INFLUENCE OF PHYSIOLOGICAL STATE OF INOCULUM ON VOLATILE ACIDITY PRODUCTION BY *SACCHAROMYCES CEREVISIAE* DURING HIGH SUGAR FERMENTATION

INFLUENCE DE L'ÉTAT PHYSIOLOGIQUE DE L'INOCULUM SUR LA PRODUCTION D'ACIDITÉ VOLATILE PAR *SACCHAROMYCES CEREVISIAE* AU COURS DE LA FERMENTATION DE MOÛTS RICHES EN SUCRE

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Abstract: An approach consisting of controlling yeast inoculum to minimize volatile acidity production by *Saccharomyces cerevisiae* during the alcoholic fermentation of botrytized must was investigated. Direct inoculation of rehydrated active dry yeasts produced the most volatile acidity, while a yeast preparation pre-cultured for 24 hours reduced the final production by up to 23 %. Using yeasts collected from a fermenting wine as a starter must also reduced volatile acidity production. The conditions for preparing the inoculum affected the fermentation capacity of the first generation yeasts: fermentation duration, sugar to ethanol ratio, and wine composition. A pre-culture medium with a low sugar concentration (< 220 g/L) is essential to limit volatile acidity production in high sugar fermentations.

Résumé : L'influence de la préparation de l'inoculum de levure sur la production d'acidité volatile au cours de la fermentation alcoolique de moûts riches en sucre, tels que les moûts de raisins atteints par la pourriture noble, a été étudiée. Trois méthodes de préparation du levain ont été comparées : une inoculation directe avec 20 g/hL de levures sèches actives (LSA) réhydratées, une inoculation avec des levains obtenus par préculture sur moût de raisins sains ou botrytisés, dilués ou non, et une inoculation à l'aide de moût en pleine fermentation (4 jours) comme pied de cuve. La souche *Saccharomyces cerevisiae* Zymaflore ST est utilisée dans ce travail. Cette étude met en évidence que l'inoculation directe avec des LSA est la modalité qui engendre la plus forte production d'acidité volatile, par contre un levain issu d'une pré-culture permet de réduire cette production jusqu'à 23 %. Le pied de cuve permet la même réduction après deux repiquages successifs. La composition du milieu de la préculture affecte le déroulement de la fermentation. Une pré-culture de 24 h dans un milieu peu riche en sucre (<220 g/L) tel un moût sain ou un moût issu d'une vendange botrytisée dilué de moitié est préconisée pour la meilleure maîtrise de la production d'acidité volatile mais également pour réduire la durée de fermentation, le rendement sucre/alcool. Ce travail montre que l'état physiologique des populations levuriennes au moment de l'inoculation joue un rôle essentiel sur le déroulement de la fermentation des moûts de raisins botrytisés. Les préparations industrielles sont beaucoup plus sensibles aux inhibiteurs de la fermentation alcoolique que des levains obtenus par préculture sur un moût dont la concentration en sucre n'excède pas 220 g/L. Dans nos conditions expérimentales, la concentration en levure en début de fermentation n'affecte pas la production finale en acidité volatile.

Key words: *Saccharomyces cerevisiae*, inoculum, botrytized must, volatile acidity, enology

Mots clés : *Saccharomyces cerevisiae*, inoculum, moût botrytisé, acidité volatile, oenologie

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INTRODUCTION

Volatile acidity, mainly acetate, can play a significant role in wine aroma and an excessive concentration of this alcoholic fermentation by-product is highly detrimental to wine quality. The amount of volatile acidity produced is usually low (0.25 to 0.50 g/L expressed in acetic acid) but may be higher under certain fermentation conditions. In particular, during fermentation of high-sugar media, such as botrytized musts, the volatile acidity content may be 1.8 g/L or even higher, i.e. over the EEC legal limit of 1.5 g/L.

Several authors have studied the origins of volatile acidity production by *Saccharomyces cerevisiae* under usual winemaking conditions. This production is affected by the yeast strain (MILLAN and ORTEGA,1988 ; SHIMAZU and WATANABE, 1981), the composition of the medium, and fermentation conditions, such as temperature variations (MONK and COWLEY, 1984). Other studies have demonstrated the stimulating effect of insoluble materials on fermentation, reducing the production of volatile acidity by providing the yeasts with saturated and unsaturated fatty acids (ALEXANDRE *et al.*,1994 ; DELFINI and COSTA,1993 ; LAVIGNE, 1996).

