The Effect of Nitrogenous Substances on the Time of Flocculation of *Saccharomyces cerevisiae*

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SUMMARY

The point in the growth cycle at which a strain of brewer's yeast became potentially flocculent could be delayed by supplementing the medium with ammonia, basic amino acids, glutamine, asparagine, γ -aminobutyric acid or urea. Other amino acids were ineffective. β -Alanine and 2-chloro-4-aminobenzoic acid led to an abnormally early appearance of potential flocculence. No development of flocculence occurred in the absence of glucose. It is suggested that the maintenance of non-flocculence is dependent upon the presence in the cell wall of a nitrogenous compound; potential flocculence will develop when this compound is not synthesized at a rate sufficient to maintain its concentration in the wall. The nitrogenous nutrients which delay flocculation would then act by enhancing this rate of synthesis.

Brewer's yeast cells are freely dispersed in the early stage of logarithmic growth, but as this continues they tend to adhere to each other, forming clumps or flocs. The tendency to flocculate varies with different strains and is, to some extent at least, under genetic control (Gilliland, 1951; Thorne, 1951). Cells which have become flocculent may be reversibly separated by a variety of treatments, even washing in saline will suffice. But such redispersed cells differ from the disperse cells of the early logarithmic phase, in that when the former are transferred to a suitable medium containing calcium, they will flocculate at once, whereas the latter will not. Clearly the two types of cells must have structural differences. Those cells which have been reversibly separated by simple changes of environment may be called potentially flocculent, in distinction from those which are dispersed as a consequence of their structure, and which may truly be called non-flocculent.

Three distinct areas of study may be recognized in approaching the phenomenon of flocculation and it is necessary that information obtained in one field should not be confused with that from another: (i), there is the inherited nature of the cell; (ii) there is the nature of the change from non-flocculence to potential flocculence, together with the stimulus which provokes this change; (iii) there is the nature of the interactions of the cells with each other and with the environment, which manifest potential flocculation in the actual formation of flocs.

The present paper is concerned with the second of these fields of study, more especially with the nature of the stimulus which leads to a change to potential flocculence. It will be shown that this change is connected with a specialized part of the nitrogen metabolism of the organism.

METHODS

Yeast. A Strain 7002 of *Saccharomyces cerevisiae* was used throughout. This was a typical class III yeast (Gilliland 1951) and was obtained by Mr J. C. Holdaway from a single cell isolated from a stout-brewing strain.

Media. Glucose yeast-extract salts ammonium medium (GYSA) consisted of 75 g. glucose, 5 g. Difco yeast extract, 2 g. $(\text{NH}_4)_2\text{SO}_4$, 12.75 ml. 2M- CH_3COOH , 12.25 ml. 2M- CH_3COONa and 100 ml. salts solution (see below) in 1 l. distilled water, adjusted to pH 4.6 with dilute HCl.

Glucose yeast-extract agar consisted of a 2% (w/v) solution of agar in GYSA medium.

Glucose peptone growth-factor (GPF) medium consisted of 150 g. glucose, 10 g. 'Oxoid' mycological peptone, 25.5 ml. 2M- CH_3COOH , 24.5 ml. 2M- CH_3COONa , 200 ml. salts solution, 8 mg. calcium pantothenate, 4 mg. inositol, 2 mg. thiamine and 2 mg. pyridoxine HCl in 1 l. distilled water, adjusted to pH 4.6. This medium was always diluted with an equal volume of water or test solution for use, and 0.4 ml. of a solution of biotin (25 $\mu\text{g.}/\text{ml.}$ 95% (v/v) ethanol in water) was added to each litre of working medium, after sterilization and just before inoculation.

All media were sterilized by autoclaving for 15 min. at 120°.

The salts solution contained: 50 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g. NaCl, 10 g. KH_2PO_4 , 5 mg. $(\text{NH}_4)_2\text{MoO}_4$, 10 mg. MnCl_2 , 5 mg. FeCl_3 , 5 mg. ZnSO_4 , 5 mg. H_3BO_3 , 5 mg. KI, 5 mg. CuSO_4 ; made up to 1 l. in distilled water.

