On the Doublet Formation in the Flocculation Process of the Yeast Cells

S. Stan*
Faculty of Natural Sciences,
Center for Malting and Brewing Science,
K.U. Leuven, Kardinaal Mercierlaan 92,
B-3001 Heverlee, Belgium

F. Despa
Faculty of Natural Sciences,
Department of Physics,
K. U. Leuven, Celestijnenlaan 200D,
B-3001 Heverlee, Belgium

February 9, 2008

Abstract

The combination of single cells to form doublets is regarded as the rate-limiting step of flocculation and requires the presence of surface proteins in active form. The process of activation of the flocculation proteins of yeast cells is described in the frame of the autocrine interaction regime (Cantrell, D. A. and Smith, K. A., 1984, Science 224, 1312-1316). The influence of several effectors (the cell efficiency to use sugars, the calcium content in the external medium and the probability that free cells collide each other under thermal motion conditions) on the initial rate of flocculation and on the fraction of remaining free

*Corresponding author (the present address: Department of Food Sciences and Technology, Oregon State University, Wiegand Hall 240B, Corvallis, OR 97331-6602, USA)
cells in the steady state is briefly discussed in the paper. The present model offers an useful tool for further quantitative investigations in this topic. Also, it indicates qualitatively a way in which the regulation of flocculation might be controlled at the level of the expression of cell-surface activation abilities.

*Keywords*: flocculation; yeast; autocrine binding; lectin hypothesis
1 Introduction

Flocculation is a well-known example of natural, active aggregation and is defined as the reversible aggregation of cells into flocs (Stradford, 1992; Straver et al., 1993). Particularly, the flocculation process is important in industrial processes related to fermentation technology (brewing, wine-making, bioconversions).

In essence, flocculation is an ongoing process in which a yeast cell population, initially consisting entirely of single cells, is transformed into two fractions, flocculated and single cells. This results in a bimodal floc-size distribution (Davis and Hunt, 1986), which means that there are single cells and flocs with a clear dividing line between them, not a whole spectrum of sizes of miniflocs spreading themselves over large distances in the solute volume. Also, the visual examination of single cells and small flocs falling under gravity showed that large fast-moving flocs did not collide with single cells in their path but swept them aside in mass flows of liquid ahead of the particles (Stradford, 1992). The only collision observed, and subsequent adhesion, were between floc particles of approximately similar sizes.

In the light of this evidence, Stratford (1992) proposed the cascade theory to approach the flocculation process. According to this theory, the particles only succeed in colliding with particles of similar size. The rate-limiting step of the process is the combination of single cells to form doublets. Flocs then rapidly build up in size by combining with similar-sized flocs until they reach the maximum size limit imposed by shear forces of agitation. As a consequence of rapid formation of large flocs, the relocation of the remaining single cells and floc compression elapses. The overall effect is that flocs become progressively less dense and incorporate more empty space as size increases (Davis and Hunt, 1986). In the empty spaces of the large floc structure, the remaining single cells form small clusters. Such a system showing local symmetry repeated at different enlargements and scales is a fractal structure (Mandelbrot, 1990). The fractal dimension measured for yeast flocs confirms the supercluster nature of the floc structure (Davis and Hunt, 1986), and also indicates that the structure was formed by a diffusion-limited process (Schaefer, 1989). Thus, the properties of the floc structure are strongly related to their microstructured morphologies resulting from a specific agglutination and growth process.

Since major macroscopic features of the flocculation process seem to be
well understood, several questions regarding the microscopic aspects of the process still remain open. For example, it is of a crucial importance to know how two cells form a dimer structure? How will factors in the external cellular medium prevent or induce the dimer formation and which are the main external factors intervening in the mechanism of dimer formation?

Within a fully developed yeast-culture, most cells are flocculating or retain the option to flocculate when activated by calcium ions. The flocculation process of the yeast cells requires the presence of surface proteins and mannan receptors. If these are not available, or masked, blocked by binding specific sugars or generally inhibited or denatured, flocculation can not occur. Flocculation, once developed, is an intrinsic property of the cell wall. To sum up at this point, sugar-binding proteins, lectins and flocculation share the characteristics of inhibition by specific sugars and a requirement for calcium ions. Also, for flocculation to be expressed, receptor groups must be available on the other yeast walls to allow bonding by the flocculation protein (Stradford, 1992).

