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ORIGINAL PAPER

Modification by glucose of the flocculent phenotype of a *Kloeckera apiculata* wine strain

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Abstract We have evaluated the induction of the flocculent phenotype of *Kloeckera apiculata* by glucose mc1 and propose a pathway involved in carbohydrate flocculation induction. Pulses of glucose were given to cells growing in glucose-poor medium (2 g l^{-1}) and the flocculation percentage was measured. To elucidate the mechanism involved in flocculation induction, cycloheximide was injected into the cultures 120 min before the glucose pulse. 2,4-Dinitrophenol or cAMP was added to the media instead, or simultaneously with glucose, while a protein kinase A (PKA) inhibitor was added 30 min before the glucose pulse. With 20 and 50 g l^{-1} glucose pulse, the yeast flocculation percentage arises to 55 and 65%, respectively. The quantity of proteins and the reflocculating capacity of a lectinic protein extract from the yeast cell wall increase as the concentration of glucose pulse was higher. Cycloheximide prevented the glucose-induced flocculation, while cAMP or 2,4-dinitrophenol increased it 4- and 5-fold, respectively. PKA inhibitor completely prevented the glucose induction flocculation. The flocculent phenotype of *K. apiculata* mc1 was induced by glucose and the mechanism seems to imply de novo protein (lectin) synthesis via the PKA transduction pathway. This work contributes to the elucidation of the mechanism involved in flocculation induction by glucose of a non-*Saccharomyces* wine yeast, *K. apiculata*, which has not been reported. The induction of flocculation by glucose could be a biotechnological tool for the early removal of the indigenous microorganisms from the grape must before the inoculation of a selected starter strain to conduct the alcohol fermentation.

Keywords *Kloeckera apiculata* · Flocculation · Flocculent phenotype · Glucose induction · PKA pathway · cAMP effect · Wine yeasts

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Introduction

Flocculation can be defined as the phenomenon where yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended [16, 29, 30]. Various theories have been proposed to explain the aggregation of yeasts. The "lectin-like" theory of flocculation proposes that specialized cell wall proteins called "lectins", present only on flocculent cells, recognize and interact with carbohydrates (receptors) on the neighboring cell wall. Calcium ions enable the adhesins to achieve their active conformation [20, 30]. Several yeast species such as *Kluyveromyces bulgaricus* [1], *Kluyveromyces lactis* [6], and *Saccharomyces cerevisiae* [13, 33], show flocculating abilities which involve lectinic mechanisms.

The flocculation phenomenon could be affected by many genetic [31, 34, 35], physiological and environmental factors such as pH and temperature [8, 14, 15, 26, 27], as well as by the composition of the growth culture medium such as carbohydrate source [24, 28].

In the brewing and winemaking industries, the flocculation process has been traditionally used as an easy and cheap process of cell separation after fermentation has finished. Considering that the fermentation medium in both industries

is a complex mixture of different fermentable sugar, it is important to know the role of the carbohydrates in the regulation of the flocculation. In *S. cerevisiae,* the main yeast involved in winemaking, there are several well-known signaling cascades regulate flocculation expression in response to environmental changes [9, 19, 23, 25, 40]. Regulation of the flocculation genes by glucose is achieved by transcriptional controls and different pathways have been described [5, 36, 42]. One of them, the Ras/cAMP/PKA pathway requires glucose or sucrose for activation [9, 39, 41].

In non-*Saccharomyces* wine yeasts, which have become increasingly useful in modern fermentation biotechnology, limited information is available regarding the effect of the carbohydrates in the regulation of flocculation. Which signals trigger the flocculation and how they affect the lectin synthesis or activation is not known in detail. In *K. bulgaricus*, it has been reported that glucose is an activator of the flocculent phenotype and involves a PKA transduction signal that leads the flocculation [11]. *Kloeckera apiculata* is one of the predominant non-*Saccharomyces* yeast species in grape berries; it can participate in the early stages of natural wine fermentation and contributes to the characteristics of the wine [22]. Farías and Manca de Nadra [7] reported that *K. apiculata* mc1, growing in YMPG medium, present a flocculent phenotype mediated by galactose-specific lectins and stabilized by Ca^{2+}. Glucose seemed to modify the flocculation phenotype since this strain was weakly flocculent in the presence of low glucose concentration (2 g l^{-1}). This observation prompted us to examine the role of glucose on the *K. apiculata* mc1 flocculation and to propose a possible pathway involved in the carbohydrate induction of yeast cell aggregation.

