invited paper

G.G. Stewart

Brewer's yeast research – a keynote paper – part 2

Following a general review of current developments in brewer's yeast species and strains in Part 1, Part 2 considers more specific aspects. Flocculation, centrifugation, genetic manipulation and spontaneous mutation aspects of a broad spectrum of brewer's yeast strains are reviewed in detail. In particular, the complexities of a flocculation phenomenon as they are affected by genetic, physiological and environmental factors (nature-nurture effects) are considered. The prevalent hypothesis, the lectin-like theory of flocculation, is discussed in detail together with the genes that control this phenomenon.

Descriptors: centrifugation, flocculation, genetic manipulation, lectins, mitochondria, respiratory deficiency, spontaneous mutation.

1 Harvesting and cropping yeast – flocculation

As well as the various aspects of brewer's yeast fermentation already discussed in this journal, another important fermentation objective is to produce crops that can be harvested, stored and subsequently re-pitched into a subsequent brew [58]. Flocculation is a complex phenomenon that is affected by genetic [62], physiological [50] and environmental factors –nature-nurture effects [61]. A number of hypotheses have been proposed to explain the mechanisms of flocculation in both *S. cerevisiae* and *S. pastorianus* strains [17]. The prevalent hypothesis is the lectin-like theory of flocculation [33]. According to this model, a specific protein (lectin), only present in flocculent yeast cells, is secreted into the outer extremities of the cell wall. The N-terminal part of the protein binds two mannose residues present in the cell walls of neighbouring flocculent and non-flocculent yeast cells [23] (Fig. 1). In this process, calcium ions are necessary for lectin activation [71]. The term floc derives from the Latin word floccus, which means a tuft of wool, while cultures that are not able to form flocs are known as non-flocculent or powdery [60] (Fig. 2, see page 95). *S. cerevisiae* and *S. pastorianus* cultures can be found aggregated in different ways and not to be confused with flocculation, such as sexual aggregation [13], co-flocculation [64] (Fig. 3), biofilm formation [30] (Fig. 4, see page 96), and chain (pseudohyphae) [18] formation, where daughter cells do not physically separate from their mother cell (Fig. 5, see page 96).

The characteristics of flocculation are illustrated by the concentration of yeast in suspension during the course of a static wort fermentation (Fig. 6, see page 97). The reasons for, and advantages of, yeast flocculation (particularly, but not only, during the brewing process) have been extensively studied and debated [62]. Flocculation may enhance the survival of yeast cells during adverse (for example, starvation) conditions. Flocculation is an important characteristic in an environment with limited nutrients because the death and autolysis of the cells inside flocs can provide further nutrients to viable cells in the surrounding environment. It has been shown that many highly flocculent cultures lost their flocculation characteristics during the early stages of growth, in the presence of nutrients (particularly wort sugars), and recovered this property towards the end of the exponential growth phase coinciding with nutrient depletion.

Individual strains of brewer's yeast (both ale and lager) differ considerably in their flocculating characteristics. At one extreme, there are highly non-flocculent strains [82] and at the other, there are very flocculent strains (Fig. 6). The latter strains tend to separate early out of suspension from fermenting wort, often resulting in an under-attenuated, sweeter, and less fully fermented beer. Beers of this nature, because of the presence of fermentable sugars (usually maltotriose and maltose), can be liable to biological instability.

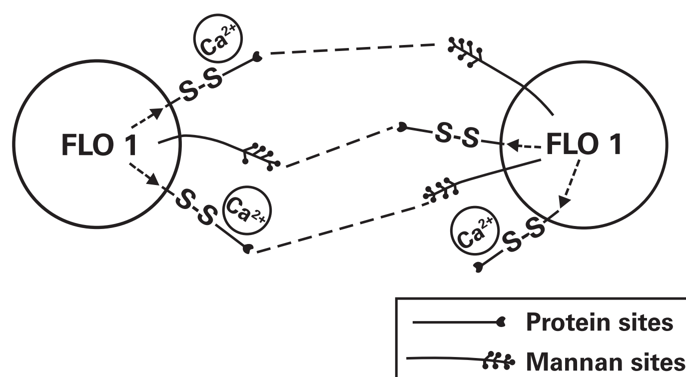

Fig. 1 Lectin theory of flocculation. Protein lectins on the yeast cell surface interact with either mannose containing and/or glucose containing carbohydrate determinants on the cell walls of adjacent cells only in the presence of calcium

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Authors

Graham G. Stewart, The International Centre for Brewing and Distilling, Heriot Watt University, Riccarton, Edinburgh, Scotland, corresponding author: profggstewart@aol.com

In contrast, poorly flocculent (non-flocculent or powdery) yeasts produce a dry, fully fermented beer with enhanced biologically stability in which cell clarification is slow, leading to filtration difficulties and the possible development of yeasty off-flavours [68].

The disadvantages presented by the two flocculation types of brewer's yeast strains are particularly relevant to traditional wort fermentation systems where the fermentation process is dependent upon the yeast's sedimentation characteristics. Contemporary brewing technology has largely reversed this situation, as sedimentation characteristics are now applied to fermenter design. The efficiency, economy, and speed of batch fermentations have improved with the use of cylindro-conical fermentation vessels (Fig. 7, see page 97), which are often (but not always) employed in tandem with a culture's flocculent characteristics. There is no doubt that the differences in the flocculation characteristics of various yeast cultures are primarily manifestation of the culture's cell wall structure.

Yeast flocculation requires the presence of cell surface protein and mannan receptors (Fig. 1). If these receptors are not available, masked, blocked, inhibited or denatured, flocculation will not occur. Brewer's yeast flocculation is an off-cost process which does not require significant energy input. However, cooling at the end of fermentation does facilitate yeast separation and this normally requires some energy. It is considered that flocculation increases process efficiency and reduces the energy consumption associated with cell separation with, for example, the use of a centrifuge which can be energy inefficient. Indeed, yeast flocculation in brewing is an example of Lean Manufacturing [12].

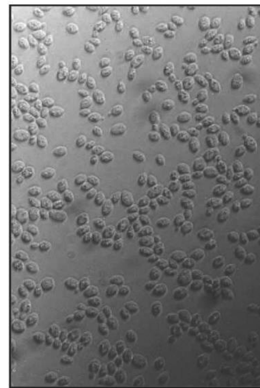
There is a dire need for some degree of standardisation in the measurement of yeast flocculation. There are a plethora of flocculation tests that can roughly be divided into three groupings:

- Sedimentation methods (for example, the Helm Sedimentation Test) [22] (Fig. 8, see page 98);
- Direct observation of floc formation in the growth/fermentation medium. This method permits routine flocculation determinations of a large number of cultures and has been extensively employed for segregation studies during FLO gene mapping studies [66]. An alternative measurement of flocculation is to microscopically examine the flocs and determine the percentage of cells in a number of flocs compared to un-flocculated cells;
- Static fermentation methods where the concentration of yeast in suspension is determined during the course of static fermentation (Fig. 6).

The first two methods for measuring yeast flocculence can be viewed as artificial tests for flocculation due to the fact that they are conducted under in vitro conditions in relation to the viewing process. The latter method is an in vivo style test because it is carried out under conditions more closely akin to the fermentation conditions encountered in a typical brewery [58] (Fig. 6).

Differences in the cell surface morphology between flocculent and non-flocculent brewer's yeast cultures have been extensively studied [6]. The wall comprises an inner layer composed predominantly of β-glucan and chitin together with an outer fimbrilar layer on flocculent cells (Fig. 9, see page 99), consisting primarily of α-mannan (highly glycosylated) fimbriae associated with proteins [10].

