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Flocculation and cell surface characterization of *Kloeckera apiculata* from wine

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ABSTRACT

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Aims: To characterize and analyze the flocculation phenomenon of *Kloeckera apiculata* mc1 from Agentinian wine to understand the cell–cell interaction pattern.

Methods and Results: *Kloeckera apiculata* mc1 possess intense cell–cell interactions in MYPG medium (0·5% malt extract, 1% yeast extract, 2% glucose, 2% peptone), pH 5·5 by shaking at 25°C. Optimum flocculation is observed at pH 4·5 in the presence of 3 mmol l^{-1} Ca^{2+}. The flocculation is induced by peptone and malt extract and not by yeast extract and is reversed by 50 mmol l^{-1} galactose or lactose. The flocculation is highly susceptible to pronase, chymotrypsine and proteases types IV and XXVII and is partially resistant to trypsin. The electronic microscopy shows that the cells are attached to each other along their sides by fine hair-like threads.

Conclusions: The mechanism of flocculation of *K. apiculata* mc1 is mediated by protein–carbohydrate interaction, stabilized by Ca^{2+}.

Significance and Impact of the Study: The use of selected pure yeast inocula of known ability is preferred to wine elaboration, therefore the indigenous flora must be avoided and the flocculation of *K. apiculata* could be an economic method to do it.

Keywords: electronic microscopy, flocculation, interactions, *Kloeckera apiculata*, wine quality.

INTRODUCTION

Flocculation is the term used to describe the property expressed by certain yeasts, when they spontaneously aggregate to form flocs with sediment in the culture. During the past decade, there has been a resurgence of interest in the subject of yeast flocculation, both in *Saccharomyces cerevisiae* and other yeast genera. This has stemmed, in part, from commercial interest in immobilized-yeast fermentations, but also from the search for economic methods for the separation of cells from media in the downstream processing of fermentation products. Flocculence in yeast is interesting as an example of interaction between the surfaces of the cells and is subject to control by a complex variety of genetic, environmental and metabolic factors. Several mechanisms have been proposed to explain this interaction. One hypothesis is that anionic groups of cell wall components are linked by Ca^{2+} ions (Mill 1964). Another hypothesis implicates glycoproteins specific to flocculent strains acting in a lectin-like manner to cross-link the cells; in this case, Ca^{2+} ions acting as ligands would promote flocculence by conformational changes (Miki *et al.* 1980).

Yeast flocculation has for many years been associated solely with brewing strains of *S. cerevisiae* and *S. uvarum* (Miki *et al.* 1982). However, flocculation has also been found in several other non-*Saccharomyces* yeast genera, such as *Hansenula* (Moriya *et al.* 1990; Saito *et al.* 1990), *Kluyveromyces* (Hussain *et al.* 1986) and *Schizosaccharomyces*

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(Johnson *et al.* 1988; El-Behhari *et al.* 2000). *Kloeckera apiculata*, one of the predominant species of grape berries, can participate in the early stages of natural wine fermentation contributing to the characteristic of the wine. In large-scale wine production, the needed to obtain consistent wine flavour and predictable quality, the use of selected pure yeast inocula of known ability is preferred and therefore, the indigenous flora must be avoided. The flocculation phenomenon is an important and economical method to do it.

The aim of this study was to characterize and to analyse the flocculation and deflocculation of *K. apiculata* mc1 from Argentinian wine to understand the cell–cell interaction pattern.

MATERIALS AND METHODS

Micro-organism

Kloeckera apiculata mc1 was isolated from Argentinean wine.

Culture media

The yeast was grown aerobically in MYPG medium (0·5% malt extract, 1% yeast extract, 2% glucose, 2% peptone) with a pH of 5·5 by shaking in conical flasks at 25°C. Growth was monitored by absorbance at 620 nm after the addition of 10 mmol l^{-1} EDTA, and in the logarithmic phase of growth, the cells were harvested by centrifugation at 1000 *g* and washed twice with distilled water.

