Effect of cultural and nutritional conditions on the control of flocculation expression in *Saccharomyces cerevisiae*

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The effect of cultural (temperature and pH) and nutritional conditions (nitrogen and carbon source) on the flocculation expression of three strains was studied. The strains' flocculation ability was determined by placing the cells in a stationary phase of growth in standard flocculation conditions. The flocculation ability of strain NCYC 1195, recently classified in the literature as the NewFlo phenotype, was more sensitive to growth temperature than Flo1 phenotype strains (NCYC 869 and NRRL Y265). The initial pH of the culture medium did not affect the flocculation ability of Flo1 phenotype strains but in the case of strain NCYC 1195 flocculation was repressed when the initial pH of the culture medium was below 3.5. Flocculation in strain NCYC 1195 was also repressed in defined culture medium; this inhibition was not related to a deficiency in any particular nitrogen source, but rather to the poor buffering capacity of the defined medium. All strains showed strong flocculation when grown in glucose, but were nonflocculent in glycerol. It was clearly demonstrated that the phenotypic expression of flocculation could be induced or repressed by changing cultural and nutritional conditions. Two distinct behaviours were also displayed with regard to the effect of the cultural conditions upon flocculation, namely the effect of pH. These different behaviours can be used to distinguish the two flocculation phenotypes.

Key words: *Saccharomyces cerevisiae*, flocculation, growth temperature, pH, nitrogen source.

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Nous avons étudié chez trois souches de *Saccharomyces cerevisiae* l'effet de conditions de culture (température et pH) et de conditions nutritives (source d'azote et de carbone) sur l'expression de la flocculation. Ce phénomène a été étudié plaçant les cellules en phase stationnaire dans des conditions habituelles de flocculation. La température de croissance a eu plus d'influence sur le pouvoir de flocculation de la souche NCYC 1195, classée récemment dans la littérature comme phénotype NewFlo, que chez les souches NCYC 869 et NRRL Y265 de phénotype Flo1. Le pH initial du milieu de culture n'avait pas d'influence sur la flocculation des souches du phénotype Flo1, mais dans le cas de la souche NCYC 1194 une répression était observée lorsque le pH était inférieur à 3,5. Cette répression de la flocculation chez la souche NCYC 1195 a aussi été observée dans un milieu de culture défini. Cette inhibition n'était pas reliée à un besoin spécifique d'azote mais à un faible pouvoir tampon de ce milieu défini. Toutes les souches ont fortement floculé lorsque cultivées dans le glucose mais pas dans le glycérol. Il est clairement démontré que l'expression phénotypique de la flocculation pouvait être induite ou réprimée par un changement des conditions de culture ou de nutrition. Nous avons observé deux comportements distincts conséquents à l'effet des conditions de culture sur la flocculation, particulièrement du pH. Ces comportements différents pourraient servir à distinguer deux phénotypes de flocculation.

Mots clés : *Saccharomyces cerevisiae*, flocculation, température de croissance, pH, source d'azote.

[Traduit par la Rédaction]

Introduction

The property known as flocculation, whereby yeast cells aggregate in clumps that rapidly sediment in the culture medium (Stewart 1975), has received considerable attention owing to its industrial application, mainly in the brewing industry (Stewart and Russell 1981).

Flocculation is under genetic control (Johnston and Reader 1983). *FLO* genes, their supressors, and mutations giving rise to flocculation were recently reviewed (Stratford 1994). Recent studies report the cloning and sequencing of the *FLO1* gene (Teunissen et al. 1993*a*, 1993*b*; Watari et al. 1994).

There are many reports that correlate flocculation with the cultural conditions and the composition of the growth medium. According to several authors, flocculation can occur in synthetic media, which means that exogenous substances, namely wort proteins, are not necessary for the development of flocculation (Eddy 1955; Jansen and Mendlik 1951). However, many strains flocculate only in complex media (Beavan et al. 1979), and require a peptide inducer, absent in minimal media (Stewart et al. 1973, 1975).