A Non-flocculent



B Flocculent

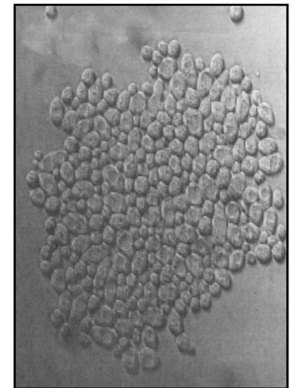


Fig. 2 Flocculation characteristics of brewer's yeast strains. Non-flocculent (A) culture and flocculent (B) culture

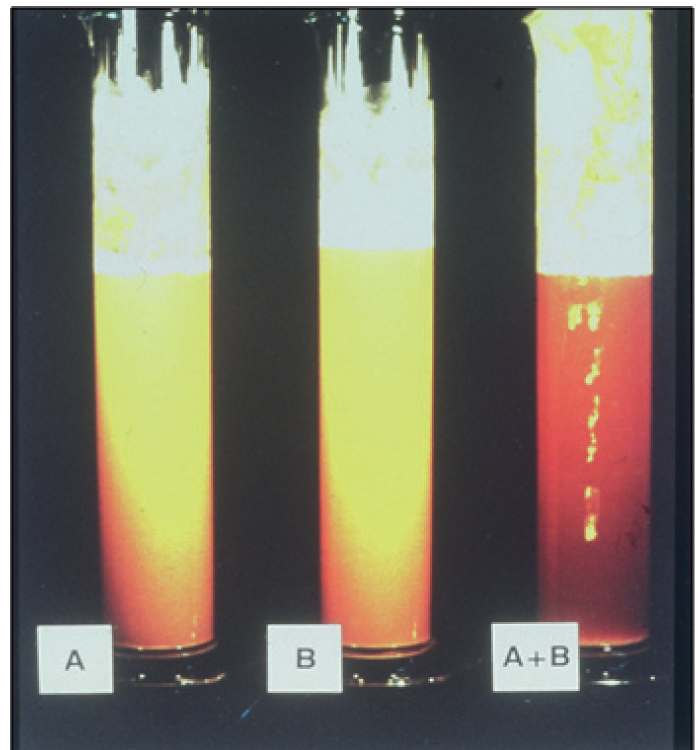


Fig. 3 Ale yeast co-flocculation – 2 L cylinder wort fermentation test

Several factors can affect yeast flocculation [68]. Flocculin proteins are encoded by members of the FLO group of genes [52]. The genetic background (nature effects) with regard to FLO genes greatly varies amongst various types of brewer's yeast (ale or lager) and other strains. Strains contain a variety of FLO gene combinations [81], resulting in a spectrum of flocculation characteristics [48,78].

Flocculation is affected by the physiological environment (nurture effects). This includes the pH, availability of appropriate metal ions, and wort nutrients during the growth phase [72]. The pH will influence the cell surface charge which will have an effect on the

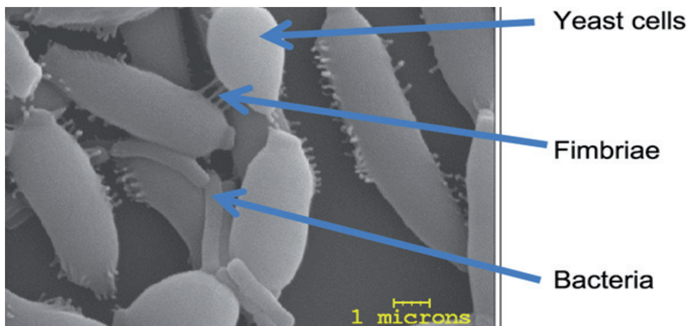


Fig. 4 Micrograph of a biofilm on the inner surface of a beer dispensing line

flocculation phenotype. Changes in pH may also modify the ionisation of functional groups in flocculin proteins, which will modify their conformation. Cells detect the expression of FLO genes and their translation into Flo proteins, and their location in the cell wall, which is influenced by a number of environmental factors [83].

The physical environment affects flocculation and the hydrodynamic (liquids in motion) conditions must be favourable and promote collision rates between cells. However, agitation must not be sufficiently violent to cause flocs to disperse. In addition, there must be sufficient cells in suspension in order to result in cell collisions to form flocs. Factors that increase cell walls' hydrophobic characteristics (cell surface hydrophobicity) and factors that decrease repulsive negative electrostatic charges on cell walls (cell surface charge), result in more intense flocculation, because they facilitate cell-cell contact [68].

Flocculation, as such, is not absolutely necessary for brewer's yeast cells to sediment out of wort towards the end of primary fermentation. This is because the size and density of yeast cells can overcome the Brownian motion that maintains cells in suspension. Under these circumstances, the sedimentation rate is slow, which is dependent on the particle size. Smaller particles settle more slowly than larger particles of similar density, because they are more retarded by friction (overall viscosity). Older (larger) yeast cells settle faster than younger, smaller cells. Nevertheless, the sedimentation of single cells is too slow to be of practical importance during wort fermentation, where flocculation is necessary to achieve the sedimentation of most yeast cultures during the later stages of a typical fermentation cycle. An appropriate concentration of yeast cells is required for effective flocculation and some cells often remain in suspension even following the bulk sedimentation of yeast flocs. As with many aspects of brewer's yeast metabolism, yeast flocculation is primarily strain dependent.

The following conditions are favourable for the sedimentation of yeast cultures during the later stages of fermentation when most of the wort sugars have been taken up by the yeast:

- Carbon dioxide production is much slower than during the fermentation's exponential phase;
- Flocculation ability is high but usually not too high;
- Wort attenuation is approaching completion – most of the wort fermentable sugars have been removed by the yeast culture, including: glucose, fructose, sucrose, maltose, and finally maltotriose [15,87];
- Yeast concentration in suspension is maximal but as soon as flocculation intensifies it will decrease.

Specifically, there are several factors that influence the rate of floc sedimentation out of wort:

- The manner that the yeast cells are packed into flocs;
- The floc size, shape and density;
- Nurture factors that include wort properties which encompass environmental factors such as: concentration (gravity), viscosity, density, and turbulence [61] [the issue of premature yeast flocculation (PYF)] [27] is a separate, but related phenomenon;
- Higher gravity worts, following fermentation, results in "green" (immature) beers which have higher alcohol viscosity and density. These factors will also retard yeast sedimentation leading to increased osmotic pressure and ethanol before dilution to the fermented wort's sales gravity at an appropriate alcohol concentration.

Co-flocculation, also known as mutual aggregation or mutual flocculation, is a heterotypic aggregation process (while flocculation is homotypic) amongst two separate ale yeast strains [64]. One strain is non-flocculent and the other strain is often weakly flocculent. When these strains are mixed together in the presence of Ca^{++} ions, flocs form and the culture rapidly settles out of suspension (Fig. 10, see page 99).

Co-flocculation between lager strains has not been identified [64]. The focus of our studies on co-flocculation was conducted with

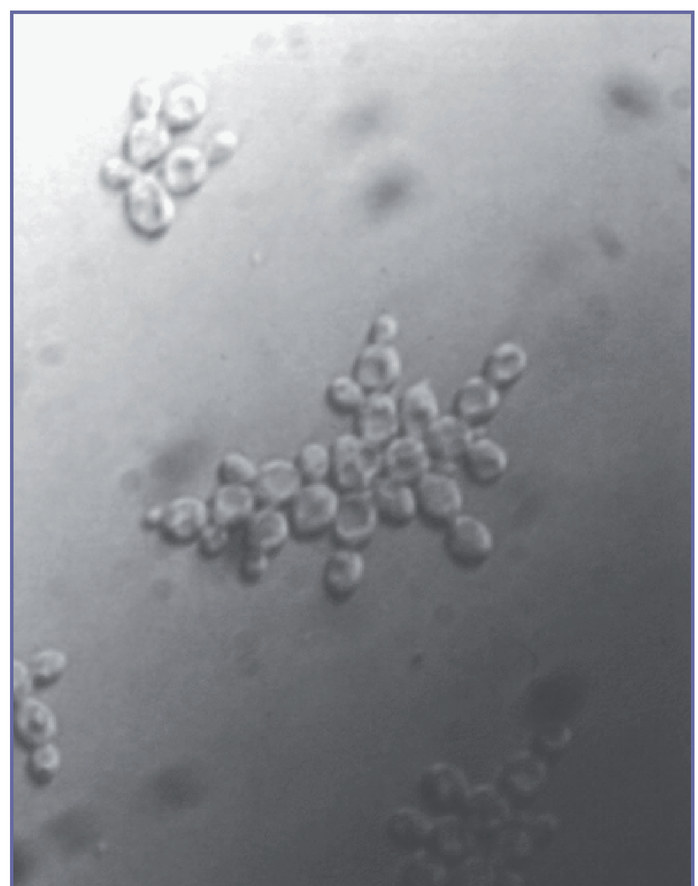


Fig. 5 Yeast chains – pseudohyphae

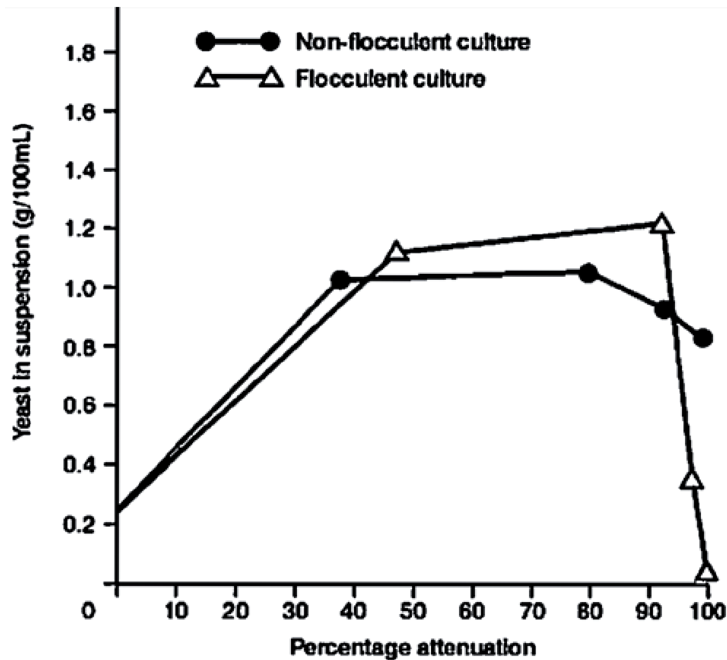


Fig. 6 Static fermentation flocculation (sedimentation) characteristics of brewer's yeast strains

