PHYSICAL DYNAMICS OF SLUDGES DURING WINE FERMENTATION IN LIQUID PHASE

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Abstract

Aims: The aim of this work was to study the dynamics of the sludge particles during wine fermentation in liquid phase.

Methods and results: Ninety L of Sauvignon blanc enriched with 1.8 % (v/v) of sludges, inoculated with a commercial strain of *S. cerevisiae*, were fermented in a transparent 100 L-fermentation tank. Results showed that the physical behavior of the sludge particles is dependent on the yeast CO_2 production activity. Sludge dynamics can be divided in three phases clearly linked to the yeast fermentative activity. Although a complete dispersion of the sludges occurred during the fermentation, it could be observed that either at the surface or at the bottom of the tank, the medium turbidity never ranges the initial turbidity level, due to the disruption of the medium/gross particles into fine particles during the growth phase. Fine particles rapidly re-aggregated into medium/gross particles during the stationary phase.

Conclusions: From these results, it appears that the evolution of must turbidity during fermentation is much more complex than previously expected. Sludges are only dispersed in the fermenting must during a very short phase after the initiation of cell growth.

Significance and impact of the study: This study raises the questioning of the relationship between initial must turbidity and nutrient availability in trouble musts, since sludges are completely deposited at the onset of both cell growth and fermentation. Further research works are therefore needed to study the factors which can impact sludge particle size modification and consequently sludge nutrient accessibility for yeast growth.

Key-words: sludges, particles, wine fermentation, liquid phase

Résumé

Objectif : L'objectif de cette étude consistait à étudier le comportement physique des particules composant les bourbes durant la fermentation en phase liquide.

Méthode et résultats : Une fermentation a été réalisée dans une cuve transparente de 100 L sur du moût de Sauvignon blanc enrichi avec 1.8 % (v/v) de ses bourbes et inoculé avec une souche commerciale de *S. cerevisiae*. Les résultats montrent que le comportement physique des particules de bourbes est dépendant de la production de CO_2 par les levures et se décompose en trois phases étroitement liées à l'activité fermentaire des levures. Bien que les bourbes soient complètement dispersées durant la fermentation, la turbidité du milieu mesurée à la surface et au fond de la cuve n'a jamais atteint la valeur initialement déterminée. Ceci s'explique par la désagrégation des particules moyennes et grosses en particules plus fines durant la phase de croissance. Ensuite, pendant la phase stationnaire, ces petites particules se ré-agrègent rapidement pour reformer des particules moyennes et grosses.

Conclusion : Ces résultats indiquent que l'évolution de la turbidité d'un moût pendant la fermentation est beaucoup plus complexe qu'initialement prévue. Les bourbes ne se remettent en suspension dans l'intégralité de la cuve qu'après initiation de la croissance cellulaire et pendant une durée limitée.

Signification et impact de l'étude : Cette étude remet en question la disponibilité des nutriments contenus dans les bourbes pour la croissance levurienne. Des travaux complémentaires doivent être menés pour étudier les facteurs pouvant influer sur la taille des particules des bourbes et, par conséquent, sur l'accessibilité des nutriments aux levures.

Mots-clés : bourbes, particules, fermentation du vin, phase liquide

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INTRODUCTION

During white wine making (liquid phase fermentation), sludges may play an important role in the fermentation process. These sludges, mainly composed of heterogeneous particles kept in the liquid phase after grape pressing, are composed in average of (w/w) 76 % polysaccharides, 8 % lipids and about 3 % nitrogen (Alexandre *et al.*, 1994), and then represent an important source of nutriments for fermenting yeasts (Ribéreau-Gayon *et al.*, 1975). They may also contain indigenous yeasts. According to the enologist's needs, sludges may be eliminated before or after the alcoholic fermentation, with huge variations in the extent of this clarification step (Feuillat, 1995). There are several reasons for the clarification of white juices prior to fermentation (Boulton *et al.*, 1996): i) a major proportion of oxidative enzyme activities (plant cell polyphenol oxidase or mold laccase, that could deplete oxygen in the juice) as well as elemental sulfur and other vineyard residues (source of formation of H_2S by yeasts during fermentation) are associated with the pulp and skin fragments, ii) there is evidence of an esterase activity within grape tissue which reduces the accumulation of esters produced by the yeast during fermentation.