We are entering an exciting phase within which links are being forged between transduction events at the plasma membrane and the surface cell receptors (carbohydrate and proteins), which contribute to the onset of flocculation. Indeed, there are several transduction steps to elucidate in order to understand how the regulatory factors (antigens) act on the genes involved in the protein secretion, and how the secreted proteins become surface cell receptors and bring about the onset of flocculation. Nevertheless, the precursor stage of dimerization, after the flocculation proteins are fully expressed in the cell wall but not activated yet for the flocculation onset, is rather complex. Further investigation at this stage is one pressing issue in the general problem of understanding how the flocculation effectors govern the dynamics of the process and how can the regulation of flocculation can be controlled at the level of the expression of cell-surface activation abilities.

With this background we can state the main issues of this paper. What is the precursor stage of the dimer formation and how do we characterize it? What is the physical mechanism of the process and what mathematical equation governs this process?

To address these topics the objective of this study was twofold: First, we aimed to demonstrate that the process of activation of the flocculation proteins can be seen in a more general context of cellular processes, that is the autocrine interaction regime (Cantrell and Smith, 1984). In turn, this
would mean flocculation has a kinetic base and informations can at least be mathematically attainable on a computer (Despa and Despa, 1997). A second objective was to explore quantitatively the influence of the flocculation effectors on the rate of the dimer formation. Beyond this was the idea of varying both the cell intrinsic parameters and external effectors to see how modifying their range would affect, within the limitations of the present approach, the tendency of cells to form dimers.

2 Theoretical model

In order to achieve a rapid progress in describing flocculence, we will use in the following a more simplified model which, indeed, retains the important features observed so far.

Suppose that the conditions for binding between specific surface lectin proteins of flocculent cells and carbohydrate receptors on nearby cells are fulfilled satisfied. This is the lectin-like binding hypothesis (Miki et al., 1982). The surface proteins need active conformations (lectin properties) in order to bond to the corresponding carbohydrate receptors. This is fully ensured by bonding $Ca^{2+}$ ions to the flocculation proteins, which lead consequently to their lectin (active) conformation. Note that, another hypothesis of the doublet formation assumes the leading role to the calcium ions (the calcium-bridging hypothesis (Harris, 1959; Mill, 1964)). There, the divalent calcium ions form bridges between surface carboxyl or phosphate groups on different cells. The calcium-bridging hypothesis falls to explain the inhibition of flocculation by sugars while, the lectin-like binding hypothesis succeeded. The lectin-like binding hypothesis gets further support by the observation that various non-flocculent strains of yeast are able to co-flocculate when mixed together (Stradford, 1992a).

We recall from above that the onset of the flocculation process needs a primary insertion of the flocculation proteins from the plasma membrane into the cell wall and is based on the calcium-dependent interaction between lectins and cell wall mannan receptors. The mechanisms leading to the activation of the flocculation proteins (this implicitly assumes correct lectin conformation by $Ca^{2+}$ binding) are largely unknown. In the following, we propose an activation mechanism for the flocculation proteins much in the same manner as it has used to describe the activation of helper $T$-cells by
IL – 2 growth factors (Cantrell and Smith, 1984; Despa and Despa, 1997). There, the interaction between the helper T− cells and their corresponding IL – 2 growth factors obey a self-interaction mechanism within the limit of the autocrine binding regime.

Similarly, we assume here that the activation mechanism of the flocculation proteins and the onset of the flocculation process involve an autocrine binding regime for sugar radicals and Ca\(^{2+}\) ions. It implies a self-interaction phenomenon, which means the following: The presence of sugars (and the other nutrients) in the autocrine region leads primarily to its consumption and consequent production of metabolic energy. Production of metabolic energy is vital at all stages of the cell development including the protein encoding by flocculation genes (Novick et al., 1981). Once the flocculation proteins developed, these are inserted in the cell wall and, afterwards, activated by Ca\(^{2+}\) binding. Concomitantly, the activated surface proteins may be blocked to onset flocculation by the inhibitory action of sugars. Lectins may have great affinity for wort sugars but the interaction in the cell wall mannan of the cell with other sugar residues may be also possible. So that, on one hand the sugar promotes flocculation by providing the cell with the metabolic energy for flocculation protein formation and, on the other hand, it fetters the ongoing process by specific binding to the active flocculation proteins.

