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Carbohydrate carbon sources induce loss of flocculation of an ale-brewing yeast strain

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ABSTRACT

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Aims: To identify the nutrients that can trigger the loss of flocculation under growth conditions in an ale-brewing strain, *Saccharomyces cerevisiae* NCYC 1195.

Methods and Results: Flocculation was evaluated using the method of Soares, E.V. and Vroman, A. [*Journal of Applied Microbiology* (2003) **95**, 325]. Yeast growth with metabolizable carbon sources (glucose, fructose, galactose, maltose or sucrose) at 2% (w/v), induced the loss of flocculation in yeast that had previously been allowed to flocculate. The yeast remained flocculent when transferred to a medium containing the required nutrients for yeast growth and a sole nonmetabolizable carbon source (lactose). Transfer of flocculent yeast into a growth medium with ethanol (4% v/v), as the sole carbon source did not induce the loss of flocculation. Even the addition of glucose (2% w/v) or glucose and antimycin A (0·1 mg l^{-1}) to this culture did not bring about loss of flocculation. Cycloheximide addition (15 mgl^{-1}) to glucose-growing cells stopped flocculation loss.

Conclusions: Carbohydrates were the nutrients responsible for stimulating the loss of flocculation in flocculent yeast cells transferred to growing conditions. The glucose-induced loss of flocculation required *de novo* protein synthesis. Ethanol prevented glucose-induced loss of flocculation. This protective effect of ethanol was independent of the respiratory function of the yeast.

Significance and Impact of the Study: This work contributes to the elucidation of the role of nutrients in the control of the flocculation cycle in NewFlo phenotype yeast strains.

Keywords: carbon source, flocculation, NewFlo phenotype, protein synthesis, *Saccharomyces cerevisiae*.

INTRODUCTION

Yeast flocculation has been traditionally exploited in brewing industry (Stewart and Russell 1981). However, this characteristic might also be useful in modern biotechnology as it is an easy and cheap way of separating the cell biomass from the media at the end of fermentations.

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According to lectin-like theory, flocculation occurs as a consequence of the interaction between the specific flocculation proteins (lectins) present only in the flocculent cells and the carbohydrate residues (receptors) of the walls of neighbouring cells (Miki *et al.* 1982); in this process, Ca^{2+} seems to induce the correct conformation of the lectins (Miki *et al.* 1982; Stratford 1989). Four phenotypes were described taking into account pH and sugar inhibition sensitiveness or the requirement of ethanol to induce flocculation, they were: Flo1 phenotype, the flocculation of these strains was only inhibited by mannose; NewFlo phenotype, inhibited by

mannose and glucose; mannose insensitive (MI) phenotype and ethanol-dependent flocculation (Stratford and Assinder 1991; Masy *et al.* 1992b; Dengis and Rouxhet 1997). The majority of brewery yeasts belong to NewFlo phenotype (Stratford and Assinder 1991), which flocculates in the stationary phase of growth, while almost all Flo1 phenotype strains are constitutively flocculent (Stratford and Assinder 1991; Stratford and Carter 1993; Soares and Mota 1996).

Flocculation is an inheritable characteristic of yeast strains. Thus, several dominant flocculation genes, suppressors and mutations giving rise flocculation have been implicated in this phenomenon; for a review see references (Stratford 1992; Teunissen and Steensma 1995).

The ideal brewing yeast grows as discrete cells and flocculate after sugar depletion from the media, leaving clear beer (Stewart and Russell 1981). Premature flocculation leads to an incomplete attenuation of the wort, while later flocculence or the failure of the strain to flocculate impairs the separation of the cells from the fermented wort and off-flavours can often result owing to yeast autolysis (Stewart 1975).

Despite the commercial importance of yeast flocculation, little is known about the regulation of this characteristic. In lager brewer's yeast strains, nitrogen (Smit *et al.* 1992) or oxygen limitations (Straver *et al.* 1993) trigger flocculation, while in ale strains, there is a correlation between the end of exponential growth, when the minimum glucose level in the culture medium was attained, and the onset of flocculation (Soares and Mota 1996).

