125th Anniversary Review: Yeast Flocculation and Sedimentation in Brewing

Virve Vidgren* **and John Londesborough**

ABSTRACT

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Flocculation is prerequisite for bulk sedimentation of yeast during brewery fermentation. Although single yeast cells gradually sediment in green beer, this sedimentation rate is too slow without formation of large yeast flocs. The present review concerns the major determinants of yeast flocculation and sedimentation in brewery fermentations. Flocculation characteristics of yeast are strongly strain-dependent and largely defined by which *FLO* genes are functional in each strain. In addition to the genetic background, several environmental factors affect flocculation. These can be, somewhat arbitrarily, classified as physiological factors, such as the calcium availability, pH, temperature and ethanol and oxygen concentrations in the medium or physical factors, such as cell surface hydrophobicity, cell surface charge and the presence of appropriate hydrodynamic conditions for the formation of large flocs. Once yeast flocs are formed, their size, shape and density and the properties of the surrounding medium affect the rate at which the flocs sediment. Higher gravity worts usually result in green beers with higher viscosity and density, which both retard sedimentation. Moreover, environmental factors during yeast handling before fermentation, e.g., propagation, storage and cropping, influence the flocculation potential of yeast in subsequent fermentation. Premature yeast flocculation (PYF) and the role of PYF factors are discussed. In conclusion, some potential options available to adjust yeast flocculation are described.

Key words: Brewer's yeast, Flo1, flocculation, *FLO* genes, NewFlo, PYF, sedimentation.

INTRODUCTION

Yeast flocculation is the aggregation of single yeast cells into flocs consisting of thousands of cells. After formation, these flocs usually sediment rapidly from the medium to the bottom of the fermentation tank or in some cases, e.g., ale yeasts in traditional fermentors, rise to the surface. Flocculation is a prerequisite for the bulk sedimentation of yeast during brewery fermentations, so that alterations in flocculation properties generally also mean profound changes in sedimentation behaviour. In the fermentation process, timing of flocculation is important. Flocculation should not take place too early, before the wort is completely attenuated, because premature flocculation causes sluggish or stuck fermentation and final beers with high residual sugars and unsatisfactory flavour characteristics[81]. Instead, strong and virtually complete flocculation at the end of the fermentation is desired, providing a cheap, effective and environmentally friendly way to remove nearly, but not quite, all yeast cells from the green beer.

Sometimes brewer's yeast strains exhibit undesirable changes in their flocculation characteristics with time, e.g., when serial repitching of fermentations is carried out[67,70,113]. An ideal brewing yeast should exhibit constant flocculation characteristics during consecutive rounds of fermenting, cropping, storing and repitching. Improved and reproducible flocculation is currently important because many breweries are using increasingly high-gravity worts, which are believed to be associated with sub-optimal yeast sedimentation[92]. The present review targets practical aspects of yeast flocculation that are relevant to brewer's yeast strains under industrial brewery conditions. Earlier reviews include Verstrepen et al., 2003[103], Verstrepen and Klis, 2006[106] and Soares, 2010[73] and a recent symposium contains relevant articles[76].

The most common mechanism of yeast flocculation is generally accepted to be the lectin-mediated adhesion of adjacent yeast cells to form large cell clusters[51,81,82]. Possession of lectin-like (sugar binding) proteins, i.e. flocculins, at the cell surface of yeast, is required for this kind of flocculation to take place. Flocculins of one cell bind to mannose residues in the cell wall of adjacent cells, and so link the yeast cells into clusters that may contain thousands of cells[51,81,82]. Calcium is essential for lectin-mediated flocculation[80]. It has been suggested that Ca^{2+} binds to flocculin proteins and is necessary for flocculin to obtain the correct structural conformation[51]. A recent crystallization and structural study of flocculins showed that Ca^{2+} is directly involved in carbohydrate binding[101]. Flocculation is a reversible phenomenon, so that cell flocs can be dissociated, e.g., by adding a chelating agent that removes calcium ions or by adding mannose, which competitively displaces cell wall mannose residues from flocculin binding sites.

Several kinds of factors affect yeast flocculation. First is the genetic background of the strain. Flocculin proteins

VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044, VTT, Finland.
* Corresponding author. E-mail: virve.vidgren@vtt.fi

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are encoded by members of the *FLO* group of genes[95]. The genetic background in regard to *FLO* genes varies greatly among brewer's yeast strains, i.e. different strains contain different combinations of *FLO* genes[99], resulting in different flocculation characteristics[69,86].

Second, flocculation is affected by the physiological environment, e.g., the pH and availability of metal ions and nutrients[19]. The pH influences the charge of the cell surface, which in turn has an effect on flocculation. Changes in pH may also alter ionization of functional groups in flocculin proteins and so change their conformation[32]. The environment is sensed by yeast cells and expression of *FLO* genes, their translation to Flo proteins and localization of Flo proteins to the cell wall are all influenced by environmental factors[106,107]. Third, the physical environment affects flocculation. The hydrodynamic conditions must be favourable and promote a sufficient collision rate between cells, but agitation must not be violent enough to break up cell clusters. The concentration of yeast cells in suspension must be sufficient to cause the number of collisions necessary to form flocs[96]. Factors that increase the hydrophobic character of the yeast cell walls (cell-surface hydrophobicity) and factors that decrease the repulsive negative electrostatic charges on the cell walls (cell surface charge) cause stronger flocculation, presumably because they facilitate cell-cell contact[32,114].

Even without flocculation, yeast cells gradually sediment in green beer. This is because the size and density of yeast cells overcome the Brownian motion that would keep them suspended[81]. This sedimentation rate is slow, especially when the medium is agitated, for example by gas bubbles formed during fermentation. The sedimentation rate is also dependent on particle size: smaller particles generally settle more slowly than larger particles of the same density, because they are relatively more retarded by friction (viscosity). Therefore older yeast cells sediment faster than younger, smaller cells. However, the sedimentation of single cells is too slow to be of practical importance in brewery fermentations, where flocculation is required to achieve sedimentation of most yeast during, typically, less than one day as the fermentation approaches completion.

In flocculating strains, cells that are not entrapped in flocs have not necessarily lost the ability to flocculate[78,102]. Instead, there is a dynamic equilibrium between flocs and free cells resulting in a continuous exchange between cells entrapped in flocs and free cells. Single cells are constantly set free from the flocs while new cells are entrapped. Loosening of cells from the flocs occurs because of hydrodynamic forces. This results in a dynamic equilibrium similar to the equilibria of ordinary chemical reactions[29,85]. Thus, dilution favours an increased proportion of free cells, which is why a sufficient concentration of yeast cells is needed for effective flocculation and one reason why some cells remain in suspension even after bulk sedimentation.