In the following, we consider that the surface proteins are preformed at an earlier stage of development and inserted in the cell wall. The abundance of flocculation proteins in the cell wall is directly related to the cell efficiency to use sugars (nutrients). We also assume that the calcium ions have the physical access to the flocculation proteins. The active conformation of the flocculation proteins is achieved by bonding Ca\(^{2+}\) ions. Accordingly, we propose the following rate equation

\[
\frac{dW}{dt} = \alpha + N_{\text{bound}}(t) - \alpha_{-1}W(t),
\]

(1)

to describe the activation of the surface proteins. The first term in the right side of this equation represents the cell efficiency to use sugars (nutrients) in order to produce the metabolic energy needed for the activation of the flocculation proteins. (Actually, the ratio of concentration of signal nutrient to sugar may conceivably influence flocculation (Stradford, 1992).) The effective value of this term accounts for the sugar depletion in unit time. The
second term, \( N_{\text{bound}}(t) \), represents the time-depending number of receptors (flocculation proteins) where the \( Ca^{2+} \) ions are bound. This depends on the size of the autocrine region (i.e., the region close to the cell in which any \( Ca^{2+} \) ion will be undoubtedly bound to the surface protein). The third term represents the self-interaction term, which induces a saturation effect due to the extra-sugar content (\( \alpha \equiv 1 - \alpha \), in the absolute value).

In such a way, we may observe that the external concentration of sugar controls both early events, prior the activation, (starting the signaling cascade of proteins encoding by structural genes) and later events (e.g., the inhibition of the surface cell receptors). \( Ca^{2+} \) has the secondary role to promote the lectin properties of the surface proteins. Other cumbersome effects related to the cofactors action on the flocculation proteins (proteolytic cleavage, physical exposure) are disregarded in the present model.

Further on, let us consider a suspension of cells at the moment of time \( t \), each of them having an average number \( W_0 \) of surface proteins uniformly distributed on the cell surface. We assume that the calcium ions having the concentration \( n_{Ca^{2+}} \), move diffusively around the yeast cells. The surface proteins bind calcium ions from the autocrine region resulting flocculation proteins in active (lectin) forms. The bonding process has a certain probability \( P(t) \). The simplest choice for the binding probability in unit time is

\[
P(t) = \frac{[W_0 - N_{\text{bound}}(t)]}{W_0},
\]

\( W_0 - N_{\text{bound}}(t) \) is the number of the available receptors (surface proteins) at the moment of time \( t \). Consequently, the time variation of the number of surface proteins (receptors) where calcium ions are bound (receptor occupation), \( N_{\text{bound}}(t) \), is given by the following equation

\[
\frac{d}{dt} N_{\text{bound}}(t) = n_{Ca^{2+}} V_{ar} P(t),
\]

where \( V_{ar} \) is the volume of the autocrine region. For numerical applications we may approximate the radial dimension of the autocrine region by the characteristic Debye length (\( \lambda_D \)). The Debye length measures the size of the ionic cloud which surrounds the (charged) yeast cell. Doing so, \( V_{ar} \) results in

\[
V_{ar} \approx \frac{4\pi}{3} [(R + \lambda_D)^3 - R^3],
\]

\[7\]
where $R$ is the radius of (spherical) cell. The size of the ionic cloud surrounding the cell is undoubtedly related to the $pH$ value of the medium. Any change of the $pH$ value does affect both surface charge and Debye length ($\lambda_D$). Implicitly, the equilibrium value of the calcium concentration around the cell is changed.

To obtain the solution of eq. (1), which gives us the number of activated surface proteins, ready for flocculation, we have to integrate numerically the equations system composed from eqs. (1), (2) and (3). In the assumptions of the present model, $W_0$ entering eq. (2) has a constant value.

Before embarking on other details, we see that the activation of the surface proteins which, in turn, promotes flocculation, depends in the present kinetic model on: the total number of surface proteins ($W_0$), the average concentration of calcium ions ($n_{Ca^{2+}}$), the rate and/or efficiency of the cell to use sugars and the degree of saturation of sugar content in the external medium ($\alpha_{-1}$), and on the specificity of the medium (temperature, viscosity, charge density on the cell membrane) by the net value of the Debye length.