the Labatt ale yeast culture. This ale culture possessed classical top-cropping properties. It also exhibited intermittent premature flocculation characteristics, resulting in under-fermented worts containing residual sugars (mainly maltotriose). This was a particular problem in Canada where the alcohol specification was (and still is) a legal regulation (Federal Health Protection Board Regulations) and the beer's alcohol composition was (and also still is) specified on the label of the bottle, can, or keg with a ± 0.2 % (v/v) alcohol specification permitted. This problem was exacerbated during HG brewing trials of ales [59]. Consequently, it was important to enumerate the number of strains in this ale culture and characterise them.

This Canadian brewery's top cropping ale yeast culture's strain composition showed that two morphologically different colony types were present when cultured on wort-gelatin media (Figs. 11 A and B, see page 100) [64], that were coded LAB A/69 and LAB B/69, with the former strain being ~ 75 % of the ale culture and the latter strain comprising ~ 25 % of this culture. A production-scale fermentation trial with the ale strain LAB A/69 was conducted in a 200-hL (20,000 L) open wood fermenter with a 12 °Plato wort at 21 °C. Although this fermentation was under-pitched it progressed rapidly, and all of the wort's five fermentable sugars were metabolised in less than 96 h. It was then that problems began! The expected top-crop failed to develop on the fermentation's surface (which had occurred with the original two strain ale mixed culture) and most of the yeast culture remained in suspension. As the brewery in question (at that time) did not possess a centrifuge, it was not possible to harvest the yeast for reuse in a subsequent fermentation and the fermented wort (all 200 hL of it) had to be discarded into the sewer with the cost charged to the brewery's effluent budget!

The two-strain composition of the Labatt co-flocculent production ale culture was deemed to be undesirable, particularly because of its tendency for premature flocculation and the consequent wort under-attenuation that occurred. This was particularly the case with high gravity worts (> 14 °Plato) [59]. Production trials with the LAB A/69 ale strain were conducted with both regular (sales) (12 °Plato) and higher gravity (16 °Plato) worts. This strain proved to be capable of successfully fermenting both gravity worts but, because of its non-flocculent property, centrifugation was required in order to harvest the culture for yeast removal, beer clarification and yeast collection for reuse. This strain (LAB A/69) has been employed for ale production by Labatt with high gravity worts (> 16 °Plato) for the past 40 years and was one of the reasons for the introduction of centrifuges into Labatt plants – further details regarding the use of centrifuges and their effect on brewer's yeast strains later.

It has already been discussed that co-flocculation has only been observed with ale strains [64] (Fig. 8). There are reports (including extensive studies in the Labatt research laboratories) of attempts to identify co-flocculation between non-flocculent lager yeast strains but to no avail [64]. Another type of co-flocculation, that has been described, occurs when an ale yeast strain has the ability to aggregate and co-sediment with contaminating bacteria such as *Hafnia protea*, *Lactobacillus brevis*, *Pediococus sp.*, or *Lactobacillus fermentum* (Fig. 12, see page 100) [86]. The *Lactobacillus fermentum* strain was isolated from a fuel alcohol molasses fermentation in Brazil and its co-flocculation characteristics with an ale yeast strain studied in Canada.

Fig. 7 Cylindroconical fermenters

Table 1 Current view of flocculation phenotypes

Genes	Character	Sugars that Inhibit Flocculation
FLO1, FLO5, FLO9, FLO10	Strong Flo1 phenotype	Only mannose
FL08	Regulation of other FLO genes	Unknown
Lg-FLO1	NewFlo phenotype	Mannose, glucose, sucrose, maltose, and maltotriose (not galactose)
FLO11	Chain formation – pseudohyphae	No inhibition by sugars
FLONL, FLONS	Like NewFlo phenotype	Mannose, glucose, sucrose, maltose, maltotriose, and galactose
Not known	Mannose-insensitive (MI) flocculation (Ca-independent)	No inhibition by sugars

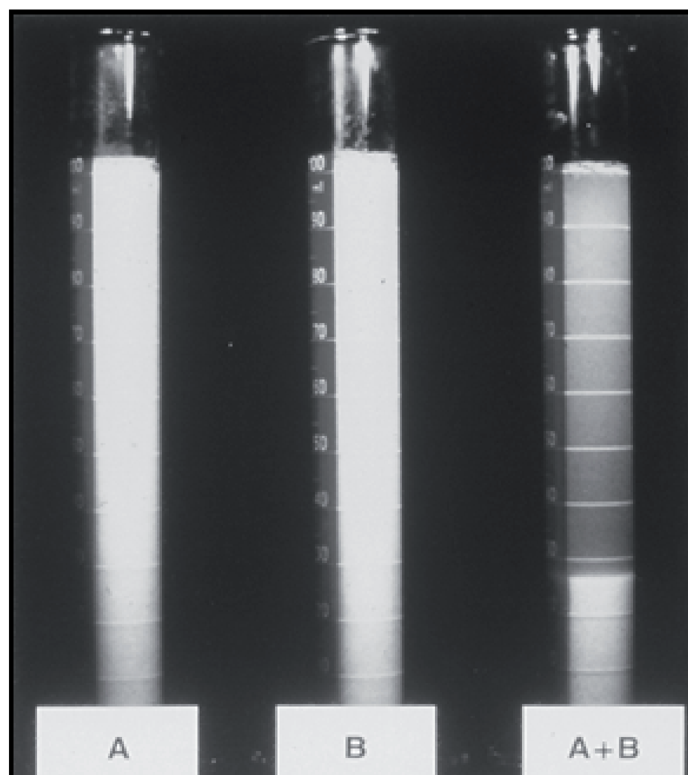


Fig. 8 Helm's sedimentation in vitro test – co-flocculation

Latterly, co-flocculation has been studied from a different perspective [34,72]. They viewed co-flocculation as a process to separate non-flocculent yeast cells from a fermentation medium. The possibility of this process being employed for the cell separation of different yeast species was assessed. The fission yeast *Schizosaccharomyces* pombe was used as the control, since these cells are unable to be aggregated by flocculent cells of *S. cerevisiae*, due to a lack of compatible receptors with *S. cerevisiae* flocculation lectins. However, this strain of the yeast *Kluyveromyces marxianus* can exhibit co-flocculation, it consequently separates this culture from suspension by settling using flocculent cells of *S. cerevisiae* to enhance it. The different degrees of co-flocculation amongst non-flocculent strains were the consequence of the different composition and structure of the yeast cell wall, particularly subtle differences in the composition and structure of its yeast cell wall, especially the mannan architecture.

Cell adhesion phenotypes are complex and are influenced not only by genetic factors which determine the composition and properties of the cell wall, but also by a number of environmental parameters, which impacts upon its adhesion behaviour. The data provides, for the first time, genetic insights into the phenomenon of co-flocculation in *S. cerevisiae*. Further studies should include additional yeast species and strains under various experimental conditions in order to comprehensively investigate the yeast co-flocculation concept. In addition, the relevance of yeast species adhesion phenotype to microbial interactions in natural ecosystems requires further detailed investigation.

Genetic studies on yeast flocculation began over 70 years ago. Such studies with brewing yeast strains, are fraught with difficulties due to their frequent triploid, polyploid or aneuploid nature [38]. It has been confirmed that *S. cerevisiae* flocculation is controlled by a number of dominant genes that have been termed FLO genes. The first flocculation gene to be identified from this yeast species was FLO1, which has been studied in detail [28]. Early studies focussed on laboratory haploid and diploid flocculent and non-flocculent strains which confirmed that the flocculent character of FLO1 was dominant and stable.