Flocculation will lead to sedimentation if the turbulence intensity and fluid velocities in the tank are low enough. Turbulent shear force caused by CO_2 evolution during fermentation is an important factor restricting sedimentation[78]. Speers et al.[78] showed that an ale strain with the NewFlo phenotype started to flocculate after 24 h of fermentation, but most flocs remained in suspension until 60 h, when the average turbulent sheer rate caused by CO_2 evolution declined to below 8 s^{-1}.

At the end of fermentation, conditions are favourable for sedimentation: carbon dioxide production is low, flocculation ability is high and yeast concentration is maximal[96]. These conditions favour the formation and settling out of large flocks. Although floc formation takes only a few seconds, the settling-out time in a large industrial brewery fermentation is measured in hours[96]. Several factors influence the rate at which flocs sediment. These include the way cells pack into flocs and the resultant floc size, shape and density, and medium properties such as viscosity, density and turbulence[84]. In general, higher gravity worts result in green beers with higher viscosity and density, which both retard sedimentation. As settling out proceeds, the floc size decreases because the concentration of yeast cells still in suspension decreases. In turn, the smaller flocs settle out more slowly[96].

MEASUREMENT OF FLOCCULATION

The ability of a particular batch of yeast to flocculate (its flocculation potential) is nearly always assessed by measuring the rate at which yeast sediments under static conditions[77]. A homogeneous suspension of the yeast cells is allowed to stand, and after a defined time the concentration of yeast remaining in the upper part of the suspension is estimated by some means (cell count, optical density, etc.) and used to calculate what proportion of the total cells has sedimented. Less attention has been paid to assaying how complete sedimentation will eventually be attained or to how strongly the cell flocs are held together (which can be estimated by thermal deflocculation[93]), though the reader is directed to work modelling the kinetics of cell collision and capture (the orthokinetic capture coefficient)[27,78]. The sedimentation rate is apparently simple to measure and of immediate practical importance to brewers. The so-called Helm's test uses this principle and is the standard flocculation test recommended by brewing societies such as the ASBC (American Society of Brewing Chemists). There are several modifications of the Helm's test. These modifications generally aim to make the test more relevant to actual brewing conditions by producing the yeast under conditions closer to brewery practice and carrying out the test in a medium more closely resembling a partially fermented wort. Lawrence and Smart[42] provide an extensive list of these modifications (which the interested reader should consult) and themselves argue in favour of growing yeast anaerobically before assaying its flocculation ability. D'Harcourt and Smart[13] have modified the Helm's test by extending the growth time from 48 to 74 h to allow NewFlo phenotype cells (see below) time to develop active flocculins. The same authors suggested that flocculation should be measured in a medium containing 4% ethanol, since flocculation is affected by ethanol concentration, which is around 4% in brewery fermentations when flocculation commences.

YEAST FLOCCULATION PHENOTYPES

Two common flocculation phenotypes have been observed for flocculin-mediated adhesion i.e. Flo1 and NewFlo phenotypes. These phenotypes differ in the sugar-binding ability of the corresponding flocculin proteins. In Flo1 phenotype strains, flocculation is inhibited by mannose but not by glucose, sucrose, maltose or galactose, suggesting that of these five sugars only mannose can be bound by the flocculins on the surface of Flo1-type cells. However, in NewFlo phenotype strains flocculation is inhibited by mannose, glucose, sucrose, maltose and maltotriose (also by galactose in some strains) suggesting that flocculin proteins in these cells are able to bind this wider range of sugars[67,86]. Most brewer's yeast strains (both ale and lager) have been reported to possess the NewFlo phenotype[9,78,86]. This makes them particularly suitable for brewing since flocculation is delayed until the fermentable wort sugars (glucose, maltose and maltotriose) that inhibit this kind of flocculation have been largely consumed as the fermentation approaches completion.

Further complexity has been introduced by identification of another flocculation mechanism, which does not seem to be flocculin-mediated. This third flocculation phenotype has the characteristic that flocculation is not inhibited by mannose (or other sugars) and it has been called the mannose-insensitive (MI) phenotype[12,35,49]. In addition, this MI phenotype is not dependent on calcium for flocculation. These properties strongly suggest that flocculation of MI phenotypic strains is not lectin-mediated, but occurs by some other, as yet unidentified, mechanism. Onset of flocculation in the MI strains has been suggested to be controlled by both a change in cell surface hydrophobicity and an increase in ethanol concentration[12]. Although the MI phenotype is much less common than the Flo1 and NewFlo phenotypes, both ale and lager strains of MI phenotype have been described[12,49].

GENETIC BACKGROUND

FLO genes

There are at least nine genes i.e. *FLO1*, *FLO5*, *FLO9*, *FLO10*, *FLO11*, *FLONL*, *FLONS* and *Lg-FLO1* in *S. cerevisiae* or *S. pastorianus* that encode flocculin proteins. It is characteristic of all these genes that their sequences include long tandem repeats (see below). The flocculin encoded by *FLO11* differs from others in that it is involved in filamentous growth, adhesion to solid surfaces and flor formation[3,18,20,21] rather than flocculation. Another *FLO* gene, *FLO8* encodes a transcriptional factor regulating the expression of other *FLO* genes[5]. Each strain usually possesses several *FLO* genes in its genome. For example, the completely sequenced laboratory yeast strain, S288C, contains six *FLO* genes (*FLO1*, *FLO5*, *FLO8*, *FLO9*, *FLO10* and *FLO11*) and, in addition, four non-functional *FLO* pseudogenes. The presence of a functional Flo8 transcriptional factor is essential for other *FLO* genes to be expressed[17,45]. For strain S288C, possession of a mutant *FLO8* gene results in a non-flocculent phenotype[45] which can be rendered flocculent by introducing a wild type *FLO8*[17]. When expressed individually, each of the four genes *FLO1*, *FLO5, FLO9* and *FLO10* was found to confer a strongly flocculating Flo1 phenotype[95,110]. The amino acid sequences of Flo5, Flo9 and Flo10 proteins are 96, 94 and 58% identical, respectively, to Flo1p. As expected, Flo11p is the most distantly related to other flocculins, with only 37% identity to Flo1p[116].

Lg-FLO1, FLONL and *FLONS* encode flocculin proteins conferring the NewFlo phenotype[36,46,47] and they are found in brewing strains but not in laboratory strains. It is supposed that the *Lg-FLO1* gene originated from a recombination event between *FLO5* on chromosome VIII and a *FLO1* pseudogene on chromosome I[36,55]. The amino acid sequence of Lg-Flo1p shares approximately 60% identity with the corresponding region of Flo1p[36].

The DNA sequences of *FLONL* and *FLONS* are very similar to that of *FLO1*, but compared to *FLO1* they have lost some of the internal tandem repeats. More tandem repeats were lost in *FLONL* than in *FLONS*. Deletion of these repeats appears to have converted the flocculation phenotype from the Flo1 to the NewFlo phenotype[47]. Moreover, the NewFlo flocculation phenotype conferred by *FLONS* and *FLONL* is also inhibited by galactose[46], whereas the usual NewFlo phenotype is insensitive to galactose. This indicates that the sugar binding properties of the flocculins are dependent upon the number of the tandem repeats present in the flocculin gene. These different flocculation phenotypes are summarised in Table I.