In a previous work, it was found that a highly flocculent ale-brewer strain rapidly lost its flocculation ability (Soares and Mota 1996), when transferred to fresh rich medium yeast extract peptone dextrose (YEPD). However, when incubated for up to 12 h in the absence of a nitrogen or carbon source, the loss of flocculation did not occur, although the addition of the missing nutrient triggered a fast loss of flocculation (Soares and Duarte 2002). When this strain was incubated for a long period (48 h) in the presence of fermentable carbon sources, it showed a progressive loss of flocculation (Soares and Vroman 2003). These observations prompted us to examine, in some detail, which are the nutrients that trigger the loss of flocculation during the beginning of the growth phase of an ale-brewer strain of *Saccharomyces cerevisiae*, in a chemically defined medium yeast nitrogen base (YNB). Additionally, the effect of different carbon sources and protein synthesis in the loss of flocculation of a NewFlo phenotype strain was also investigated.

MATERIALS AND METHODS

Strain, media and culture conditions

The flocculent ale-brewing strain of *S. cerevisiae* NCYC 1195 was used in this work. The original strain was obtained from the National Collection of Yeast Culture (NCYC; UK), and is characterized as NewFlo phenotype (Stratford and Assinder 1991).

The strain was routinely maintained at 4°C on YEPD slopes containing (per litre of water): yeast extract, 10 g; peptone, 20 g; glucose, 20 g; agar, 20 g.

The strain was grown in YEPD broth and in YNB broth [6·7 g l^{-1} yeast nitrogen base (Difco) plus 20 g l^{-1} glucose (or other carbon sources, see below), 5·9 g l^{-1} succinic acid and 0·46 g l^{-1} $CaCl_2{\cdot}2H_2O$]; pH was adjusted to 5·9 with NaOH.

Precultures were prepared in 40 ml of YEPD broth in 100-ml Erlenmeyer flasks. Cells were incubated at 25°C on an orbital shaker (Braun Certomat S, Melsungen, Germany), at 150 rev min^{-1}. After 48 h of growth, flocculent cells were harvested by centrifugation (2000 *g*, 5 min) and washed twice with 30 mmol l^{-1} EDTA solution to ensure floc dispersion. Finally, cells were washed and suspended in deionized water.

Cultures in YNB medium [with different carbon sources, all at a final concentration of 2% (w/v) or 4% (v/v) in the case of ethanol] were prepared by inoculating 0·5 l of culture medium, in 1-l Erlenmeyer flasks, with *ca* 2×10^6 cells ml^{-1} from precultures. For glucose pulse addition to ethanol-growing cells experiments, 2 l of the culture medium in 3-l Erlenmeyer flasks were used. In all cases, cells were grown at 25°C on an orbital shaker at 150 rev min^{-1}.

In glucose pulse experiments, after 72 h of growth, the culture was aseptically divided in four aliquots of 300 ml into 1-l Erlenmeyer flasks. To the 300 ml aliquots filter-sterilized glucose (at a final concentration of 20 g l^{-1}), and/or antimycin A (Sigma) (at a final concentration of 0·1 mg l^{-1}), were added, according to the experiment; in the case of glucose and antimycin A addition, glucose solution and antimycin A (solubilized in ethanol) were injected simultaneously.

In the experiments with cycloheximide (Sigma), yeasts cultures were treated with filter-sterilized cycloheximide (dissolved in deionized water), at a final concentration of 15 mg l^{-1}. Cycloheximide stops yeast protein synthesis at 4 mg l^{-1} and completely inhibits yeast growth at 5 mg l^{-1} (Stratford and Carter 1993). Control experiments with the strain of *S. cerevisiae* NCYC 1195 in YNB with 2% (w/v) glucose and 15 mg l^{-1} cycloheximide, showed a complete inhibition of yeast growth (data not shown). Addition of a 15-mg l^{-1} cycloheximide pulse to growing cells allowed a little increase in the number of cells (1·2 and 1·5 times after 3 and 20 h, respectively) (data not shown).