The chromosome location of this gene was determined (mapped). Nuclear DNA in *S. cerevisiae* consists of 16 chromosomes. Classical gene mapping techniques involve: mating, sporulation, micromanipulation, tetrad analysis, spore germination and multiple flocculation tests according to the methods discussed by Sherman and his colleagues [46]. It has been shown that FLO1 is located on the right-hand side of chromosome I, 33cM from the centromere [43]. Since the successful mapping of this gene, novel genetic techniques have been developed, the principal of which began with the sequencing of the *Saccharomyces* genome [19] with the haploid yeast strain S288C. This has significantly expanded our knowledge regarding the genetic control of flocculation. As a result of the reversible inhibition of flocculation by sugars, salts, a low pH environment, or protease sensitivity, two main flocculation phenotypes have been distinguished: Flo1 and New Flo phenotypes [75]. The Flo1 phenotype includes strains in which flocculation is specifically inhibited by the monosaccharide mannose and its derivatives. The NewFlo phenotype contains the majority of brewing ale strains. In this phenotype, flocculation is reversibly inhibited by mannose, maltose, glucose, and sucrose, but not galactose. Newflo phenotype strains are more sensitive to inhibition by cations, low pH conditioning, and hydrolysis by trypsin or proteinase [75]. Also, phenotypes display different sensitivities to culture conditions such as temperature, pH, ions and nutrient availability [52].

There are a number of dominant and recessive flocculation genes as well as activator or suppressor genes. At least nine FLO genes, with FLO1 being the most studied [84], have been identified. The other eight are: FLO5, FLO8, FLO9, FLO10, FLO11, FLONL,

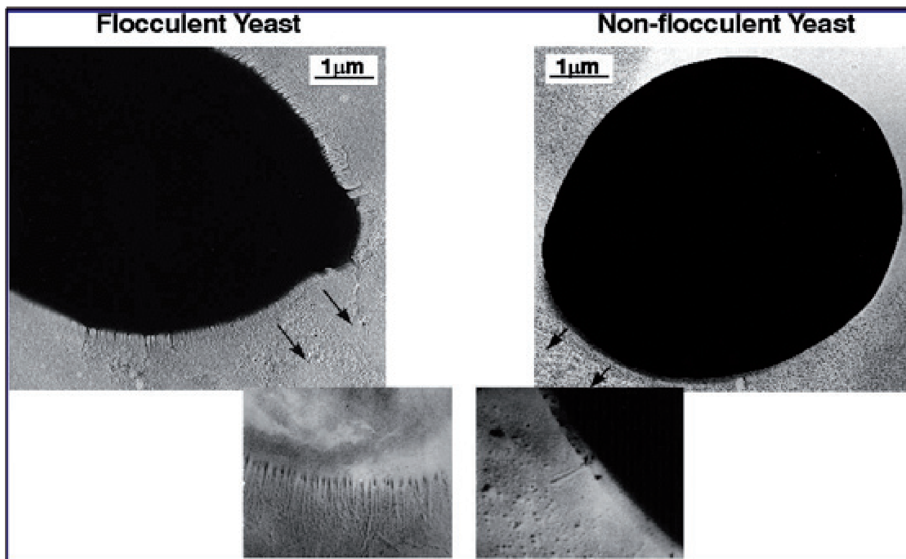


Fig. 9 Electron photomicrograph of *Saccharomyces cerevisiae* flocculent and non-flocculent strains shadow-cast with tungsten oxide showing fimbriae on the surface of a flocculent yeast culture

FLONS and LgFLO in both *S. cerevisiae* and *S. pastorianus* strains that encode flocculin proteins (lectins) (Table 1). The flocculin encoded by the FLO11 gene differs from the other genes because it is involved in filamentous growth (pseudomycelia), adhesion to solid surfaces, and flor formation rather than calcium induced flocculation per se [3]. Another gene (FLO8) encodes for a transcription factor that regulates the expression of other FLO genes [4]. For example, the first completely sequenced yeast strain (the haploid-S288C strain [19]) contains six FLO genes (FLO1, FLO5, FLO8, FLO10 and FLO11) as well as four non-functional FLO pseudogenes genes. The amino acid sequence of Flo5, Flo9 and Flo10 proteins are 96 %, 94 %, and 58 % identical, respectively, to Flo10p. As expected, Flo11p is the most distantly related to other flocculins, it is only 37 % identical to Flo10p [3].

The sedimentation performance of a brewer's yeast strain often changes during repeated cropping and re-pitching in a brewery. In principle, this could be due to irreversible or reversible genetic changes. Alternatively, it could be due to long-lasting physiological effects caused by external or environmental factors that switch genes on and affect how cells retard genes and respond to modifications in yeast-handling and fermentation environments (for example, HG worts [59]). When a genetic change, conferring a non-flocculent phenotype, occurs in a yeast culture, it will gradually become a mixture of flocculent and non-flocculent cells [45]. Often within a production lager yeast population exhibiting moderate flocculent characteristics, a more flocculent variant from the culture can be isolated. An example of this development occurred when a Canadian brewing company began brewing its lager beer, under contract, in breweries located in the United Kingdom. Most of the contracted UK breweries employed vertical fermenters (Fig. 7). However, at that time, Canadian breweries predominantly employed horizontal tanks (as both fermentation and maturation vessels). This difference in tank geometry influenced the yeast culture's sedimentation characteristics. In vertical fermenters, the Canadian yeast culture was too non-flocculent, with a considerable number of yeast cells remaining in suspension at the conclusion of fermentation (centrifuges were not available in the UK breweries at the time). It was thought that possibly this culture contained a spectrum of isolates exhibiting differing flocculation intensities. Consequently, one of the variants from the strain, with more intensive flocculation characteristics, was successfully isolated and employed in the vertical fermenters. The result was less yeast in suspension at the end of fermentation. However, care had to be taken to ensure that the flocculent variant used was not too flocculent because underfermented wort (maltose and maltotriose remaining) and residual undesirable flavours (for example, diacetyl and other VDKs) could have been the result.

Spontaneous changes in flocculation behaviour as a result of repeated re-pitching in wort during brewery fermentations have been studied by a number of research groups and the changes in flocculation intensity [67] observed. Cropped ale yeast, during 30 successive fermentation cycles, has been studied. During the first seven cycles, flocculation intensity increased from 50 % to 100 % of the original cycle (first cycle). Between the ninth and 23rd cycle, flocculation remained high. Then between the 24th and 32nd cycles, flocculation ability and cell viability diminished [68]. A long-term study in a number of breweries showed that flocculation intensity tended to decrease whilst at the same time other fermentation parameters (wort fermentation rate, esters and production of higher alcohols, etc.) usually remained constant. However, studies by the author with HG worts (16 – 18 °Plato) did not confirm this fact – rather, it is proposed that many observations of this nature must be strain-dependent [59].

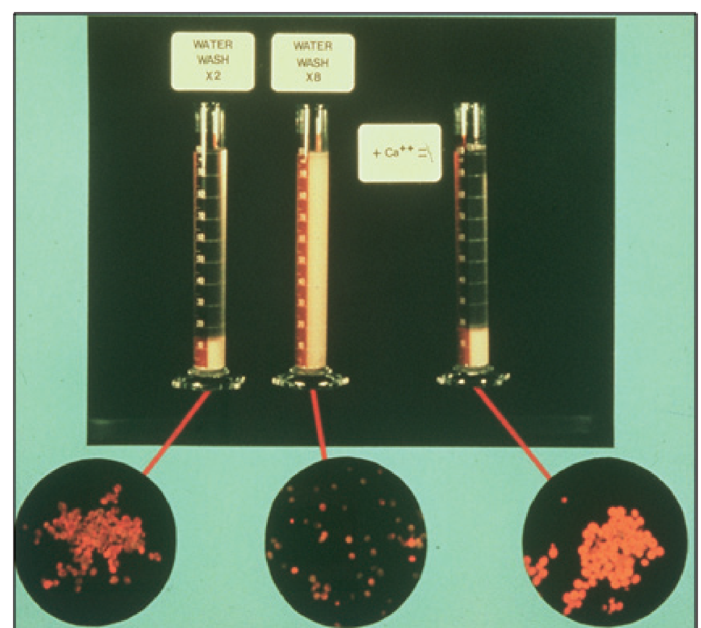


Fig. 10 De-flocculation of a yeast culture as a result of repeated water washing and subsequent re-flocculation following the addition of calcium ions

Yeast cropping methods favour the enrichment of certain cell types. Few modern lager breweries currently recycle their yeast culture more than 20 times (many breweries operate less than 10 yeast cycles) [59]. Ale brewing operators often (not all) recycle their yeast culture many more times – a few do not even enumerate the number of yeast cycles! However, as the original wort gravity increased (high gravity brewing operations), the number of yeast cycles has been reduced. Typically, with a 16 °Plato wort, the number of cycles is 10 times or less [59]. The results of these studies suggest that it is more likely that a change in flocculation behaviour due to modification in process conditions or raw materials (for example, a malt leading to changes in wort composition also with increased adjunct levels), especially if changes in the culture composition persists when freshly propagated yeast is introduced to the process.