The FLO family genes are unstable

FLO genes are very long (up to 4.6 kbp) because of tandem repeated DNA sequences of about 100 nucleotides that are repeated 10-20 times in each gene[104,105]. Tandem repeated DNA sequences in the *FLO* genes are highly dynamic components of the genome, i.e. they change more rapidly than other parts of the genome. They enable rearrangements both between and within flocculin genes. Several studies have shown that the longer the Flo protein (carrying more repeats) the stronger is the flocculation ability it confers[30,105,111]. Accordingly, *FLO1* was observed to be the longest *FLO* gene, with the most repeats and to confer the strongest flocculation phenotype[104]. In addition to quantitative variation, tandem repeat size variation is known to also create qualitative alterations to phenotypes,

Table I. Current view of flocculation phenotypes.

Genes	Character	Sugars that inhibit flocculation
FLO1, FLO5 FLO9, FLO10	Strong Flo1 phenotype	Only mannose
Lg-FLO1	NewFlo phenotype	Mannose, glucose, sucrose, maltose and maltotriose (not galactose)
FLONL, FLONS	Like NewFlo phenotype	Mannose, glucose, sucrose, maltose, maltotriose and galactose
Not known	Mannose-insensitive (MI) flocculation (Ca-independent)	No inhibition by sugars

e.g., to change the sugar substrate range of flocculins (see above[46,47]).

Another reason for increased instability of the *FLO* genes is their near-telomeric localization. All *FLO* genes except *FLO11* are located close to telomeres[21,55] and thus are prone to rearrangement events such as deletions, duplications and translocations[6]. The *Lg-FLO1* gene itself represents a translocation event between *FLO* genes in different chromosomes (see above). In addition, near-telomeric genes (and also *FLO11*[21]) can become transcriptionally repressed by an epigenetic process known as telomeric silencing. This is caused by an alteration in chromatin structure near the telomeres leading to the silencing of genes located in that region. This effect can be long lasting. For example, the epigenetic state of *FLO11* is heritable for many generations[21]. The strength of this telomeric silencing varies greatly between different telomeres in yeast[48]. This is probably because the number of subtelomeric repeat elements also varies between different chromosome ends. Significant variation has also been detected between different strains[61]. The COMPASS complex is involved in telomeric silencing in yeast[52]. *FLO* and *MAL* genes near to telomeres were found to be silenced in some strains whereas in other strains inactivating the COMPASS complex did not affect expression of these genes. This is consistent with the finding of significant variation in the strength of the silencing effect between different chromosome ends and in different strains. In strains where the COMPASS complex had a strong silencing effect, genetic inactivation of this complex increased the expression of *FLO1*, *FLO5* and *FLO9* genes[14]. Compared to wild type cells, these mutants displayed enhanced flocculation during high-gravity fermentation, flocculating earlier and forming much larger aggregates[14]. These findings are of considerable interest concerning the regulation of flocculation, although it is not yet clear how they can best be applied to improve the control of flocculation in industrial fermentations.

FLO genotypes and phenotypes in brewer's yeast strains

Lager strains are allopolyploid hybrids of *Saccharomyces cerevisiae* and a *Saccharomyces bayanus*-like yeast[15,53] that was recently identified as *Saccharomyces eubayanus* sp. nov.[44]. Lager strains possess *FLO* genes derived from both their parents, and so have an even larger number and greater diversity of *FLO* genes in their genomes than ale (*S. cerevisiae*) strains. Correlation of *FLO* genotypes to Flo phenotypes in brewer's yeast strains is still only possible to a limited extent because it appears that the *FLO* genotype of no brewer's yeast strain has been completely characterised. Even though the genome of the lager strain WS34/70 has been completely sequenced[53], its *FLO* genes have not been studied in detail and it is not known which of them are expressed in a physiologically functional way.

Studies of ale and lager strains of NewFlo phenotype have revealed that in addition to one or more of the NewFlo type genes, *Lg-FLO1*, *FLONS* and *FLONL,* they possess also *FLO* genes such as *FLO1, FLO5, FLO9* and *FLO10*[11,99], which are usually linked to a strong Flo1 phenotype. *A priori,* it is expected that the Flo1 phenotype (flocculation not inhibited by wort sugars) would be dominant over the *Lg-FLO1*-encoded NewFlo phenotype (flocculation inhibited by glucose, maltose and maltotriose), so what is the mechanism that gives these yeasts a NewFlo phenotype? It has been suggested that genetic or epigenetic mechanisms prevent more than one or a few *FLO* genes from being expressed simultaneously in a yeast cell[107]. On the other hand, gene expression analysis confirmed that tested flocculent lager strains strongly expressed all four flocculin-encoding genes studied, *Lg-FLO1*, *FLO1*, *FLO5* and *FLO9* simultaneously (whereas non-flocculent yeasts showed nearly no expression)[23]. One or more flocculin(s) were detected in the cytoplasm and cell wall fraction of the flocculent strains (much less in the non-flocculent strains) by Western analyses using rabbit antiserum that did not discriminate between the four gene products, Lg-Flo1p, Flo1p, Flo5 and Flo9p. Peptide analysis of the excised bands detected a 14 amino acid sequence unique to Lg-Flo1p[23], but no evidence for (or against) the presence of the other three flocculins. Possibly some mechanism ensures that Lg-Flo1p is the dominant flocculin even when genes encoding Flo1 type flocculins are strongly expressed. Or perhaps the presence in the cell wall of NewFlo-type flocculins, which are blocked by wort sugars, hinders flocculation even when non-blocked, Flo1 type flocculins are also present. Conceivably, sugars bound to NewFlo type receptors might cause steric hindrance of cell-cell linking. However, there is no experimental evidence bearing on this hypothesis.

Genetic instability of flocculation characteristics in brewer's strains

The sedimentation performance of a brewer's yeast sometimes changes during repeated repitching in a brewery. In principle, this could be due to irreversible genetic change or to reversible but long lasting physiological, perhaps epigenetic, responses to (changes in) yeast-handling and fermentation environments. When a genetic change conferring a non-flocculent phenotype occurs in a yeast culture, the culture gradually becomes a mixture of flocculent and non-flocculent cells[67]. Jibiki et al.[30] studied this using a PCR-based method to detect *FLO5* genes (see also below). All 48 single cell colonies isolated from the stock culture of a lager strain exhibited the PCR band. After repeated recycling of the yeast in brewery fermentations, single cell colonies were again isolated from batches of yeast that now showed poor sedimentation performance. In one case, most (75%) single cell colonies failed to yield the *FLO5* PCR band, indicating that a genetic mutation (loss of intact *FLO5*) had occurred and spread through the population, and it was necessary to replace the brewery yeast with fresh culture. In the other case, nearly all (>90%) of the single colonies still showed the *FLO5* PCR band, indicating that the poor sedimentation behaviour did not have a genetic basis, but was due to the physiological condition of the yeast. In this case, the flocculation ability gradually recovered during repeated use of the yeast in the brewery. It was apparently not investigated whether the reversibly altered flocculation behaviour had an epigenetic basis or was due to some temporary change in the brewery environment.

