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# Behaviour of *Kloeckera apiculata* Flocculent Strain in Coculture with *Saccharomyces cerevisiae*

*Oscar A. Sosa*[1], *María C. Manca de Nadra*[1,2] *and Marta E. Farías*[1,2]*

[1]CERELA – National University of Tucumán, Chacabuco 145, San Miguel de Tucumán, Argentina

[2]Career Investigator of National Scientific and Technical Research Council (CONICET), Argentina

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**Summary**

The behaviour of the flocculent *Kloeckera apiculata* strain in coculture with the non-flocculent *Saccharomyces cerevisiae* strain, both yeasts isolated from wine, has been analyzed. Pure culture of *Kloeckera apiculata* (apiculate yeast) exhibits 75 % flocculation in the yeast extract/peptone/glucose (containing 20 g/L of glucose, $YPG_{20}$) medium after 24-hour incubation at 28 °C. This yeast expresses a weak flocculent phenotype in glucose-poor medium (10 % of flocculated cells). Increasing the glucose concentrations in this medium induces yeast flocculation. When the apiculate yeast was co-inoculated with a nonflocculent strain of *Saccharomyces cerevisiae* (elliptic yeast), an increase of the number of elliptic cells that settled at the bottom of the culture was observed. Electron microscopy observations of the aggregates formed in the mixed culture confirmed that apiculate and elliptic yeasts can interact and establish a binding between them through homogeneous mucus. However, a lower percentage of *Kloeckera apiculata* flocculation with respect to the pure cultures was observed, this behaviour being correlated with a higher rate of glucose consumption by *Saccharomyces cerevisiae*. The prompt coflocculation of *Kloeckera apiculata* with *Saccharomyces* strains and the induction of this phenomenon by glucose could be considered an important biotechnological tool for the early decreasing of indigenous saccharomycetic flora from the media, before the inoculation of a selected starter strain to carry out a more controlled alcohol fermentation.

*Key words*: *Kloeckera apiculata*, *Saccharomyces cerevisiae*, flocculation, wine strains

## Introduction

Flocculation is a highly complex phenomenon that can occur in a few yeast strains and it results from the nonsexual aggregation of single cells into multicellular masses, which then settle at the bottom of the medium (*1*). Cell separation technology and yeast flocculation have been reviewed (*2*,*3*). This capacity of yeast cells has been traditionally used by the lager brewing industry where, after fermentation is complete, suspensions of single-celled yeast gather into clumps and sediment rapidly from the beer.

More recently, there has been considerable interest in flocculation as a cheap and 'natural' method of cell separation in other industrial fermentations. According to the lectin hypothesis, 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 neighbouring cell walls (*4*,*5*). In this process $Ca^{2+}$ seem to induce the correct conformation of the lectins. Taking into account the pH sensitivity and sugar inhibition, two phenotypes have been described: Flo1 phenotype, which is only mannose-sensitive, and NewFlo phenotype, which is sensitive to glucose, maltose and mannose (*6*,*7*). Other

*Corresponding author; Phone: ++54 381 4311 720; Fax: ++54 381 4005 600; E-mail: mfarias@cerela.org.ar

flocculation phenotypes have been described, namely mannose-insensitive flocculation (*7*) or strains whose flocculation only occurs in the presence of sufficiently high ethanol concentration (*8*).

So far, the literature on yeast flocculation reports mainly results obtained with *Saccharomyces* strains (*9–11*). Few studies deal with flocculent yeasts belonging to other genera although several, among them *Kluyveromyces* (*12,13*), *Hansenula* (*14*), *Schizosaccharomyces* (*15*) and *Saccharomycodes* (*16*), have become increasingly useful in modern fermentation biotechnology. We determined that *Kloeckera apiculata* strain, a non-*Saccharomyces* yeast isolated from wine, possesses intense cell-cell interactions in a yeast extract/malt extract/peptone/glucose ($YMPG_{20}$) medium. The flocculation mechanism is mediated by a protein-carbohydrate interaction stabilized by $Ca^{2+}$ (*17*). *K. apiculata* is one of the predominant yeast species in grape berries; it can participate in the early stages of natural wine fermentation and contributes to the characteristics of the wine (*18–20*). Thus, the flocculent phenotype of *K. apiculata* could influence the growth and metabolism of *S. cerevisiae*, the main yeast responsible for alcohol fermentation during winemaking. The premature flocculation of the non-*Saccharomyces* yeast could bring fermentation to a premature end. This quantitative study has been undertaken to investigate the behaviour of a flocculent *K. apiculata* strain in a coculture with *S. cerevisiae*.