Materials and methods

Strain, media and culture conditions

Kloeckera apiculata mc1 flocculent strain was isolated from Argentinean wine. The yeast was grown in YMP liquid medium (20 g l^{-1} peptone, 10 g l^{-1} yeast extract and 2.5 g l^{-1} malt extract). When indicated, the medium was supplemented with 2, 20 or 50 g l^{-1} glucose. The initial pH was adjusted to 5.5 with NaOH. The cells were incubated at 25 °C during 72 h. Cultures were prepared by inoculating 5 ml of the medium in 10-ml shaker flask and aerated by stirring on a rotary shaker (150 rev min^{-1}).

Effect of glucose on flocculation induction

After 18 h of growth, cells precultured in glucose poor medium (2 g l^{-1}) were harvested by centrifugation (3,000×*g*, 5 min) and washed twice with 10 mmol l^{-1} of EDTA solution to ensure floc dispersion of the yeast-forming flocs.

Finally, cells were washed twice and the optical density ($OD_{620\ nm}$) was adjusted to 0.8 with deionized water. Cultures were prepared by inoculating 50 ml of YMP plus 2 g l^{-1} glucose, in 100-ml test tubes, with about 1×10^6 cells ml^{-1} from precultures. Cells were grown at 25 °C with magnetic stirring. Growth of the cells was estimated by measuring the absorbance of the culture at 620 nm after checking that there was a linear relationship between absorbance and biomass. After 8 h of growth (exponential growth phase), when the optical density of the cultures reached approximately 0.5 (about 12.5 mg dry weight or 2.18×10^6 cells ml^{-1}), aliquots of filter (0.22 μm) sterilized glucose solution (400 g l^{-1}) were added under sterile conditions to obtain a final concentration of 20 or 50 g l^{-1}. The flocculation percentage was measured at different times after the glucose pulse.

Lectin isolation from yeast cells

Yeast cells growing in YMP medium plus 2 g l^{-1}, and induced by 20 and 50 g l^{-1} glucose were harvested after 12 h of incubation by centrifugation at 3,000×*g* and was extensively washed with a phosphate buffer (10 mmol l^{-1} Na_2HPO_4, 10 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} NaN_3), pH 7.4. Cells were then suspended at a concentration of 4% (w/v) in the same buffer supplemented with 5 mmol l^{-1} EDTA and incubated under slight stirring at 37 °C for 5 h. After centrifugation at 3,000*g* for 10 min, the supernatant was dialyzed at 4 °C against distilled water for at least 48 h then lyophilized.

Effect of antibiotics, metabolites and toxic agents on flocculation

Several biochemical and chemical compounds used were, cycloheximide, cyclic 3′,5′-adenosine monophosphate (cAMP), 2,4-dinitrophenol (2,4-DNP) and PKA inhibitor (cell permeable cAMP-dependent protein kinase inhibitor fragment 14–22). All the products were purchased from Sigma, USA. All the compounds were filter-sterilized and added under sterile conditions in liquid basal medium when the optical density of the yeast culture was near 0.5.

2,4-DNP (solubilized in ethanol) and cAMP (dissolved in deionized water) were added to the yeasts cultures, according to the experiment described for glucose, to obtain a final concentration of 0.05 and 0.1 mmol l^{-1} for cAMP and 10 mmol l^{-1} for 2,4-DNP. In other experiments, cAMP or 2,4-DNP were added simultaneously with glucose.

PKA inhibitor (solubilized in deionized water) was added to the yeast cultures, 30 min before addition of the glucose pulse, to obtain a final concentration of 12.5 and

25 mg l^{-1}. Flocculation percentage was measured at different times after the compounds introduction.

Cycloheximide (dissolved in deionized water) was added 2 h before the glucose pulse to obtain a final concentration of 20 mg l^{-1}.

Measurement of flocculation ability

At defined intervals of growth, yeast cells were harvested by centrifugation (3,000×*g*, 5 min), dispersed washing twice with 10 mmol l^{-1} of EDTA solution (deflocculated cells) and washed twice with distilled water. Subsequently, the cells (about 20 mg dry weight, corresponding to a final optical density of 0.8) were resuspended in 10 ml of 50 mmol l^{-1} acetate buffer, pH 4.5, containing 3 mmol l^{-1} of calcium ions.