The presence of sludges during the fermentation exerts an important effect on the lipid status of the yeasts (Valero *et al.*, 1998 ; Cabanis and Flanzy, 1998), by giving yeast the possibility to assimilate non soluble grape phytosterols adsorbed on solid particles (Luparia *et al.*, 2004) to compensate oxygen deficencies (Andreasen and Stier, 1953), and long chain fatty acids (Cabanis and Flanzy, 1998), leading to an higher cell viability at the end of fermentation. Several studies (Ribéreau-Gayon, 1985; Houtman and Duplessis, 1986; Feuillat *et al.*, 1989) evidenced the negative effect of an excessive must clarification. Beside this effect on yeast nutrition, sludges, as any substances of particulate nature, may exert a physical effect on fermentation by favoring CO_2 nucleation (Groat and Ough, 1978; Axcell *et al.*, 1988). Dissolved CO_2 is able to adsorb on fine particles on specific nucleation sites, where CO_2 bubbles grow before leaving the particles as free CO_2 bubbles (Kuhbeck *et al.*, 2007). By this mechanism, the level of dissolved CO_2 is lower in the liquid phase, and is then less toxic to yeasts (Jones and Greenfield, 1982).

The maintenance of sufficiently high levels of turbidity (50-150 NTU) is thus essential for white wine production, solid particles acting as a source of lipid compounds and providing nucleation sites for decreasing the concentration of dissolved CO_2. However, no bibliographic data are available on the dynamics of the particles constituting sludges in relation with the progress of fermentation during winemaking in liquid phase. With the aim to get new insights in the behavior of such particles, we studied in the present work the evolution of their physical properties as well as their main size at different levels of a 100 L-scale tank during fermentation of an actual must in liquid phase.

MATERIALS AND METHODS

1. Yeast strain

The commercial enological yeast strain *Saccharomyces cerevisiae* VIN13 (Anchor, Eppindust, South Africa) was used.

2. Experimental winemaking

A Sauvignon blanc must from Languedoc-Roussillon (France) was cold-settled overnight at 2 °C in the presence of Rapidase® X-Press (Gist-Brocades France, Seclin) pectolytic enzyme preparation (2 g hl^{-1}). The deposited sludges were then harvested by simple decantation and homogenized, before keeping them freezed at - 20 °C. Their density was 1.066 at 20 °C. The settled must was then flash-pasteurized (72 °C, 20 s). This must contains 200 g L^{-1} fermenting sugars and 188 mg L^{-1} free assimilable nitrogen (FAN) and exhibits a very low turbidity of 3 NTU. Ninety liters of this must were poured into a transparent acrylic 100 L^{-1} fermentation tank. This tank was wired to earth in order to impede any electrostatic interferences. The must was supplemented or not with its original sludges (1.8 %, v/v), in order to reach a potential must turbidity of 205 NTU, as measured at the laboratory scale by serial dilution with the same must. The tank was then inoculated with 10 g of rehydrated VIN13 *S. cerevisiae* strain. Fermentation was conducted at 18 °C, and this temperature was maintained until the end of fermentation. Fermentation kinetics were followed by automatic measurement of CO_2 release by the means of a Brooks 5810 TR series gas flowmeter (Brooks Instrument, PA, USA). Fermentation was completed (residual sugars < 2 g L^{-1}) in about 300 h.

3. Sampling

Sampling was carried out at 4 locations: surface (1 cm below surface level), top, middle and bottom (corresponding to 3/4, 1/2 and 1/4 of the filled tank height, respectively).