## Materials and Methods

### *Microorganisms, culture media and growth conditions*

Two yeast strains were used: *K. apiculata* flocculent strain and *S. cerevisiae* non-flocculent strain. Both microorganisms were isolated from Argentinean wine.

The yeasts were grown in a broth containing 10 g/L of yeast extract, 20 g/L of peptone and different concentrations of glucose: 2 ($YPG_2$), 20 ($YPG_{20}$), 50 ($YPG_{50}$) or 100 g/L ($YPG_{100}$). The pH was adjusted to 5.5 with NaOH. Cells were incubated aerobically at 28 °C on an orbital shaker at 150 rpm, then harvested by centrifugation (2500×*g*, 5 min) and washed twice with 10 mM EDTA solution (Merck) to ensure aggregation dispersion of the yeast forming flocs. Finally, cells were washed twice and suspended in deionized water.

Mixed cultures were performed by mixing *K. apiculata* and *S. cerevisiae* in a ratio of 1:1. Controls were carried out with pure cultures. The growth was monitored spectrophotometrically (at 620 nm) after the addition of 10 mM EDTA solution to prevent cell aggregation.

Pure cultures of both yeasts were counted on the $YPG_{20}$ agar plates in triplicate. The plates were incubated at 28 °C for 3 days and then examined for counts of individual yeasts. $YPG_{20}$ medium supplemented with 1 μg/mL of cycloheximide (Sigma) was utilized for differential enumeration of both yeasts in the mixed culture. This antibiotic concentration was enough to inhibit *S. cerevisiae* (*21*), but not *K. apiculata*. Both yeasts were completely inhibited at 5 μg/mL of cycloheximide.

### *Determination of flocculation percentage*

Two methods were used for estimation of the flocculation degree of the yeasts. The spectrophotometric method was based on that of Bendiak (*22*), which is a modification of Helm's flocculation test (*23*). At defined times of growth, the flocculating yeast was washed twice with 10 mM EDTA, and then twice with distilled water. Washed cells (about 20 mg of dry mass, corresponding to a final absorbance of 0.8) were placed in 10 mL of 50 mM acetate buffer, pH=4.5, containing 3 mM of calcium ions. The degree of flocculation was measured after the yeast cells were suspended by vigorous shaking. Absorbance (*A*) at 620 nm was measured immediately ($A_0$) and after 10 min ($A_1$) at room temperature. The percentage of cells that sediment was calculated as follows:

$$\text{Flocculation}=[(A_o-A_1)/A_o)]\times 100 \qquad /1/$$

With the aim to elucidate the type and number of cells in the flocs from a mixed culture, aggregate-forming cells were also calculated by differential counts of total and free viable cells of both yeasts. After different incubation periods, mixed yeast cultures were agitated for 20 s in a vortex mixer at maximum speed and allowed to settle at the bottom of the tube. After 10 min, 1 mL of the upper phase of the culture was taken and differential counts of free viable cells (nonfloc-forming cells) were carried out. The remaining culture was centrifuged at 2000×*g* and washed twice with distilled water. The cell pellets were deflocculated with 10 mM of EDTA and used for the differential counts of total viable cells in the cultures. Controls were carried out with pure cultures. The percentage of flocculation was also calculated as follows:

$$\text{Flocculation}=[(\log \text{CFU/mL}_{\text{total}}-\log \text{CFU/mL}_{\text{free}})/ /\log \text{CFU/mL}_{\text{total}}]\times 100 \qquad /2/$$

### *Induction of flocculation by glucose*

*K. apiculata* was cultured in glucose-poor medium (with 2 g/L of glucose). After growth for 8 h, when the culture reached the early exponential growth phase, a glucose solution (400 g/L), sterilized through a filter of 0.2-μm pore size, was added to the culture to reach a final concentration of 20, 50 and 100 g/L of glucose. Cell cultures not induced by glucose were used as control. The flocculation percentage was measured at different times after glucose addition.