Flocculation and deflocculation measurement

The flocculating yeast was washed with 10 mmol l^{-1} of EDTA, and then twice with distilled water. Washed cells [about 20 mg dry weight, corresponding to a final optical density (O.D.) of 0·8] were placed 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. The O.D. at 620 nm was measured immediately (D_0) and after 10 min (D_1). The ratio $R = D_1 \times 100/D_0$, representing the percentage of cells still in suspension, was calculated.

Flocculation pH range

The pH ranges over which yeast flocculated was assessed by resuspending the flocculent yeast in a composite buffer with a very wide buffering range. This buffer contained 50 mmol l^{-1} Tris, 50 mmol l^{-1} succinic acid, 100 mmol l^{-1} KOH and 3 mmol l^{-1} calcium ions. Solutions were adjusted to pH with 5 mol l^{-1} HCl.

Enzyme treatments

Stationary phase cells were harvested by centrifugation, washed twice with distilled water, twice with 10 mmol l^{-1} of EDTA, and twice again with distilled water. One gram of the washed cells was added to 40 ml of 0·1 mol l^{-1} Tris-HCl buffer (pH 6·8), containing enzyme. A control was carried out without enzyme. All flasks were incubated with shaking at 25°C. Samples were taken at the required times, centrifuged for 1 min in a micro-centrifuge, and the pellets of cells were resuspended in 50 mmol l^{-1} acetate buffer pH 4·5 with calcium ions. Enzyme treatments were performed in 0·1 mol l^{-1} Tris–HCl buffer (pH 6·8) at the following concentrations: pronase E, 200 μg ml^{-1}; chymotrypsin, 100 μg ml^{-1}; trypsin, 100 μg ml^{-1}; protease type IV and XXVII, 200 μg ml^{-1}; and α-amylase 200 μg ml^{-1}.

Sugar treatments

The deflocculating effect of sugars on fully flocculating yeasts was determined at different concentrations of mono-, di- or oligosaccharide in acetate buffer at pH 4·5. After shaking for a few seconds, the O.D. was measured immediately and again after 20 min.

Electron microscopy

The surface structure of the cells was examined by a scanning (SEM) and transmission (TEM) electron microscope. For the SEM, the samples were fixed with 3% glutaraldehyde in 0·1 mol l^{-1} of phosphate buffer at pH 7·4. They were then postfixed in 1% osmium tetroxide, dehydrated with alcohol followed by acetone and critical point dried. The samples were then observed with a JEOL JSM 35CF (Akishima, Japan). The TEM samples were fixed and dehydrated in the same way. They were then impregnated with Spurr's epoxy resin, 60 nm ultra-thin sections were cut and observed with a Zeiss EM109 (Oberkochen, Germany).

RESULTS

Flocculation

Flocculation of *K. apiculata* mc1 is characterized by intense interaction between yeast cells, which results in the formation of large cell aggregates or flocs at the bottom of MYPG culture medium. The progress and extent of flocculation were followed by a decrease in turbidity of a cell suspension on addition of $CaCl_2$.

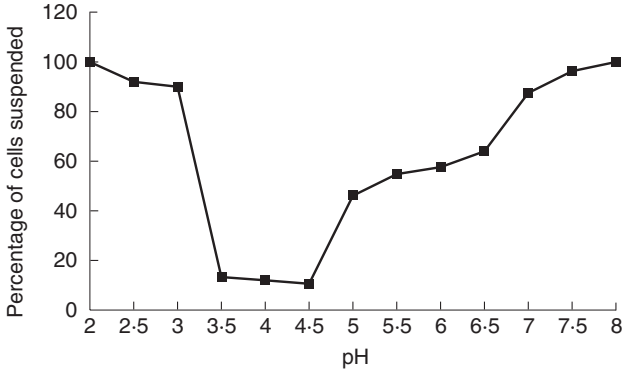

Fig. 1 Effect of pH on yeast flocculation. Values are mean of three independent experiments