Occasionally, certain malts can cause premature flocculation, leaving the wort under-fermented with sugars still in solution and consequently preventing the achievement of the beer's alcohol specification. This phenomenon has been termed premature yeast flocculation (PYF) [26] and is detected by way of a static fermentability test [27]. Most PYF studies have not focussed on yeast per se. They have focussed on the malt employed to produce the wort used during the fermentation studies. Nevertheless, nature-nurture interactions are critical [61]. Also, the need for detailed PYF studies with a number of brewer's yeast strains (both ale and lager) have been conducted. No relationship between PYF and specific yeast strains has been reported.

What is PYF [79]? It has been noted that PYF behaviour results in:

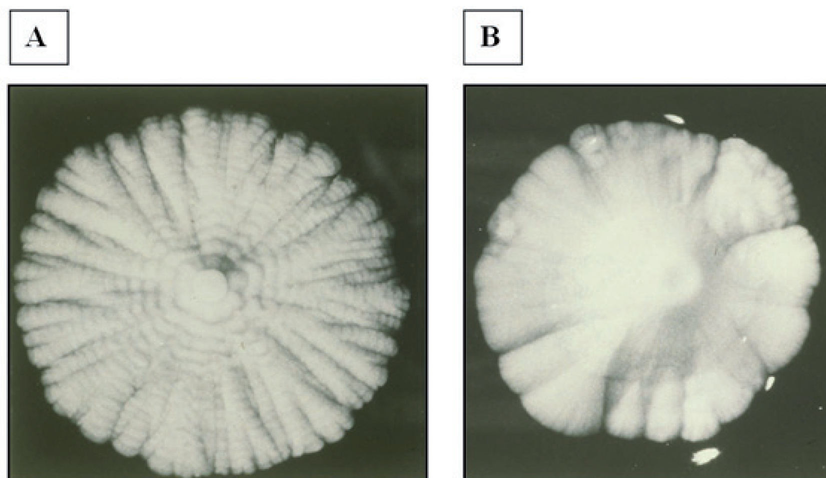
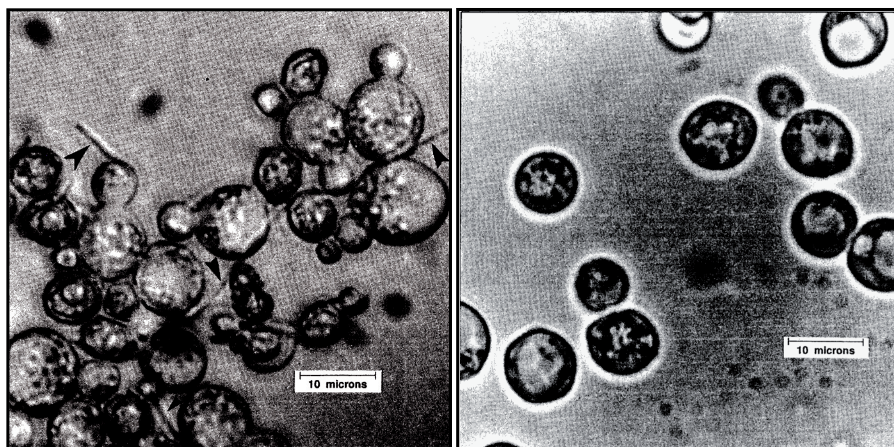


Fig. 11 Giant colony morphologies of co-flocculent ale strains A and B cultured on wort gelatin media

+ Bacteria **- Bacteria**



***Lactobacillus fermentum,* strain 125**

Arrows indicate bacterial bridges

Fig. 12 Co-flocculation between an ale yeast strain and a *Lactobacillus sp.* strain – bacteria induced yeast flocculation

- Rapid yeast flocculation out of suspension, resulting in elevated apparent extract values relative to a "normal" malt (depending on the fermentation vessel employed);
- Typically, normal fermentations exhibit parabolic yeast-in-suspension curves. They proceed in a normal and parabolic manner to a peak and then decline in a concave fashion.

Two theories dominate the literature regarding the development of PYF factors with respect to barley and malt:

- It is believed that increased microbial loads, particularly during wet seasons, leads to the production of PYF factors [80]. Barley husk is the main carrier of microorganisms [5]. The microflora consists of bacteria, wild yeasts and filamentous fungi;
- The second theory regarding PYF mechanisms is that during antimicrobial mechanisms peptide factors inhibit or negatively affect yeast metabolism. This would initiate flocculation earlier to a greater extent than normal. It has been shown that a PYF-positive malt leads to a minor decrease in wort sugar (particularly maltose and maltotriose) metabolism by yeast [79]. However, it is unclear whether this reduction in sugar metabolism is due to insufficient yeast in suspension (due to PYF) or direct inhibition of yeast metabolism.

In addition to the genetic characteristics (nature) of brewing yeast strains (FLO genes together with their suppressors and activators), and number of nurture parameters will also affect yeast flocculation [59]:

- Cations have similar roles in both ale and lager yeast flocculation with calcium ions being recognised as the most effective

ions for the promotion of flocculation [4,33]. Calcium can be removed from the yeast cell surface by washing with deionised water, through which many flocculent cultures will be de-flocculated. A de-flocculated culture will become flocculent again when calcium ions are added (Fig. 10). Some flocculent yeast strains are not de-flocculated by washing with water and the cells need to be treated with a solution of a chelating agent such as 10mM EDTA [59] followed by washing with water to remove the EDTA. This treatment de-flocculates these cultures, and the flocculation phenotype is restored upon the re-addition of calcium ions.

- Zn^{++}, Mg^{++} and Mn^{++} have also been described as inducers of flocculation [39,65]. Second to calcium, the effect of zinc on flocculation has received greatest attention regarding yeast flocculation. In addition, considerable research has been devoted to a study of the effects of Zn^{++} on enzyme activity (particularly, but not only, alcoholic dehydrogenase) and fermentation efficiency [40]. The flocculation – de-flocculation behaviour of *S. cerevisiae* is strongly dependent on the concentration of Zn^{++} in the fermentation medium (for example, wort) and it is strain-specific. However, *S. pastorianus* is not affected by the presence of Zn^{++}, which suggests another useful method for distinguishing between lager and ale flocculent strains. However, this zinc effect requires considerable further study regarding overall yeast metabolism including this lager/ale yeast difference.

Cations, such as Ba^{++}, Sr^{++}, and Pb^{++}, competitively inhibit flocculation because of the similarity of their ionic ratio to Ca^{++}. It is possible that these ratios compete for the same "calcium site" of flocculation lectins, but are not able to induce the appropriate conformation of lectins. At low concentrations, Na^{+} and K^{+} most likely induce flocculation because of a reduction in the electrostatic repulsive forces with the yeasts and/or they stimulate leakage of intracellular Ca^{++} [20].

Medium pH values can also have a profound effect on the yeast flocculation phenotype. With many laboratory and industrial strains, flocculation occurs over a wide pH range (2.5–9.0), while many brewing strains (a sub-group of the NewFlo phenotype) only flocculate within a narrow pH range (3.5–5.7) [53]. In both cases, the optimum flocculation pH value takes place between 3.0 and 5.0 according to the brewing yeast strain studied. Extreme pH values promote a reversible dispersion of yeast flocs. The modification of the pH value affects the ionisation of lectin amino acids with the consequent change in its conformation [24].

Most ale strains *S. cerevisiae* do not flocculate following growth in a chemically defined medium such as yeast nitrogen base (YNB) [67]. It could be that these strains also exhibit a narrow pH range in order to flocculate. Peptone, certain peptides, and wort also play an important role because ale cultures grown in a peptone-containing medium or wort exhibited the flocculation phenotype. This does not only occur in the culture medium. Following cell harvesting and washing, the cells were still flocculent in an in vitro flocculation test such as the Helm's Flocculation (Sedimentation) Test (Fig. 8) [22].