Measurement of flocculation ability

At defined times, indicated in the figures, yeasts were harvested by centrifugation (2000 *g*, 5 min), washed and

resuspended on ice-cold deionized water and stored at 4°C for a maximum of 72 h. Before flocculation measurements, cells were washed twice in 30 mmol l^{-1} EDTA solution. Flocculation ability was monitored in standard conditions using a micro flocculation technique previously described (Soares and Vroman 2003).

Growth

Cells were diluted appropriately in 100 mmol l^{-1} EDTA solution to prevent cell aggregation. Growth was monitored spectrophotometrically (at 600 nm) or by direct microscopic counting, using a counting chamber.

Glucose and ethanol determinations

Cells were harvested by centrifugation, at 2500 *g*, 5 min, and the supernatant was collected and immediately stored in the freezer until glucose and ethanol determinations. The glucose concentration in culture medium was measured enzymatically with glucose oxidase–peroxidase method [using the Adaltis (Adaltis, Bologna, Italy) glucose reagent kit]. Ethanol concentration was measured enzymatically with alcohol dehydrogenase method (using the Merck alcohol cell test).

RESULTS

We have previously shown that when flocculent cells of *S. cerevisiae* NCYC 1195 were exposed to fresh rich medium with glucose (YEPD), they rapidly grew and lost flocculation (Soares and Mota 1996). Here, it can also be seen that yeast cells lost flocculation when incubated in minimal defined medium (YNB) with glucose (Fig. 1). As yeast cells can grow on a variety of fermentable and nonfermentable carbon sources, it was tested if other carbon sources will also stimulate the loss of flocculation. As can be seen in Fig. 1, fructose, maltose and sucrose induce fast growth and loss of flocculation. The rate of loss of flocculation differed slightly for different carbon sources but was particularly slower for cells incubated in the presence of galactose (Fig. 1); with this carbon, a slow, but progressive, loss of flocculation occurred (Fig. 2). Lactose, a fermentable sugar that cannot be metabolized by *S. cerevisiae* cells, did not induce the loss of flocculation (Figs 1 and 2), even when the incubation time was prolonged to 48 h. These results suggest that fermentable carbon source is the only nutrient that stimulates the loss of flocculation, as the cells remained fully flocculent over a period of time (48 h) (Fig. 2), when exposed to other essential nutrients present in YNB (nitrogen, phosphate and sulphur sources as well as micronutrients and vitamins).

Growth in a nonfermentable carbon source (ethanol) did not induce the loss of flocculation – neither in the first 20 h of growth (data not shown) nor when the incubation time was expanded to 104 h (Fig. 3). The addition of a glucose pulse [in a final concentration of 2% (w/v)] to growing cells in ethanol (desrepressed cells) triggered growth (80% of glucose was consumed in the next 8 h after glucose pulse – data not shown), but did not induce the loss of flocculation (Fig. 3). Similarly, no loss of flocculation occurred in cells grown in 1% (v/v) of ethanol and when a glucose pulse of 4% (w/v) was added (data not shown). The presence of 0·1 mg l^{-1} antimycin A, blocked the growth and consump-