LAFON-LAFOURCADE and RIBEREAU-GAYON (1977) showed that raising the initial sugar content from 189 to 391 g/L increased the volatile acidity concentration from 0.56 to 1.46 g/L in wines made from botrytized grapes. *S. cerevisiae* responds to increased external osmolarity by enhanced production and intercellular accumulation of glycerol to counterbalance the osmotic pressure (BLOMBERG and ALDER, 1989-1992 ; MAGER and VARELA, 1993). This regulation mechanism is due to the expression of certain osmoresponsive genes include glycerol 3-phosphate dehydrogenase which is encoded by GPD1 (ALBERTYN *et al.*, 1994 ; ATTFIELD and KLETSAS, 2000), and also two types of aldehyde dehydrogenase encoded by ALD2 and ADL3 (NAVARRO-AVINO *et al.*,1999 ; ERASMUS *et al.*, 2003 ; PIGEAU and INGLIS, 2005). In order to maintain the intracellular redox balance, yeast cells regenerate an equimolar amount of cytoplasmic NADH. This requirement seems to be partially met by a decreased reduction of acetaldehyde to ethanol, on the one hand, and an increased oxidation to acetate, on the other (BLOMBERG and ALDER, 1989). Thus, it is clear that an overproduction of acetate cannot be avoided in high sugar fermentation (CARIDI *et al.*, 1999). Recently, BELY *et al.* (2003) reported that production of volatile acidity was inversely correlated to the assimilable nitrogen concentration of the must during high sugar fermentation. They suggested that, by stimulating cell growth, nitrogen addition provides NADH in the redox-equilibrating process, which in turn reduces volatile acidity formation.

Substances secreted by *Botrytis cinerea* inhibit yeast growth (RIBÉREAU-GAYON *et al.*, 1979), thus contributing to the formation of large quantities of volatile acidity in botrytized musts.

MILLAN *et al.* (1991) described the influence of the physiological state of the inoculum on acetic acid production under standard conditions. Thus, yeast showed a higher specific rate of acetic acid production during the exponential growth than in the declining phase. On the other hand, little data are available on high sugar fermentation conditions.

The aim of this work is to assess the impact of yeast inoculum preparation on fermentation behavior and volatile acidity production in sweet botrytized wine.

MATERIALS AND METHODS

I - YEAST INOCULATION

Figure 1 illustrates the various techniques used to prepare yeast inoculum: i) musts were inoculated with 200 mg/L industrial active dry yeast (ADY) immediately after rehydration in diluted must, as recommended on the packaging (A); ii) musts were inoculated with a wine inoculated using procedure A (B1) or B1 (B2) that had been fermenting for four days (pied de cuve), and, finally, iii) musts were inoculated with yeast cells previously grown in botrytized musts (diluted with water 1:1 vol. or not) or in non-botrytized musts at 23 °C in flasks without agitation for 24 hours (C, D, and E). These pre-cultures were inoculated with 200 mg/L ADY.

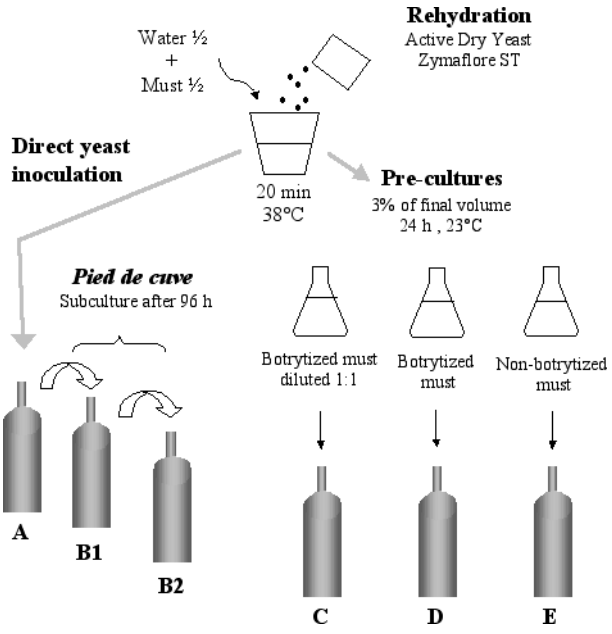

Figure 1 - Inoculum preparation protocols

Protocoles de préparation des inoculums

II - CELL POPULATION DETERMINATION

Cell population was determined by measuring absorbance at 610 nm, one absorbance unit corresponding to 10^7 cells/mL.