Several other groups have studied spontaneous changes in flocculation behaviour during repeatedly repitched

brewery fermentations. Increased[70,94,113], stable[60] and decreased[22,67,112] flocculation have been observed. Smart and Whisker[70] studied cropped yeast during 30 successive fermentations by an ale strain. During the first seven rounds, flocculation ability increased from 50% to 100%. Between rounds 9 and 23, flocculation remained high. Between rounds 24 and 32 both flocculation ability and cell viability decreased and other changes occurred. Wightman et al.[113] and Teixeira et al.[94] reported increased flocculation ability of lager strains during serial repitching. Powell and Diacetis[60] reported stable flocculation behaviour of an ale yeast during 98 rounds of serial repitching and suggested that in some yeast strains the flocculation character is genetically stable. Sato et al.[67] report the tendency for flocculation of a lager strain to decrease after serial repitching. A long term study in production plants showed that flocculation tended to decrease while other properties of the lager strain remained the same. A commercial lager strain and eight single-colony isolates from it were used in the study. Samples were taken from fermentation plants during several years. For five of the eight single colony isolates the flocculation ability decreased with time and for three strains it remained unchanged. Genetic changes, such as chromosome deletion or loss of the *Lg-FLO1* gene, were detected in some strains with decreased flocculation ability and were proposed as reasons for the declining flocculation[67].

In these studies, different fermentation conditions, media, strains, etc. have been used. The different results may reflect strain-dependent differences or experimental differences. For example, some cropping methods favour the enrichment of certain cell types. In the above studies, only Powell and Diacetis[60] reported the cropping method: after rejection of trub (containing 1% of the yeast), all the rest of the sedimenting yeast was collected. The lack of any fractionation of the yeast crop is consistent with the stable behaviour they found. Probably few modern breweries recycle their yeast more than 20 times, though the trend seems to be to increase the number of cycles. On the whole, the results of these studies suggest that, although genetic and perhaps epigenetic changes in flocculation behaviour sometimes occur under industrial brewery conditions, it is more likely that a change in flocculation behaviour is due to a change in process conditions or raw materials, especially if the change persists when freshly propagated yeast is taken into use.

Jibiki et al.[30] suggest that their PCR-based method to detect *FLO5* genes (mentioned above) can be used for the early detection of non-flocculent mutants in brewery fermentations. They tested more than 30 different industrial lager strains with PCR primers designed to detect *FLO5*. The PCR product varied in size from about 4.8 to 2.3 kb, with more flocculent strains showing larger products (> 4 kb) and less flocculent strains showing smaller products (2.3 kb in a non-flocculent strain). Cell surface hydrophobicity also decreased with decreasing size of the PCR product. Thus, the appearance of non-flocculent mutants during a series of brewery fermentations could be tested by PCR analyses. Interestingly, PCR primers designed for Lg-*FLO1*, *FLO1* or the regulatory gene, *FLO8*, did not have this predictive power. In strains failing to form a PCR product with the *FLO5* primers, southern hybridization after chromosome fingerprinting using a *FLO5* fragment as probe showed that *FLO5* was missing from chromosome VIII, whereas hybridization to chromosome I (the site of *FLO1*) still occurred. The sequences of the successful *FLO5* PCR primers were not revealed, so we do not know whether they recognized only *FLO5* (which is 96% identical to *FLO1*).

THE EFFECT OF CELL SURFACE HYDROPHOBICITY (CSH) AND CELL SURFACE CHARGE

Several studies indicate that cell surface hydrophobicity (CSH) and surface charge change when flocculation commences, i.e. an increase of CSH and decrease of cell surface charge occur at the onset of flocculation[2,33,71,78,91]. Cell surface hydrophobicity is reported to increase rapidly as cells pass through the exponential phase and to reach high, stable levels in stationary phase[78]. The low CSH in exponential phase can be explained by the presence of many young daughter cells, which are significantly less hydrophobic than older cells[58]. It is suggested that CSH plays an important role in maintaining the correct conformation of flocculin molecules[33], so that the flocculins present in the stationary phase are more 'active'. The yeast cell surface has an overall negative charge, which is mainly determined by the phosphate groups in cell wall mannoproteins and is greater when the pH of the surrounding medium is higher[8,64]. A decrease in cell surface charge has been suggested to promote flocculation by decreasing the electrostatic repulsion between cells[64]. The cell surface charge of brewing strains has been shown to vary during growth[97]. However, no clear relationship between cell surface charge and the onset of flocculation has been observed[12,71,97]. Several environmental factors affect CSH. Higher ethanol concentrations[32], lower growth temperatures[98] and higher pitching rates[33] all increase CSH. It has recently been proposed that hydrophobic oxylipins located at the cell surface of flocculating cells are a cause of increased CSH[88,90]. It has also been observed that cell surface hydrophobicity rises when *FLO* proteins are expressed[30,102] on the cell surface. The increase in CSH observed at the onset of flocculation may be a consequence of the presence of flocculins on the yeast cell surface rather than a cause of yeast flocculation[97].

Ale strains were found to be systematically more hydrophobic and less negatively charged than lager strains[2]. As well as contributing to floc formation, the greater hydrophobicity of ale strains probably explains why the flocs of ale strains associate with CO_2 bubbles and rise to the beer surface during traditional ale fermentations, whereas the flocs of lager strains sink to the bottom[12]. In many modern breweries, ale strains also sink to the bottom of fermentations in large cylindroconical vessels (CCVs). Probably the greater hydrostatic pressure in large CCVs restricts the size of CO_2 bubbles attached to yeast flocs and the increased turbulence may detach CO_2 bubbles from the flocs, but also sedimenting mutants of ale strains have been either deliberately or accidentally selected to facilitate the more convenient process of bottom cropping.

CO-FLOCCULATION

In co-flocculation, a mixture of two strains forms flocs containing cells of both strains under conditions in which neither strain or only one strain would flocculate by itself. Co-flocculation involving two or more different ale strains is relatively common, but the phenomenon has not been reported for pairs of lager strains. This may reflect the fact that in many ale breweries the yeast has been a more or less stable mixture of two or more strains that have been repetitively cropped together for a very long time. Stewart and colleagues (described by Stewart[79]) showed that the Labatt brewery ale yeast was a mixture of two strains, which were both non-flocculent when cultured alone in wort, but which flocculated well towards the end of wort fermentations pitched with equal amounts of the two strains. To our knowledge, there is not yet a satisfactory explanation for this behaviour. It is difficult to understand how the lectin-mannose binding that underlies both *FLO1*-type and NewFlo-type flocculation can account for the co-flocculation of two strains that are each non-flocculent in pure culture. It would seem to require that only one strain has functional flocculins, whereas only the other strain contains accessible mannose residues in its cell wall.