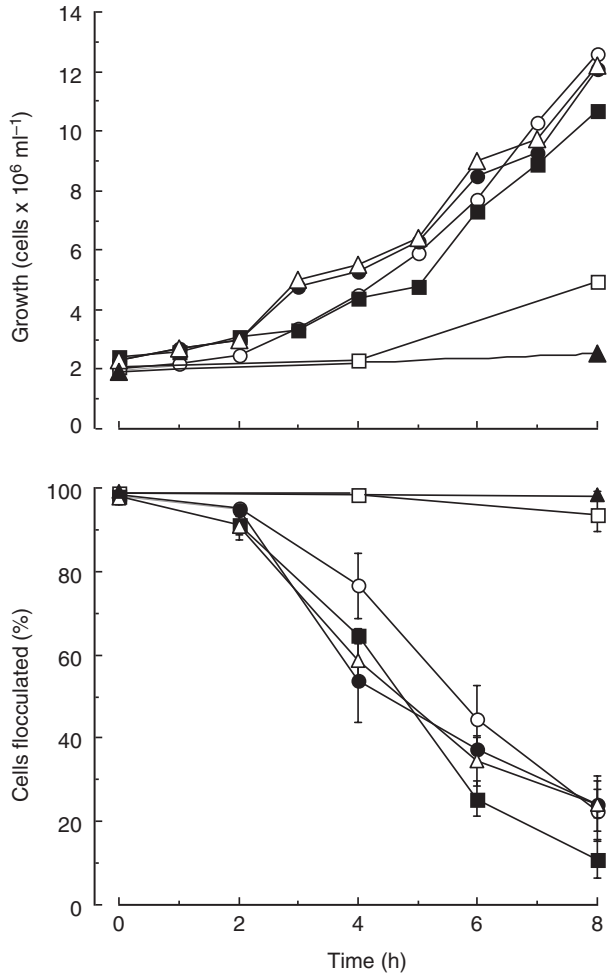

Fig. 1 Growth and loss of flocculation of the strain *Saccharomyces cerevisiae* NCYC 1195 in different carbon sources. Flocculent cells were inoculated in YNB containing 2% (w/v) of the following carbon sources: (○) glucose, (●) fructose, (□) galactose, (■) maltose, (△) sucrose and (▲) lactose. At times indicated in the figure, cells were collected, washed and suspended in citrate buffer (pH 4·0, 50 mmol l^{-1}) containing Ca^{2+} (8 mmol l^{-1}) for measuring flocculation ability; for more details see 'Materials and methods'. Each point represents the mean of two independent experiments performed in duplicate; standard deviations are presented ($n = 4$). In growth graphic, error bars are not shown for pictorial clarity

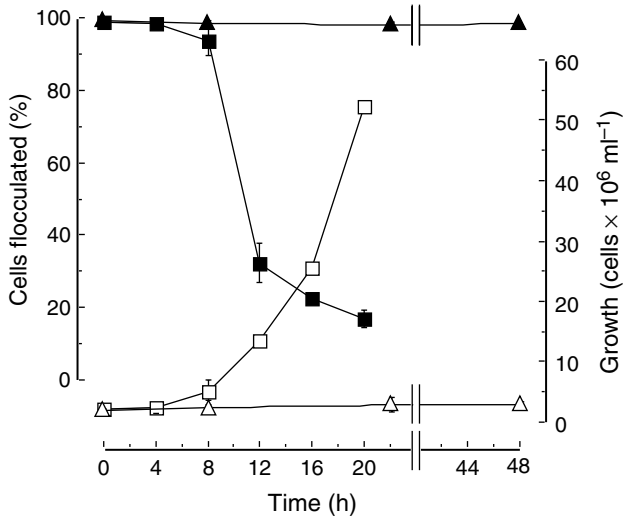

Fig. 2 Growth and flocculation of the strain *Saccharomyces cerevisiae* NCYC 1195. Flocculent cells were inoculated in YNB containing 2% (w/v) of galactose (□) or lactose (△); growth (open symbols), flocculation (closed symbols). Each point represents the mean of two independent experiments performed in duplicate; standard deviations are presented ($n = 4$)