The degree of flocculation was measured after the yeast cells were suspended by vigorous shaking for 20 s using a vortex mixer at the maximum speed (2,500 rpm). The optical density at 620 nm was measured immediately (OD_0); after a pause of 10 min at room temperature, the optical density of the suspension was again measured (OD_1). The flocculation percentage was calculated according to the expression: % Floc = $[(OD_o - OD_1)/OD_o] \times 100$ [3, 12].

Flocculation induced by extracted lectin was assayed by mixing 2-fold serial dilutions of 1% lectin extract solution in Helm's buffer with 1% *K. apiculata* deflocculated cell suspension in the same buffer. A positive reaction was determined by the formation of aggregates estimated by visual observation and by optical microscopy [6]. The yeast reflocculating titre corresponded to the reciprocal of the highest dilution of the lectin solution giving detectable aggregation of deflocculated yeasts.

Reproducibility of the results

All the experiments were repeated independently, three times. Although, absolute data were not comparable in the experiments performed on different days, the observed trends were fully consistent among the independent experiments. The data reported for flocculation percentage are the mean values and standard deviations, performed in quadruplicate for each experiment.

Results and discussion

We have previously reported that flocculent cells of *K. apiculata* mc1 showed an intense flocculent phenotype in glucose-rich medium (YMPG) [7]. Here, it was observed that yeast cells growing in the YMP medium with 2 g l^{-1} showed a lower expression of the flocculent phenotype (18%), which remained constant during yeast growth (Fig. 1).

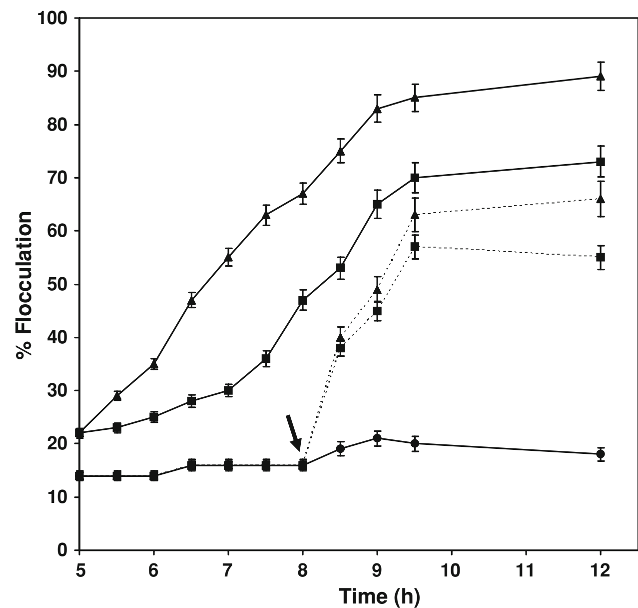

Fig. 1 Induction of *K.apiculata* flocculation during the grown in glucose poor medium (2 g l^{-1}) by addition of glucose. Arrow indicates time of glucose addition into the medium. Flocculation percentage of *K. apiculata* grown in basal medium with 2 g l^{-1} glucose (*filled circle*), 20 g l^{-1} glucose (*filled square*) and 50 g l^{-1} glucose (*filled triangle*); and after induction of *K. apiculata* growing in glucose-poor medium by addition of 20 g l^{-1} glucose (*filled square*) and 50 g l^{-1} glucose (*filled triangle*). *Vertical bars* represent standard deviation

In the media with 20 or 50 g l^{-1} of initial glucose concentration, a higher flocculation percentage was observed (70 and 90%, respectively). As can be seen in Fig. 1, when a pulse of glucose was given 8 h after inoculation of *K. apiculata* cells growing in the media with the lower sugar (2 g l^{-1}) concentration, a rapid increase of the flocculation was observed. Ninety minutes after the glucose pulse, the percentage of flocculation rose to 55 and 65% for 20 and 50 g l^{-1}, respectively. After glucose pulses, the maximal yeast flocculation percentages rise was lower than that observed in the media with 20 and 50 g l^{-1} initial glucose concentration. Glucose seemed to be a stable activator of the flocculation phenotype, since this flocculation degree was constant during growth. The flocculation phenotype can be activated by diverse environmental triggers, such as nitrogen and/or carbohydrate depletion [24]. However, glucose or sucrose can activate the RAS/cAMP pathway, whose targets include key proteins involved in the control of the flocculation [9]. Our results are in agreement with those observed in other non-*Saccharomyces* yeast, *K. bulgaricus* [11]. The authors showed an increase of the flocculation degree when the yeast cells were treated with glucose. In contrast, the flocculation of *Saccharomyces* strains was generally reported as induced by glucose depletion. Amri et al. [2] described that *Saccharomyces uvarum* flocculation was inhibited when the yeasts were grown in

glucose and fructose. Soares and Mota [27] found that in an ale-brewing *Saccharomyces* strain, the depletion of glucose in a rich culture medium was coincident with the onset of flocculation.