Strong flocculation was observed when cells were grown in the presence of various sugars, namely glucose, fructose, and sucrose (Nishihara et al. 1976; Bonaly et al. 1981). On the other hand, flocculation was inhibited in a strain grown in glucose and

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TABLE 1. Characteristics of strains used in this study

Strain	Genotype	Phenotype (flocculation)	Source	Reference
NCYC 869	*MATα FLO4*	Flo1	NCYC	Stewart and Russell 1977; Stratford and Assinder 1991
NRRL Y265	Unknown	Flo1	NRRL	Taylor and Orton 1973, 1978
NCYC 1195	Unknown (brewer's yeast)	NewFlo	NCYC	Stratford 1989; Stratford and Assinder 1991

NOTE: All strains used were *Saccharomyces cerevisiae*. Genes *FLO1*, *FLO2*, and *FLO4* are allelic, and are now called *FLO1* (Russell et al. 1980); the two flocculation phenotypes (Flo1 and NewFlo phenotype) were distinguished by sugar inhibition studies (Stratford and Assinder 1991). NCYC, National Collection of Yeast Cultures; NRRL, Northern Regional Research Laboratory.

fructose (Amri et al. 1979). Flocculence increased in a strain containing the dominant gene for flocculence *FLO4*, after growth on maltose compared with growth on glucose (Stewart et al. 1976). The authors proposed that this was due to a decrease in catabolic repression of respiration. However, Holmberg and Kielland-Brandt (1978) replaced glucose by glycerol, sodium lactate, and ethanol and observed no flocculation.

Hayduck (1905) and Schönfeld (1910), quoted by Jansen (1958), were the first to describe flocculation repression in brewery yeast by the action of high temperatures (30°C) during fermentation, or by propagation of a flocculent culture at high temperature (25–30°C). Later studies by Gilliland (1951) and Lund (1951) confirmed the above results. In more recent years, Holmberg and Kielland-Brandt (1978) described a temperature-sensitive mutant of a flocculent strain that is nonflocculent when grown at 30°C, and Williams et al. (1992) observed an initial inhibition of flocculation when the strain in their study was grown at 37°C.

Several authors (De Clerck and van Roey 1951; Gilliland 1951; Hartong 1951) reported that the effect of pH on flocculation is a minor factor under brewery conditions. However, Nishihara et al. (1976) observed significant inhibition of flocculation in cells grown in a medium at an initial pH below 3.0. An extended review of the effects of cultural and nutritional factors in flocculation may be found in Calleja (1987).

The molecular mechanism of yeast flocculation has not yet been completely explained. The "lectin-like" theory (Taylor and Orton 1978; Miki et al. 1982*a*, 1982*b*) proposes the presence of a specific lectin that binds to α-mannan of adjoining cells, using calcium ions to ensure the correct conformation of the lectin. Stratford and Assinder (1991) proposed two lectin-like mechanisms, the Flo1 and NewFlo phenotypes, on the basis of inhibition by sugar, salt, and low pH, and protease sensitivity. In a recent review, Calleja (1994) proposed a model (Velcro model) for visualizing yeast flocculation. Complementary perspectives of yeast flocculation were reviewed by Calleja (1994), Moradas-Ferreira et al. (1994), Mota and Soares (1994), Rose (1984), and Stratford (1992*a*, 1992*b*).

In this paper, the effects of growth temperature, initial pH of the culture medium, and medium composition (nitrogen and carbon sources) upon expression of flocculation of three strains, are reported. Additionally, the overall behaviour of the strains in response to different cultural and nutritional conditions was examined. The possibility of distinguishing between the Flo1 and NewFlo phenotypes on the basis of the difference in sensitivity to cultural conditions is discussed.

Materials and methods

Microorganisms

The strains used in this work are listed in Table 1, together with their genotypic (if known) and phenotypic flocculation characteristics and the study in which these characteristics were reported. Strains were obtained from the National Collection of Yeast Cultures, Norwich, U.K. (NCYC), and the Northern Regional Research Laboratory, Agricultural Research Services, Peoria, Illinois, U. S. A. (NRRL).

Media

Yeasts were routinely maintained at 4°C on YEPD slopes containing (per litre of water): yeast extract (Difco), 10 g; Bactopeptone (Difco), 20 g; glucose (Merck), 20 g; agar (Difco), 20 g. Yeasts were grown in the following four media: YEPD broth, YM broth (yeast extract (Difco), 3 g/L; malt extract (Difco), 3 g/L; Bactopeptone (Difco), 5 g/L), YNB broth (Bacto yeast nitrogen base (Difco), 1.7 g/L), and YNB w/o aa broth (Bacto yeast nitrogen base without amino acids (Difco), 1.7 g/L). Unless stated otherwise, glucose was used as the carbon source (20 g/L).