III - MUST

The musts were obtained from botrytized Sauvignon and Semillon grapes harvested in vineyards in the Sauternes and Barsac appellations (2000 to 2003 vintages). They were collected in the cellar after settling. The initial sugar concentration varied from 345 to 365 g/L.

IV - FERMENTATION CONDITIONS

Active dry *Saccharomyces cerevisiae* Zymaflore S.T (Laffort Œnologie, Floirac, France) was used. This strain was selected by the laboratory from native microflora during spontaneous fermentation of a botrytized must for its low production of SO_2 binding compounds (BARBE *et al.*, 2000; MASNEUF and DUBOURDIEU, 2000). Yeast strain implantation was verified by comparing the initial industrial yeast karyotype with the biomass using pulsed field electrophoresis (FREZIER and DUBOURDIEU, 1992). All the inoculated yeasts were well implanted.

1) In the laboratory

Fermentations were carried out in 375 mL sterile bottles (330 mL must per bottle) at 23 °C. Fermentations were monitored by CO_2 release: the amount of CO_2 released was determined by measuring weight loss every 24 hours.

2) In the winery

Musts were placed in identical 225 L barrels filled to 90 % vol. Alcoholic fermentations were monitored daily by measuring the density. The fermentation temperature was not controlled and ranged from 17 to 25 °C. Every experiment was duplicated.

Under both laboratory and winery conditions, fermentation was stopped by adding sulfur dioxide (300 mg/L) when the required alcohol concentration of 14 % vol. was reached. Residual sugars ranged from 100 to 120 g/L, depending on the initial concentration.

V - ANALYTICAL METHODS

1) Assimilable nitrogen

The assimilable nitrogen concentration in musts, including ammonium salts and α-amino acids, was estimated by the Sörensen method (AERNY, 1996 ; MASNEUF and DUBOURDIEU, 1999). This method is based on the reaction of formaldehyde with amino functions. The variation coefficient was < 5 %.

2) Wine analysis

Ethanol concentration (% vol.) was measured by infrared reflectance (Infra Analyser 450; Technicon, Plaisir, France). Sugar (g/L) and volatile acidity (expressed in g/L acetic acid) were determined chemically by colorimetry (A460 nm) in continuous flux (Sanimat, Montauban, France). These analyses were carried out in the SARCO laboratory (Floirac, France) and followed a quality assurance protocol approved by the French Accreditation Committee « Réseau Cofrac ».

The quantity of residual sugars (g/L) was used to calculate the sugar to ethanol ratio S/E (g/L.%vol), widely used in enology.

3) Statistical analysis

Volatile acidity concentrations were subjected to single factor variance analysis with three repetitions ($\alpha = 0,01$) followed by a Newman-Keuls average comparison test ($\pm = 0,05$) (ANOVA, Statbox Software©, Grimmer Logiciels).

RESULTS AND DISCUSSION

I - IMPACT OF INOCULUM PREPARATION ON VOLATILE ACIDITY AND FERMENTATION BEHAVIOR

Several fermentations were carried out under laboratory conditions at 23 °C (table I) using the various techniques for preparing yeast inoculum (figure 1). Initial sugar concentrations varied from 345 to 365 g/L for botrytized musts and from 210 to 220 g/L for non-botrytized musts. The volatile acidity concentration in the wine and the sugar to ethanol ratio, calculated from residual sugar and alcohol values (not communicated), varied according to the yeast inoculum preparation. Direct inoculation without pre-culture (A) or with 24 h pre-culture in botrytized must (D) always produced the least favorable results in both series of experiments. On the contrary, yeast cells previously grown in diluted half-botrytized musts (C) produced 12 % (must 1), 9.4 % (must 2), 19.8 % (must 3), and 17.3 % (must 4) lesss volatile acidity, compared to direct inoculation. Furthermore, the sugar/ethanol ratios were always lower. In this experiment, the difference between direct inoculation (A) and pre-cultured preparation C corresponded, in extreme cases (must 1), to a remarkable difference in alcohol production for the same amount of residual sugars, i.e. as much as 0.9 percent less alcohol in wine produced by direct inoculation (results not communicated).