The cell walls of *Schizosaccharomyces pombe* yeasts contain galactomannans, in which the mannose residues involved in lectin interactions in other yeasts are shielded by galactose residues. Consequently, *S. pombe* does not take part in *FLO1*-type or NewFlo-type flocculation. However, co-flocculation of *S. pombe* and *S. cerevisiae* strains has been reported and attributed to the presence of 3-OH oxylipins on the cell surfaces of both yeasts[89]. Conceivably, 3-OH oxylipins might be involved in the co-flocculation of non-flocculent ale strains described by Stewart[79], but we know of no relevant experimental evidence.

Co-flocculation of brewer's yeasts with certain bacteria, including *Acetobacter*, *Lactobacilli* and *Pediococcus damnosus* has been reported[56]. Often these bacteria are encountered as beer spoilage organisms, so that this co-flocculation is a harmful phenomenon in breweries, but it may be an advantage in the manufacture of some speciality lambic and geuze beers, where the activity of lactic acid bacteria is required. Co-flocculation of bacteria and yeast seems to involve, at least sometimes, yeast mannans and bacterial lectin-like proteins. In some cases, mannoproteins excreted into the medium by the yeast are enough to cross-link the bacterial cells resulting in their flocculation, whilst in other cases the presence of yeast cells is required to cause flocculation of the bacteria[56].

ENVIRONMENTAL FACTORS INFLUENCING FLOCCULATION

Oxygen content

It has been reported that poor wort aeration results in early and incomplete flocculation, whereas normal saturation with oxygen both delays and intensifies flocculation. The poor growth and flocculation observed after yeast was pitched into wort lacking oxygen were reversed by addition of ergosterol and oleic acid. This indicates that oxygen probably does not act directly on flocculation but rather indirectly through the synthesis of sterols and unsaturated fatty acids[91]. Sterols and unsaturated fatty acids might be necessary, for example, in the delivery and stabilization of flocculins to the cell surface.

Lawrence and Smart[42] measured flocculation (by a modified Helm's test) during growth of two industrial, NewFlo phenotype, lager strains either aerobically or under strictly anaerobic conditions with accumulating CO_2 (as in a brewery fermentation). The two strains showed completely different responses. For one (SCB2) the flocculation ability was low (<5%) after 48 h aerobic growth and much higher (50%) after 48 h anaerobic growth. By 96 h, similar flocculation abilities (30–35%) were observed aerobically and anaerobically. The other strain (SCB3) showed constant, high (ca 85%) flocculation ability during aerobic growth, but only modest ability (60% at 48 h, 30% at 96 h) during anaerobic growth. No precise explanation for this brutal difference between strains is available. The authors[42] suggest that differences in both oxygen availability and pH (which became lower during anaerobic growth) may affect expression levels of flocculation-related genes. Presumably strains SCB2 and SCB3 contain different assortments of these genes.

Differential expression of cell wall mannoproteins under aerobic and anaerobic conditions[1] has been shown in laboratory yeast. It has been postulated that this modified expression may alter the composition and structure of the cell wall, favouring lectin-receptor interactions after growth-limitation by oxygen deficiency[42,43].

Temperature

The effect of temperature on flocculation also seems to be strain-dependent. In general, Flo1 phenotype strains flocculate over a wider temperature range than NewFlo phenotype strains. For Flo1 phenotypes, flocculation did not change markedly between 5 and 25°C, whereas NewFlo phenotypes flocculated moderately to strongly at temperatures between 10 and 25°C but weakly at 5°C[32]. Neither continuous exposure to mild heat stress (37°C for 24 h) nor a short heat shock (52°C for 5 min) followed by rapid cooling and incubation at 25°C stimulated the onset of flocculation compared to control cells[9].

pH

Helm et al.[24] studied the pH-dependence of flocculation of four brewer's yeasts (flocculent and poorly flocculent strains each of ale and lager yeasts). Flocculation was maximal at strain-dependent pH values between 2.5 and 4.5. Studies of electrophoretic mobility[4,50] indicate that the isoelectric point of *S. cerevisiae* cells, the pH where they have no net electrical charge, is ≤pH 3. (Isoelectric focussing in the presence of ampholytes yielded much higher values between pH 5.2 and 6.4, which are hard to understand unless, e.g., ampholytes with net positive charge bound to the yeast cells[68].) For pI ≤pH 3, yeast cells are negatively charged at the pH (about 4-5) of fermenting wort and become increasingly negative at higher pH. This is expected to cause stronger electrostatic repulsion between cells, and thus decrease flocculation. Higher pH values may also alter the ionization of functional groups

in surface proteins, such as flocculins, and so change their conformation and activity[32]. The pH optimum for flocculation seems to be strain-dependent and differs significantly between Flo1 and NewFlo phenotype cells. The flocculation behaviour of Flo1 cells has been reported to be insensitive to pH[32,83] and to occur over the range from pH 1.5 to 10[83]. In contrast, many, though not all, NewFlo phenotype brewer's yeasts flocculate within only a narrow pH range[12,83]. Stratford studied 12 NewFlo type ale strains and found that 9 of them flocculated only between pH 2.5 and 5.0, whereas 3 of them flocculated over the broad pH range of 2.5 to 9.0[83].

Ethanol concentration

It has been suggested that ethanol affects the cell wall conformation and decreases cell-cell electrostatic repulsion, thus promoting flocculation[32]. However, the influence of ethanol on flocculation seems to be strain-dependent. Negative, positive and null effects have been reported[12,13]. There are general differences observed between Flo1 and NewFlo phenotype strains, i.e. the flocculation behaviour of Flo1 cells was found to be insensitive to ethanol over the range 0-10%, whereas NewFlo cells were found to exhibit significantly increased flocculation when ethanol concentrations increased from 1% towards 10% [32,72]. The positive effect of ethanol on flocculation has been observed only at moderate ethanol concentrations. Already 10% ethanol was found to be toxic and, perhaps therefore, to prevent flocculation[9].

Fermentable sugars and other nutrients

The presence or absence of fermentable sugars is a major factor influencing flocculation by NewFlo phenotype strains[78]. As long as glucose, maltose or maltotriose are present in sufficient amounts, flocculation is inhibited because these sugars occupy the flocculins, so inhibiting binding to mannose residues of adjacent cells. Addition of glucose (20 g/L) rapidly dissociated flocs of a starved ale yeast with NewFlo phenotype[75]. This might be expected because glucose blocks the mannose-binding sites of NewFlo-type flocculins, but the authors suggested that loss of flocculation requires energy[75].

