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Influence of Culture and Nutritional Conditions on Self-Flocculation of a *Kloeckera apiculata* Wine Strain

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Summary

The current work examines the environmental conditions to control the expression of self-flocculation of *Kloeckera apiculata* mc1 isolated from Argentinian grapes with proven beneficial effect on wine aroma. Temperature had a direct effect on the expression of flocculation resulting in an increase in the number of zymolectin sites determined with a BSA--Gal-FITC probe and quantified with the Scatchard plot. Agitation had a positive effect on yeast flocculation and the effect did not correlate with an increase in the hydrophobic index. The addition of glucose, fructose or ethanol to yeast nitrogen base medium without amino acids or ammonium sulphate stimulated flocculation. Assimilative nitrogen sources had a negative impact on flocculation in the absence of an energy source. During winemaking, control of flocculation by environmental factors such as temperature and the presence of nutrients could be applied in order to optimize the fermentation process and hence the quality of the final product. Knowledge of the effect of different parameters on flocculation of the wine yeast *K. apiculata* mc1 allows prediction of the behaviour of this yeast during fermentation.

Key words: Kloeckera apiculata, wine yeast, flocculent phenotype, environmental conditions

Introduction

Cell flocculation only occurs in a few yeast strains and results from the non-sexual aggregation of single cells into multicellular clumps, which then sediment to the bottom (*1–3*). The mechanisms underlying yeast flocculation have been extensively examined, especially regarding *Saccharomyces cerevisiae*, and it has been found that cellular interaction is mediated by cell wall proteins (*4,5*). Yeast flocculation in *S. cerevisiae* is a complex process that depends on the expression of several specific genes such as *FLO1*, *FLO5*, *FLO8* and *Lg-FLO1*. Also, *FLO11*-encoded flocculin is required for a variety of important phenotypes in *S. cerevisiae*, including flocculation, adhesion to agar and plastic, invasive growth, pseudohyphae formation and biofilm development (*1–3,5–12*), and for the calcium-dependent flocculation in a strain-dependent manner (*13*). The ability of *Saccharomyces* yeast cells to aggregate is of considerable importance to the brewing industry as it is an economical method for the separation of cells from media in the downstream processing of fermentation products. Flocculation has also been found in several non-*Saccharomyces* yeast genera. Some *Kluyveromyces* spp. have been shown to possess a flocculation phenotype which is well-correlated with certain cell wall proteins (*14–16*). *Kluyveromyces marxianus* ATCC 10022 accumulates a glyceraldehyde-3-phosphate dehydrogenase isoform in the cell wall of flocculent cells and the en-

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zyme was shown to be involved in the flocculation phenotype (*17,18*). *Kloeckera apiculata* mc1, isolated from Argentinian grapes, exhibited a flocculent phenotype which was mediated by galactose-specific zymolectins and stabilized by Ca^{2+} while grown in yeast extract-malt extract--peptone-glucose (YMPG) broth (*19*). This flocculent phenotype was induced by glucose and the mechanism seemed to imply *de novo* protein (zymolectin) synthesis *via* the PKA (cyclic AMP-dependent protein kinase A) transduction pathway (*20*). The yeast was able to coflocculate with a *Saccharomyces* strain, a phenomenon that was also induced by glucose (*21*). Flocculation is a very strain-specific phenomenon affected by numerous parameters such as nutrients, dissolved oxygen, pH, fermentation temperature, yeast handling and storage conditions, and this complexity makes it difficult to predict specific responses. Therefore, a comprehensive knowledge of the strain behaviour is essential in order to gain maximal control over flocculation.

The present study explores the influence of various carbon and nitrogen sources and environmental conditions in order to control the expression of self-flocculation of *K. apiculata* mc1 from wine.

Materials and Methods

Isolation and identification of the yeast strain

Kloeckera apiculata mc1, a flocculent yeast, was isolated from Argentinian grapes. The strain was selected for its high flocculating capacity using the method previously described by Smit *et al.* (*22*). Identification was carried out by morphological examination and classical biochemical and physiological tests (*23*). The taxonomic identity of the isolated yeasts was subsequently confirmed by the analysis of partial nucleotide sequence of the large subunit ribosomal RNA gene (*24–26*). The sequence determined in this study was registered in the GenBank Data Library under accession number FJ800030.