Table I - Impact of yeast inoculum preparation on volatile acidity (g/L acetic acid), fermentation duration (h), and sugar/alcohol ratio (g/L.%vol.) in laboratory conditions at 23 °C.

Inoculum preparations: A, B1, B2, C, D and E (figure 1). ND: non-determined

Influence de la préparation de l'inoculum sur la production d'acidité volatile et la durée de fermentation en conditions de laboratoire.

Inoculum A, B1,B2,C,D et E (figure 1). ND : non-déterminé

		Direct inoculation	Pied de cuve : repeated subcultures		Pre-cultured yeast		
		A	B1	B2	C	D	E
Must 1	Volatile acidity	0.67 **b***	0.62 **b**	0.60 **a**	0.59 **a**	0.65 **b**	ND
	Fermentation duration	190	205	205	190	190	ND
	Sugar/alcohol	18.8	17.6	18.2	17.4	17.5	ND
Must 2	Volatile acidity	1.06 **b**	ND	ND	0.96 **a**	1.09 **b**	0.96 **a**
	Fermentation duration	240	ND	ND	240	250	190
	Sugar/alcohol	19.1	ND	ND	18.3	18.5	18.3
Must 3	Volatile acidity	0.86 **c**	0.78 **b**	0.72 **ab**	0.69 **a**	0.84 **c**	0.70 a
	Fermentation duration	170	180	180	170	170	170
	Sugar/alcohol	19.2	18.2	18.3	18.1	18.2	17.7
Must 4	Volatile acidity	1.04 **b**	ND	ND	0.86 **a**	1.02 **b**	ND
	Fermentation duration	385	ND	ND	290	385	ND
	Sugar/alcohol	17.9	ND	ND	17.2	17.6	ND

* Means of duplicate or triplicate fermentations.
Values followed by different letters are statistically different (Newman-Keuls test, $\alpha = 0.05$).

The subcultures produced from fermenting must (B1 and B2) and pre-culture E (using non-botrytized must) also reduced volatile acidity production in the wine. After two successive subcultures (B2), volatile acidity concentrations were the same as the lowest level obtained using pre-culture C (diluted botrytized must). The sugar/alcohol ratios for both subcultures were better than those obtained by direct inoculation.

Compared to direct inoculation, musts inoculated with yeast cultured in diluted must also decreased fermentation duration in case of sluggish fermentation (must 4).

To confirm all these results, other experiments were carried out in a winery using two techniques: direct inoculation (A) and yeast pre-cultured in botrytized must diluted by half (C). It was not possible to calculate the sugar/alcohol conversion ratios due to lack of accurate data. The results (tableau II) confirmed that direct inoculation was less favorable in terms of volatile acidity production. Wines made with pre-cultured yeast in diluted must contained 16.5 % (must 5), 16.1 % (must 6), and 9.4 % (must 7) less volatile acidity. Moreover, fermentation times were systematically shorter.

II - IMPACT OF PHYSIOLOGY STATE OF INOCULUM

As previously stated, yeasts pre-cultured in diluted must gave positive results, in terms of both volatile acidity production and sugar/alcohol conversion ratio. One advantage of pre-culture is to increase the yeast concentration compared to direct inoculation. Under our laboratory conditions, the cell population in pre-cultures increased from 2.10^6 cells/mL (corresponding to 200 mg/L ADY) to 8.10^6 cells/mL after 24 h. Additional experiments were carried out to determine whether this increase in yeast concentration in the inoculum was responsible for the reduction in volatile acidity production. Table III presents volatile acidity levels in wines inoculated with different yeast doses. The maximum cell populations obtained after 3 days' fermentation were not significantly different, i.e. $2.7x10^7$ cells/mL in all samples. The yeast concentration in the inoculum did not affect volatile acidity production by *S. cerevisiae*. The pre-cultured yeast preparation produced 23.6 % less volatile acidity in wine and fermentation times were shorter, thereby confirming the previous results. These findings suggest that the pre-cultured yeast preparation not only increased the yeast concentration in the inoculum, but also modified its physiological state, compared to direct inoculation with ADY (A). Figure 2 presents the kinetics of volatile acidity production and cell growth in experiments 2 and 4. The inoculum preparation did not affect the cell growth rate. Volatile acidity was produced essentially during cell growth. When the final population was reached, at least 70 % of the volatile acidity had already been released. This is consistent with previous results (BELY *et al.*, 2003). It is worth pointing out that, in the case of direct inoculation, volatile acidity production was higher than in pre-cultured samples from the onset of fermen-