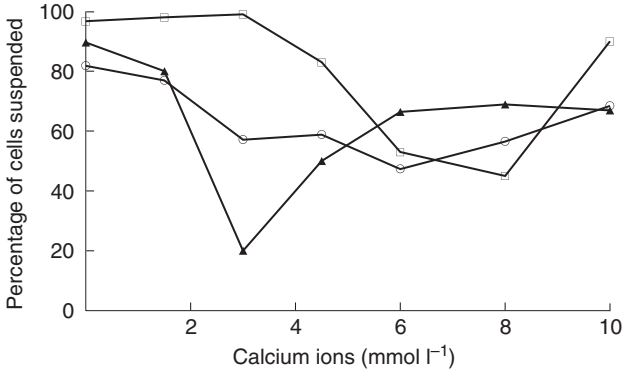

Fig. 2 Effect of Ca^{2+} ions on yeast flocculation at pH 3·0 (□), 4·5 (▲) and 6·5 (○). Values are mean of three independent experiments

Table 1 Inhibition of *Kloeckera apiculata* mc1 flocculation by sugars

Sugars (5 mmol l^{-1})	Relative percentage of aggregated cells	Inhibition
Control	93·3	–
D-glucose	90·0	–
2-Deoxy-D-glucose	92·0	–
D-galactose	30·7	+++
D-mannose	87·3	–
L-arabinose	84·0	–
D-fructose	90·3	–
Lactose	46·5	++
Cellobiose	96·5	–
Trehalose	89·8	–
Maltose	90·7	–
Melibiose	46·8	++

Values are mean of three independent experiments.

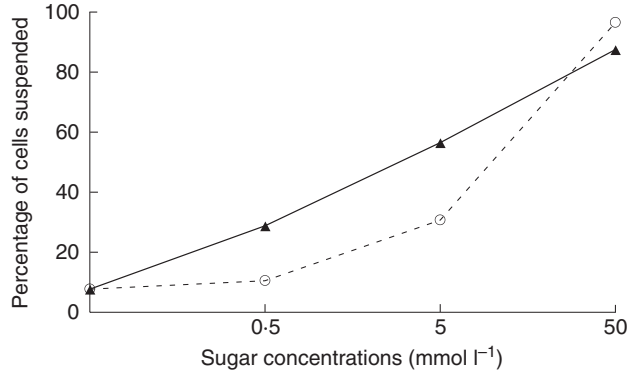

Fig. 3 Inhibition of yeast flocculation by different galactose (○) and lactose (▲) concentrations. Values are mean of three independent experiments

Combined effect of pH and calcium

Floc stability was influenced by the pH of the buffer. Optimal aggregation of *K. apiculata* mc1 occurred across a pH range of 3·5–4·5. Low and high pH values reversibly inhibited the flocculation (Fig. 1). About 3 mmol l^{-1} of Ca^{2+} was necessary to obtain maximal flocculation at pH 4·5. Above and below this pH, higher cation concentrations (8 and 6 mmol l^{-1} for pH 3·0 and 6·5, respectively) were necessary for maximal aggregation (Fig. 2). This cation cannot be substitute by Mg^{2+} or Mn^{2+} ions.

Influence of sugars

Flocculation of *K. apiculata* mc1 was inhibited by some sugars. The monosaccharide found to inhibit flocculation were D-galactose (Table 1). Disaccharide bearing a D-galactose in a terminal non-reducing position, such as lactose also reversed the yeast flocculation.

Inhibition of yeast flocculation by galactose and lactose were tested by addition of increasing amount of mono and disaccharide to a suspension of flocculating yeasts in acetate buffer pH 4·5. An important reversion of flocculation was observed with 50 mmol l^{-1} of both sugars (Fig. 3). By washing the cells with distilled water, flocculation was restored.

Effect of medium composition

The influence on the flocculation was studied in three different media: a rich medium (MYPG), Sabouraud and basal grape juice. *Kloeckera apiculata* flocculated strongly in MYPG medium ($R = 5{\cdot}7\%$), with a lesser degree in Sabouraud ($R = 24\%$) and in the basal grape juice ($R = 72\%$). The direct influence of the culture medium composition on flocculation was investigated by submitting

Table 2 Effect of MYPG medium components on the flocculation

Treatment*	Cells in suspension (%)
MYPG	5
MYPG diluted twice	13
MYPG + 2 or 10 mmol l^{-1} Ca^{2+}	0
Peptone (20 g l^{-1})	8
Peptone (5 g l^{-1})	13
Peptone (2 g l^{-1})	15
Malt extract (5 g l^{-1})	0
Malt extract (1·25 g l^{-1})	2·5
Malt extract (0·5 g l^{-1})	4
Yeast extract	100

*Deflocculated cells were submitted to treatment with the different components and then the degree of flocculation was determined.