Culture media

The yeast cells were grown statically in 50-mL conical flasks in 25 mL of YMPG broth containing (in g/L): yeast extract 10, malt extract 5, peptone 20, glucose 20, at pH=5.5 and 25 °C. Growth was monitored by measuring the absorbance at 620 nm after the addition of 50 mM EDTA to ensure floc dispersion, and cells were harvested during the logarithmic phase by centrifugation at 1000×*g*. Cell pellets were washed twice and suspended in deionized water.

Effect of carbon and nitrogen sources

Experimental cultures were inoculated with 10^6 CFU/mL from the suspension prepared according to the procedure described before and incubated aerobically at 25 °C. The following media were used to determine the effect of the carbon source on flocculation: YNB (1.7 g/L of Difco yeast nitrogen base with amino acids or ammonium sulphate), YNB w/o AA (1.7 g/L of Difco yeast nitrogen base without amino acids or ammonium sulphate) and YNB w/o AA supplemented with 20 g/L of fermentable sugars: glucose (YNB w/o AA+glu), fructose (YNB w/o AA+fru), galactose (YNB w/o AA+gal) and lactose (YNB w/o AA+lac) or with 50 g/L of a non-fermentable carbon source: ethanol (YNB w/o AA+eth). The effect of nitrogen was determined with: YCB_m (11.7 g/L of Difco yeast carbon base, additionally supplemented with glucose to obtain a final concentration of 20 g/L) and YCB_m supplemented with 20 g/L of peptone (YCB_m+pep) or with 5 g/L of ammonium sulphate ($YCB_m+NH_4^+$). At different incubation times the cells were collected and evaluated for the degree of flocculation.

Determination of flocculation

Flocculation ability of the *K. apiculata* mc1 was monitored under standard conditions, using a technique previously described by Soares and Mota (*27*) with some modifications. After different incubation periods, yeast cultures were harvested by centrifugation (2500×*g*, 3 min) and deflocculated with a 50-mM EDTA solution. Subsequently, cells were washed twice and resuspended in deionized water at a final concentration of ~$2 \cdot 10^9$ cells/mL. The number of cells was determined spectrophotometrically (Boeco S-20, Hamburg, Germany) at 620 nm, using a calibration curve (number of viable cells *vs.* absorbance that was constructed beforehand). Cell suspensions were placed in 5 mL of 50 mM acetate buffer, pH=4.5, containing 3 mM of calcium ions (*19*), in test tubes of 12 mm in diameter and 100 mm of height, at a final concentration of $5 \cdot 10^7$ cells/mL (CFU/mL_{total}). The tubes were sealed, stirred vigorously in a vortex for 10 s and agitated in a horizontal position for 2 h on an orbital shaker at 200 rpm. After agitation, the tubes were allowed to stand undisturbed for 60 s, in a vertical position, after which samples (200 μL) taken from just below the meniscus of the liquid medium were dispersed in 50 mM EDTA solution. Subsequently, the free viable cell counts (non-floc-forming cells) were carried out (CFU/mL_{free}). Yeast cultures were counted in triplicate on YMPG plates. The plates were then incubated at 28 °C for 2–3 days and afterwards examined for individual yeast counts. The percentage of flocculated cells was calculated by subtracting the fraction of the cells remaining in the suspension after 60 s of shaker stopping from the total cell count, using the following equation:

$$\text{Degree of flocculation} = [(CFU/mL_{total} - CFU/mL_{free})/CFU/mL_{total}] \cdot 100 \quad /1/$$

Effect of temperature and agitation on flocculation

To determine the optimal flocculation temperature, the flocculent cells were resuspended in Helm's buffer (*28*) and incubated at 16, 25 and 32 °C. To evaluate the effect of agitation, yeast cells grown in YMPG medium were incubated statically or with agitation on an orbital shaker at 100, 200 and 300 rpm at 25 °C. In all cases, the flocculation percentage was measured at different time intervals during 48 h.

Effect of temperature on galactose-specific zymolectin density of K. apiculata mc1