### *Glucose determination*

Cell-free samples were obtained by centrifugation of the growth medium at 2500×*g* for 10 min. The supernatant was collected and immediately stored at –20 °C until analysis. The glucose concentration in the culture medium was measured utilizing the glucose oxidase--peroxidase-*o*-dianisidine enzymatic assay (Sigma).

### *Electron microscopy*

The surface structure of the floc-forming yeast was examined by a scanning electron microscopy (SEM). After 24 h of growth at 28 °C in $YPG_{20}$ medium, *K. apiculata* in pure culture or cocultivated with *S. cerevisiae*

was harvested by centrifugation (2000×*g*, 5 min) and washed twice with deionized water. After washing, the cells were fixed for 1 h at 4 °C with 3 % glutaraldehyde in 0.1 M phosphate buffer at pH=7.4, with very gentle agitation. They were then postfixed in 1 % osmium tetraoxide for 1 h at room temperature. The samples were then dehydrated with alcohol followed by ascending concentrations of acetone, and dried at a critical point. The samples were examined with a JOEL JSM 35CF microscopy (Akishima, Japan).

## Results

### *Flocculation of K. apiculata induced with glucose*

Fig. 1 shows the progress and extent of the flocculation profile of pure cultures of *K. apiculata* determined by a spectrophotometric method (see Materials and Methods). The yeast showed to possess cell-cell interactions in $YPG_{20}$ medium, resulting in the formation of a layer at the bottom of the tube. After shaking, the layer broke up into large aggregates that settled rapidly with a flocculation percentage of 75 % after 24 h of incubation. This yeast, when cultured in a glucose-poor medium (with 2 g/L of glucose), expressed a flocculation of 10 %, which remained constant during the growth of the yeast.

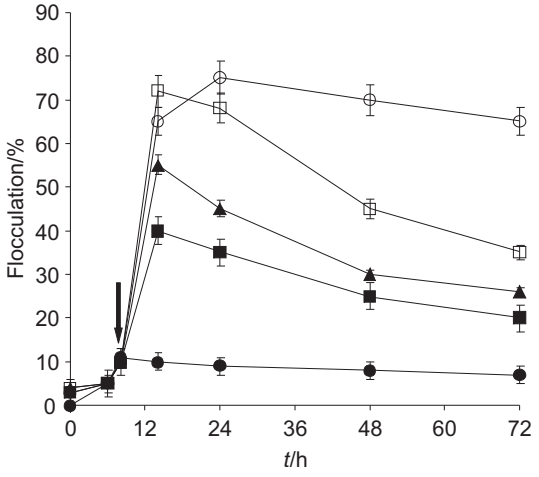


**Fig. 1.** Flocculation profile of pure cultures of *K. apiculata* on $YPG_{20}$ medium (○) and $YPG_2$ medium (●). Arrow indicates the addition of glucose pulse to the $YPG_2$ medium to obtain a final concentration of 20 (■), 50 (▲) or 100 g/L (□). Flocculation was evaluated by the spectrophotometric method (see Materials and Methods). Each point indicating flocculation percentages represents the mean of two independent experiments performed in duplicate. Vertical error bars represent standard deviations (*N*=4)

After 8 h of incubation, when increasing glucose concentrations (20, 50 and 100 g/L) were added to the culture, an induction of the flocculation was observed in direct correlation with the sugar concentrations.

### *Flocculation of K. apiculata in coculture with S. cerevisiae*

Fig. 2 shows the flocculation percentage of *K. apiculata* and *S. cerevisiae* in pure and mixed culture. Mixed