There are a number of publications that discuss why moderate aeration is beneficial for yeast flocculation. However, the principal rate of oxygen input, during the initial stages of wort fermentation, does not focus on flocculation. It acts as a catalyst during the synthesis of unsaturated fatty acids and sterols, whose primary role is to participate in the structure of membranes, particularly the plasma and mitochondrial membranes [29]. Membrane structure is important for the stress protection of yeast cultures during the

Fig. 13 Disc stack centrifuge (5 hL/hour)

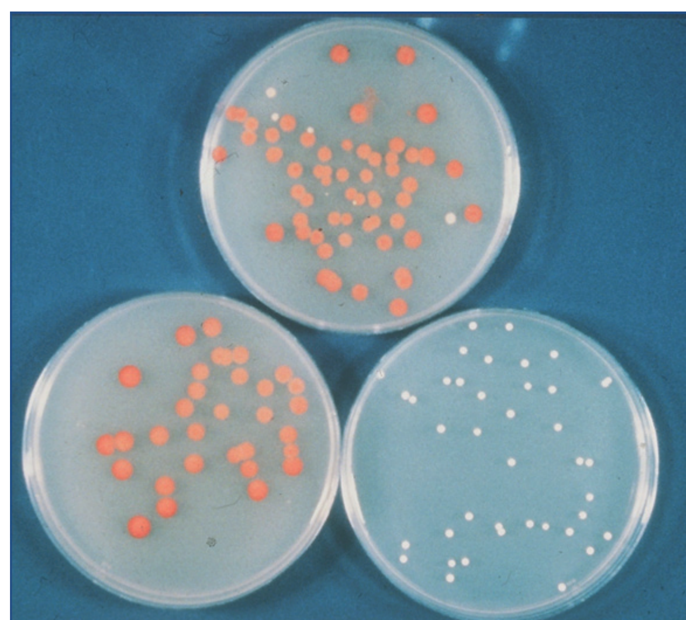


Fig. 14 Respiratory sufficient (RS) and respiratory deficient (RD) mutants – triphenyl tetrazolium chloride overlay test. RS colonies dark (grande) and RD colonies white (petite)

adverse environmental conditions that prevail during HG wort fermentations [59].

Cell wall mannoproteins are expressed differently under aerobic and anaerobic conditions [1]. The transition from semi-aerobic to anaerobic conditions, which occurs during the course of brewing fermentations, is probably associated with the expression of genes that regulate and/or encode for flocculation lectins. Cells pitched into oxygen-depleted wort flocculated relatively early during the wort fermentation cycle, but to a limited extent. The addition of ergosterol or Tween80 (a non-toxic surfactant emulsifier often used in foods and cosmetics) to the same wort restored the normal flocculation behaviour of the culture. This study concluded that lack of oxygen would inhibit ergosterol and unsaturated fatty acids (UFAs) synthesis. This limits membrane growth and results in the early onset of stationary growth phase and cell flocculation [56].

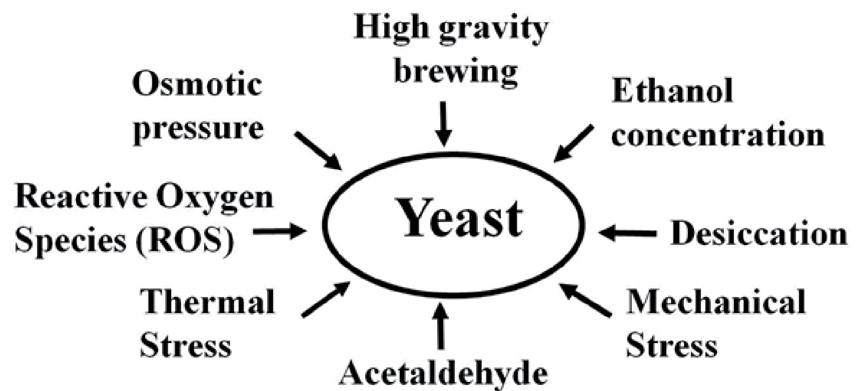


Fig. 15 Major stress factors that negatively affect yeast activity

Incubation temperature at different levels can affect the expression of the flocculation phenotype. Lower growth and fermentation temperature results in decreased yeast metabolism and CO_2 production. Consequently, there is a reduction in turbulence which will favour yeast sedimentation. During wort fermentation, the agitation (shear force – details later) temperature can also affect yeast flocculation by acting on cell-cell interactions. A temperature rise to 50 – 60 °C, for only a few minutes, promotes the reversible dispersion of flocs [77], probably because of denatured flocculation lectins. Indeed, the incubation of yeast strains above optimum ale yeast growth temperatures (35 – 37 °C) leads to reduction [9] or impairment of yeast flocculation. It is probable (but not confirmed) that heat stress (< 37 °C) acts directly on mitochondrial activity [63] and indirectly on plasma membrane structure. This affects lectin secretion with a consequent reduction in flocculation intensity.

The presence or absence of fermentable sugars is a major factor influencing flocculation by NewFlo phenotypic strains. As long as glucose, maltose or maltotriose are present in sufficient concentrations, flocculation is inhibited because these sugars bind to the flocculins which inhibits the binding to the mannose residues of adjacent cells. It has been suggested that loss of flocculation requires energy. Six aspects regarding the stimulation of flocculation loss with an ale NewFlo phenotype under growing conditions have been identified [51]:

- Carbohydrate sources are nutrients that stimulate flocculation loss in a defined growth medium [for example, yeast nitrogen base (YNB)] [67];
- All metabolisable carbon sources (for example, glucose, fructose, galactose, maltose and sucrose) induce the loss of flocculation in YNB, which ethanol does not;
- The rate of sugar-induced flocculation appears to be associated with the rate of sugar metabolism;
- The rate of sugar-induced flocculation loss most likely requires energy and this process is blocked by ethanol;
- Growth does not always trigger flocculation loss because cells grown in an ethanol medium remain flocculent;
- Glucose-induced loss of flocculation requires de novo protein synthesis - cycloheximide addition (an inhibitor of protein synthesis) to glucose-growing cells impairs the loss of flocculation [31].

As well as cell-cell adhesion (flocculation) in brewing (and wine) yeast cultures, biofilm formation is often exploited as a convenient and cost effective way to separate biomass from fermentation products (for example, fermented wort and wine must). Fermentation with biofilms appears to be an adaptive mechanism because it usually ensures access to oxygen and permits continued growth of substrates such as nonfermentable ethanol. Biofilm-adhering cells have been shown to have elevated and/or modified lipid contents as well as increased surface hydrophobicity [83].

2 Centrifugation

The incentive to optimise brewing operating costs while reducing processing times is imperative for the commercial survival of the process. Breweries, together with other manufacturing industries, continuously search for ways to enhance production efficiency [59] and lean technology concepts [12]. As a consequence, the disc stack centrifuge has become a popular component of yeast process management systems in order to reduce fermentation, maturation, and clarification times as well as to control effluent treatment costs [59] (Fig. 13). Although, the use of centrifuges in breweries (not by this author and his colleagues) was initially viewed with misgivings by many brewers [47]. However, the advantages and disadvantages are now much clearer [7]. Although centrifuges have a number of applications specifically, they can be used with brewing yeast cultures for:

- Cropping of non-flocculent yeast cultures at the end of primary fermentation;
- Reducing the yeast from immature (green) beer before the start of secondary fermentation/maturation;
- Beer recovery from cropped yeast;
- Removal of cold break (precipitated protein, polyphenols, etc.) and yeast at the end of maturation;
- Separation of the hot break after wort boiling.

The uses of centrifuges during brewing are diverse. One effect that centrifugation has on brewer's yeast is damage to the cell and the

resulting impact that this has on beer quality and stability together with yeast viability and vitality. Studies at Heriot Watt University have focused on a better understanding of the effects of passing brewing yeast cultures through a 5 hL/h disc stack centrifuge (Fig. 13) [8] as part of yeast cropping procedures. In order to confirm that the effects on yeast were from centrifugation, an extensive number of centrifugation cycles operating at two different G-forces (high and low) were employed. The centrifuged ale yeast exhibited decreasing cell viability (determined with methylene blue staining) during repeated cycles. Also, the same cultures had a higher percentage of respiratory deficient (RD) petite mutants, determined with the triphenyl tetrazolium chloride overlay method [36] (Fig. 14). The increasing RD level and decreasing viability were due to centrifugation when the exit temperature was 30 °C [60]. Fermented wort produced with this yeast exhibited reduced fermentation rates and was incomplete. Closer study of the fermented wort revealed reduced wort maltose and maltotriose uptake rates, with residual sugars when fermentation ceased. Consequently, the fermented wort's alcohol specification was not achieved. In addition, diacetyl (butterscotch-like flavour) and other vicinal diketones (VDK) were elevated at the end of fermentation because of difficulties with VDK reabsorption by yeast at the end of fermentation and during maturation [25]. Also, yeast autolysis occurred, resulting in reduced foam stability due to excreted intracellular proteinase, which hydrolysed (inactivated) foam-inducing polypeptides [59] elevated unfilterable haze (mainly mannoproteins) from disrupted cell walls, and autolysed yeast off-flavours [73]. When the bowl of the centrifuge was cooled and the exit temperature reduced to 20 °C, the cell viability increased, the culture's RD level decreased, the wort fermentation characteristics returned to normal, and the beer was drinkable again [60].

