles, C.A. and Fink, G.R. (1996) *Saccharomyces cerevisiae* S288C has a mutation in *FL08* a gene required for filamentous growth. *Genetics* **144**, 967–978.

Liu, N., Wang, D.L., Wang, Z.Y., He, X.P. and Zhang, B.R. (2007) Genetic basis of flocculation phenotype conversion in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7**, 1362–1370.

Lo, W.-S. and Dranginis, A.M. (1998) The cell surface flocculin flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. *Mol Biol Cell* **9**, 161–171.

Machado, M.D., Santos, M.S.F., Gouveia, C., Soares, H.M.V.M. and Soares, E.V. (2008) Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: the flocculation as a separation process. *Bioresour Technol* **99**, 2107–2115.

Machado, M.D., Janssens, S., Soares, H. and Soares, E.V. (2009) Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: advantages of using dead biomass. *J Appl Microbiol* **106**, 1792–1804.

Machado, M.D., Soares, E.V. and Soares, H.M.V.M. (2010a) Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: chemical speciation as a tool in the prediction and improving of treatment efficiency of real electroplating effluents. *J Hazard Mater* **180**, 347–353.

Machado, M.D., Soares, E.V. and Soares, H.M.V.M. (2010b) Removal of heavymetals using a brewer's yeast strain of *Saccharomyces cerevisiae*: application to the treatment of real electroplating effluents containing multielements. *J Chem Technol Biotechnol* **85**, 1353–1360.

Machado, M.D., Soares, H.M.V.M. and Soares, E.V. (2010c) Removal of chromium, copper and nickel from an electroplating effluent using a flocculent brewer's yeast strain of *Saccharomyces cerevisiae*. *Water Air Soil Pollut* **212**, 199–204.

Masy, C.L., Henquinet, A. and Mestdagh, M.M. (1992) Flocculation of *Saccharomyces cerevisiae*: inhibition by sugars. *Can J Microbiol* **38**, 1298–1306.

Matsumoto, T., Fukuda, H., Ueda, M., Tanaka, A. and Kondo, A. (2002) Construction of yeast strains with high cell surface lipase activity by using novel display systems based on the Flo1p flocculation functional domain. *Appl Environ Microbiol* **68**, 4517–4522.

Miki, B.L.A., Poon, N.H., James, A.P. and Seligy, V.L. (1982a) Possible mechanism for flocculation interactions governed by gene *FLO1* in *Saccharomyces cerevisiae*. *J Bacteriol* **150**, 878–889.

Miki, B.L.A., Poon, N.H. and Seligy, V.L. (1982b) Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*. *J Bacteriol* **150**, 890–899.

Mortier, A. and Soares, E.V. (2007) Separation of yeasts by addition of flocculent cells of *Saccharomyces cerevisiae*. *World J Microbiol Biotechnol* **23**, 1401–1407.

Mulders, S.E.V., Christianen, E., Saerens, S.M.G., Daenen, L., Verbelen, P.J., Willaert, R., Verstrepen, K.J. and Delvaux, F.R. (2009) Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **9**, 178–190.

Nishihara, H., Toraya, T. and Fukui, S. (1982) Flocculation of cell-walls of brewers-yeast and effects of metal-ions, protein-denaturants and enzyme treatments. *Arch Microbiol* **131**, 112–115.

Ogata, T., Izumikawa, M., Kohno, K. and Shibata, K. (2008) Chromosomal location of Lg-*FLO1* in bottom-fermenting yeast and the *FLO5* locus of industrial yeast. *J Appl Microbiol* **105**, 1186–1198.

Paula, L. and Birrer, F. (2006) Including public perspectives in industrial biotechnology and the biobased economy. *J Agric Environ Ethics* **19**, 253–267.

Pilkington, P.H., Margaritis, A., Mensour, N.A. and Russell, I. (1998) Fundamentals of immobilised yeast cells for continuous beer fermentation: a review. *J Inst Brew* **104**, 19–31.

Pizarro, F., Vargas, F.A. and Agosin, E. (2007) A systems biology perspective of wine fermentations. *Yeast* **24**, 977–991.

Powell, C.D. and Diacetis, A.N. (2007) Long term serial repitching and the genetic and phenotypic stability of brewer's yeast. *J Inst Brew* **113**, 67–74.