Inoculum preparation

The inoculum was prepared by transferring a loop of cells in 20 mL of YEPD to a 100-mL Erlenmeyer flask. The preculture was incubated at 30°C on an orbital shaker (Braun Certomat R) at 150 rpm. After 48 h growth, flocculated cells were harvested by centrifugation (4500 × *g*, 5 min), and washed once with distilled water and twice with 30 mM EDTA (ethylenediaminetetraacetic acid) to ensure floc dispersion. Cells were washed and suspended in deionized water. Inoculation was performed at a level of about $2–5 \times 10^5$ cells/mL.

Culture conditions

In the experiments on the influence of growth temperature, each strain was inoculated in 1.7 L of YM or YEPD broth, both containing 60 g/L of glucose, in 2-L mechanically stirred (250 rpm) Biolab or Biostat M fermenters (Braun, Melsungen, Germany). The cultures were constantly aerated with sterilized air at 10.2 L/h, at 10, 20, 30 and 35°C. In all other experiments the strains were grown at 30°C in 150 mL of culture medium in 250-mL Erlenmeyer flasks, on an orbital shaker at 150 rpm.

When the cultures reached the stationary phase of growth, the cells were harvested by centrifugation (4500 × *g*, 4°C, 5 min) and vigorously washed twice with EDTA solution (250 mM), to ensure floc dispersion. The cells were then washed with a NaCl solution (250 mM) at pH 2, followed by washing with a NaCl solution (250 mM) at pH 4.5. Cells were finally suspended in NaCl solution (250 mM), at pH 4.5, at 1×10^8 cells/mL.

Measurement of flocculation ability

The flocculation ability of the cells was determined using a modification of a Helm sedimentation test (Soares et al. 1992). Cell suspensions (24 mL) in NaCl solution (250 mM), at pH 4.5, were placed in a 25-mL cylinder. The suspension was adjusted to 4 mM $CaCl_2$ with the addition of $CaCl_2$ solution (1 mL from a stock solution of 100 mM, at pH 4.5), and then agitated (18 inversions of the cylinder) to promote flocculation. At defined intervals, samples were taken from a fixed position (the level corresponding to 20 mL), and dispersed in NaCl solution (250 mM) at pH 2. The cell concentration was determined spectrophotometrically at 620 nm using a calibration curve as reference.

Growth measurement

Growth was monitored with a Hitachi U 1100 spectrophotometer at 620 nm using a calibration curve as reference, or by direct cell counting (with a hemacytometer), after appropriate dilution in NaCl solution (250 mM) at pH 2 to prevent cell aggregation.