According to the cascade theory of flocculation the rate-limiting step of the process is the combination of single cells to form doublets (Stradford, 1992). The doublets combine to form groups of four, and on to eight, 16, 32, 64, etc. (cascade theory). Subsequent collisions between pairs of increasingly larger particles are energetically easier and are therefore not rate-limiting.

The rate of the dimer formation is in a direct proportion with the concentration of free cells ($c_0 - c_f$) (where $c_0$ is the initial concentration of free cells and $c_f$ stands for the dimers at the moment of time $t$), with the relative number of activated proteins in unit time $\frac{1}{W_0} \frac{dW}{dt}$ and, indeed, depends on the probability that free cells collide each other under thermal motion conditions $\nu$. Therefore, this can be written as

$$\frac{dc_f}{dt} \cong \frac{1}{W_0} \frac{dW}{dt} (c_0 - c_f) \nu .$$

The number of collisions which occur between molecules in a fluid system can be calculated from a complex function (Chapman and Cowling, 1970) depending, mainly, on the temperature $T$ and on the viscosity of the medium. This resolves to

$$\nu = aT^{\frac{1}{2}} ,$$

where $a$ is a constant measured in appropriate units.
3 Results

The present model assumes that yeast flocculation is a kinetic process depending on several effectors. The effectors may change both the initial rate of flocculation and the net value of the content of remaining free cells. Their influence will be examined in the following. The initial number of free cells was set at $10^9$ per litter and any cell division was disregarded at the present level of approximation. Also, we assumed that each yeast cell has approximate $10^6$ surface proteins. The cell is considered as having a spherical form with the radius equal to about $5 \mu m$.

3.0.1 Cell efficiency to use sugar/nutrients

We proceeded to integrate numerically (5) over a wide interval of time, between 0 and 100 (arbitrary) time units. The calcium content is maintained at a constant value $n_{Ca^{2+}} = 3.1 \times 10^{21}$ ions per unit volume. The Debye length was set at $\lambda_D = 0.1 \mu m$ and, for simplicity, $\nu$ equal to unity. Generally, we observed that the flocculation process proceeds from a high initial rate which progressively declines until a steady state is reached where no further flocculation occurs, leaving a small fraction of free cells. This general behavior agrees with the experimental observations (see Stradford, 1992 and references therein). Specifically, in Fig. 1 we may see the behavior of the free cells concentration, $c_0 - c_f$, for two different values of the $\alpha$ parameter. For $\alpha = 0.9$, which means an almost ideal efficiency of cell to use sugar/nutrients, we can see a high initial rate of flocculation. The steady state is achieved relatively soon and is characterized by a small fraction $\left(\frac{c_0-c_f}{c_0} \simeq 10^{-4}\right)$ of free cells. (The last result is not evident from Fig. 1.) Lowering the efficiency of yeast cell to use sugar/nutrients to $\alpha = 0.5$, the value of the remaining free cells fraction is drastically enhanced about three orders of magnitude $\left(\frac{c_0-c_f}{c_0} \simeq 10^{-1}\right)$. In the latter case, the initial rate of flocculation is dramatically changed, the slope being modified with about 20%.

3.0.2 Calcium content

In the following, we will keep the efficiency parameter at a constant value ($\alpha = 0.5$). The concentration of the calcium ions is now varied while, all the parameters in above remain at their previous values. We increased the
calcium content of three times, from $3.1 \times 10^{21}$ ions to $9.3 \times 10^{21}$ ions per unit volume. Looking at Fig. 2, we may observe that the initial rate of flocculation is strongly influenced by the calcium content, as we just expected. Supplying the calcium content at the initial stage of evolution of the cell culture, the activation rate of the surface proteins is sped up resulting in a more rapid flocculation process. The steady state free cells fraction is almost the same for both calcium contents.

The same effect, as above, can be achieved by changing the Debye length and, implicitly, the volume of the autocrine region. (In the practicality the Debye length should be related to the $pH$ value in the external medium.)