Powell, C.D., Quain, D.E. and Smart, K.A. (2003) The impact of brewing yeast cell age on fermentation performance, attenuation and flocculation. *FEMS Yeast Res* **3**, 149–157.

Powell, C.D., Quain, D.E. and Smart, K.A. (2004) The impact of sedimentation on cone yeast heterogeneity. *J Am Soc Brew Chem* **62**, 8–17.

Pretorius, I.S. (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16**, 675–729.

Pretorius, I.S. and Bauer, F.F. (2002) Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends Biotechnol* **20**, 426–432.

Queller, D.C. (2008) Behavioural ecology. The social side of wild yeast. *Nature* **456**, 589–590.

Rando, O.J. and Verstrepen, K.J. (2007) Timescales of genetic and epigenetic inheritance. *Cell* **128**, 655–668.

Sampermans, S., Mortier, J. and Soares, E.V. (2005) Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients. *J Appl Microbiol* **98**, 525–531.

Sato, M., Watari, J. and Shinotsuka, K. (2001) Genetic instability in flocculation of bottom-fermenting yeast. *J Am Soc Brew Chem* **59**, 130–134.

Sato, M., Maeba, H., Watari, J. and Takashio, M. (2002) Analysis of an inactivated Lg-*FLO1* gene present in bottom-fermenting yeast. *J Biosci Bioeng* **93**, 395–398.

Seong, K.T., Katakura, Y., Ninomiya, K., Bito, Y., Katahira, S., Kondo, A., Ueda, M. and Shioya, S. (2006) Effect of flocculation on performance of arming yeast in direct ethanol fermentation. *Appl Microbiol Biotechnol* **73**, 60–66.

Shen, H.Y., Moonjai, N., Verstrepen, K.J. and Delvaux, F.R. (2003) Impact of attachment immobilization on yeast physiology and fermentation performance. *J Am Soc Brew Chem* **61**, 79–87.

Shuler, M.L. and Kargi, F. (2002) Recovery and purification of products. In *Bioprocess Engineering* ed. Amundson, N.R. pp. 329–384. New Jersey: Prentice Hall PTR.

Silva, C.L.C., Vianna, C.R., Cadete, R.M., Santos, R.O., Gomes, F.C.O., Oliveira, E.S. and Rosa, C.A. (2009) Selection, growth, and chemo-sensory evaluation of flocculent starter culture strains of *Saccharomyces cerevisiae* in the large-scale production of traditional Brazilian cachaca. *Int J Food Microbiol* **131**, 203–210.

Sivakumar, G., Vail, D.R., Xu, J.F., Burner, D.M., Lay, J.O., Ge, X.M. and Weathers, P.J. (2010) Bioethanol and biodiesel: alternative liquid fuels for future generations. *Eng Life Sci* **10**, 8–18.

Smart, K.A. (2007) Brewing yeast genomes and genome-wide expression and proteome profiling during fermentation. *Yeast* **24**, 993–1013.

Smit, G., Straver, M.H., Lugtenberg, B.J.J. and Kijne, J.W. (1992) Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Appl Environ Microbiol* **58**, 3709–3714.

Smukalla, S., Caldara, M., Pochet, N., Beauvais, A., Guadagnini, S., Yan, C., Vinces, M.D., Jansen, A. *et al.* (2008) *FLO1* is a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell* **135**, 726–737.

Soares, E.V. and Duarte, A.A. (2002) Addition of nutrients induce a fast loss of flocculation in starved cells of *Saccharomyces cerevisiae*. *Biotechnol Lett* **24**, 1957–1960.

Soares, E.V. and Mota, M. (1996) Flocculation onset, growth phase, and genealogical age in *Saccharomices cerevisiae*. *Can J Microbiol* **42**, 539–547.

Soares, E.V. and Mota, M. (1997) Quantification of yeast flocculation. *J Inst Brew* **103**, 93–98.

Soares, E.V. and Seynaeve, J. (2000a) The use of succinic acid, as a pH buffer, expands the potentialities of utilisation of a chemically defined medium in *Saccharomyces cerevisiae* flocculation studies. *Biotechnol Lett* **22**, 859–863.

Soares, E.V. and Seynaeve, J. (2000b) Induction of flocculation of brewer's yeast strains of *Saccharomyces cerevisiae* by changing the calcium concentration and pH of culture medium. *Biotechnol Lett* **22**, 1827–1832.