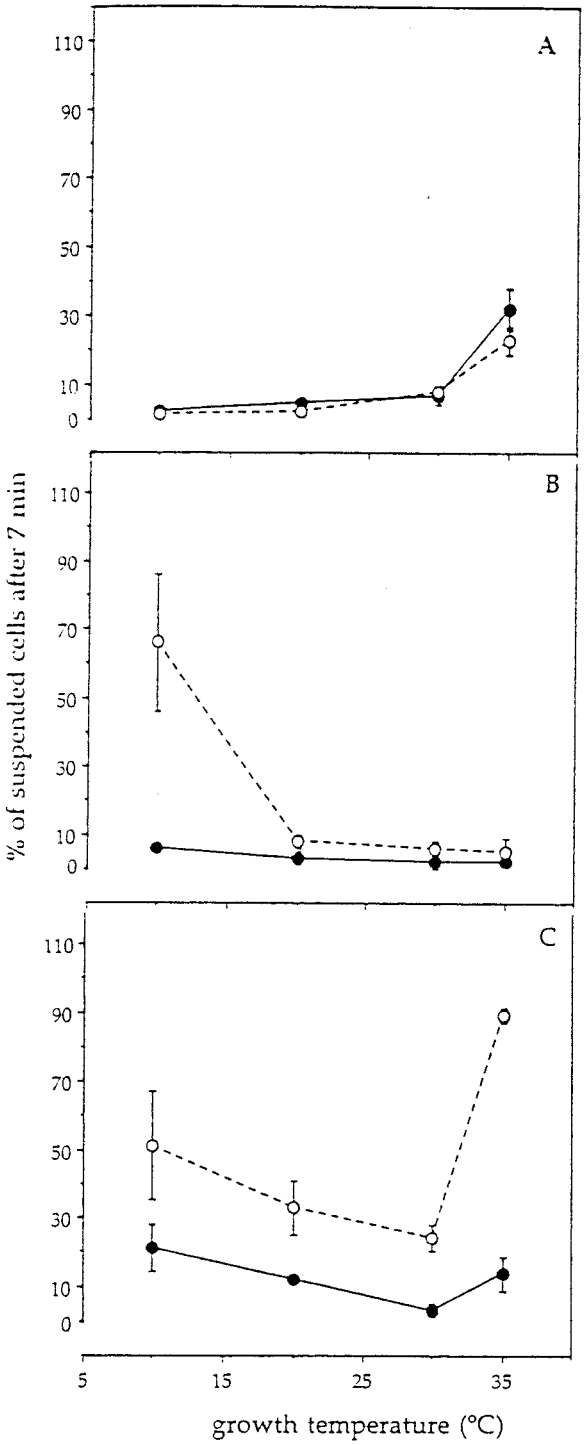

FIG. 1. Effect of growth temperature on expression of flocculation in strains NCYC 869 (A), NRRL Y265 (B), and NCYC 1195 (C), in YEPD (●) or in YM (○). Yeast cells (1×10^8cells/mL) were suspended in NaCl solution (250 mM), at pH 4.5, and flocculation was initiated by adding 4 mM $CaCl_2$ (for more details see Materials and methods). The flocculation ability was determined, in quadruplicate, of cells grown in three independent cultures ($n = 12$). Where no error bars are shown, confidence limits (95%) are within the points.

pH measurement

All pH measurements were made using a Metrohm 620 or 691 pH meter.

Results

The effect of growth temperature on the expression of flocculation was studied by growing the yeasts at four temperatures, 10, 20, 30, and 35°C; these results are shown in Fig.1. All strains were very flocculent when cultured in YEPD broth at 30°C. In this culture medium, growth temperature did not seem to have a very strong effect on the expression of flocculation of the three strains. However, lower or higher temperatures than 30°C did strongly modify the flocculation ability of strain NCYC 1195 (Fig. 1C), in YM broth. In contrast, the expression of flocculation in strains NCYC 869 and NRRL Y265 seemed to be less influenced by growth temperature. In the case of strain NRRL Y265 (Fig. 1B), flocculation expression was only affected at 10°C, in YM broth. Strain NCYC 869 was strongly flocculent in both culture media, at all growth temperatures studied except 35°C, at which a diminution was observed (Fig. 1A).

The influence of medium composition was studied using three different media: a rich medium (YEPD), a medium containing malt extract (YM), and a defined medium (YNB). Strains NCYC 869 and NRRL Y265 flocculated strongly and showed essentially the same behaviour in all media tested (Fig. 2). On the other hand, strain NCYC 1195 flocculated only in YEPD and to a lesser degree in YM. As YEPD is more rich in peptone than YM, it was decided to investigate the effect of peptone concentration on the flocculation expression of strain NCYC 1195. For this, a defined medium (YNB) was supplemented with 5 or 20 g/L peptone. This defined medium supplemented with 5 g/L peptone did not induce flocculation; but when supplementation reached 20 g/L peptone, flocculation was strong (Fig. 3).

Figure 4 shows the influence of the initial pH of the culture medium on flocculation. Cells were grown in rich culture medium (YEPD) with the initial pH adjusted by HCl addition. The flocculation expression of strains NCYC 869 and NRRL Y265 did not seem to be affected in the pH range tested. In contrast, flocculation was repressed in strain NCYC 1195 at pH 3.5. In medium with a low initial pH (3.5 for strain NCYC 1195 and 2.5 for strains NCYC 869 and NRRL Y265), the cells grew very slowly. In the case of strain NCYC 1195, growth was completely inhibited in the culture medium with an initial pH below 3.0.

The effect of the buffering capacity of the medium on the expression of flocculation in strain NCYC 1195 was also tested. In this experiment, the strain was grown in different media, all buffered with 50 mM phosphate at pH 5.9. The strain was very flocculent in all media, including YNB w/o aa, whose only nitrogen source was ammonium sulphate (data not shown).

The effect of carbon source on the expression of flocculation was studied using glucose and glycerol. It is interesting to note that the same overall behaviour of flocculation was found with all flocculent strains tested (Fig. 5). The strains showed strong flocculation in the culture medium with glucose as carbon source (YEPD) and nonflocculation in the culture medium with glycerol (YEPG).