Although centrifugation can exhibit negative effects, the positive influence of controlled centrifugation on beer production and effluent control cannot be overstated. However, yeast cultures are subject to numerous factors that individually and collectively impose stresses on yeast cells (Fig. 15) [59]. The effects of environmental conditions and beer production equipment have often been underestimated (and sometimes ignored). A more complete understanding of yeast's biological response to interactions with cell physiology and brewing and analytical equipment are important criteria in maintaining process efficiency on beer quality. It is worth noting that the advent of flow cytometry and confocal imaging has introduced novel analytical methods to yeast research that have broadened the scope of research in this (and other) areas.

3 Genetic manipulation of brewer's yeast strains

Although extensive research has been conducted on the development of brewing yeast cultures through genetic engineering techniques during recent decades, the use of genetically modified yeast strains for brewing fermentations are not common. This is principally the result of both regulation and public opinion [54]. The European Union (EU) has a complicated regulatory framework, and this is slowing the approval process of novel genetically modified microorganisms. Currently, labelling of GM organisms (GMO) is mandatory in all Member States. However, the USA, in which GMO labelling is not mandatory, continues to lead developments in the biotechnology of microorganisms (including yeast), crops, biopharmaceuticals, biomaterials and bioenergy. Nevertheless, the use of novel GMO techniques with yeast for the production of potable ethanol and baker's yeast has not been extensively employed anywhere! However, yeast strains to be employed in both the USA and Brazil for industrial ethanol production (for example, fuel ethanol) have been the subject of novel GMO techniques [85].

Brewing yeast strain development is not just driven by a demand for faster fermenting strains with controlled sedimentation properties but also with stress tolerant properties. In addition, during the past few decades, the demand for brewing yeast strains that produce novel and distinct flavour profiles has increased. The brewing industry is being driven by an increasing requirement for craft and speciality beers that possess unique aromas and novel fermentation characteristics and the use of unique yeast strains for this purpose is one route to this objective [37].

The importance of molecular biology with *S. cerevisiae* strains and its closely related lager species *S. pastorianus* is emphasized in many relevant publications. The past 30 years or so have witnessed unprecedented developments in the molecular biology of yeast and many other microorganisms [2]. However, the expectation that genetically manipulated yeast strains would soon be extensively employed in both the brewing and distilling industries has not yet been realised [37]. *S. cerevisiae* was the first microorganism to be employed for the production of many foods and non-alcoholic beverages. In 1996, the complete DNA sequence of a haploid *S. cerevisiae* strain was reported [19]. Six thousand genes were identified in this strain but five thousand of them are thought to be non-essential for the functioning of the yeast cell. As well as hybridization with *a* and α mating type cells [70], spheroplast (protoplast) fusion [44] and rare mating [55] have been employed as means to introduce foreign genetic material into the yeast's genome. However, with the exception of spontaneous mutation (details later) these GM methods have not been successfully applied to brewery yeast strains [76].

Recombinant DNA (rDNA) presents the possibility of introducing

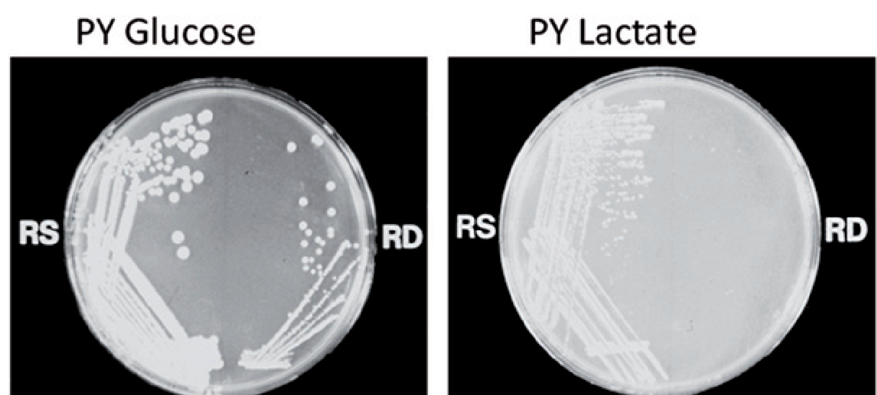


Fig. 16 Growth of respiratory sufficient (RS) and respiratory deficient (RD) cultures on fermentable (glucose) and non-fermentable (lactate) carbon sources

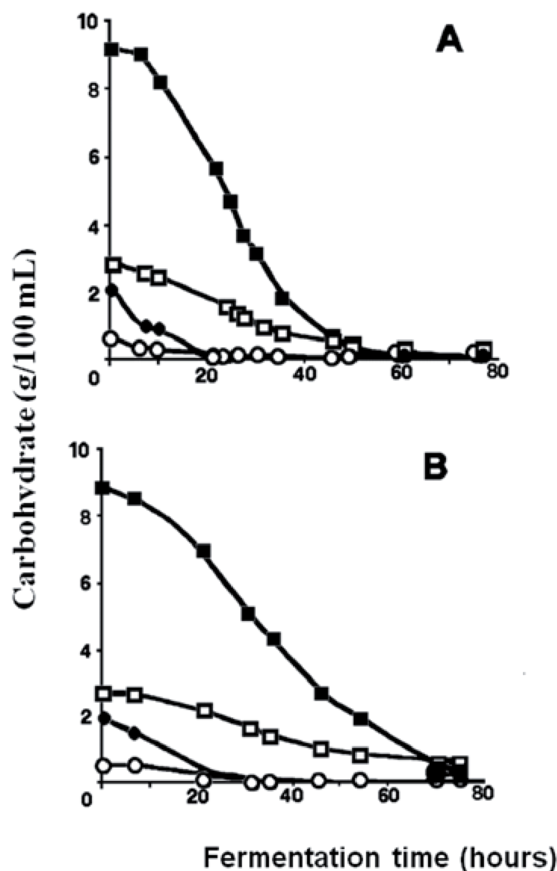


Fig. 17 Maltose (■), maltotriose (□), glucose (●), and fructose (○) uptake during the fermentation of 16 °Plato wort by brewing lager strain 3021 RS (A) and its spontaneously generated respiratory deficient mutant RD (B). Fermentations were conducted in 30 L static fermenters at 15 °C

additional DNA characteristics into yeast strains in a controlled manner. It allows the transfer of genetic information between yeast strains and species and also between completely unrelated organisms. With improvement in transformation procedures and the advent of PCR techniques to amplify DNA in order to generate millions of copies of a particular DNA sequence, genetic engineering of yeasts (and other microorganisms) has become a critical part of mainstream molecular biology research. These developments have led to automated DNA sequencing, high-throughput "omics" technologies (for example, genomics, proteomics and metabolomics), bioinformatics and enhanced contemporary tools which have enabled molecular biologists acquire the equipment to rewrite, reshuffle and edit genomes. These twenty-first century molecular biology tools have reinvented "genetic engineering" and led to the evolution of this field into "genome engineering".

Multiple gene editing with techniques such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) [74] enables rapid strain engineering of both wild type and industrial yeasts such as alcohol producing cultures. CRISPR technology has advantages over conventional marker-based genome editing. It enables rapid strain engineering of both wild-type and industrial yeast strains. It also allows performing multiple genome editing simultaneously and is independent of marker cassette integration.

To enable extensive adoption of CRISPR, the following limitations need to be addressed:

- Design of efficient and specific targeting for different yeast species;
- Elimination of a necessity for cloning;
- Large-scale multiplexing can occur;
- Intellectual property (IP) questions must be resolved – indeed, resolution of the ownership of CRISPR technology is delaying its adoption for industrial biotechnology.

4 Spontaneous mutations in brewer's yeast strains

In brewer's yeast, a number of spontaneous mutations can occur and the most frequently identified spontaneous mutation is the respiratory deficient (RD) or cytoplasmic "petite" mutation, also known as the rho-mutation that occurs in mitochondria. The reason why this type of mutation is called "petite" is because colonies (not individual cells) of such a mutant are usually much smaller than unmutated wild type respiratory sufficient (RS) colonies (also called "grande") (Fig. 14). This mutation has already been briefly discussed in this article. The reason for the French terminology is that this mutation was first identified and described in the Pasteur Institute in Paris by two Polish scientists – *Ephrussi* and *Slonimski* [14].

One of the unique features of yeast mitochondria is that they contain their own DNA – this is termed mitochondrial (mt)DNA. This mtDNA genome ranges in size from 65 to 80 kilobase pairs (Kbp) and makes up less than 0.2 % of the total cellular DNA with the remaining 99 % plus DNA being located within the nucleus. Deletions and amplifications of the mtDNA occur spontaneously in yeast cells at an incidence of approximately 1 %. The frequency of this type of mutation can be increased by stressing cells [59], for example with heat, ethanol or centrifugation plus heat in combination with other stresses already discussed.