Soares, E.V. and Vroman, A. (2003) Effect of different starvation conditions on the flocculation of *Saccharomyces cerevisiae*. *J Appl Microbiol* **95**, 325–330.

Soares, E.V., Teixeira, J.A. and Mota, M. (1991) Influence of aeration and glucose concentration in the flocculation of *Saccharomyces cerevisiae*. *Biotechnol Lett* **13**, 207–212.

Soares, E.V., Teixeira, J.A. and Mota, M. (1994) Effect of cultural and nutritional conditions on the control of flocculation expression in *Saccharomyces cerevisiae*. *Can J Microbiol* **40**, 851–857.

Soares, E.V., De Coninck, G., Duarte, F. and Soares, H.M.V.M. (2002) Use of *Saccharomyces cerevisiae* for Cu^{2+} removal from solution: the advantages of using a flocculent strain. *Biotechnol Lett* **24**, 663–666.

Soares, E.V., Vroman, A., Mortier, J., Rijsbrack, K. and Mota, M. (2004) Carbohydrate carbon sources induce loss of flocculation of an ale-brewing yeast strain. *J Appl Microbiol* **96**, 1117–1123.

Sousa, M.J., Teixeira, J.A. and Mota, M. (1992) Differences in the flocculation mechanism of *Kluyeromyces marxianus* and *Saccharomyces cerevisiae*. *Biotechnol Lett* **14**, 213–218.

Speers, R.A., Wan, Y.-Q., Jin, Y. and Stewart, R.J. (2006) Effects of fermentation parameters and cell wall prperties on yeast flocculation. *J Inst Brew* **112**, 246–254.

Stewart, G.G. (2009) The Horace Brown Medal lecture: forty years of brewing research. *J Inst Brew* **115**, 3–29.

Stewart, G.G. and Russell, I. (1981) Yeast flocculation. In *Brewing Science, Food Science and Technology* ed. Pollock, J.A.R. pp. 61–92. New York: Academic Press.

Stratford, M. (1989) Yeast flocculation: calcium specificity. *Yeast* **5**, 487–496.

Stratford, M. (1992a) Lectin-mediated aggregation of yeasts-yeast flocculation. *Biotechnol Genet Eng Rev* **10**, 283–341.

Stratford, M. (1992b) Yeast flocculation – a new perspective. *Adv Microb Physiol* **33**, 1–71.

Stratford, M. (1992c) Yeast flocculation – receptor definition by *mnn* mutants and concanavalin-A. *Yeast* **8**, 635–645.

Stratford, M. (1993) Yeast flocculation: flocculation onset and receptor availability. *Yeast* **9**, 85–94.

Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value. *FEMS Microbiol Lett* **136**, 13–18.

Stratford, M. and Assinder, S. (1991) Yeast flocculation: Flo1 and newFlo phenotypes and receptor structure. *Yeast* **7**, 559–574.

Stratford, M. and Bond, C.J. (1992) Selective separation of microorganisms by lectins – yeast and concanavalin-a as a model system. *Biotechnol Bioeng* **40**, 835–843.

Stratford, M. and Carter, A.T. (1993) Yeast flocculation: lectin synthesis and activation. *Yeast* **9**, 371–378.

Straver, M.H., Aar, P.C.V.D., Smit, G. and Kijne, J.W. (1993) Determinants of flocculence of brewer's yeast during fermentation in wort. *Yeast* **9**, 527–532.

Strehaiano, P., Ramon-Portugal, F. and Taillandier, P. (2006) Yeasts as biocatalysts. In *Yeasts in Food and Beverages* ed. Querol, A. and Fleet, G.H. pp. 243–283. Heidelberg: Springer.

Strittmatter, A.W., Fischer, C., Kleinschmidt, M. and Braus, G.H. (2006) *FLO11* mediated filamentous growth of the yeast *Saccharomyces cerevisiae* depends on the expression of the ribosomal RPS26 genes. *Mol Genet Genomics* **276**, 113–125.

Taylor, N.W. and Orton, W.L. (1975) Calcium in flocculence of *Saccharomyces cerevisiae*. *J Inst Brew* **81**, 53–57.

Teixeira, J.A., Mota, M. and Goma, G. (1990) Continuous ethanol production by a flocculating strain of *Kluyveromyces marxianus*: bioreactor performance. *Bioprocess Eng* **5**, 123–127.

Teunissen, A.W.R.H. and Steensma, H.Y. (1995) Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast* **11**, 1001–1013.