Inocula. The yeast was maintained on GYSA agar slopes. Inocula were always prepared from slopes which had been incubated for 40–70 hr at 25°. The yeast from one of these was suspended in 10 ml. GYSA medium, and then shaken at 25° for 6 hr. The cells were then collected by gentle centrifugation and resuspended in distilled water. The concentration of the suspension was estimated in an EEL colorimeter. This suspension was used at once to inoculate the cultures, the equivalent of about 9 mg. dry wt. cells being added to each litre of medium.

Cultures. Cultures were grown in 500 ml. conical flasks containing 200 ml. of medium; these were agitated on a rotatory shaker at 25°.

Measurements of cell concentration. Cell concentrations were estimated by centrifuging a sample of the culture, resuspending the yeast in 0.01N-HCl and measuring the opacity of the suspension in an EEL colorimeter with a Chance filter OB4. The colorimeter was calibrated with dilutions, in 0.01N-HCl, of a suspension of washed cells harvested during the early phase of logarithmic growth. The dry weight of the cells in this suspension was determined after heating a sample at 95° until an approximately constant weight was obtained. Flocculent cells harvested after prolonged growth gave a very similar curve, provided that the comparisons were made on the basis of dry weights. Comparisons based on cell numbers gave considerable differences.

Measurements of potential flocculence. Potential flocculence was determined with cells suspended in a 0.05 M-sodium acetate buffer (pH 4.6) containing 0.1% CaCl_2 . The gross rate at which the cells sediment in this buffer is almost exclusively dependent on the size of the flocs. In general, cells obtained by centrifuging a culture were washed, first in water and then in the buffer; they were then resuspended in the buffer at a concentration equivalent to about 4 mg. dry wt./ml. The suspension

was agitated vigorously and then poured quickly into the sample tube of the EEL colorimeter. Readings of the colorimeter were taken at timed intervals and the readings converted to apparent dry wt. cells in the light path of the instrument. A graph of these apparent cell dry-wt. values against time could then be constructed; this was approximately sigmoid in shape, and the steepest negative slope of this graph was taken as a measure of the sedimentation rate (SR). The logarithm of this (log SR) was considered to provide a good measure of the relative flocculence of the cells in the buffer, and hence of the potential flocculence of the cells in the culture. Values of SR obtained, ranged from equiv. 10 μ g. dry wt./ml. suspension/min., for a completely non-flocculent culture, to about equiv. 15,000 μ g. dry wt./ml./min. for a very flocculent one. The inaccuracies of this procedure are of little significance in comparison with the enormous changes encountered during the development of a culture.

RESULTS

Figure 1 shows the development of flocculence in a culture of the yeast in GPF medium. The potential flocculence increased rapidly in the later half of the logarithmic phase of growth. When considering why the development of flocculence should occur at this time, at least three possible reasons, or stimuli, come to mind. First, is there an internal clock mechanism which leads the yeast through cycles of flocculence and non-flocculence? This possibility is presumably ruled out by the well-known observation that in continuous culture the yeast may remain either flocculent or non-flocculent, depending upon the particular conditions.

Table 1. *The potential flocculence of yeast cells incubated in mixtures of media components*

Components present in (+), or absent from (-), the incubation mixture			Potential flocculence (log SR)	
Glucose (1.25 %)	Yeast extract (0.25 %)	Ammonium succinate (0.06 M)	After 4 hr	After 24 hr
+	-	-	2.37	2.82
+	+	-	2.47	2.92
+	+	+	1.42	2.05
+	-	+	1.42	1.70
-	+	+	1.32	1.20
-	+	-	1.32	1.20
-	-	+	1.20	1.20
-	-	-	1.27	1.20

All the mixtures of media components also contained 10 % (v/v) of salts solution.
The cells had a log SR of 1.20 before incubation.

Secondly, does the yeast flocculate as a response to the accumulation in the medium of some substance, e.g. a product of its metabolism? A culture of yeast was prepared in GYSA medium and during early logarithmic phase the yeast was separated by centrifugation. It was divided into three equal portions, one of which was resuspended in one-third of the original culture fluid, another in a similar volume of fresh medium and the third in a similar volume of 5 % glucose solution. As growth continued potential flocculence first appeared in the culture in glucose

alone, later in the culture in the used medium and finally in the culture in the fresh medium. This rules out any simple explanation based on the accumulation of a metabolite in the medium, for on such a basis flocculation should have occurred first with the culture in the used fluid.