Quantification of the density of galactose receptor sites on the cell surface of *Kloeckera apiculata* mc1 was performed using the spectrofluorimetric method according to Masy *et al.* (*29*) with some substantial changes. The apiculate yeasts grown in YMPG for 12 h at differ-

ent temperatures (16, 25 and 32 °C) were harvested and suspended in sodium acetate buffer supplemented with 3 mM $CaCl_2$, pH=4.0, at a concentration of $2{\cdot}10^6$ cells/mL. Then, aliquots of 2980 μL of yeast suspension were transferred to a 4-mL clear, four-sided cuvette (Agilent Technologies, Palo Alto, CA, USA) and treated with 20 μL of appropriate dilutions of a BSA-Gal-FITC stock solution (bovine serum albumin α-D-galactopyranosylphenyl isothiocyanate labelled with fluorescein isothiocyanate; Sigma-Aldrich, St. Louis, MO, USA; dissolved in sodium acetate buffer) to reach the final concentrations between 5 and 30 μg/mL. The mixture (containing the probe bound to the yeast cells, free probe and buffer) was vortexed for 10 s and fluorescence was measured immediately at an excitation wavelength of 495 nm and an emission wavelength of 520 nm with 10 nm slit widths with a spectrophotofluorimeter (Cary Eclipse, Agilent Technologies). The cuvette was wrapped in Parafilm™ (American National Can, Greenwich, CT, USA) and centrifuged at 3000 rpm for 3 min to settle the yeast cells and bound probe. Then the fluorescence intensity of the supernatant fluid (containing the free probe and buffer) was examined under the same conditions. Samples were determined in duplicate along with blanks which consisted only of yeast cells suspended in sodium acetate buffer. Nonspecific binding of the probe to yeast cells (background) was determined with a BSA-FITC fluorescent probe (bovine albumin-fluorescein isothiocyanate conjugate, Sigma-Aldrich) following the procedure described above. Free and bound probe fluorescence intensities were converted into concentrations (μg/mL) by comparing the values obtained with a calibration curve prepared for the BSA-Gal-FITC probe. The yeast-bound probe concentration was calculated from the difference between total and free probe concentrations using the following equation:

$$c(\text{fixed probe})/\mu\text{M} = c(\text{total probe})/\mu\text{M} - c(\text{free probe})/\mu\text{M} \qquad /2/$$

The data collected were then analyzed according to the method by Scatchard (*30*) and modified by Tinoco Jr. *et al.* (*31*), to estimate the density or number of lectin sites per cell.

Cell surface hydrophobicity assay of flocculating cells

Calculation of cell surface hydrophobicity (CSH) was based on the microbial adhesion to hydrocarbons (MATH). Following a modified procedure by Rosenberg (*32*), apiculate yeasts grown statically or under agitation on an orbital shaker (200 rpm) in YMPG medium for 12 h at 25 °C were harvested by centrifugation at 3000 rpm. Cell pellets were deflocculated with 0.1 mM EDTA-NaP_i buffer, pH=7.5. Yeasts were washed twice with 0.1 mM K_2HPO_4–KH_2PO_4 buffer, pH=7.1, and resuspended in the same buffer at an absorbance of 1.0 at 620 nm (A_{before}). Then, 2 mL of hydrocarbon (*n*-hexadecane, *o*-xylene or toluene) were added to 4 mL of the sample (yeast cell suspension in buffer). The mixture was vortexed for 2 min to ensure complete mixing of the two phases and then allowed to settle for exactly 15 min to make sure that two distinct layers appeared before further analysis. During this time the yeast cells divided between the two phases. The aqueous (lower) layer was carefully removed with a Pasteur pipette, transferred to a cuvette, and absorbance was measured again at 620 nm (A_{after}). A blank (buffer) was used in both cases and all measurements were carried out in triplicate. The percentage of microbial adhesion to each hydrocarbon (MATH) was calculated as follows:

$$\text{MATH}=[1-(A_{after}/A_{before})]\cdot 100 \qquad /3/$$

where A_{before} is the absorbance before partition and A_{after} the absorbance after partition. A higher MATH value corresponds to a more hydrophobic cell surface.

Reproducibility of the results

All experiments were repeated, independently, three or four times. In general, absolute values of the independent experiments were not alike, but the trend was considerably consistent among the experiments performed on different days. The data reported for growth and flocculation are the mean values of triplicate or quadruplicate repetitions, respectively.

Results and Discussion

Effect of incubation temperature

Flocculation is an interesting example of interaction between cell surfaces controlled by genetic, metabolic and environmental factors. Sosa *et al.* (*20*) determined that this phenomenon in *K. apiculata* mc1, isolated from Argentinian wine grapes, and culture conditions were related, observing a strong flocculent phenotype in glucose-rich medium. In the present work the effect of different environmental conditions on the flocculent phenotype of *K. apiculata* mc1 was examined. Temperature can affect the development and expression of flocculation. However, there is an apparent contradiction in the literature; several authors found deflocculation with the increase of temperature while others noted an increase in flocculation with increased temperature (*33–36*). Fig. 1 shows the effect of different growth temperatures (16, 25 and 32 °C) on *K. apiculata* mc1 flocculation. Independent of