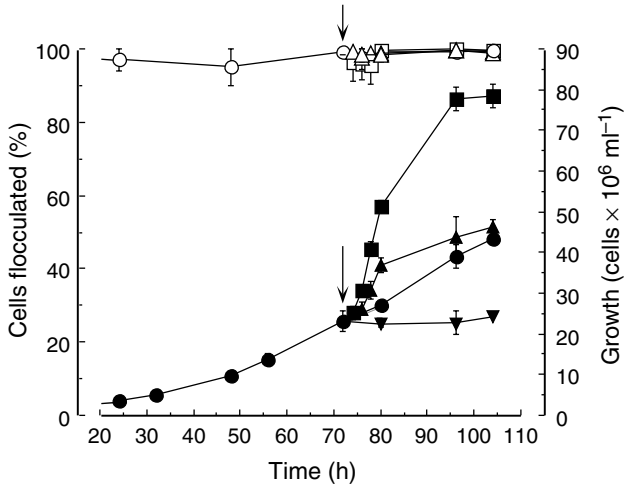

Fig. 3 Growth (●) and flocculation (○) of the strain *Saccharomyces cerevisiae* NCYC 1195 in YNB containing 4% (v/v) of ethanol. At the time indicated by the arrow, it was added: a glucose pulse at a final concentration of 2% (w/v) (□); a glucose pulse at a final concentration of 2% (w/v) and 0·1 mg l^{-1} antimycin A (△); 0·1 mg l^{-1} antimycin A (control) (▽). Growth (closed symbols) and flocculation (open symbols) were evaluated. Each point represents the mean of two independent experiments performed in duplicate; standard deviations are presented ($n = 4$)

tion of ethanol (data not shown, for ethanol); additionally, the presence of antimycin A reduced the growth in glucose/ethanol growing cells (Fig. 3). In both cases, the strain remained completely flocculent (Fig. 3), suggesting that the presence of ethanol seems to block the triggering of loss of flocculation induced by glucose. Control experiments showed that 0·1 mgl^{-1} antimycin A provoked a slower growth and deflocculation rate (compared with the control), but did not impair the loss of flocculation induced by glucose, the flocculation ability being in control after 8 h (absence of antimycin A) similar to that observed in the presence of antimycin A (data not shown). The slowdown in glucose effects on growth and flocculation can be explained by the decrease in energy produced by the metabolization of glucose, as in the presence of antimycin A (a respiratory inhibitor) all the energy was obtained by a fermentative pathway. These observations, together, reinforce the suggestion of the protective role of ethanol on the flocculation loss. However, the ethanol protective effect seems not to be associated with the respiratory metabolism, as antimycin A did not block the ethanol effect in the cells exposed simultaneously to ethanol and glucose (Fig. 3).

To test whether the loss of flocculation induced by glucose requires protein synthesis, high flocculent cells were inoculated in YNB with glucose and at defined period of time, cycloheximide was added at a final concentration of 15 mg l^{-1}. As a consequence of cycloheximide addition, the growth was arrested and loss of flocculation was stopped (Fig. 4), indicating that glucose-induced loss of flocculation is dependent on protein synthesis.

DISCUSSION

Reversible inhibition of flocculation by specific sugars such as mannose, maltose, glucose or sucrose, which causes cell dispersing of the flocs affecting directly the flocculation bonding, by competition with the sugars of the yeast cell wall for flocculation lectins, has been described (Miki *et al.* 1982; Stratford and Assinder 1991; Masy *et al.* 1992b). In this work, we examined the effect of the principal mono- and disaccharides found in wort (glucose, fructose, maltose and sucrose) as well as the effect of other carbohydrates (galactose and lactose) and a nonfermentable carbon source (ethanol) on the loss of yeast flocculation of a NewFlo phenotype strain, in growing conditions. In the studies carried out in the present paper, we investigated the effect of the sugars on yeast flocculation, as nutrients able to modify flocculation via the metabolism of yeast. Thus, to distinguish from the dispersing effect of sugars reported above, flocculation was always determined in washed cells, in standard conditions; in this way, it is possible to correlate the flocculation capacity with the presence of active surface lectins on yeast cell walls, as it is described that flocculation receptors are present in all phases of growth (Stratford 1993; Soares and Mota 1996). The correlation between the flocculation intensity and the presence of the zymolectins