Table 3 Enzymes effect on *Kloeckera apiculata* mc1 flocculation

Enzymes	Cells in suspension (%)
Control*	5·7
Pronase E	
30 min	100
Chymotrypsin	
30 min	100
Proteases IV and XXVII	
30 min	100
Trypsin	
30 min	50
60 min	82
120 min	100

*Without treatment.
Values are mean of three independent experiments.

the cells to a flocculation test in which, instead of using acetate buffer containing calcium ions, the cells were resuspended in fresh MYPG medium or in the solutions of the constituents of the medium. Table 2 shows that the cells resuspended in fresh medium flocculated again with $R = 5\%$. By dilution of the medium a lesser extent of flocculation was achieved. The addition of 2 or 10 mmol l^{-1} Ca^{2+} increased the flocculation ($R = 0\%$). When deflocculated cells were treated with 20 g l^{-1} of peptone, 8% remained in suspension. This percentage increased to 13 and 15% when peptone concentration diminished to 5 and 2 g l^{-1}, respectively. All deflocculated cells treated with 5 g l^{-1} of malt extract flocculated again ($R = 0$). About 2·5 and 4% of cells remained in suspension when treated with 1·25 and 0·5 g l^{-1} of malt extract, respectively. Yeast extract did not cause flocculation of deflocculated cells.

Enzyme treatment

The *K. apiculata* mc1 floc-forming ability was irreversibly lost by 30 min incubation with pronase E, chymotrypsin and proteases VI and XXVII, and 120-min incubation was necessary to achieve the complete inhibition by trypsin (Table 3).

Electron microscopy

Using SEM (Fig. 4), it is possible to observe the cell surface of a flocculent *Kloeckera* strain. The cells were attached to each other normally along their sides by fine hair-like threads, which emanate from both cell walls.

The TEM (Fig. 5a) shows the fibrilar attachments between flocculent yeast cells. The extensions from the cells surfaces intermingle with those of the other cells. The effect of trypsin on flocculent cells is shown in Fig. 5b.

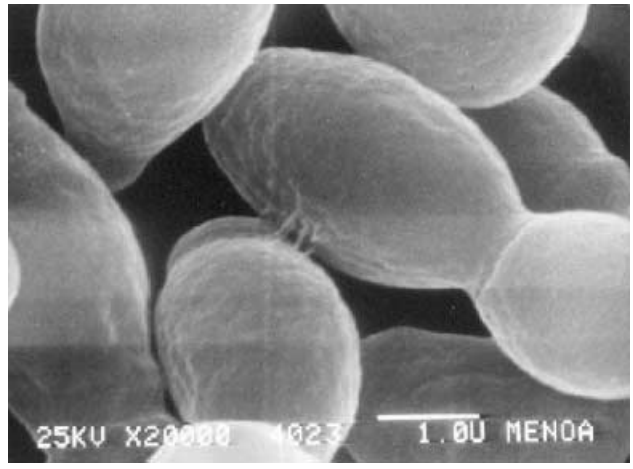

Fig. 4 SEM micrograph of *Kloeckera apiculata* mc1 flocculent cells attached by fine hair-like threads

The enzyme produced the lost of the capacity to flocculate with a total cell elongations loss after 120-min incubation.

DISCUSSION

Kloeckera apiculata mc1 flocculating over a narrow pH range and was optimal at pH 4·5. The aggregation inhibition without calcium ions might results from changes in lectin conformation following calcium ion loss at these pH values. This behaviour is typical of Ca^{2+} dependent lectin (Stratford 1996). Induction of flocculation by pH change may be used to separate cells from media at any stage during fermentation.