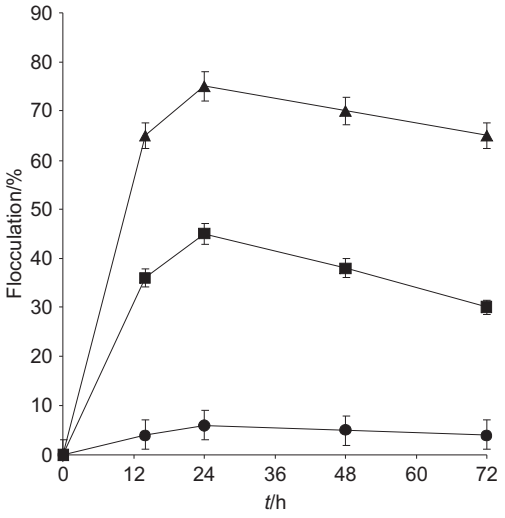


**Fig. 2.** Profile of flocculation of pure cultures of *K. apiculata* (▲), *S. cerevisiae* (●) and mixed cultures (■) grown on $YPG_{20}$ medium. Flocculation was evaluated by the spectrophotometric method (see Materials and Methods). Each point indicating flocculation percentages represents the mean of two independent experiments performed in duplicate. Vertical error bars represent standard deviations (*N*=4)

cultures were performed by mixing *K. apiculata* and *S. cerevisiae* in a ratio of 1:1. The elliptic yeast was unable to flocculate in the single culture. When it was cocultured with *K. apiculata* in $YPG_{20}$ medium, an apparent inhibition (40 %) of the apiculate yeast flocculation (assayed by spectrophotometric method) was observed after incubation for 24 h.

In order to find out whether the decrease in *K. apiculata* flocculation in the mixed culture was due to an inhibition of the cell-cell aggregation of the apiculate yeast or to an increase in the nonflocculent *S. cerevisiae* cells in the supernatant, we carried out a differential count of the total viable and free cells (nonfloc-forming cells) of both yeasts in the culture (see Materials and Methods). The results shown in Table 1 indicate that the percentage of free apiculate cells that remained in suspension in a mixed culture after 24 h of incubation was higher (95 %) than in the pure culture (65 %) with respect to total *K. apiculata*. Under these conditions, *S. cerevisiae* showed an increase in the cells that settled at the bottom of the culture with respect to total elliptic cells (from 5 to 21 % in the pure and mixed culture, respectively).

Table 1. Differential count of *K. apiculata* and *S. cerevisiae* in pure and mixed cultures

| Yeast | log CFU/mL* | | | |
|---|---|---|---|---|
| | Pure culture | | Mixed culture | |
| | Total cells | Free cells | Total cells | Free cells |
| *K. apiculata* | 6.08±0.20 | 3.95±0.09 | 5.11±0.25 | 4.85±0.18 |
| *S. cerevisiae* | 6.36±0.17 | 6.05±0.28 | 6.44±0.27 | 5.09±0.15 |

*Differential enumeration of each yeast after the 24-hour incubation. The values represent the mean of two independent experiments performed in duplicate (*N*=4)

