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Population dynamics of flocculating yeasts

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Abstract: The problem of understanding the recognition and specific interactions in a population of yeast flocculating cells is discussed. The biochemistry, physiology and genetics of flocculation is briefly reviewed. Yeast flocculation requires the expression of a specific protein (lectin) on flocculent cells, and carbohydrate (receptors) on neighbouring cells. Adhesion experiments performed with cells whose flocculation is repressed by growth conditions, indicating that the inhibition of flocculation is due to inhibition or inactivation of 'lectin-like' component. Additionally, using adhesion experiments, it is demonstrated that cells of non-flocculent strain interact by establishing a true bond with flocculent cells rather than by entrapment inside the floc matrix. As phenotypic expression of flocculation, for several strains, is shown to be repressed, modulated or induced by modifying growth conditions, the constitutiveness and inducibility of flocculation are also discussed.

Key words: Flocculation; Yeast; Environmental conditions; Cell–cell interactions; *FLO* genes

Introduction

The word flocculation derives from the latin 'flocculus' meaning a 'tuft of wool'. Stewart [1] defined flocculation as the "phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended." According to Calleja [2], terms such as adhesion, agglomeration, agglutination, association, clumping and flocculence are widely used as synonyms of flocculation. However, this terminology is used by several authors to describe different phenomena.

More recently, the concept of flocculation in culture or fermentation is being considered different of flocculence. As a matter of fact, and according to Amory et al. [3], flocculence concerns the ability of cells to exhibit flocculation under optimum and standard conditions.

In order for cells to express flocculation, a number of conditions must be fulfilled: (i) physical movement, i.e. cells must be put in such a situation that they can collide among themselves – that is why Stratford and Keenan [4,5] stress the importance of mechanical agitation before assessing flocculence; (ii) presence of calcium [3,6–9]; and (iii) cell concentration [10]. In a recent review, Speers et al. [11] discussed the current methods employed for the assay of brewing yeast flocculation.

Flocculation has received quite a significant amount of attention, particularly from brewers. This phenomenon has also been studied by other groups, since flocculent cultures can be grown to

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very high cell densities [12,13], leading to rather high productivities [14].

Factors affecting flocculation have been extensively studied through the years, e.g. wort components, oxygen concentration, pH, temperature and cations. An extended review of this matter may be found in Calleja [2].

However, an amount of interfering factors precluded the arrival to a conclusion on those effects. Indeed, uncontrolled factors such as diversity of strains; variability of media and other environmental conditions, led often to divergent conclusions. The use of defined strains, culture media, environmental conditions and standardized flocculation tests are now becoming generalized, thereby enabling a better understanding of this phenomenon.

Molecular mechanism of flocculation

Yeast flocculation would appear to involve the external surface of cells. Thus, isolated walls from flocculent cells retained the ability to form flocs [15].

The structure of yeast cell walls is complex and still unclear. Three main types of polymers have been recognized: β-glucans, α-mannans and chitin [16]. The α-mannans (probably the best characterized and most studied component of yeast cell wall) are associated with proteins (mannoproteins), which are exposed on the external surface. A detailed review of cell wall structure can be seen in Fleet [17].

Flocculation is blocked by cyclohexamide [18], which implicated protein synthesis in development of flocculation. Additionally, chemical and enzymatic treatments irreversibly inhibit the flocculation [6,15,19,20], suggesting the involvement of cell wall proteins in the expression of flocculation. Nevertheless, all yeast cells possess mannoproteins on their cell walls, and still there are many powdery strains. Therefore, specific proteins linked to cell aggregation must be involved.

Lectins located on cell surface have been associated with cell–cell interactions [21], in a wide range of microbial aggregation phenomena [22]. Several authors claimed the isolation of specific 'flocculins' from flocculating strains; Stewart et al. [23] and Holmberg [24] for *Saccharomyces cerevisiae*, Teixeira et al. for *Kluyveromyces marxianus* [25] and Saito et al. [26] for *Hansenula anomala*. Recently, Fernandes et al. [27] reported that, in the case of *K. marxianus*, the protein induced in the cell wall of flocculent cells was a glycoprotein highly identical to cytosolic glyceraldehyde-3-phosphate dehydrogenase from *Saccharomyces cerevisiae*.