tation. As the growth curves were similar, the specific production of volatile acidity, i.e. per unit cell, was higher following direct inoculation. This is due to the fact that the sugar concentration of the medium in which the first generations grow after rehydration affects their metabolism. Active dry yeasts are produced by aerobic metabolism, in the presence of very low sugar concentrations. At the end of the production process, yeasts are usually maintained in the desired dormant state to minimize metabolic activity during storage by lyophilization, which causes major physical and physiological changes. BEKER and RAPOPORT (1987) reported that ADY had damaged cytoplasm and plasma membranes. A rehydration period of 20 min is sufficient for the cellular membranes to repair themselves, as described by SAULITE *et al.*, (1986) but not sufficient to acclimatize yeasts to the high sugar concentration of a botrytized must (> 350 g/L). The higher the sugar concentration in the must to be inoculated, the more the yeasts have to respond to increased external osmolarity. Alcoholic fermentation is partially diverted towards the production of by-products, resulting in a lower sugar/alcohol conversion ratio and higher volatile acidity levels. Pre-culture for 24 h in low sugar concentrations (<220 g/L), e.g. musts diluted by half (C) or non-botrytized musts (E) is apparently crucial for the yeast to adapt to high sugar fermentation. Excessive sugar levels in pre-culture (D, table I) prevent this adaptation.

It was recently established that the initial assimilable nitrogen content affected volatile acidity production in sweet wines inoculated with pre-cultured yeast preparation (C) (BELY *et al.*, 2003). It seemed important to study the impact of yeast preparation methods combined with assimilable nitrogen adjustment. As shown in figure 3, irrespective of the method used to prepare the inoculum, there was a correlation between volatile acidity production and the assimilable nitrogen content of the must. The higher the nitrogen concentration, the less volatile acidity was released. As mentioned above, direct yeast inoculation (A) gave the least positive results, while pre-culture in diluted must (C) was the most advantageous, irrespective of the nitrogen concentration. These results show the advantage of using the right yeast preparation and adjusting assimilable nitrogen to minimize volatile acidity production during the fermentation of botrytized must. A 68 % reduction was obtained by adding nitrogen to obtain 200 mgN/L in a must with a low initial assimilable nitrogen concentration (25 mgN/L) and inoculating with pre-cultured yeasts rather than ADY. Other yeast strains were also tested to verify the impact of preculture on reducing volatile acidity (unpublished data).

CONCLUSION

Fermenting grape musts with pure selected yeast cultures offers several advantages. Fermentation is better

Table II - Impact of yeast inoculum preparation on volatile acidity and fermentation duration in winery conditions. Inoculum preparations : A and C (figure 1)

Influence de la préparation de l'inoculum sur la production d'acidité volatile et la durée de fermentation en conditions de chai. Inoculum A et C (figure 1)

	Direct inoculation	Pre-cultured yeast
	A	C
Must 5		
Volatile acidity (g/L acetic acid)	1.82 b*	1.52 a
Fermentation duration (h)	800	600
Must 6		
Volatile acidity (g/L acetic acid)	1.24 b	1.04 a
Fermentation duration (h)	430	385
Must 7		
Volatile acidity (g/L acetic acid)	1.06 b	0.96 a
Fermentation duration (h)	410	360

* Means of triplicate fermentations.
Values followed by different letters are statistically different (Newman-Keuls test, $\alpha = 0.05$).