Flocs of flocculating *K. apiculata* cells were deflocculated when resuspended in 50 mmol l^{-1} galactose and disaccharide lactose bearing a D-galactose in a terminal non-reducing position. Thus, this system in *K.apiculata* mc1 is deemed to

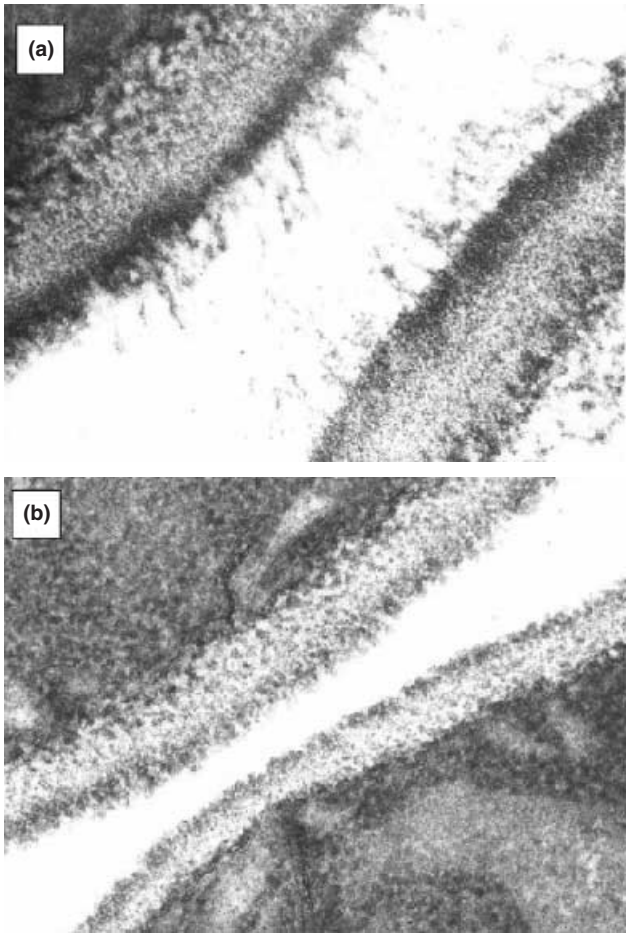

Fig. 5 TEM micrograph of fibrilar links between flocculent cells (a) and after 120 min of trypsin treatment (100 μg ml^{-1}) (b)

be mediated by lectin-type interaction wherein galactomanan in the wall interacts with the flocculum protein. In *Kluyveromyces bulgaricus* and *Kluyveromyces lactis*, Al-Mahmood *et al.* (1991) and Bellal *et al.* (1995) demonstrated that the self-flocculation involves galactose-specific lectins. Stratford and Pearson (1992) indicated that aggregation of the yeast *Saccharomycodes ludwigii* NCYC 734 was specifically inhibited by monosaccharides that are similar in structure, differing only at the carbon-6 position (D-galactose, L-arabinose and D-fucose), and disaccharides containing non-reducing terminal galactose residues (lactose, lactulose and melibiose).

Proteinase treatments of flocculent cells indicated an important role of the cell wall proteins. This fitted well with the calcium-bridging theory, in that carboxyl groups are normally found on proteins. Proteins on *K. apiculata* flocculent cells appear to have all the characteristics of lectins, i.e. proteins that bind specific sugars and that require metallic ions. Stratford and Assinder (1991) proposed that yeast surface lectins may originate from one of two sources, either from within the yeast (Flo1 phenotype) or from the media in which the yeast are grown (NewFlo phenotypes). Our results appear to agree with the NewFlo phenotype for *S. cerevisiae*, as *K. apiculata* mc1 flocculated intensely in rich medium and in solutions of peptone or malt extract.

The SEM and TEM images show a clear correlation between the presence of surface fibrils and the ability of a culture to flocculate. It appears likely that the active groups responsible for flocculation are located in these fibrils on the surface of the *K. apiculata* mc1. These fibrils would form the complex protein-galactose involved in the flocculation.

In brief, the flocculation phenomena of *K. apiculata* mc1 occurs in accordance with the lectin theory of flocculation: (i) yeast cells are surrounded by cell walls containing galactose residues, (ii) flocculent cells form surface lectins, are activated by the presence of calcium ions, and then bind to sugar receptors on neighbouring cell walls, and (iii) the flocs are progressively built up by the simultaneous interbinding of many cells.

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