EDTA extraction of lectinic proteins and flocculation activity of crude extract

Kloeckera apiculata mc1 shows a flocculent phenotype mediated by protein (lectin)-carbohydrate interaction [7]. The lectinic factor involved in flocculation was extracted from the yeast cell wall using EDTA as reported by Al-Mahmood et al. [1]. Table 1 shows that the extraction yield and quantity of protein achieved with the cation chelating agent were higher as the concentration of glucose pulse increases in the culture medium. Also, the reflocculating capacity of *K. apiculata* extracts on the deflocculated yeast cells was correlated with the sugar concentrations (from titre 2 for cells from YMP with 2 g l^{-1} to 32 and 64 for cells induced with 20 and 50 g l^{-1} glucose, respectively). The reflocculating activities were irreversibly abolished when the EDTA extracts were previously heating at 100°C for 10 min. These results suggested that lectinic proteins are directly involved in the induction of the flocculation by the sugar.

Studies of the mechanism involved in the induction of *K. apiculata* flocculation by glucose

To evaluate the requirement of de novo protein (lectin) synthesis during the induction of the flocculation by glucose, cells of *K. apiculata* in medium with 2 g l^{-1} glucose were exposed to 20 mg l^{-1} cycloheximide 120 min before the glucose pulse (Fig. 2). The antibiotic hindered the flocculation induced by 20 or 50 g l^{-1} glucose. The flocculation prevention by cycloheximide of glucose-induction in *K. apiculata* indicates that this process is dependent on protein (lectin) synthesis. This behavior could explain the higher yeast flocculation percentage observed in media

Table 1 Protein concentrations and reflocculating capacity of EDTA lectinic extracts

Concentration of glucose pulse[a] (g l^{-1})	Extraction yield (mg g^{-1} wet yeast)	Proteins (mg g^{-1} crude extract)	Reflocculation titre[b]
0	6.6 ± 0.3	81 ± 0.9	2 ± 0.1
20	10 ± 0.4	130 ± 5.3	32 ± 1.3
50	18 ± 0.06	307 ± 4.4	64 ± 2.6

[a] To cells growing in YMP plus 2 g l^{-1} after 8 h growth

[b] Test were performed upon deflocculated cells of *K. apiculata* cultivated in YP medium with 50 g l^{-1} glucose and treated with EDTA