TABLE III - Volatile acidity production and fermentation duration in wines inoculated with different cell concentrations in laboratory conditions at 23°C. Inoculum preparations: A and C (figure 1)

Production d'acidité volatile et durée de fermentation en fonction du taux d'inoculation. Inoculum A et C (figure 1)

	Direct inoculation		Pre-cultured yeast		
	A		C		
Experiment number	1	2	3	4	5
Initial cell concentration (cells/mL)	1.10^6 (10 g/hl)	2.10^6 (20 g/hl)	1.10^6	2.10^6	8.10^6
Volatile acidity (g/L acetic acid)	1.05 **b***	1.10 **b**	0.85 **a**	0.84 **a**	0.84 a
Fermentation duration (h)	360	340	265	265	240

(*) Means of triplicate fermentations.
Values followed by different letters are statistically different (Newman-Keuls test, $\alpha = 0.05$).

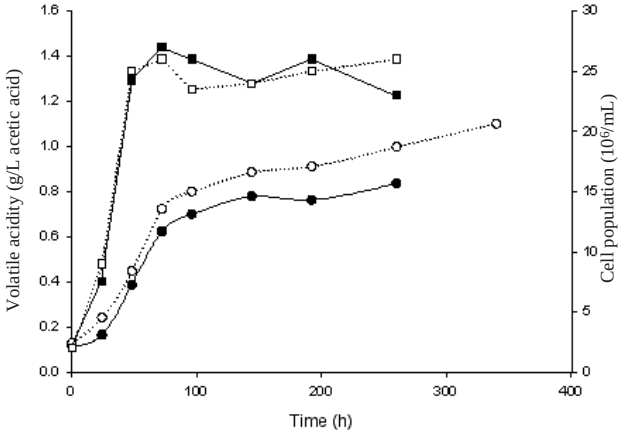

Figure 2 - Kinetics of volatile acidity production (circle) and cell growth (square) at 23 °C.
Inoculum preparations: A(open symbol) and C (filled symbol) (figure 1)

Évolution de l'acidité volatile (cercle) et de la croissance cellulaire (carré) au cours de la fermentation à 23 °C.
Inoculum A (symbole ouvert) et C (symbole plein) (figure 1)

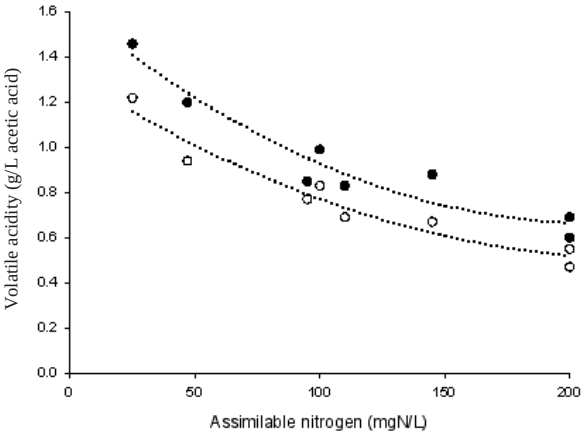

Figure 3 - Effect of assimilable nitrogen content in must on volatile acidity production at 23 °C.
Inoculum preparations A (filled symbol) and C (open symbol) (figure 1)

Incidence de la concentration en azote assimilable du moût sur la production d'acidité volatile à 23 °C.
Inoculum A (symbole plein) et C (symbole ouvert) (figure 1)

controlled and the risk of negative organoleptic effects resulting from the metabolism of indigenous yeasts is reduced. The yeast strain used for these experiments, *S. cerevisiae* Zymaflore ST, was selected for its low production of SO_2 binding compounds during high sugar fermentation. However, the outcome of fermentation may also be affected by the physiological state of the yeast used. Thus, yeasts from a 24 h pre-culture in a reduced-sugar medium (<220 g/L) were better adapted to fermenting a high-sugar must (> 350g/L) than active dry yeast inoculated immediately after rehydration. The composition of the pre-culture medium affected the synthesis of the products excreted into the wine, particularly those synthesized in the first few days, such as volatile acidity. The direct consequence was a decrease in fermentation time and the sugar to ethanol ratio.

The advantage of inoculation with yeast collected from fermenting wine (pied de cuve) was also shown up in this study. These yeast cells have also an opportunity to acclimatize to the medium. This preparation is easy to envisage as botrytized grapes are harvested over a fairly long period.

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