In brewing yeast strains, the RD mutation normally occurs at frequencies between 0.5 and 5.0 % [49]. However, RD mutation levels up to 50 % have been reported for the same strain depending on temperature and culture conditions. Information on physiological and morphological differences between RS strains and their RD mutants is important to aid the understanding of mitochondrial gene expression effects in yeast. In addition, the use of mutants with modified physiological characteristics, such as sugar uptake, metabolite production, flocculation and reduced stress tolerance are important for brewing and other industrial ethanol production processes [57].

It has already been discussed that deficiencies in mitochrondrial function results in diminished ability to function aerobically. Consequently, these yeasts are unable to metabolise non-fermentable carbon sources such as lactate, glycerol or ethanol but are able to metabolise fermentable sugars such as glucose (Fig. 16). RD mutants are much more difficult to store viable in a culture collection compared to the RS culture. However, liquid nitrogen at – 196 °C and refrigeration at – 70 °C have both been found to be

effective storage procedures for yeast RD cultures [42].

Studies with brewer's yeast RD cultures have been reported since the early 1970s [44] but their overall characteristics are still not fully understood. This is particularly the case with respect to the involvement of mtDNA deletion in a yeast strain's overall characteristics. Specifically, with respect of brewer's yeast strains, the following areas have been considered:

- Effects of RD mutants on the uptake of wort sugars – particularly maltose and maltotriose, beer flavour congeners and on overall wort fermentation rate (Fig. 16);
- Effects of RD mutants on yeast sedimentation (flocculation) characteristics;
- Influence of stress conditions (centrifugation and heat) on the formation of RD mutants [21].

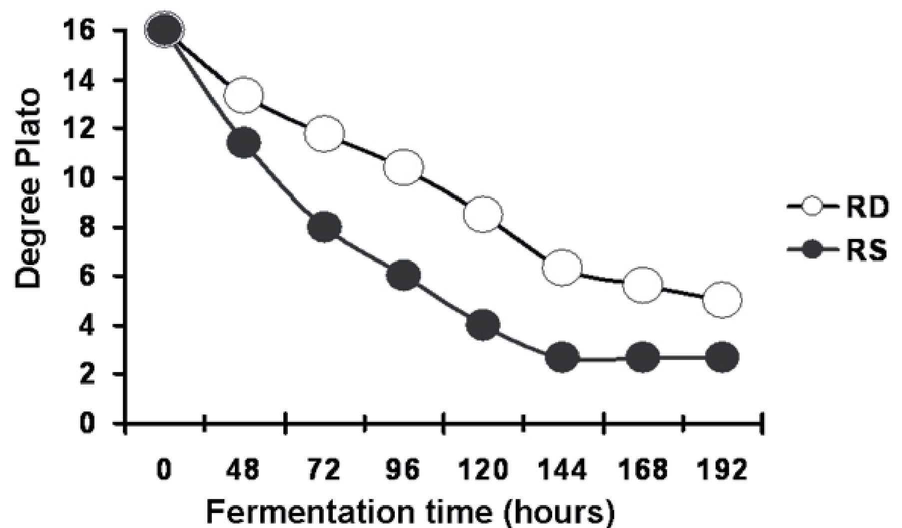


Fig. 18 Specific gravity decrease of a 16 °Plato wort by a lager brewing strain respiratory RS (●) and its spontaneously generated respiratory deficient RD (○) mutant. Fermentations were conducted in 30 L static fermenters at 15 °C

Fermentations with 16 °Plato wort were conducted at 15 °C on the 30 liter scale in stainless steel fermenters with an RS lager yeast strain and its RD spontaneous mutant. This mutant was characterized by its inability to grow on lactate and its ability to grow on glucose (Fig. 16). Also, the difference in colony size between the wild type culture (RS-grande) and its RD mutant (petite) was another factor that confirmed their identity. At specified times during the wort fermentation, a sample of the cell suspension was withdrawn, the cells removed and the supernatant stored frozen prior to analysis. Further, samples were taken from the fermentation in order to determine the concentration of yeast in suspension and the RS and RD characteristics of the culture confirmed.

The RS cells metabolised most of the wort sugars more efficiently than the RD mutant and the wort gravity decreased more rapidly (Figs. 17 and 18). Although, the uptake pattern of glucose in the two fermentations (RS and RD) was similar, the uptake of maltose and maltotriose was more rapid in the RS fermentation compared to the RD fermentation. This confirms the differences between sugars that require an active process (energy) (maltose and maltotriose) to be taken into the cell and those that employ passive transport uptake systems (for example, glucose). RD cultures contain reduced concentrations of metabolic energy (mostly as ATP) compared to their wild type RS cultures. As a result, maltose and maltotriose uptake is inhibited in RD cells whereas glucose (and fructose – data not shown) uptake in the two sets of fermentations (RS and RD) were very similar (Fig. 17).

It has already been discussed (Part 1) that many factors contribute to beer flavour [32] and of paramount importance amongst these is yeast performance and stability. Spontaneously generated RD yeasts have been reported to adversely affect beer flavour [49]. It has already been discussed that appropriate amounts of esters, fusel oils, and carbonyls and sulphur compounds (organic and inorganic) are important in determining beer flavour. In particular, esters constitute an important group of beer volatiles due to their strong, penetrating fruity flavors [69].

The concentration of carbonyls (particularly acetaldehyde), esters and fusel oils (higher alcohols) (in lager beer produced by RS and RD cultures during a 16 °Plato wort fermentation have been compared. The RD culture wort fermentation produced considerably higher amounts fusel oils (particularly propanol, isobutanol and isoamyl alcohols) compared to the RS culture. However, lower amounts of esters (ethyl acetate and isoamyl alcohol concentrations) were detected in beer produced by the RD mutant. This was also the case with acetaldehyde – acetaldehyde gives beer a characteristic grassy or "green apple" immature flavour.

The importance of flocculation during brewing wort fermentation has already been discussed. However, mitochondria in yeast cells have been reported to have a controlling influence on its cell wall characteristics. Also, the absence of distinct mtDNA regions in the genome leads to loss of flocculation [16]. The RS lager brewing strain already discussed here exhibited strong flocculation characteristics. However, the spontaneously arising RD (petite) mutant was nonflocculent. In a static 16 °Plato wort fermentation, the RS culture grew considerably faster than the RD culture and towards the end of fermentation the RS culture sedimented more rapidly out of suspension than the RD cultures. This illustrated that the RD culture's cell wall structure has been modified as a result of changes in the characteristics of the mtDNA. This resulted in loss of this mutant's flocculation intensity and also in RD mutants' inability to withstand stress [21].

Although beyond the scope of this review, information on the effects of yeast mutants on wort fermentation, damage and the subsequent dysfunction of mitochondria are important factors regarding a range of human diseases that influence animal cellular metabolism. Fundamental studies on *Saccharomyces sp.* have provided invaluable basic, information on mitochondrial malfunction. These can be extrapolated to human neurological disorders and also myopathy, diabetes, liver and heart disease and a variety other manifestations [11]. Diseases induced by mitochondrial disorders may be considered by many to be unrelated to the influence

of yeast mitochondria on brewing yeast fermentations. However, our understanding of yeast mitochondrial problems has assisted our knowledge of human mitochondrial function and disease. Current research involves removing the mtDNA from the ovaries of a diseased patient and replacing it with unmutated (healthy) mtDNA from a donor to produce a non-diseased zygote that can be inserted by in vitro fertilisation into a female recipient. However, caution regarding the clinical application of this technique has recently been expressed. The UK's Human Fertilization and Embryology Authority have published the following statement [41] "There are still hurdles to overcome before these techniques could be used clinically and it won't be a suitable treatment option for everyone at the risk of having a child with a mitochondrial disease. As in every area of medicine, moving from research into clinical practice always involves a degree of uncertainty. Experts should be satisfied that the results of further safety checks are reassuring and long term studies are critical. Every patient will need to carefully weigh up the risk and benefits for them".

5 Conclusion

Four important aspects of brewer's wort have been reviewed here. They are: flocculation, centrifugation, spontaneous mutation and genetic manipulation. In the later stages of primary fermentation, yeast is harvested from the fermented wort and used again in subsequent wort fermentations. This harvesting can be accomplished by employing the flocculation characteristics of the yeast culture or by centrifuging the culture. A number of spontaneous mutations can occur to the yeast during brewing fermentations, with the most frequently identified of them being the respiratory deficient mutation (also called the 'petite' mutation). This reflects effects on the structure of the mitochondrial DNA (mtDNA). Developments in molecular biology have facilitated brewing yeast strains improvement as a result of metabolic engineering. There are concerns about public health and environmental safety of genetically modified organisms (GMO) including yeast. This represents a hurdle for the successful introduction of manipulated brewing yeast strains in the production practice. However, the advent of CRISPR technology permits multiple gene editing and marker cassette integration.

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