One curious feature common to three of these studies is that the apparent molecular mass obtained in each case for the protein extracted from three different genera is similar, about 37 kDa. Nowadays, there is better evidence that calcium plays a important role in flocculation. At first, calcium was thought to act simply as a charge carrier, bridging two cells [7,28]. Several workers suggested also that anionic groups implicated in the bridging are carboxyls [7,29]; others claimed that interacting surface groups were phosphodiester groups of wall mannan [30,31]. Recently, it was demonstrated that this is not the case, and that its role is of a conformation inducer [6,8,32], at least in the case of *Saccharomyces cerevisiae*. On the other hand, authors who have worked with different flocculent strains, from which they have prepared cell walls, thereby preventing an eventual calcium efflux from the cytoplasm, reported different effects when using other polyvalent cations [15,33].

The calcium-bridging theory cannot explain the reversible inhibition of flocculation by certain sugars as mannose, sucrose, maltose and glucose [7,34–36]. Reversible inhibition of flocculation by mannose and its derivatives [32,37] suggests the involvement of the α-mannan in the flocculation. Mannan blocking and chemical modification also prevents flocculation [6,38], which suggests that α-mannan can act as a recognition and binding site in cell–cell interactions [6]. Therefore, it has been proposed the 'lectin-like' theory [6,10,37]. According to this theory, a specific lectin binds to α-mannan of the adjoining cells, using calcium cations to ensure the correct conformation of the lectin. Probably, 'lectin-like' interactions are mediated by hydrogen bonds between the macromolecular components.

Recent work performed by Stratford and Assinder [39] verified two 'lectin-like' mechanisms: a Flo1 phenotype, containing all strains bearing Flo genes, in which flocculation is inhibited by mannopyranoses; and a NewFlo phenotype, containing many top ale strains, where flocculation is inhibited by manno and glucopyranoses. Flo1 and NewFlo phenotypes were further distinguished by being inhibited by high concentrations of salt, low pH values, and by sensitivity to protease.

Many questions still remain to be answered. For instance, in what measure can flocculent and non-flocculent cells interact, i.e. co-flocculate? Have the non-flocculent cells the ability to establish a true stable binding with flocculent cells, or does the co-flocculation correspond to a simple entrapment of non-flocculent cells inside the floc matrix?

In our laboratory, we have blended flocculent and non-flocculent stained cells in different proportions. Flocculence was assessed for each blend, and microscopic observations were made as well. The results showed that, even with a proportion of flocculent cells as low as 20%, 50% of the whole population will settle down after 7 min (see Fig. 1) [40]. This means that monitoring the flocculating ability of a culture, such as it is currently performed in brewing, i.e. with the help of the Helm's test [41], may lead to distorted conclusions as far as the flocculating state of the culture is concerned. In that work [40], it was also shown that the non-flocculent cells are able to compete for the binding sites of flocculent cells. Our observations [40], corroborated with microscope observations, clearly demonstrated that non-flocculent cells with receptors interact and establish a true binding with flocculent cells, i.e. they are adhered and not simply entrapped in the flocs.

A recent review of the mechanism of yeast flocculation may be found in Stratford [42].

Regulation of flocculation

Another question also to arise concerns constitutiveness and inducibility of flocculation, viz.