4 Final remarks

Although sporadic flocculation may appear even from an earlier stage of the yeast culture development, it is actually initiated after the growth process ceased. The quantity of free cells in solution decreases sharply after a certain time has elapsed. Under brewing conditions, the initiation of this process is triggered after the growth limitation proceeded by a limited oxygen supply (i.e., oxygen saturation of the wort at the beginning of fermentation), as it was shown recently (Straver et. al, 1993a). The $CO_2$ formation during the fermentation produces a natural agitation among the suspended cells that is a causal factor in flocculation. Agitation causes rapid and progressive flocculation but, if at any time, due to various reasons, agitation ceased, flocculation stopped (Stradford, 1987). In the present model, the above observation can be easy correlated with the appropriate number of collisions $\nu$, which occur between cells in the suspension. By increasing $\nu$ the rate of flocculation goes into higher values.

On the other hand, the delay in the initiation of flocculence has been seen as an expression of the fact that the synthesis of the lectin involved in flocculation of brewer’s yeast is not regulated during the growth process (Straver et. al, 1993a). This observation indicates that the regulation of flocculation might be controlled at the level of the expression of cell-surface activation abilities. Characterization and regulation of flocculation at this level of surface cell activation is a real challenge in the development of industrially feasible methods for manipulating yeast-cell components in order to control flocculence during fermentation. The theoretical model of floccula-
tion developed in the present paper offers, at a certain extent, an useful tool for further investigations on this line.

Moreover, a sum of other similar biological processes can be described by using the present kinetic approach. For example, the adhesion of yeast cells to carbon dioxide bubbles (flotation) in the wine-making technology or the binding of cells to the matrix and to microorganisms that have already adhered can be subject of the present approach.
REFERENCES

Cantrell, D. A. and Smith, K. A., 1984, The Interleukin-2 T-Cell System: a New Cell Growth Model, Science 224, 1312-1316.

Chapman, S. and Cowling T.G., 1970, The Mathematical Theory of Non-Uniform Gases, 3rd ed. (Cambridge University Press) pp. 235-236.

Davis, R.H. and Hunt, T.P., 1986, Modeling and Measurement of Yeast Flocculation, Biotechnology Progress 2, 91-97.

Despa, S.-I., and Despa, F., 1997, Diffusion Model for Growth Factors-Cell Receptors Interaction, BioSystems, 44 59-68.

Harris, J.O., 1959, Possible Mechanism of Yeast Flocculation, Journal of the Institute of Brewing 65, 5-6.

Mandelbrot, B.B., 1990, Fractals - a Geometry of Nature, New Scientist 127, 38-43.

Miki, B.L.A., Poon, N.H., James, A.P. and Selegy, V.L., 1982, Possible Mechanism for Flocculation Interactions Governed by the Gene FLO1 in Saccharomyces cerevisiae, J. Bacteriol. 150, 878-889.

Mill, P.J., 1964, The Nature of the Interactions between Flocculent Cells in the Flocculation of Saccharomyces cerevisiae, Journal of General Microbiology 35, 61-68.

Novick, P., Ferro, S. and Schekman, R., 1981, Order of Events in the Yeast Secretory Pathway, Cell 25, 461-469.

Schaefer, D.V., 1989, Polymers, Fractals, and Ceramic Materials, Science 243, 1023-1027.

Stratford, M., 1992, Yeast Flocculation: A New Perspective, Adv. Microb. Physiol. 33, 2-71.

Stratford, M. 1992a, Yeast Flocculation: Reconciliation of physiological and Genetic Viewpoint, Yeast 8, 25-38.

Stratford, M. and Keenan, M.H., 1987, Yeast Flocculation: Kinetics and Collision Theory, Yeast 3, 201-206.

Straver, M.H., Kijne, J.W. and Smith, G., 1993, Cause and Control of Flocculation in Yeast, Trends in Biotechnology 11, 228-232.

Straver, M.H., Smit, G. and Kijne, J.W., 1993a, Determinants of Flocculence of Brewer’s Yeast During Fermentation in Wort, Yeast 9, 527-532.
FIGURE CAPTIONS

Fig. 1 - The influence of the efficiency of the yeast cell to use the sugar/nutrients on the flocculation process.

Fig. 2 - The influence of the calcium content on the flocculation process.
This figure "FLOC11.jpg" is available in "jpg" format from:

http://arXiv.org/ps/physics/0105062v1
This figure "FLOC22.jpg" is available in "jpg" format from:

http://arXiv.org/ps/physics/0105062v1