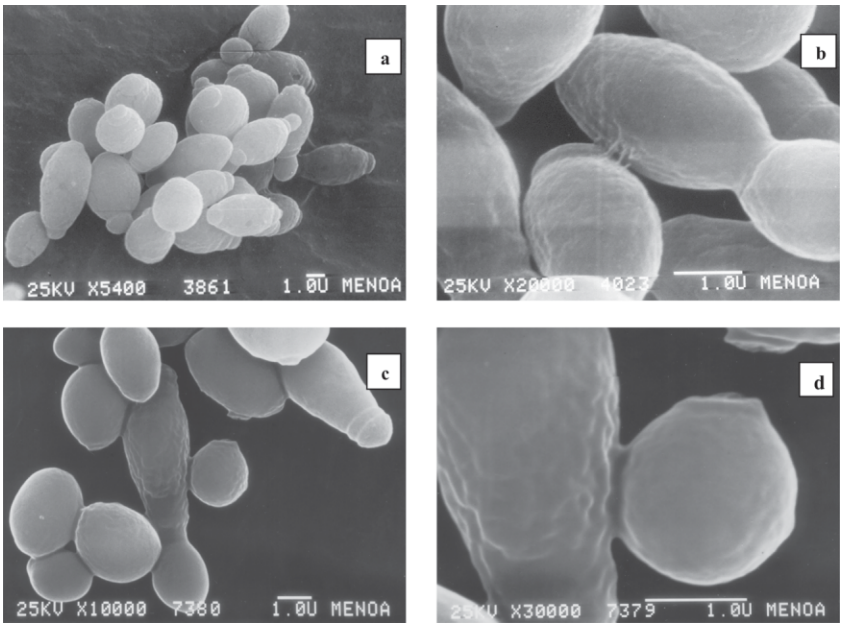


**Fig. 3.** SEM photomicrographs of (a) a floc formed during the growth of *K. apiculata* in $YPG_{20}$ medium; (b) apiculate flocculent cells are attached by fine hairlike threads. (c) Flocs built during the growth of *K. apiculata* with a nonflocculent *S. cerevisiae* in $YPG_{20}$ medium; (d) cells are attached by intercellular mucus between both yeasts

### *Scanning electron microscopy of the flocs formed by pure and mixed yeast cultures*

Figs. 3a and b show the SEM micrographs of a floc observed in the pure culture of *K. apiculata* grown in $YPG_{20}$ medium. The cells are attached to each other along their sides by fine hairlike threads. Pure cultures of *S. cerevisiae* did not form aggregates. The electron microscopy observation of the aggregates formed in mixed cultures shows apiculate and elliptic yeast cells adhered in combined flocs, connected by intercellular homogeneous mucus between both yeasts (Figs. 3c and d).

### *Effect of glucose on the flocculation of K. apiculata in mixed cultures with S. cerevisiae*

The effect of different glucose concentrations on the degree of flocculation of *K. apiculata* in mixed cultures is shown in Table 2. Higher percentage of flocculation was observed as the glucose concentration increased in the cultures. After 24 h of incubation at 28 °C, the percentage of flocculent cells with respect to the total apiculate yeast was 5, 25 and 65 % for 20, 50 and 100 g/L of glucose, respectively. However, the degree of flocculation was lower than in pure culture of *K. apiculata* (35, 54 and 80 % for 20, 50 and 100 g/L of glucose, respectively). *S. cerevisiae* under coculture conditions showed a lower percentage of cells that remained in the supernatant as the glucose concentration increased. In the pure culture, *S. cerevisiae* did not modify significantly its settling profile at the glucose concentrations assayed.

Table 2. Influence of glucose on the aggregation of *K. apiculata* and *S. cerevisiae* in pure and mixed cultures

| Culture media | Flocculation/%* | | | |
|---|---|---|---|---|
| | *K. apiculata* | | *S. cerevisiae* | |
| | Pure culture | Mixed culture | Pure culture | Mixed culture |
| with 20 g/L glucose | 35±1.9 | 5.0±0.3 | 4.0±0.2 | 10±0.7 |
| with 50 g/L glucose | 54±3.0 | 25±0.9 | 5.0±0.5 | 20±0.9 |
| with 100 g/L glucose | 80±3.9 | 65±1.2 | 4.0±0.4 | 25±0.8 |

*Calculated by the differential enumeration of each yeast with respect to the total counts of the respective yeast after 24-hour incubation (Eq. 2). The values represent the mean of two independent experiments performed in duplicate ($N$=4)

Fig. 4 shows the settling profile of pure and mixed cultures with respect to glucose consumption with the most effective glucose concentration assayed being 100 g/L. After incubation for 72 h, the pure culture of *K. apiculata* consumed 55 % of glucose and 92 % flocculation was observed. Under coculture conditions, an inhibition of *K. apiculata* flocculation was observed from 24 h of incubation onwards. The rate of glucose removal from the medium was higher as a consequence of the greater sugar fermentation power of the elliptic yeast. After incubation for 72 h, 89 % of the sugar was consumed and the flocculation of the apiculate yeast was 10 %.

## Discussion

Yeast flocculation is correlated with several culture conditions such as pH and temperature (*24*,*25*), as well as with the composition of the culture medium (*26*). Strong yeast-yeast aggregation in *S. uvarum* is observed when cells are cultured in the presence of various sugars, such as glucose, fructose and sucrose (*27*). We determined that *K. apiculata* (an apiculate yeast) possesses intense cell–cell interactions in a $YPG_{20}$ medium. In the presence of 2 g/L of glucose in the medium, yeast floc-