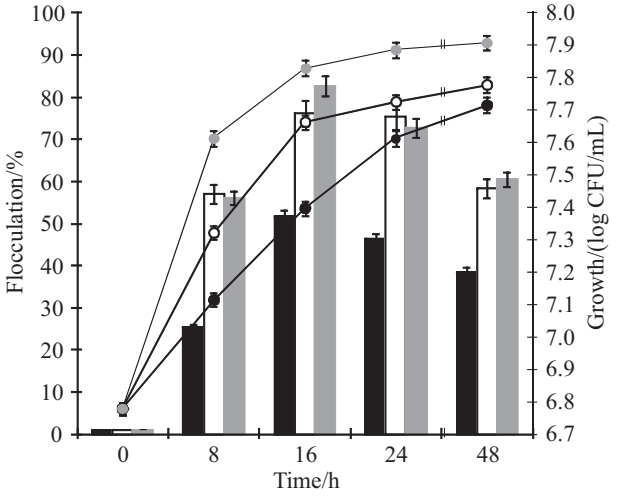

Fig. 1. Growth (circles) and flocculation ability (bars) of *K. apiculata* mc1 wine strain grown in YMPG medium at the following temperatures: 16 °C (black symbols), 25 °C (white symbols) and 32 °C (gray symbols). Each point represents the mean of two independent experiments performed in duplicate; standard deviation is shown (*N*=4)

the temperature, the highest percentage of flocculation was observed after 16 h of growth, being maximal at 32 °C. Similar results have been obtained by Soares *et al.* (*33*). These authors demonstrated that *S. cerevisiae* NewFlo was sensitive to the incubation temperature and observed the highest flocculation at 30 °C. Rhymes and Smart (*34*) reported that the ale brewing yeast NCYC 2593 exhibited highest flocculation at 25 °C. Another study confirmed that flocculation of lager strains was optimal above 10 °C and decreased dramatically below 5 °C (*35*). In other cases, flocculation was repressed at 25 °C and cell sedimentation was optimal at lower temperatures (5 °C) (*36*). This variation in the optimal temperature of flocculation clearly indicates the strain-specificity of flocculation, as well as the importance of secondary factors that are not always known or controllable. *K. apiculata* mc1 revealed a flocculent phenotype mediated by protein (zymolectin)-carbohydrate interaction (*19,20*). As the yeast secretory pathway is prone to temperature--sensitive mutations (*36*), the temperature could affect the availability of the Flo protein rather than having a direct effect on flocculation. The temperature effect on the degree of flocculation could be the result of a modification of the synthesis process or secretion of zymolectins involved in cell-cell aggregation. Besides, in order to prove that the temperature effect relates to an increase in lectin density on the cell surface, the number of lectin sites was determined using the BSA-Gal-FITC probe and quantification was carried out using the Scatchard plot. The results show that the number of lectin sites increased with increased incubation temperature (Table 1). The fluorescent probe assays confirm our previous findings that cell surface receptors of flocculent *K. apiculata* cells are galactose-specific (*19*) and that the number of lectin sites increases with the temperature. Similar results have been reported by Fernandes *et al.* (*37*) for a *Kluyveromyces marxianus* strain. The authors indicated that flocculation could be induced by a temperature upshift and this effect would be related to an increase in the synthesis and subsequent accumulation of a protein in the cell wall. The results in Table 1, indicating that the number of lectin sites/cell steadily increases with age, seem to disagree with the data presented in Fig. 1, which show that flocculation is maximal in a 16-hour-old culture and then declines. However, this apparent contradiction is probably explained by the technique used to determine the percentage of flocculated yeast. The observed differences could be attributed to the fact that in older cultures the number of dead yeast is constantly increasing. These nonviable cells are not taken into account in determining the degree of flocculation by the method of counting viable yeast (Fig. 1). By contrast, fluorescent glucidic probes used to quantify the density of lectin sites (Table 1) bind specifically to lectin receptors on the surface of flocculent yeasts independently of the physiological state of the cells (*38,39*).