Thirdly, does the yeast flocculate as a response to the exhaustion of a factor present in the fresh medium? A similar experiment was performed in which the freshly harvested yeast was added to solutions of glucose, yeast extract and ammonium salts, in various combinations. No flocculence occurred in the absence

Fig. 1. Growth (solid lines) and potential flocculence (broken lines) of *Saccharomyces cerevisiae* in (a) GPF medium, $\square$; (b) GPF medium + 0.012 M-ammonium succinate, $\times$. Log SR = log equivalent $\mu\text{g. dry wt. organism/ml./min.}$

of glucose. Flocculence developed rapidly in the presence of glucose alone or glucose + yeast extract, but was much delayed when ammonia was also present (Table 1). Experiments were therefore devised to follow this effect more closely and to test a number of nitrogenous compounds in this system.

The effect of nitrogenous compounds on the development of potential flocculence

Media were prepared by adding the double strength (GPF) mixture to equal volumes of solutions of the substances being tested, and growth and the development of potential flocculence were followed in shaken cultures. Controls using the unsupplemented (GPF) medium were included and all cultures were duplicated. Figure 1, curve b, shows the effect obtained with 0.012M-ammonium succinate in such a system. There was a marked delay in the onset and development of potential flocculence (expressed as log SR).

To evaluate the efficacy of various substances in such a system, the amount of growth which had occurred by the time the culture reached an arbitrarily selected potential flocculence was noted, and expressed as a percentage of the corresponding

Table 2. *The effects of some α -amino acids and ammonia on the stage of growth at which potential flocculence of yeast cells developed*

Compound tested	Concentration (M)	Growth as a % of that in control at log SR = 2.70
Ammonium succinate	0.012	230
L-Glutamine	0.025	220
L-Glutamic acid	0.025	100
L-Asparagine	0.025	210
L-Asparagine	0.012	200
L-Aspartic acid	0.025	100
L-Lysine	0.025	210
L-Arginine	0.025	170
L-Ornithine	0.025	180
DL-Methionine	0.025	110
L-Cysteine*	0.025	—
DL-Threonine	0.025	110
L-Serine	0.025	110
L-Phenylalanine	0.020	100
DL-Tyrosine	Sat. soln.	110
DL-Tryptophan	0.020	110
DL-Proline	0.020	100
L-Histidine	0.020	100
Glycine	0.025	100
L- α -Alanine	0.025	100
DL-Valine	0.025	100
DL-Leucine	0.025	110

* Cysteine inhibited growth almost completely at this concentration.

Table 3. *The effect of certain nitrogenous substances on the stage of growth at which potential flocculence of yeast cells developed*

All substances were tested at a concentration of 0.025 M, except pantothenate 100 μ g./ml. and folic acid 25 μ g./ml.

Substance tested	Growth as a % of that in control at log SR = 2.70	Substance tested	Growth as a % of that in control at log SR = 2.70
β -Alanine	60	Orotic acid	100
γ -Aminobutyrate	150	5-Methylorotic acid	100
Glucosamine	100	8-Azaguanine	100
Pantothenate	100	2,6-Diaminopurine sulphate	100
2-Chloro-4-aminobenzoate	60	Oxamic acid	100
p-Aminobenzoate	100	Urea	200
Folic acid	100	Barbiturate	120

amount of growth attained by the control cultures when they had reached the same potential flocculence. A value of log SR = 2.70 was chosen for the comparison, since this value is reached soon after the increase in the potential flocculence of the

culture becomes apparent; moreover, the measurement of potential flocculence is most readily carried out at about this value. The comparison of cultures on a basis of the amount of growth which has taken place by the time that a standard potential flocculence has been reached, rather than by a direct measurement of the time required to attain this flocculence, eliminates difficulties associated with synchronizing the growth of different cultures. It was found in practice that supplementation of GPF medium with nitrogenous substances had little effect on growth itself, although in a few cases a prolongation of the logarithmic phase was noted. Such a prolongation cannot account for the results described here.

Over a period of about 6 months, the replicated controls to these experiments showed a standard deviation of 140 about a mean of equiv. 1500 μ g. dry wt./ml. at $\log SR = 2.70$ (i.e. about 10%). Variations from the control outside 70–130 % were therefore considered to be highly significant, falling outside three standard deviations ($P < 0.01$). Table 2 presents results obtained in this way with ammonium succinate and some amino acids. Significant delays in the development of potential flocculence were found only with ammonia, the basic amino acids and the amides of the dibasic acids. Such a delay occurred with asparagine for example, but not with aspartic acid, even when the amount of total nitrogen supplied was the same.