Some authors have found nitrogen starvation to play a major role in the onset of flocculation[66]. In a defined standard cultivation medium, changing the initial amount of nitrogen was observed to shift the onset of flocculation[71]. However, Straver et al.[91] found that supplementation of wort with amino acids did not change the flocculation behaviour of the lager strains tested.

Calcium ions are essential for lectin-mediated flocculation. Calcium cannot be replaced by other cations[80]. However, some other cations (Na^+, Mg^{2+}, Ni^{2+} and even $Tris^+$) trigger a release of intracellular Ca^{2+} from yeast cells into the medium in sufficient amounts to support flocculation. In addition to this phenomenon, some cations other than Ca^{2+} may play a role in flocculation. Magnesium has been observed to be necessary and magnesium-limited yeast cells are not able to flocculate[71]. Zinc supplementation of the medium both improves flocculation[62] and decreases the average size of the yeast flocs[117].

THE INFLUENCE OF YEAST HANDLING

Effect of cell size and generational age

During handling in a brewery, the average size of yeast cells and even the proportion of virgin cells can vary. Both these parameters have been reported to affect the flocculation potential of yeast. For three industrial yeast strains fractioned into virgin-rich and mother-rich pools, the virgin-rich pool had a lower flocculation potential (25–46%) compared to the mother-rich pool (51–87%)[58]. Several differences between the cell surfaces of virgin and replicatively aged cells may explain the difference in flocculation potential. Young virgin cells lack flocculins[74], so although they can be bound by older cells, they cannot bind each other. Virgin cells have also extremely smooth cell surfaces with very few protruding structures. A rough cell surface topography is believed to favour cell to cell adhesion during the onset of flocculation[63]. Flocculins have been reported to be localised at the site of bud formation, so that mother cells may express a greater number of flocculins[7]. Moreover, cell surface hydrophobicity, which has a positive effect on flocculation, increases with replicative age.

Yeast propagation

Aeration during propagation is used in some breweries to increase yeast yields and shorten propagation times. Aeration during propagation has been shown to affect the subsequent flocculation potential of yeast cells. Three industrial lager yeast strains, earlier characterised as low, medium or highly flocculent, were propagated either aerobically (oxygen maintained throughout propagation above 5% of saturation) or anaerobically (no further aeration after pitching aerated wort)[54]. Both propagations were in 12°P wort at 12°C. For the two strains earlier characterised as low or medium flocculent, the flocculation potential was clearly lower after aerobic propagation than anaerobic propagation. For the third strain, earlier characterised as highly flocculent, the flocculation potential was only slightly lower after aerobic propagation. Robinson and Harrison[65] obtained similar results, observing that the flocculation potential of low or medium flocculent strains was lower after aerobic propagation, whereas the flocculation potential of a highly flocculent strain was unchanged or slightly improved by aeration.

Yeast cropping

Most brewery fermentations are performed in cylindroconical fermenters, which have heights and aspect ratios that vary from brewery to brewery. Yeast cropping is facilitated by yeast sedimentation to the bottom (the cone) of the vessel. At least sometimes, there is heterogeneity between different layers of the crop regarding the yeast's physiological condition, flocculation potential, cell size and replicative age. Thus, successive cropping and repitching with a specific portion of the cone may lead to a drift in the characteristics of the cropped yeast. Different yeast fractions obtained from warm cropped or conventionally cropped vessels were observed to have considerable variation in flocculation characteristics[59]. Yeast at the

bottom of the cone, removed during the initial stages of cropping, had a flocculation potential of 77%, whereas yeast in the middle fraction of the cone had a flocculation potential of 86-89%. Yeast from the top of the cone had a flocculation potential of 79%. A similar pattern of results was obtained for yeast removed during warm cropping[59], where in a similar way highly flocculent cells were found to be preferentially located in the middle layer of the cone.

Storage conditions before repitching

In the study of Rhymes and Smart[64], an ale strain (showing optimum growth at 25°C) was stored for three days at 4, 10 and 25°C under either starved (deionised water) or fed (YPD) conditions and with or without agitation (120 rpm). Flocculation was then examined in a new fermentation. Starved cells had slightly poorer flocculation ability than fed cells at all temperatures. Agitation always promoted flocculation. The best flocculation ability was observed after storage at 25°C, second best at 4°C and poorest at 10°C.

FLOCCULIN DENSITY AND ACTIVITY IN THE YEAST CELL WALL

The binding partners for flocculins, mannose residues, have been reported to be present in the yeast cell wall at a constant level throughout wort fermentation[78]. It has also been observed that the abundance of flocculins at the cell surface did not change during fermentations, although the Helm's flocculence of cells isolated and washed at various fermentation times increased from 90 to 98%[78]. Thus, flocculation ability (flocculence) did not depend merely on the number of receptors, but rather on the appearance of active flocculins. It seems that, although present, flocculins are not always accessible or operational[82,87]. Poreda et al.[57] studied calcium concentration in the medium over four successive fermentations in 9°P wort. They found that the pattern of uptake and release of calcium was similar in successive fermentations. The concentration of calcium in the medium decreased at the end of the logarithmic phase. It was assumed that just before the termination of fermentation, calcium ions were removed from the medium by the yeast and absorbed on the cell surface, causing activation of the flocculins.

MITOCHONDRIAL ASSOCIATED YEAST FLOCCULATION

So-called 'petite' mutants of yeast have little or no mitochondrial DNA and lack respiratory function. Lack of a complete mitochondrial genome causes several deleterious cell surface characteristics, such as decreased sugar uptake and alterations in flocculation ability[115]. Mitochondrial activity (measured as the ability of mitochondria to metabolize tetrazolium hydroxide, a reagent often used to quantitate mitochondrial metabolism) was shown to be higher in strongly flocculent yeast than in weakly flocculent cells[90]. Kock et al.[37] reported the accumulation of hydrophobic carboxylic acids, i.e. 3-OH-oxylipins on the cell surfaces of flocculating cells. It is proposed that 3-OH oxylipins are produced in the mitochondria of yeast cells. The presence of 3-OH oxylipins in strongly flocculating cells and their absence in weakly flocculent cells was observed[90]. When acetylsalicylic acid, a known 3-OH oxylipin inhibitor, was added to cultures of *S. cerevisiae,* both flocculation and the production of 3-OH oxylipins were significantly inhibited[88,90]. Oxylipins may increase the flocculation via their effect to increase cell surface hydrophobicity.