Table 1. Effect of temperature on the lectin density of *K. apiculata* cell surface

Temperature °C	Galactose-specific lectin receptor/(sites/cell·10^6)*			
	Incubation time/h			
	8	12	24	48
16	4.8±0.2	7.7±0.3	8.0±0.4	8.2±0.3
25	6.5±0.2	9.5±0.7	10.4±0.9	15±1.0
32	9.0±0.5	10.3±0.4	11.6±0.8	20±0.9

*Data are mean values of three independent experiments±standard deviation

Effect of carbon and nitrogen sources

Table 2 shows that the degree of flocculation in yeast cells increased when they were grown in minimal defined medium (yeast nitrogen base without amino acids or ammonium sulphate) with glucose. Other carbon sources were also assayed for their stimulation of flocculation, but only fructose was able to stimulate flocculation of the yeast. These results are consistent with the mechanism proposed to explain glucose-induced flocculation of *K. apiculata* mc1 (*20*). The higher percentage of flocculation observed was a consequence of an increase in intracellular cAMP produced by intracellular acidification due to glucose metabolism. This phenomenon modifies the protein kinase A complex (PKA), which possibly activates the expression of the genes involved in the flocculent phenotype.

Table 2. Effect of carbon and nitrogen sources on the flocculation of *K. apiculata* mc1

Culture media	Flocculation/%			
	Time/h			
	8	12	24	48
YNB w/o AA	19±0.1	27±1.1	30±0.7	25±0.7
YNB w/o AA+glu	54±2.8	70±3.4	67±3.1	59±1.1
YNB w/o AA+fru	45±3.1	51±2.1	45±2.5	49±2.1
YNB w/o AA+eth	39±2.1	41±1.5	56±2.1	44±3.1
YNB	14±0.9	12±0.6	13±1.0	17±0.9
YCB_m	45±2.3	59±2.1	69±3.1	38±1.4
YCB_m+$(NH_4)_2SO_4$	67±3.4	66±4.1	69±2.8	69±2.7
YCB_m+peptone	98±5.1	90±6.0	99±3.9	90±4.1

Data are mean values of three independent experiments±standard deviation
YNB w/o AA: yeast nitrogen base without amino acids and ammonium sulphate medium
YNB: yeast nitrogen base with amino acids and ammonium sulphate medium
YCB_m: modified yeast carbon base medium (final glucose concentration 20 g/L)

Growth in 5 % ethanol, a nonfermentable carbon source, induced flocculation; maximum values were obtained during the stationary growth phase (24 h of incubation). Induction of flocculation by 5 % ethanol was similar to the results obtained by Dengis *et al.* (*40*), who indicated that the addition of ethanol induced flocculation of a stationary ale yeast. The stimulatory effect of ethanol and other alcohols on flocculation has also been reported by other authors (*41–45*). Jin and Speers (*46*) found that cells of *S. cerevisiae* expressing the NewFlo phenotype significantly increased flocculation with increasing ethanol concentrations while Flo1 cells were ethanol-insensitive. Smukalla *et al.* (*47*) stated that floc-

culation is a cooperative protection mechanism that shields cells from stressful environments, including antimicrobials and ethanol. Another explanation could be a relationship between flocculation behaviour and the dielectric constant of suspensions containing organic solvents at high concentrations (*44*).

Growth of *K. apiculata* in YNB w/o AA medium showed a higher flocculent phenotype related to the same medium supplemented with amino acids and ammonium sulphate (YNB). These results are in agreement with the observations of Sampermans *et al.* (*48*). These authors found that the presence of an external nitrogen source was not necessary to develop a flocculent phenotype in a NewFlo strain of *S. cerevisae.* Chen and Fink (*49*) demonstrated that nitrogen starvation induced the production of aromatic alcohols, which could act as quorum-sensing molecules to trigger the expression of *FLO11*. However, in both YNB media maximal flocculation was lower than that observed in YCB_m medium. Thus, it can be inferred that the lack of glucose, an inducer of *K. apiculata* flocculation (*20*) in YNB medium with and without amino acids and ammonium sulphate, is responsible for the lower flocculation related to YCB_m. However, cells grown in YCB_m supplemented with 5 g/L of ammonium exhibit a flocculation phenotype from the early stage which continued throughout the incubation time to reach a flocculation degree similar to that observed in YCB_m medium. The fact that flocculation was not inhibited after the addition of ammonium to this medium could indicate that the negative impact of the nitrogen source on flocculation occurs in the absence of an energy source.

The addition of 20 g/L of peptone to YCB_m greatly increased the degree of flocculation. The induction of flocculation by peptone was similar to the findings reported by Soares *et al.* (*33*). These authors indicated that *S. cerevisiae* showed strong flocculation in the presence of peptone, probably due to an increase in buffering capacity of the culture medium caused by this compound.