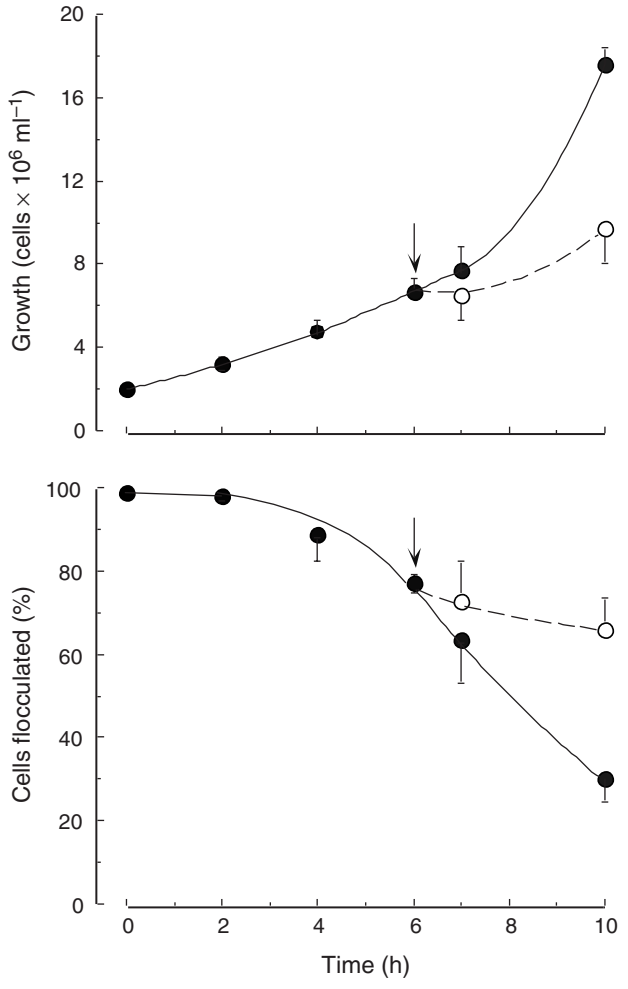

Fig. 4 Effect of cycloheximide addition on the growth and loss of flocculation of the strain *Saccharomyces cerevisiae* NCYC 1195 in YNB containing 2% (w/v) of glucose. At the time indicated by the arrow, cycloheximide was added at a final concentration of 15 mg l^{-1}. Control (●), untreated cells. Each point represents the mean of two independent experiments performed in duplicate; standard deviations are presented ($n = 4$)

has been shown in different works (Masy *et al.* 1992a; Bony *et al.* 1998; Patelakis *et al.* 1998).

While Flo1 phenotype strains are constitutively flocculent, NewFlo phenotype strains possess cyclic flocculation ability; NewFlo strains progressively lose their flocculation ability in the early growth, in rich medium with glucose (YEPD), and recover it towards the end of exponential phase of growth (Soares and Mota 1996). The results described in this work showed that in chemically defined medium the strain showed a similar behaviour to the one described with rich medium; besides glucose, other fermentable carbon sources (fructose, galactose, maltose and sucrose) also induced a flocculation loss, suggesting that the flocculation loss is an energy-dependent process. This supposition is also supported by the fact that the incubation in a nonmetabolizable (by *S. cerevisiae*) energy source (lactose) did not trigger the flocculation loss; *S. cerevisiae* cells do not use lactose because they lack the lactose permease system as well as β-galactosidase (Siso 1996). The rate of flocculation loss is similar for all the carbohydrates, except for galactose (much slower), which can be due to the fact that galactose transport is controlled by an inducible system (Barnett 1997).