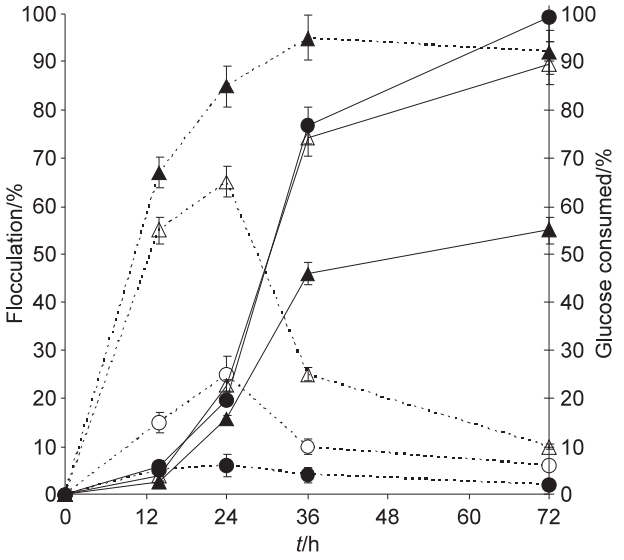


**Fig. 4.** Flocculation profile (----) and glucose consumption (——) of pure cultures (▲,●) and mixed cultures (△,○) of *K. apiculata* and *S. cerevisiae*, respectively, grown on $YPG_{100}$ medium. Aggregate-forming cells were calculated by the difference between total and free viable cells (see Materials and Methods). Each point indicating flocculation percentages represents the mean of two independent experiments performed in duplicate. Vertical error bars represent standard deviations (*N*=4)

culation is weak (10 % at the most), but the addition of different glucose concentrations during the exponential growth phase resulted in an increase of the flocculation percentage. The maximum flocculation observed during the induction with 20 g/L of glucose was lower than that observed when the cell was grown in $YPG_{20}$ medium. This behaviour could be related to the presence of an intracellular pool of proteins (lectins) involved in the flocculation when the cells grow in $YPG_{20}$ medium (precultured in the basal medium without sugar). It is important to notice that these lectins would not be present in the cells exposed to 20 g/L of glucose pulse (precultured in YP medium). This explanation is based on our investigations which indicate that glucose induces the flocculation by *de novo* synthesis of protein (lectin) *via* cAMP/PKA pathway (*28*).

This result is in agreement with those of Géhin *et al.* (*29*), who demonstrated that *Kluyveromyces bulgaricus* is very weakly flocculent in a glucose-poor medium. Géhin *et al.* (*30*) also reported that glucose is an activator of *Kluyveromyces bulgaricus* self-flocculation. This phenomenon seems to be correlated with the differential expression of cell wall proteins as a consequence of a glucose increase in the culture medium. This carbohydrate could lead to different transduction pathways in yeasts.

Electron microscopy observations showed that *K. apiculata* cells are attached to each other along their sides by fine hair-like threads. When *K. apiculata* was cocultured with a nonflocculent *S. cerevisiae* strain, a lower flocculation degree was observed with respect to the pure culture of the apiculate yeast. This behaviour was correlated with a higher rate of glucose removal from the medium by the elliptic yeast. An increase in *S. cerevisae* cells that settled at the bottom of the culture was also determined. This behaviour could be due to a higher entrapment of *Saccharomyces* into the flocs formed by the apiculate yeast or to a cell-cell interaction between the cells of both yeasts. The results from the electron microscopy observations of the floc formed under coculture conditions indicate that both genera of yeasts can coflocculate through the regular mucus formed between elliptic and apiculate yeasts. Our results are in agreement with those of Soares *et al.* (*24*), who demonstrated that nonflocculent cells can interact and establish a true binding with flocculent cells, *i.e.* they are adhered and not simply entrapped in the flocs**.**

## Conclusions

We demonstrated that *K. apiculata* can coflocculate with *S. cerevisae*, modifying the number of initial elliptic yeast cells suspended in the medium due to the formation of mixed floc that sediment at the bottom of the culture.

The prompt coflocculent capacity of *K. apiculata* with *Saccharomyces* strains and the induction of this phenomenon by glucose could be considered an important biotechnological tool for the early decreasing of indigenous saccharomycetic flora in the media, before inoculation with the selected yeast to carry out a more controlled alcoholic fermentation.

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