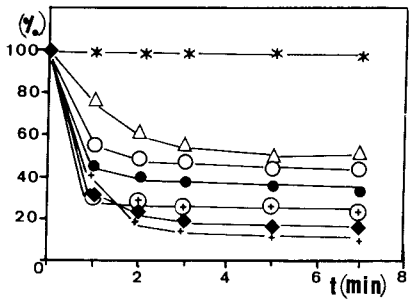

Fig. 1. Settling profiles of mixtures of flocculent cells (Saccharomyces cerevisiae NRRL Y265) with a non-flocculent strain (Saccharomyces cerevisiae var. sake). A constant amount (2×10^7 cells ml^{-1}) was mixed with various amounts of non-flocculent cells: ♦, 2×10^7 cells ml^{-1}; ⊕, 4×10^7 cells ml^{-1}; ●, 6×10^7 cells ml^{-1}; ○, 10×10^7 cells ml^{-1}; △, 14×10^7 cells ml^{-1}; Controls: +, 2×10^7 floculent cells ml^{-1}; *, 4×10^7 non-flocculent cells ml^{-1}. Cells were suspended in 15 g l^{-1} NaCl solution at pH 4 and the flocculation was initiated by the addition of 4 mM $CaCl_2$. Each point represents the mean of three independent determinations, which were made in duplicate. Reproduced from Soares et al. [40], with permission.

what are the criteria to use in order to consider a strain either as constitutive or as inducible?

An experiment run in our laboratory well illustrates the caution that must be taken when dealing with the foregoing concepts.

A reputedly constitutive strain, *Saccharomyces cerevisiae* NRRL Y265, the same strain Taylor and Orton have used in their studies [9,37,43], was grown under a set of conditions, each one corresponding to a combination of two factors in three levels: aeration rate and glucose concentration [44]. Each combination produced cells which, after collection and rinsing, exhibited, when put in standard flocculation conditions, either a powdery or a flocculent behaviour (Fig. 2).

In other words, a strain can exhibit behaviour different from the expectations, depending on the environmental conditions used for its growth. Other environmental factors, such as growth temperature and initial pH of culture medium can also affects the expression of flocculation (Soares et al., unpublished results).

Adhesion experiments similar to those performed with non-flocculent strains, were done with cells whose flocculation was repressed by growth conditions; similar results were obtained.

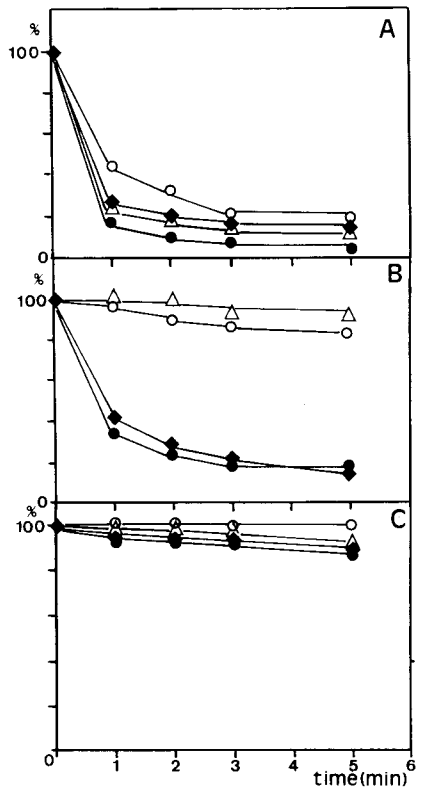

Fig. 2. Influence of aeration and glucose concentration in the flocculation capacity of Saccharomyces cerevisiae NRRL Y265. The strain was grown in different initial glucose concentrations: ○, 0.25; △, 1; ◆, 6; ●, 10% (w/v). Aeration conditions: (**A**) 0.1 vvm; (**B**) 0.5 vvm; (**C**) 5 vvm. After growth, cells were washed and suspended in 15 g l^{-1} NaCl solution at pH 4; the flocculation was initiated by the addition of 4 mM $CaCl_2$. These points are a single representation of an experience that was repeated at least three times. Reproduced from Soares et al. [44], with permission.

This suggests that the microarchitecture of cell receptors was not affected. This means that the inhibition of flocculation by adverse growth conditions was due to inhibition or inactivation of the lectin-like component.

Another situation whereby flocculation was shown to be inducible was also created in our laboratory. A strain of *K. marxianus*, in the beginning a powdery strain, was used to obtain a highly flocculent culture by means of a continuous culture technique [45]. A remarkable feature was observed: whenever a slant obtained from the flocculent population was used to inoculate a fresh medium, the new young culture returned to a powdery state. At first this was thought to be due to contamination problems. Further biochemical analysis proved that the young powdery culture was definitely *K. marxianus*. Additionally, the culture technique previously implemented gave rise again to a flocculent population.