Discussion

In this work, the influence of growth conditions was investigated by growing the cells at various temperatures in a variety of culture media with different initial pH values. As the effect of growing cells on different carbon and nitrogen sources at different temperatures and pH values will affect growth rates, all cells were collected after reaching the stationary phase of growth. Then, the flocculation ability of every sample, in

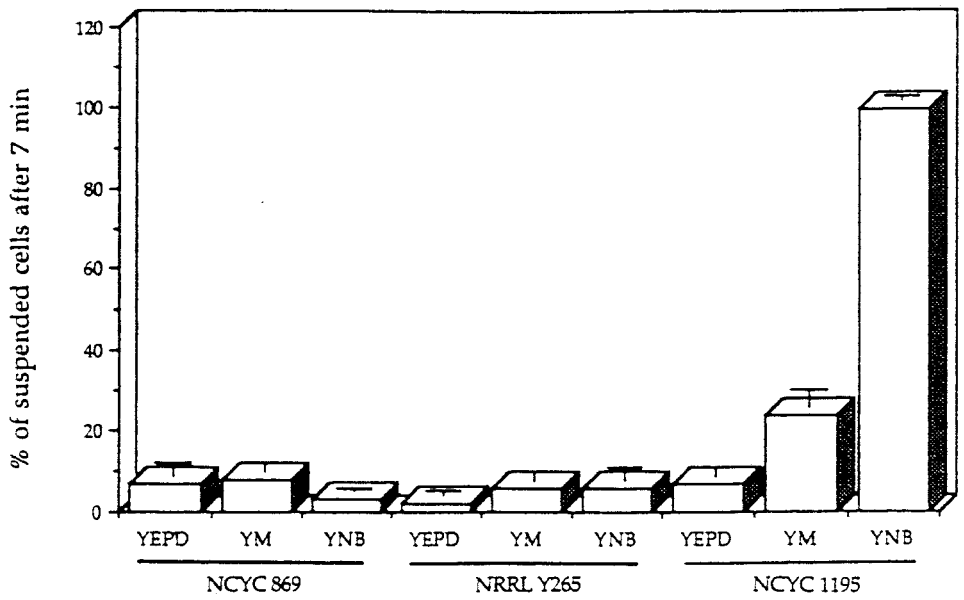

FIG. 2. Effect of medium composition on the expression of flocculation. The error bars represent 95% confidence intervals and were determined, in triplicate, for cells grown in three independent cultures (n = 9).

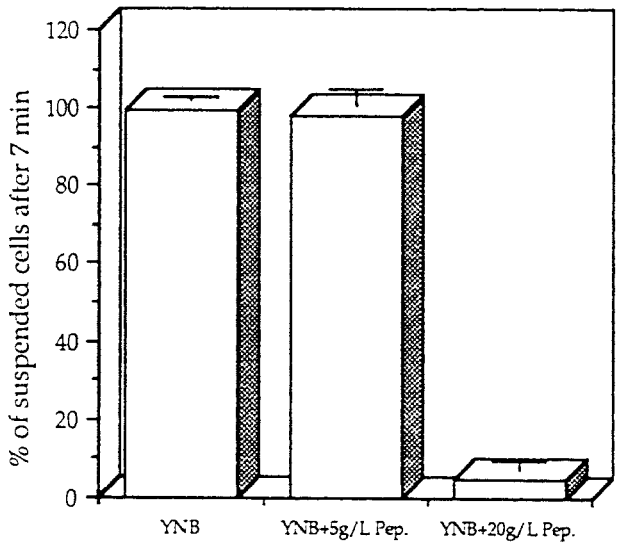

FIG. 3. Influence of peptone concentration on the expression of flocculation in strain NCYC 1195. YNB was supplemented with 5 (YNB + 5 g/L Pep.) or 20 g/L peptone (YNB + 20 g/L Pep.) The error bars represent 95% confidence intervals and were determined, in triplicate, for cells grown in three independent cultures (n = 9).

stationary phase of growth, in standard and optimal conditions (cellular density, mechanical agitation, pH, and calcium concentration) for flocculation, was evaluated.

Effect of growth temperature

According to the lectin-like theory of flocculation in *Saccharomyces cerevisiae* (Taylor and Orton 1978; Miki et al. 1982*a*, 1982*b*), lectins on flocculent cells bind sugar residues (receptors) incorporated as constituents of all cell walls. Modification in the microarchitecture of the receptors or inhibition or inactivation of the lectins can inhibit flocculation. In a previous study, it was shown that adverse environmental conditions (low glucose concentration with high aeration or in anaerobic conditions) repress flocculation (Soares et al. 1991). It was demonstrated that the suppression of flocculation was due to inhibition or inactivation of the lectin-like component, since cells whose flocculation was repressed were able to interact with flocculent cells (Soares et al. 1992).