Effect of agitation

Flocculation is not only a biochemical process, but it also implies physical interaction. Table 3 reveals that agitation on a rotary shaker (200 rpm) was advantageous for the biomass production cycle of *K. apiculata*. Also, when *K. apiculata* was grown in the agitated culture medium, maximal flocculation was higher than that observed in the same medium incubated statically, while the hydrophobicity index of the yeast shows an opposite behaviour. Therefore, the current study highlights that the improvement of the adhesion properties by stirring does not seem to be defined by general cell wall properties, such as hydrophobicity. Agitation may be necessary for cells to collide in order to bind to each other, so factors that influence these cell-cell interactions can play an important role. Similar results have been described by Robinson and Harrison (*50*), who worked with brewer's yeasts and observed that agitation caused a decrease in hydrophobicity and an increase in the flocculation capacity of the cells. Also, agitation results in a higher dissolved oxygen, which could be a determining factor for flocculation. Most cells in static culture grow under hypoxic conditions and thus the production of unsaturated fatty acids and sterols for membrane synthesis is insufficient, and yeast cells would fail to grow. So, the oxygen probably does not act directly on flocculation, but rather indirectly through its importance for the synthesis of unsaturated acids and sterols. Similar results are reported by Straver *et al*. (*51*), who found that poor wort aeration resulted in a poor growth and flocculation characteristics of yeast, which could be restored by the addition of ergosterol and oleic acid to the medium.

Conclusions

The impact of temperature, agitation, sugars, nitrogen sources and ethanol on the flocculation of *K. apiculata* mc1 was quantitatively assayed by determining flocculation, hydrophobicity index and density of zymolectin sites. It was found that the flocculation capacity of the wine yeast increased with the increase of temperature and this resulted in an increase in lectin sites. Sugars differently affected flocculation of *K. apiculata*. Glucose and fructose significantly stimulated flocculation of *K. apiculata*, which indicates that industrial culture media containing glucose can be suitable for this self-flocculating strain. Normal fermentation concentrations of ethanol facilitated flocculation of *K. apiculata*. Our results are expected to help industry to develop strategies to optimize the control of flocculation of the wine yeast strain and guarantee a more reliable winemaking process. During winemaking, control of flocculation by environmental factors such as temperature and presence of nutrients could be applied in order to optimize the fermentation process and hence the quality of the final product.

Table 3. Growth, flocculation percentage and hydrophobic index of *K. apiculata* in static or agitated YMPG medium

Time/h	Flocculation/%		Growth/(log CFU/mL)		Hydrophobicity/%	
	Static	Agitation	Static	Agitation	Static	Agitation
0	0	0	6.40±0.02	6.40±0.08	2.80±0.20	2.50±0.15
8	27.00±0.80	29.00±2.10	6.60±0.10	7.08±0.05	5.20±0.21	2.50±0.10
12	64.00±2.10	87.00±5.20	7.40±0.09	8.15±0.10	10.00±2.10	3.20±0.25
24	59.00±3.50	91.00±4.10	7.40±0.15	8.49±0.05	13.50±0.90	2.70±0.14
48	41.00±2.90	75.00±2.80	7.80±0.05	8.89±0.12	18.00±1.10	5.00±0.34
72	38.00±2.40	68.00±3.70	7.80±0.20	8.84±0.07	23.00±2.10	5.00±0.21

Propagation was realized by stirring at 200 rpm
Data are means of three independent experiments±standard deviation

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This article is dedicated to the memory of Dr Marta Elena Farías

On February 3, 2012, the National University of Tucumán (UNT) and CERELA-CONICET lost a remarkable teacher and researcher, Dr Marta Elena Farías, who died after a painful illness. She was an outstanding and skillful biochemist, an elegant and rigorous researcher, respected by colleagues, subordinates and superiors, but above all was a woman of extraordinary dignity. Her scientific and teaching career began 28 years ago, so to summarize her vast experience is not easy, neither is to put into words the feelings that her demise caused. Her work focused on the interaction of lactic acid bacteria and yeasts during vinification, on the characteristics and properties of fermented beverages and on the study of non-*Saccharomyces* yeasts. Since 1999 she guided me through my last years as a student and later became my thesis supervisor, and thus had powerful influence on my personal and professional life, so I feel the need to express my gratitude for all that she taught me. Those of us who were fortunate to have worked with her, we know the impact of her limitless energy and constant tutelage. She expected the best from herself and everyone who worked with her. And, without a doubt, her legacy will live on in the lives of the people she touched.

Dr Oscar Sosa