Data are mean values of three independent experiments ±SD

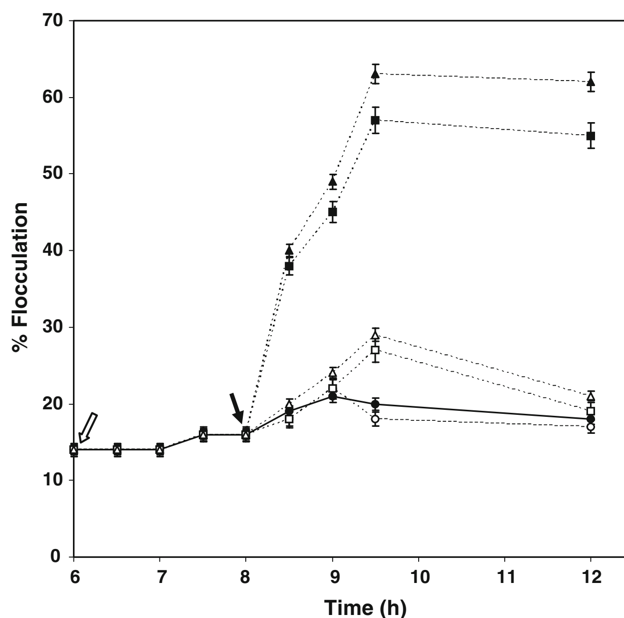

Fig. 2 Effect of cycloheximide on the induction of flocculation of *K. apiculata* by glucose. *Filled arrow* indicates time of glucose addition and *open arrow* indicates time of cycloheximide addition into the medium. Flocculation percentage of *K. apiculata* grown in 2 g l^{-1} glucose medium (*filled circle*); after addition of 20 g l^{-1} glucose (*filled square*); 50 g l^{-1} glucose (*filled triangle*); 20 mg l^{-1} of cycloheximide (*open circle*); 20 g l^{-1} of glucose in presence of 20 mg l^{-1} of cycloheximide (*open square*) and 50 g l^{-1} of glucose in presence of 20 mg l^{-1} of cycloheximide (*open triangle*). *Vertical bars* represent standard deviation

with 20 or 50 g l^{-1} initial glucose in relation to that obtained in cells exposed to glucose pulse in a initial 2 g l^{-1} glucose medium (Fig. 1). As the media with 20 or 50 g l^{-1} glucose from the beginning of growth, were precultured in the same medium, the presence of a previous intracellular pool of proteins (lectins) could be responsible of the higher flocculation percentage.

These results are in agreement with the occurrence of a continuous synthesis of lectins from the early stages of growth in *S. cerevisiae* reported by Stratford and Carter [32]. On the contrary, cycloheximide did not modify the glucose-induced flocculation in *K. bulgaricus* [11], therefore in this yeast this phenomenon did apparently not imply synthesis of new proteins.

Glucose can direct different pathways in yeast [38]. It has been shown that the addition of glucose to yeast in the glucose depletion or stationary phase induced a transient elevation of intracellular cAMP content [18]. Addition of cAMP at concentrations of 0.05 and 0.1 mmol l^{-1} to *K. apiculata* cells growing in YMP with 2 g l^{-1} led to the development of an important flocculation phenotype from 30 min onwards after introduction of the cAMP solution (Fig. 3). The flocculation percentage was nearly 4-fold higher after 90 min cAMP addition. When the cells induced

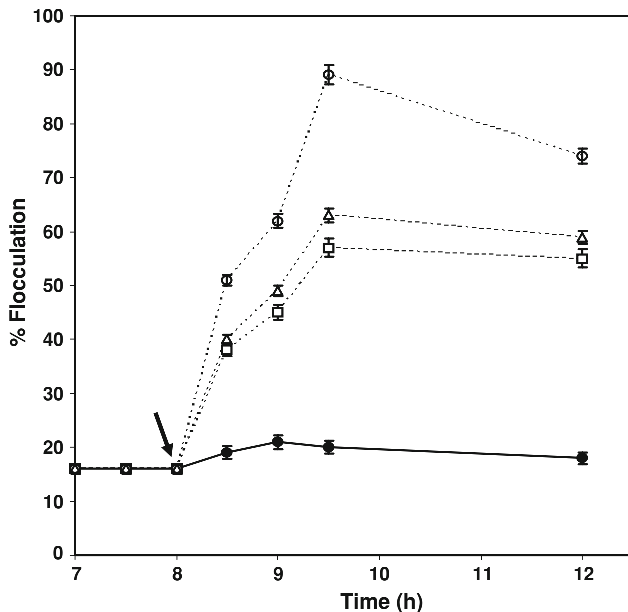

Fig. 3 Induction of flocculation of *K. apiculata* grown in 2 g l^{-1} glucose medium by addition of cAMP or 2,4-dinitrophenol. *Arrow* indicates time of the compounds addition into the medium. Flocculation percentage of *K. apiculata* grown in 2 g l^{-1} glucose medium (*filled circle*); 0.05 mmol l^{-1} of cAMP (*open square*); 0.1 mmol l^{-1} of cAMP (*open triangle*) and 10 mmol l^{-1} 2,4-DNP (*open circle*). *Vertical bars* represent standard deviation