PREMATURE FLOCCULATION ASSOCIATED WITH MALT QUALITY

Premature yeast flocculation (PYF) before fermentable sugars have been appropriately consumed leads to economic losses and undesired changes in beer character. In recent years, PYF associated with certain malt batches and certain yeast strains has become a significant problem in some breweries. The sporadic occurrence of this phenomenon and observations that it seems to affect some yeast strains much more strongly than others[31] have complicated its systematic investigation. Premature flocculation during the primary fermentation ("primary PYF") results in beers with unacceptably high levels of residual sugars. "Secondary PYF" results in sub-optimal yeast levels during secondary fermentation so that the yeast-promoted removal of undesirable compounds, such as diacetyl, is incomplete or very slow.

PYF seems to be caused by one or more factors originating in barley (see[41]). Partly because PYF is more commonly observed in years when weather conditions favour heavy fungal contaminations of barley, it is believed that fungus-derived enzymes may generate PYF factor(s) from, e.g., arabinoxylans in the barley husk. Other possibilities include that the PYF factors are derived from microbial exopolysaccharides or even antimicrobial substances produced by barley as a response to pathogens.

A suggested mechanism is that one or more high molecular weight compounds ("PYF factors") present in some malts bind cell wall flocculins and so cross-link yeast cells to form unusually large flocs, which sediment rapidly[40]. Several substances derived from barley, particularly barley husk, have been implicated as PYF factors (Table II), which may act individually or in concert.

Herrera and Axcell[25,26] suggest that the PYF factor is a high molecular weight polysaccharide or protein factor extracted from malt husks during mashing. They isolated from malt extracts a high molecular weight (>100 kDa) polysaccharide fraction that induced premature yeast flocculation and was composed of arabinose, galactose, glucose, mannose, rhamnose, xylose and an acidic sugar component[25]. This factor was shown to be present at significantly higher concentrations in wort causing premature yeast flocculation than in normal wort[26]. It could bind to the surface of flocculent yeast cells and cross-bridge adjacent cells[26]. Van Nierop et al.[100] suggest that PYF factor is generated by fungal contamination of barley in the field or during malting. They suggest that the breakdown of malt husk arabinoxylan by fungal enzymes results in formation of PYF factors. They also suggest that there is no single PYF factor, but rather a range of arabinoxylan

Table II. Factors associated with PYF (adapted from Lake and Speers[41] and Kaur[34]).

PYF-factor description	Size	References
PAS I. gum-based polysaccharide composed of arabinose (27%), xylose (17%), mannose (17%), galactose (16%), rhamnose (14%), and glucose (12%), with an acidic sugar component	>100 kDa	25, 26
Arabinoxylan products of husk degradation by endoxylanase and *Aspergillus niger*	HMW	100
Complex polysaccharide containing arabinose (31%), xylose (21%), galactose (12%), glucose (9%), rhamnose (9%), and mannose (3%)	about 40 kDa	39
High molecular weight polysaccharide with ferulic acid as an active ingredient	≥100 kDa	41
Fraction III and V –polysaccharide composed of arabinose, galactose, xylose, rhamnose, galacturonic acid, mannose, glucose and glucuronic acid	<40 kDa	38
Sanzyme 1000 enzymatic digestion products of fractions III and V	>5,000 Da	38

fractions with varying molecular weights. Koizumi[38] isolated a PYF-active polysaccharide fraction of lower molecular weight (<40 kDa). It was composed mainly of arabinose, xylose, galactose, rhamnose and galacturonic acid. Koizumi[38] suggests that this factor binds to flocculins in cell walls and cross-bridges multiple cells through Ca^{2+} ion bridges.

There are several assays to test the PYF potential of barley and malt. Most are fermentation tests that take several days to perform, such as the method described by Jibiki et al.[31], which makes use of a PYF-sensitive lager yeast. Koizumi and Ogawa[40] have developed a rapid method to measure the PYF activity of barley, malt or wort (Rapid Kirin test). The method involves preparation of extract from crushed malt or barley grain followed by precipitation of high molecular weight substances and their suspension in water and measurement of the optical turbidity. This method is fast (total analysis time of 3 h) and is claimed to be a sensitive way to determine PYF potential. However, there is still not a universal standard assay to test PYF potential. This makes it difficult to compare PYF research results. It seems that different assays do not agree well one with another, and do not correlate well with actual behaviour in breweries.

GENETIC ENGINEERING TO IMPROVE FLOCCULATION CHARACTERISTICS

In the first attempts to genetically engineer the flocculation ability of industrial brewer's yeasts, two non-flocculent strains were transformed with a *FLO1* gene under the control of an *ADH1* promoter[28,111]. Strong flocculation was achieved with apparently a single, integrated copy of *FLO1*. However, probably because of the *ADH1* promoter used, flocculation was too early, yeast concentration in suspension too low, and fermentation therefore slow compared to untransformed controls. Since then, genetic engineering of *FLO* genes has been performed mainly with laboratory strains. This may reflect the perception that consumers still have negative attitudes towards genetic engineering of brewer's yeasts, whereas laboratory strains are easier targets for genetic manipulation. Furthermore, partly because of the difficulty of working with the very long *FLO* genes containing tandem repeats (for a discussion of the long and short cloned versions of *FLO1* see[111]), a common strategy has been to modify the promoters of native *FLO* genes.

Verstrepen et al.[102] replaced the native *FLO1* promoter of a haploid, non-flocculent laboratory strain with an *HSP30* promoter. This promoter (for a heat shock protein) was chosen because it is activated at the end of fermentation (and also in response to heat or ethanol shocks). The modified strain exhibited strong flocculation at the end of fermentation. Flocculation could also be induced earlier in fermentation by heat-shock or addition of ethanol (6%). Probably because of the lower yeast concentration in suspension, slightly slower fermentation of wort was observed compared to the unmodified strain. This was overcome by increasing the pitching rate by 25%.

In the work of Govender et al.[20], *FLO1*, *FLO5* and *FLO11* were put under the control of *ADH2* or *HSP30* promoters by replacing the native promoters of these genes in a haploid, non-flocculent laboratory strain. *ADH2* or *HSP30* promoters are strongly induced when cells enter the stationary phase. Each of the resulting six promoter-gene combinations caused a characteristic flocculation behaviour in terms of timing and intensity. The *FLO1*-based constructs induced flocculation most efficiently. Larger flocs were obtained with the *ADH1* promoter than the *HSP30* promoter. *FLO5*-based constructs were less efficient but still caused significant flocculation. *FLO11*-based constructs caused only weak flocculation, but instead caused strong flor formation and invasive growth, which were not observed with *FLO1* and *FLO5* constructs[20].