Since it can be assumed that all cells, flocculent and nonflocculent, possess receptors (Miki et al. 1982*a*; Soares et al. 1992), flocculation expression probably requires de novo synthesis and secretion of lectins on the yeast cell wall. If lectin secretion is affected, flocculation can be inhibited. Stratford (1992*a*) suggested that lectin secretion was one potential target of flocculation suppression.

Therefore, the effect of low temperature (Fig. 1) on the expression of flocculation in strains NCYC 1195 (in YM) and NRRL Y265 (in YM) might be a consequence of an alteration of the secretory process and consequently of the secretion of the lectins. This hypothesis is supported by observations that indicate a modification of lipid profile with temperature (Hunter and Rose 1972), together with studies that show an active role of the phospholipids in the secretory process (Cleves and Bankaitis 1992). Additionally, Lands and Graff (1981) reported an inverse relationship between flocculation and unsaturated fatty acid concentration.

On the other hand, cell surface properties, namely flocculation, and cell membrane structure can be influenced by mitochondrial function (Evans et al. 1980; Hinrichs et al. 1988). It was proposed that mitochondria have a regulatory role in plasma membrane development (Wilkie and Nudd 1981). Furthermore, it was demonstrated that the optimal temperature for mitochondrial protein synthesis (30°C) is lower than for cytoplasmic protein synthesis (36°C) (Marmiroli et al. 1976). The inhibitory effect of a high growth temperature (35°C) on the expression of flocculation in strains NCYC 1195 (in YM) and NCYC 869 might then be explained as a consequence of an alteration in mitochondrial activity. Thermosensitive events, such as the development of mitochondrial functions and flocculation induction, have been reported in *Schizosaccharomyces pombe* (Calleja and Johnson 1979). Mitochondrial activity was also found to be essential for secretion in *Saccharomyces cerevisiae* (Spencer et al. 1981). It is therefore possible that high

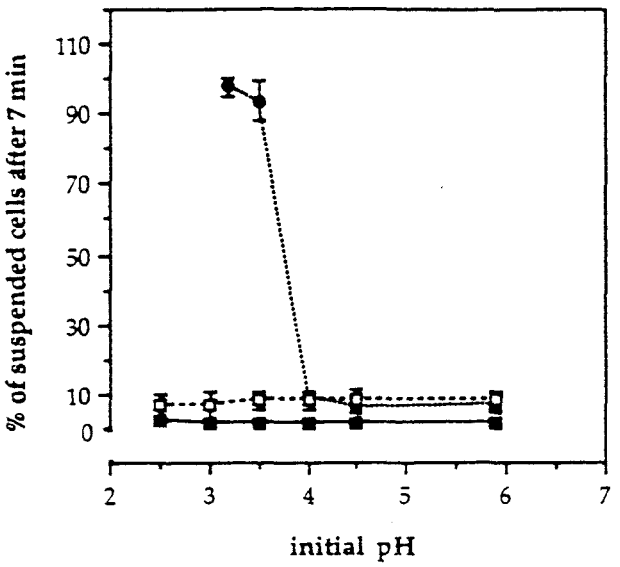

FIG. 4. Effect of initial pH of YEPD culture medium on the expression of flocculation in strains NCYC 869 (□), NRRL Y265 (■), and NCYC 1195 (●). Flocculation ability was determined, in triplicate, for cells grown in four independent cultures (n = 12). Where no error bars are shown, confidence limits (95%) are within the points.

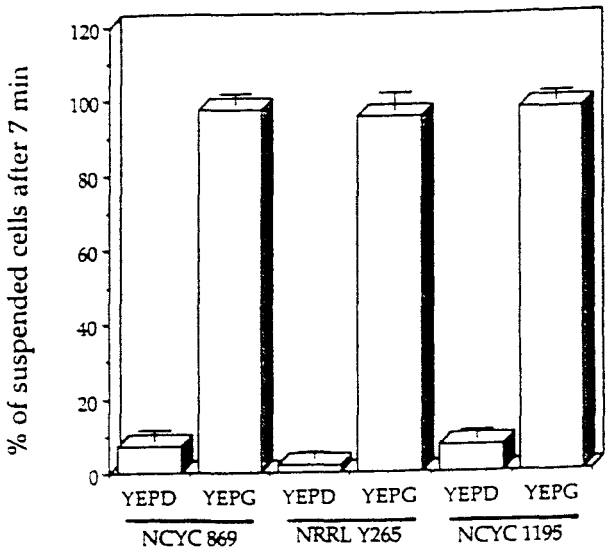

FIG. 5. Effect of carbon source on the expression of flocculation. Cells were grown in glucose (YEPD) and in glycerol (YEPG). The error bars represent 95% confidence intervals and were determined, in triplicate, for cells grown in three independent cultures (n = 9).

growth temperature (35°C), acting directly on mitochondrial activity and indirectly on cell membrane structure, may affect the secretion of flocculation lectins, with the consequent inhibition of the flocculation ability of the strains.