These experiments corroborated that, at least for some strains, flocculation is inducible and regulated by environmental factors.

The effect of environmental factors in flocculation regained the attention of many authors. Masy et al. [46] correlated the onset of flocculation with the moment at which the medium contains the exact quantity of calcium necessary for flocculation to occur. On the other hand, Smith et al. [47] reported the initiation of flocculation ability at the moment that the cells stop dividing, due to nitrogen limitation.

The existence of specific proteins connected to flocculation stimulated several authors to look at the molecular aspects of this phenomenon. The identification of correlated genes is not recent. Indeed, Gilliland [48] and Thorne [49] applied genetic crosses to establish the existence of flocculation genes.

Lewis and Johnston [50] identified one dominant flocculation gene named *FLO1*. The same authors reported the identification of another dominant gene, *FLO2* [51], and of a recessive one, *flo3* [52]. Another dominant gene, *FLO4*, was also located on chromosome I [23,53]. At last, Russell et al. [54] confirmed through tetrad analysis that *FLO1*, *FLO2* and *FLO4* were in fact allelic.

Nowadays, three genes, *FLO1* (the first to be mapped in chromosome I), *FLO5* (conferring a strong flocculation, is non-allelic with *FLO1* and has been recently mapped in chromosome I by Vezinhet et al. [55]) and *FLO8* (mapped in chromosome VIII by Yamashita et al. [56]) are unanimously recognized as the dominant ones in the case of *S. cerevisiae*. The phenotypic expression of the first two genes, which is claimed to be coupled to both temperature and α-chymotrypsin effects [57], is not evident for all strains.

Recessive genes, *flo3*, *flo6* and *flo7* were also reported [58]. Several lines of evidence suggest

that the expression of the *FLO1* locus is also negatively influenced by modified genes and may be inhibited by suppression genes: *fsu1* and *fsu2* [58,59].

Besides *FLO* genes, many mutations giving rise to flocculation have been described, namely *tup1*, *cyc8*, *abs*, *wal*, and *sfl1*. These mutations and their pleiotropic effects are listed by Stratford [60]. In the case of mutation in *TUP1* and *CYC8* loci, cells exhibit calcium-dependent flocculation [61,62].

The flocculation in yeast is not a straightforward molecular mechanism. Evans et al. [63] showed that mtDNA can control cell surface characteristics. Several authors [59,64–68], reported that *petite* strains may denote an altered flocculent behaviour when compared with the wild-type progenitor. Egilson et al. [69] also reported an inhibition of flocculation strains exposed to carcinogenes; these authors correlated the loss of flocculation with mitochondrial toxicity. The molecular analysis of mitochondrial mutants, performed by Hinrichs et al. [66] revealed two areas of mtDNA involvement in flocculation: *oli1* and *oxi2*; simultaneous deletion of both causes loss of flocculence.

However, a few questions remain unanswered: at what level do environmental factors control the expression of flocculation? Can they act by preventing the biosynthesis, transport, secretion or anchorage of 'lectin-like' molecules, or by influencing the modulation of nuclear genes? On the other hand, the interaction of mtDNA with flocculation is still to be elucidated. There the explanation for the influence of aeration rate on flocculence might lie.

Conclusion

In conclusion, we may say that in the last years real progress has been made in the understanding of flocculation. Among recent advances may be cited: the isolation and identification of flocculins, the elucidation of the role of calcium and of the co-flocculation phenomenon, significant advances in the understanding of the surface chemistry of brewery yeast, the demonstration that some strains at least are inducible and that the flocculating state can be regulated.

There still remain many points to be clarified. This is the case for the cloning and sequencing of the different dominant genes, along with the physiological studies permitting a conclusive phenotypic distinction between them; the elucidation of the role of mitochondrial DNA; the unveiling of the steps lying between the transcription and the flocculin anchorage to the cell wall; and the regulation of the expression of each particular flocculation gene.

Finally, one question that will apparently remain without a straight answer in the near future is the following: why do cells, which will suffer from nutrient limitations under flocculating conditions, keep on insisting in separating from the culture medium?

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