Cunha et al.[10] attempted to achieve flocculation at the end of a sugar cane-fuel ethanol fermentation by providing *FLO5* with a glucose-regulated promoter. The objective was to avoid the expensive centrifugation step currently used to recover yeast. With a non-flocculent laboratory strain, effective and well-timed flocculation with a high ethanol yield was achieved by using a *MOX-BB-FLO5* fusion promoter. To avoid possible legal issues associated with non-*Saccharomyces* DNA in surplus yeast (*MOX-BB* is from *Hansenula polymorpha*), they then tried the *ADH2* promoter, but this was not silent during early fermentation although it showed increased expression at the end. When regulatory sequences from the *ADH2* promoter were fused to the core promoter of *FLO5*, expression of *FLO5* correlated well with exhaustion of glucose. However, integration of this construct into an industrial strain (Fleischman yeast) resulted in markedly lower ethanol yields compared to normal Fleischman yeast. Thus, it can be difficult to achieve the desired result in practice, even when the theory seems to be clear. A promoter containing upstream activating sequences from *ADH2* was also used by Wang et al.[109], but coupled to the NewFlo type *FLONS* gene, so that the flocculin produced is inhibited by wort sugars. This construct was integrated into an industrial brewer's yeast at the α-acetolactate synthase

Table III. Potential approaches to improve flocculation characteristics.

Approach	Result	References
Over-expression of *FLO1 FLO5*, *FLO9* or *FLO10*	Strong over-expression expected to cause strong flocculation not inhibited by wort sugars (Flo1 phenotype). Approach can be used if timing of expression can be scheduled to late fermentation (promoter selection very important). Flocculation in Flo1 phenotype cells is not very sensitive to high ethanol concentrations.	10, 20, 102
Over-expression of *LgFLO1 FLONS*, *FLONL*	Causes NewFlo phenotype, which is inhibited by wort sugars. NewFlo type flocculation is more sensitive to environmental factors like high ethanol concentration.	109
Oxygenation of wort	Positive effect on flocculation. Some oxygenation is necessary for flocculation to occur.	91
Fermentation temperature change	Effect of temperature strongly strain-dependent. Changing fermentation temperature may improve flocculation of some strains.	32, 81
Supplementation of wort with zinc and magnesium	Positive effect on flocculation.	62, 117
Optimizing propagation condition	Anaerobic propagation beneficial for flocculation with low and medium flocculating strains.	54, 65
Optimizing cropping method	Yeast population in cone of the vessel is heterogeneous. A specific portion of the cone may give better results.	59
Optimizing yeast storage conditions	Nutrients and agitation are beneficial. Storage temperature affects flocculation characteristics.	64
Early detection of flocculation mutants in the brewery by PCR	Prediction of changes in sedimentation behaviour during fermentation.	30

ILV2 locus. Shake flask fermentation test showed that the flocculation ability of the integrants was increased 1.5 to 2.3-fold and that the diacetyl content of the culture media was lower than that of the original strain. However, performance of the integrant strain during wort fermentations was not fully reported.

Ellis et al.[16] designed a genetic network in which two genes mutually repressed each other and one of them repressed *FLO1*. The time required for the network to reach a new equilibrium caused a lag period of several days between a signal and resulting flocculation. In this network, *FLO1* was driven by a LacI-repressed promoter, whereas LacI was expressed from a TetR (Tn10.B tetracycline repressor)-repressed promoter and TetR was expressed from a LacI-repressed promoter. In the presence of the TetR inhibitor, anhydrotetracycline, no flocculation occurs because LacI is produced and represses *FLO1*. When anhydrotetracycline is removed, accumulation of TetR eventually inhibits LacI production so that *FLO1* is expressed and flocculation occurs. Libraries of TetR- and LacI-repressed promoters with different characteristics were synthesised. By choosing appropriate promoters, the timing and strength of flocculation after removal of anhydrotetracycline can be adjusted. In the examples provided[16] strong flocculation occurred after either 60 or 168 h. This work neatly demonstrates how flocculation can be controlled in principle, though the use of anhydrotetracycline in a brewery fermentation is not a practical option.

CONCLUSIONS

Yeast flocculation has great benefit for biotechnology as it facilitates the cheap and effective removal of yeast mass at the end of fermentation processes. The cells so removed can be collected and used again in the same or another process and down-stream processing of the liquid product (beer, wine, etc) becomes much simpler. Yeast flocculation is also exploited in bioremediation, for example to remove heavy metals from contaminated water[108]. For the yeast itself, the formation of flocs probably has evolved as a means to protect yeast cells and remove them from an environment that has become non-nutritious or toxic.

Yeast flocculation involves the complex interaction of genetic and environmental factors. The inherent flocculation ability of a yeast strain is largely defined by the *FLO* genes it contains and expresses. There are unsolved questions concerning what determines the dominance of different *FLO* genes (e.g., the Flo1 versus the NewFlo phenotype) and how the functionality ("activity") of flocculins present in the cell wall is controlled. In addition to these flocculin-based Flo1 and NewFlo flocculation phenotypes, some strains exhibit a mannose-insensitive, Ca^{2+}-independent flocculation[12,35,49], probably mediated by changes in cell surface hydrophobicity. How flocculation behaviour responds to environmental changes is usually strain-dependent. In some of the examples described above, strains responded in opposite directions to the same change in process conditions[12,13,42,54,65]. The genetic basis for these differential responses is not yet understood.

Table III lists some of the approaches that have been described to adjust the flocculation behaviour of brewer's yeasts in a brewing environment. By adjusting environmental factors significant modification of flocculation properties can be obtained. For a particular strain, it is important to determine its Flo phenotype, because NewFlo phenotype yeasts are more sensitive than Flo1 phenotype strains to environmental factors such as pH, temperature and ethanol concentration. Thus, a NewFlo phenotype strain is perhaps more likely to develop suboptimal flocculation in response to deliberate or accidental changes in process conditions in a brewery and its flocculation behaviour is more likely to be restored or improved by appropriate alterations to the process. However, from what has been said above, a trial and error approach will probably be needed.

In contrast, several studies[10,16,20,102,109] show that the timing and strength of flocculation can be successfully controlled in a planned way by genetic engineering, although it may sometimes be difficult to find the tools (promoters etc.) giving the required behaviour (see, e.g.[10]). In some cases, genetic engineering of flocculation characteristics appeared to alter other fermentation characteristics in ways not completely understood. For some applications, such as the fuel ethanol industry, the genetic engineering approach may be the preferred method, particu-

larly when a large change in yeast properties is required, such as conversion of a non-flocculent to a flocculent strain. However, it is still the case that such approaches are not acceptable in the food and drinks industries, because of the perceived negative attitude of consumers and of the industry itself. In principle, it would seem to be simple to select for spontaneous mutants with desirable flocculation properties. However, at least in this laboratory, deliberate attempts to select such mutants have not succeeded, although altered flocculation properties have sometime been obtained accidentally during selection for other characters (A. Huuskonen and J. Londesborough, unpublished results). A reason may be that selection for stronger flocculation does not isolate individual mutant cells, but flocs containing thousands of cells with different genotypes. Thus, it continues to be important to increase our understanding of the environmental factors that affect the flocculation of yeast cells with different genetic backgrounds, because controlling these factors is still the main way to control flocculation in a brewery.

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