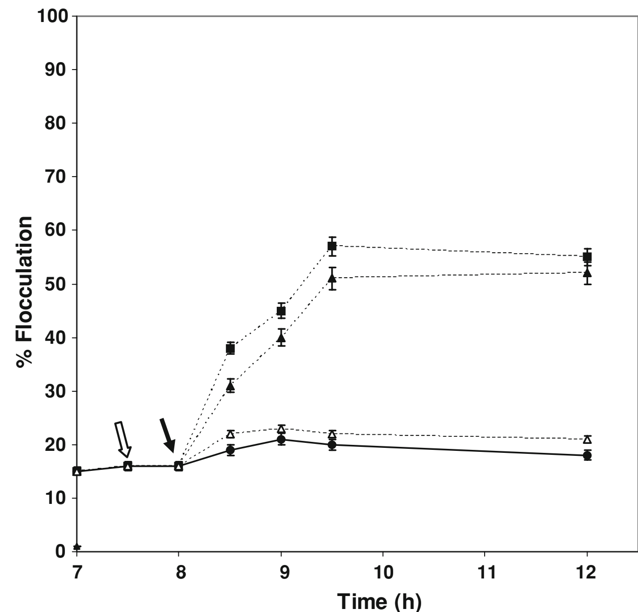

Fig. 4 Effect of PKA inhibitor on the induction of flocculation of *K. apiculata* by glucose. *Filled arrow* indicates time of glucose addition and *open arrow* indicates time of PKA inhibitor addition into the medium. Flocculation percentage of *K. apiculata* grown in 2 g l^{-1} glucose medium (*filled circle*) and after addition of 20 g l^{-1} glucose (*filled square*), 20 g l^{-1} of glucose in presence of 12.5 mg l^{-1} PKA inhibitor (*filled triangle*) and 20 g l^{-1} of glucose in presence of 25 mg l^{-1} PKA inhibitor (*open triangle*). *Vertical bars* represent standard deviation

with 20 or 50 g l^{-1} glucose (Fig. 1) were simultaneously supplemented with 0.05 mmol l^{-1} cAMP no significant modification of the flocculation degree was observed. The increase of cAMP concentration in presence of fermentable sugars could also result from intracellular acidification which could, in turn, activate the adenylate cyclase [4, 21]. With the purpose to simulate the internal acidification produced by the yeast sugars metabolism, the basal medium was supplemented with 10 mmol l^{-1} of the protonophore 2,4-DNP. At 90-min incubation, the yeast flocculation was approximately 5-fold higher than that observed in the control. When the cells induced with 20 or 50 g l^{-1} glucose (Fig. 1) were simultaneously supplemented with 10 mmol l^{-1} 2,4-DNP, no significant modification of the flocculation degree was observed. Consequently, increasing the intracellular cAMP by adding cAMP exogenous or intracellular acidification caused by the 2,4-DNP, led to an increase in the flocculent phenotype expression of *K. apiculata* mc1.

It was reported that cAMP could act on the protein kinase A complex (PKA) that inactivates Sfl1 (suppressor of flocculation) and activates the positive regulator *Flo8* [10, 17, 37] producing a stimulation of the aggregation phenomenon. To elucidate the possible role of PKA on the glucose-induced flocculation of *K. apiculata*, 12.5 mg l^{-1} of PKA inhibitor was added 30 min before 20 g l^{-1} glucose pulse (Fig. 4). A decrease of 11% in the flocculation percentage was observed after the 90-min glucose addition. When the PKA inhibitor concentration was increased to 25 mg l^{-1}, the induction of flocculation by glucose was completely prevented. Similar results were obtained for *K. bulgaricus* [11], whose report that the addition of PKA-specific inhibitors to a non-flocculent cells culture of drastically affected the induction of flocculation kinetics by glucose.

In conclusion, from the results it could be inferred that the flocculent phenotype of *K. apiculata* mc1 was induced by glucose and the mechanism seems to imply de novo protein (lectin) synthesis via the PKA transduction pathway. The higher flocculation percentage observed in *K. apiculata* mc1 as a consequence of the intracellular cAMP increase due to exogenous addition or intracellular acidification could be considered as a mechanism involved in the modification of the protein kinase A complex (PKA) that activates the flocculation genes.

Cell–cell adhesion of industrial brewing and wine yeast is often exploited as a convenient and cost-effective way to separate biomass at the end of various fermentations processes, when all the available sugars have been converted into ethanol and carbon dioxide. Thus, the ideal industrial yeasts strains should flocculate after sugar is depleted from the media. However, the glucose induction of the flocculent

phenotype of *K. apiculata* mc1 strain described in this paper, is important to consider in winemaking, due to the prevalence of this apiculate yeast in the early stages of the process when the concentration of this sugar is high. Exploiting the induction of flocculation by glucose could be a biotechnological tool for the early removal of the indigenous microorganisms from the grape must before the inoculation of a selected starter strain to conduct the desired alcohol fermentation.

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