Table 3 shows similar results with certain other nitrogenous compounds; of these, urea and γ -aminobutyric acid delayed potential flocculation, whereas β -alanine and 2-chloro-4-aminobenzoic acid advanced potential flocculation.

Figure 1 also shows a point which seems generally valid for all the effective compounds: those substances which were effective in postponing potential flocculence did not exert their action by stimulating growth until potential flocculence developed at its normal time, but actually postponed this development.

DISCUSSION

Implicit in this work is the assumption that changes in the potential flocculence of the yeast cells reflect changes in the chemical structure of their cell walls, and that these changes are a result of metabolic processes of the organism which to some extent may be controlled by its nutrition. To investigate such changes, it is essential to measure the flocculence of the yeast cells when they are supported in a carefully controlled physico-chemical environment; only thus is it possible to ensure that measured differences in flocculence truly reflect changes in the chemical structure of the cell.

Kusserow (1897) observed that asparagine inhibited the flocculation of yeast, whereas peptone enhanced it, but it is difficult now to decide whether the effects he described were due to actual changes in the cell or to changes in the suspending medium. Nielsen (1937) observed an increase in sedimentation rates of yeast grown in media containing added amino acids or peptones. Since, however, Nielsen measured the sedimentation of the washed yeast in water, without added calcium, it is difficult to be sure that his results did not reflect the availability of calcium to the cell during growth. The results described in the present paper amplify those of earlier workers. A well characterized group of compounds, namely, ammonia, the basic amino acids and the amides of the dicarboxylic amino acids, will delay the

onset of potential flocculation. This group of substances does not correspond to those compounds which form the most readily assimilable nitrogen sources of yeast (see, for example, Nielsen & Hartelius, 1938; Thorne, 1946). It is interesting to contrast the activity of glutamine and asparagine with the lack of activity of glutamic and aspartic acids. Indeed it appears that the α -amino group is not an effective source of nitrogen for this reaction. The only compounds outside of this narrow group which were found to be effective were urea, which readily yields ammonia, and γ -aminobutyric acid.

Two compounds were found to act in the opposite manner, leading to an earlier onset of flocculence, one of these was β -alanine; it is known that asparagine will antagonize the synthesis of pantothenic acid from β -alanine + pantoic acid, in certain organisms (Atkin, Williams, Schultz & Frey, 1944), and this raises the possibility that β -alanine leads to an early flocculation of yeast by antagonizing the action of asparagine. The other substance which was found to induce early flocculation was 2-chloro-4-aminobenzoic acid, a structural analogue of *p*-aminobenzoic acid which is an intermediate in the synthesis of pantothenic acid. However, there is no evidence that this connexion with pantothenate is other than fortuitous. Since β -alanine would promote, and 2-chloro-4-aminobenzoic acid antagonize, the synthesis of pantothenate, these two compounds might be expected to have opposed, and not similar, effects on flocculation were their action connected with pantothenate.

These findings can be interpreted by the hypothesis that the cell walls of non-flocculent yeasts contain a nitrogenous substance (XN) whose presence in the walls in a sufficient concentration determines their non-flocculence; so that if the concentration of XN were to decrease then the walls would show increasing potential flocculence. If now the rate of synthesis of XN by the cell were limited, then the concentration of this compound in the wall would diminish following rapid growth. The concentration of XN might also diminish as a response to the depletion in the medium, of XN itself or of materials required for its synthesis. That the change from non-flocculence to potential flocculence involves an active process is clear from the finding that no change took place in the absence of glucose. The group of substances which delay potential flocculation might then include XN itself, together with any intermediates in its synthesis, whose availability in high concentrations might increase the rate at which XN is produced. Certain corollaries should follow if this hypothesis is correct. First, there would be no definite identifiable stimulus, leading to the development of potential flocculence; for this would appear whenever a strain of yeast grew under conditions such that the rate of synthesis of XN was less than that of the other components of the cell wall. Secondly, the differences in the tendency to flocculate shown by different strains of yeast might reflect their different capabilities of synthesizing XN.

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