Effect of medium composition and initial pH

Stewart and co-workers described a group of ale strains that do not flocculate in defined medium, but require an inducer from wort or yeast extract – peptone – gelatin to flocculate (Stewart et al. 1975, 1976). Recently, Stratford and Assinder (1991) proposed two lectin-like mechanisms of flocculation: the Flo1 phenotype and the NewFlo phenotype. According to these authors, the Flo1 phenotype shows constitutive flocculence, flocculating in all media at all stages of growth. On the other hand, the NewFlo phenotype comprises many ale strains that only flocculate in the stationary phase of growth in undefined wort or in media containing yeast extract and peptone. The results presented here appear to agree with those obtained by Stratford and Assinder (1991), since strains NCYC 868 and NRRL Y265 (classified as the Flo1 phenotype) flocculated strongly in the media tested, including the defined medium (YNB). As expected, strain NCYC 1195 (classified as the NewFlo phenotype) flocculated intensely in rich medium and did not flocculate in YNB (Fig. 2).

The study of pH changes during the growth of strain NCYC 1195 in YNB (Fig. 6A) showed a lowering of the pH. At the end of the exponential phase of growth, the medium pH was near 2.5. In this case, one may consider whether strain NCYC 1195 does not flocculate in YNB owing to a lack of a specific nitrogen source or owing to the low buffering capacity of the medium. These two possibilities were tested in two different experiments. In the first one, the effect of peptone addition to a defined medium (YNB) was studied (Fig. 3). In the second one, the influence of the medium buffering capacity was evaluated. In the latter case the strain was grown in different media (YNB w/o aa, YNB, YNB supplemented with 5 or 20 g/L peptone), all buffered with 50 mM phosphate.

Defined medium supplemented with 5 g/L of peptone did not induce flocculence. However, strong flocculation was induced with 20 g/L of peptone (Fig. 3). It was also verified that the strain showed high flocculence in all buffered media (data not shown). It is now clear that strain NCYC 1195 does not need any specific nitrogen source, since high flocculence was still observed when the strain was grown in a buffered synthetic medium (YNB w/o aa), whose only nitrogen source was ammonium sulphate. The inhibition of flocculation of strain NCYC 1195 in defined medium (YNB) was due to the poor buffering capacity of the culture medium. The induction of flocculation of strain NCYC 1195 with 20 g/L of peptone was due to the high concentration of peptone, which increased the buffering capacity of the culture medium. A concentration of 5 g/L of peptone is not enough to buffer the medium.

The results of the two experiments described above were in agreement with those obtained in the study of the effect of the initial pH of culture medium on flocculation. This study was performed in YEPD because this medium includes all nutritional elements necessary for the onset of flocculation, since all strains flocculate strongly in this medium at an initial pH of 5.9. As can be seen in Fig. 4, pH did not affect the flocculation of strains NCYC 869 and NRRL Y265, but drastically inhibited the flocculation of strain NCYC 1195 below 3.5. This value was reached when strain NCYC 1195 was in the beginning of the exponential phase of growth in YNB (Fig. 6A), at the time that Stratford and Carter (1993) suggested that lectin synthesis begins in NewFlo strains. In buffered YNB (Fig. 6B), the pH of the culture medium remained above 4.0 throughout the exponential phase. This suggests that the pH of the culture medium is particularly important in this phase of growth and plays an important role in the expression of flocculation in strain NCYC 1195, which is unable to grow below pH 3.0.