It was also possible to see in this work that cell growth did not implicate the triggering of flocculation loss, as cells grow in the presence of ethanol without losing their flocculation ability; however, the rate of flocculation loss induced by sugar seems to be associated with the rate of sugar metabolization.

Gluconeogenic enzymes, which are involved in the utilization of ethanol, are repressed and inactivated by glucose and other sugars (Thevelein and Hohmann 1995). In the presence of glucose, the enzymes required to metabolize ethanol are repressed and yeasts use only glucose as carbon source (Gancedo 1998). The addition of glucose to cells of *S. cerevisiae* growing on a nonfermentable carbon source, like ethanol, triggers a rapid and transient increase in the cAMP level and an extensive series of regulatory responses at both the post-translational and transcriptional level (Thevelein and Hohmann 1995). In this work, the addition of a glucose pulse to ethanol-growing cells triggered a rapid increase in the growth rate; however, we did not observe a loss of flocculation, suggesting that the presence of ethanol seems to block the triggering of flocculation loss. Additionally, the ethanol protective effect against the glucose-induced flocculation loss seems not to be linked with the cell respiratory activity, as the presence of antimycin A did not impair the ethanol effect.

The loss of flocculation seems to be a fermentation-dependent process in growing conditions, as all metabolizable sugars tested induced the flocculation loss, while ethanol did not. Interestingly, the same behaviour was found in starved cells (Soares and Vroman 2003), which reinforces the central role of the carbohydrate carbon sources on the flocculation cycle of ale-brewing yeasts. These facts suggest that probably the molecular mechanism that triggers the loss of flocculation in growing and starvation conditions should be the same. The study of these different approaches (in starvation and growing conditions) are important because they reflect two different conditions observed by the brewers: (i) the nutrient limitations imposed to yeast cells, during the handling, after primary fermentation, and (ii) the pitching of the cells into wort to start a new fermentation.

In the yeast cell, the Tup1-Ssn6 corepressor has widespread functions in glucose repression; it was proposed

that flocculation genes are regulated at the transcriptional level by the proteic complex Tup1-Ssn6 (Teunissen *et al.* 1995; Fleming and Pennings 2001). The molecular mechanism by which glucose and other fermentable carbon sources induces loss of flocculation is unknown. However, experiments with cycloheximide performed in this work showed that protein synthesis is required in the triggering of flocculation loss. It is well documented that cycloheximide inhibits protein synthesis at ribosome level and prevents the development of flocculation (Baker and Kirsop 1972; Stratford and Carter 1993). In this work, we found that glucose-induced flocculation loss was prevented by cycloheximide addition, which is in agreement with the previous observations where the loss of flocculation glucose induced during prolonged (48 h) starvation conditions required *de novo* protein synthesis (Soares and Vroman 2003).

In conclusion, this paper examines in detail the effect of nutrients on the flocculation loss and presents evidence of six new aspects about the triggering of flocculation loss of an ale-NewFlo phenotype strain, under growing conditions. First, carbohydrate carbon sources seem to be the nutrients that stimulate the loss of flocculation in a minimal defined medium (YNB). Secondly, all metabolizable carbohydrate carbon sources (glucose, fructose, galactose, maltose and sucrose) induced the loss of flocculation in YNB, while ethanol did not. These results suggest that flocculation loss is a fermentable-dependent process. Thirdly, the rate of sugar-induced flocculation loss seems to be associated with the rate of sugar metabolization. Fourthly, the loss of flocculation most likely requires energy and this process is blocked by ethanol through an unknown mechanism. Fifthly, the growth does not implicate the triggering of flocculation loss, as cells grown in medium with ethanol remained fully flocculent. Sixthly, glucose-induced loss of flocculation requires *de novo* protein synthesis, by an unknown mechanism, as cycloheximide addition to glucose-growing cells impairs the flocculation loss. Further work should be carried out to provide a molecular understanding of flocculation loss in NewFlo phenotype strains.

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