Teunissen, A.W.R.H., Holub, E., Van Der Hucht, J., Van Den Berg, J.A. and Steensma, H.Y. (1993) Sequence of the open reading frame of the *FLO1* gene from *Saccharomyces cerevisiae*. *Yeast* **9**, 423–427.

Teunissen, A.W.R.H., Van Den Berg, J.A. and Steensma, H.Y. (1995) Transcriptional regulation of flocculation genes in *Saccharomyces cerevisiae*. *Yeast* **11**, 435–446.

Touhami, A., Hoffmann, B., Vasella, A., Denis, F.D. and Dufrêne, Y.F. (2003) Aggregation of yeast cells: direct measurement of discrete lectin-carbohydrate interactions. *Microbiology* **149**, 2873–2878.

Valles, B.S., Bedrinana, R.P., Queipo, A.L. and Alonso, J.J.M. (2008) Screening of cider yeasts for sparkling cider production (Champenoise method). *Food Microbiol* **25**, 690–697.

Van Nierop, S.N.E., Cameron-Clarke, A. and Axcell, B.C. (2004) Enzymatic generation of factors malt responsible for premature yeast flocculation. *J Am Soc Brew Chem* **62**, 108–116.

Vaughan-Martini, A. and Martini, A. (1998) Saccharomyces Meyen ex Reess. In *The Yeasts: A Taxonomic Study* ed. Kurtzman, C.P. and Fell, J.W. pp. 358–371. Amsterdam: Elsevier.

Verstrepen, K.J. and Klis, F.M. (2006) Flocculation, adhesion and biofilm formation yeasts. *Mol Microbiol* **60**, 5–15.

Verstrepen, K.J., Bauer, F.F., Winderickx, J., Derdelinckx, G., Dufour, J.P., Thevelein, J.M., Pretorius, I.S. and Delvaux, F.R. (2001a) Genetic modification of *Saccharomyces cerevisiae*: fitting the modern brewer's needs. *Cerevisia* **26**, 89–97.

Verstrepen, K.J., Derdelinckx, G., Delvaux, F.R., Winderickx, J., Thevelein, J.M., Bauer, F.F. and Pretorius, I.S. (2001b) Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59**, 69–76.

Verstrepen, K.J., Reynolds, T.B. and Fink, G.R. (2004) Origins of variation in the fungal cell surface. *Nat Rev Microbiol* **2**, 533–540.

Verstrepen, K.J., Jansen, A., Lewitter, F. and Fink, G.R. (2005) Intragenic tandem repeats generate functional variability. *Nat Genet* **37**, 986–990.

Vilanova, M. and Massneuf-Pomarede, I. (2005) Characterization of yeast strains from Rias Baixas (NW Spain) and their contribution to the fermentation of Albarino wine. *Ann Microbiol* **55**, 23–26.

Wang, J. and Chen, C. (2006) Biosorption of heavy metals by *Saccharomyces cerevisiae*: a review. *Biotechnol Adv* **24**, 427–451.

Watari, J., Takata, Y., Ogawa, M., Sahara, H., Koshino, S., Onnela, M., Airaksinen, U., Jaatinen, R. *et al.* (1994) Molecular cloning and analysis of the yeast flocculation gene *FlO1*. *Yeast* **10**, 211–225.

Weeks, M.G., Munro, P.A. and Spedding, P.L. (1983) New concepts for rapid yeast settling.1. Flocculation with an inert powder. *Biotechnol Bioeng* **25**, 687–697.

Weir, S., Ramsden, D.K., Hughes, J. and Le Thomas, F. (1993) The flocculation of yeast with chitosan in complex fermaentation media: the effect of biomass concentration and mode of flocculant addition. *Biotechnol Tech* **7**, 199–204.

Wickramasinghe, S.R., Leong, Y.K., Mondal, S. and Liow, J.L. (2010) Influence of cationic flocculant properties on the flocculation of yeast suspensions. *Adv Powder Technol* **21**, 374–379.

Xu, T.J., Zhao, X.Q. and Bai, F.W. (2005) Continuous ethanol production using self-flocculating yeast in a cascade of fermentors. *Enzyme Microb Technol* **37**, 634–640.

Zhao, X.Q. and Bai, F.W. (2009) Yeast flocculation: new story in fuel ethanol production. *Biotechnol Adv* **27**, 849–856.