The intracellular pH is a major factor controlling metabolism (Jones and Greenfield 1984). In the absence of membrane-permeable acids, internal pH is regulated in the range 5.8–6.9 for an external pH ranging from 2.8 to 7.0 (Jones and Greenfield 1984). However, at this stage, nothing is known about internal pH regulation below this value. Nevertheless, it is known that

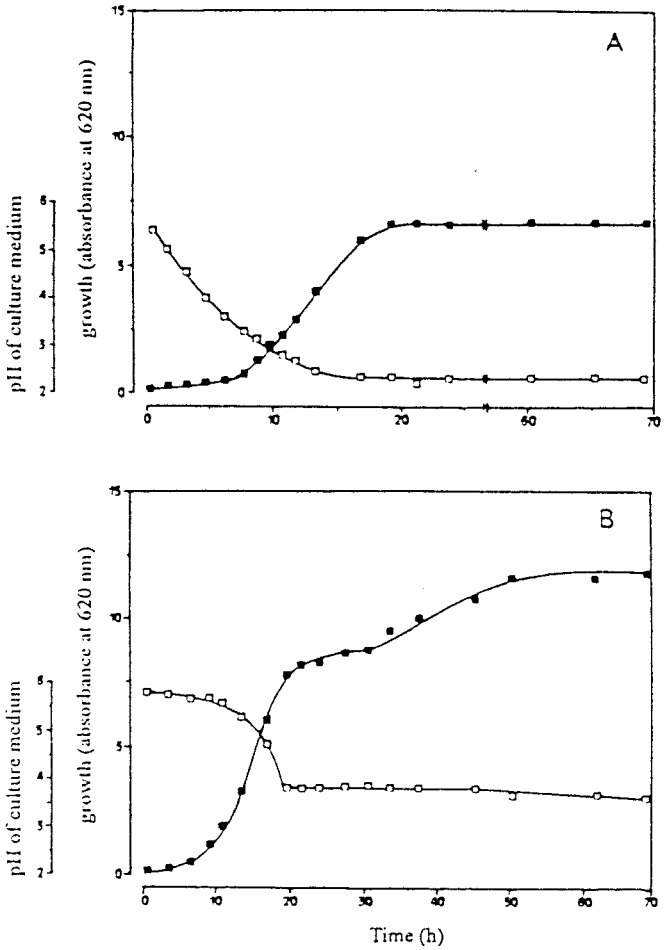

FIG. 6. Evolution of growth (■) and pH (□) of the culture medium of *Saccharomyces cerevisiae* NCYC 1195 (A) in YNB and (B) in YNB buffered with 50 mM of phosphate at pH 5.9. This is a typical example of an experiment repeated three times.

divalent cations play important metabolic and structural roles and that their uptake is significantly reduced below pH 5.0 (Fuhrmann and Rothstein 1968; Roomans et al. 1979). It is therefore probable that the synthesis or secretion of a lectin associated with flocculation of strain NCYC 1195 might be inhibited at low pH even though cell growth is possible.

Effect of carbon source

When glucose was replaced by glycerol in the culture medium, all of the strains studied used the glycerol as a carbon source and no flocculation was observed (Fig. 5). These results agree with those obtained by Holmberg and Kielland-Brandt (1978) and suggest that mitochondrial activity exerts some form of control on the expression of flocculence genes.

Stratford and Assinder (1991) reported that Flo1 phenotype strains were constitutively flocculent and consequently were fully flocculent in all media, at all stages of growth. The findings described above illustrate well the caution that must be taken when dealing with the concepts of constitutiveness and inducibility of flocculation.

Globally, the cultural conditions studied seem to affect more strongly the flocculation of strain NCYC 1195 (NewFlo phenotype) than of the Flo1 phenotype strains. The flocculation ability of strains NCYC 869 and NRRL Y265 (Flo1 phenotype) was not affected by the pH of the culture medium (in the range of pH values tested) and was less sensitive to the effect of growth temperature. On the other hand, the flocculation ability of strain NCYC 1195 was significantly affected by the pH of the culture medium. For this strain, flocculation was repressed in culture media with low buffering capacity or with an initial pH below 3.5. The expression of flocculation in strain NCYC 1195 was also more sensitive to growth temperature than that in strains NCYC 869 and NRRL Y265. These differences in sensitivity to cultural conditions, namely the clear differences in the sensitivity to the pH of the culture medium, can be useful to distinguish these two phenotypes.

Finally, it is important to point out that contrary to what might be expected, even Flo1 phenotype strains, which flocculate strongly in a wide range of cultural and nutritional conditions, are not able to flocculate in several of the conditions tested in this work.

In conclusion, our observations clearly demonstrated that the induction and repression of flocculation can be controlled by growth conditions, such as the temperature and pH of the culture medium and (or) constituents of the growth medium, namely the carbon source. Although the source of nitrogen seems to play only a minor role in the expression of flocculation in the strains studied, the source of carbon might be important.

Acknowledgments

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