4. Analytical methods

a) Cell counting

Yeast culture samples were diluted (1,000 to 2,500 times) with Isoton II® (Beckman-Coulter, Margency, France) for cell enumeration. After sonication (35 s, 10 W), cells were counted with a Coulter Z2 electonic counter (Beckman-Coulter, Margency, France)

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fitted with a 100-µm-aperture probe. Threshold settings were set in order to count particles over an hydrodynamic diameter of 9.1 μm^3.

b) Dry matter

10 mL of must sample were filtered (0.45 µm filter porosity). Retentate was dried at 103 °C during 24 h and weighted after complete drying.

c) Centrifugations and turbidity

Must samples were first centrifuged at 1, 000 xg during 7 min at 4 °C to remove yeast cells using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was then centrifuged at 3,200 xg during 30 min at 4 °C. The turbidity of the corresponding supernatant (containing fine particles) was measured with a Hach Ratio 43900 turbidimeter (Hach Company, Colorado, USA). The corresponding pellet (containing medium/gross particles) was then reconstituted to its initial volume with pure water, and the corresponding obtained turbidity measured.

d) Deposit height measurement at the bottom of the tank

Deposit height at the bottom of the tank was measured and photographed during the fermentation through the transparent tank walls.

e) Characterization of particles

Particle size distribution was studied by Dynamic Light Scattering technique, using a Malvern Autosizer 4800 Spectrometer (Malvern Instruments Ltd, Malvern, UK) on pure samples without any dilution.

RESULTS

1. Discrepancies between yeast numeration and dry matters during liquid fermentation in the presence of sludges: evidence for a solid particles gradient formation

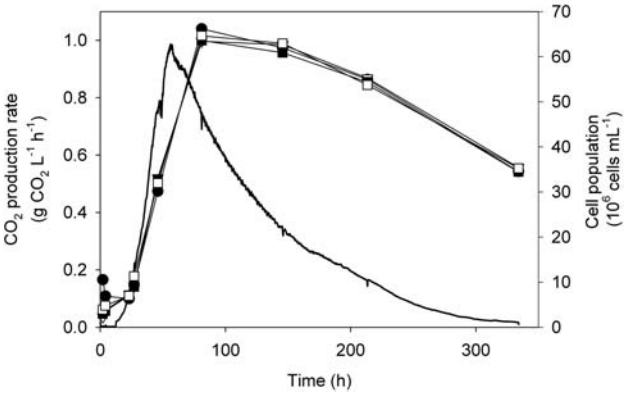


Figure 1 - CO_2 production rate (plain line, g CO_2 L^{-1} h^{-1}) and cell population (10^6 cells mL^{-1}) measured at four positions in the 100 L-tank (surface (●), top (▽), middle (■) and bottom (□)) during fermentation of a Sauvignon blanc must in the 100 L-tank at 18 °C.

Yeast numeration was achieved during fermentation by the means of an electronic particle counter, counting particles over 2.5 µm mean diameter. During almost all the fermentation, yeast cellular population was found identical in the samples harvested at the four different locations of the tank (figure 1), as previously described in the absence of added sludges (Aguera et Sablayrolles, 2005a, 2005b). The only exception concerns the first samples harvested at the surface of the tank, where cellular enumeration is overestimated due to the slow sedimentation of the added sludges in the tank which lasts about 4 hours (data not shown). On the contrary, dry matters of the same fermenting must samples clearly evidenced a gradient of solid particles in the tank that fluctuate along fermentation (table 1). Moreover, the initial deposit formed at the bottom of the tank completely disappeared when maximum fermentation rate is observed, likely due to rapid CO_2 bubbles exhaust from the fermenting must, and reappeared later during the stationary phase (table 1).

2. Differential behavior of particles in the evolution of must turbidity during fermentation

Table 1 - Dry matter (g (d.w.) L^{-1}) of the fermenting must at four positions (surface, top, middle and bottom), and deposit height (cm) at the bottom of the 100 L-tank during fermentation of a Sauvignon blanc must at 18 °C. n.d.: no data.

Fermentation progress		0.00	0.40	0.75
Fermentation time (h)		4	81	149
Dry matter (g (d.w.)L^{-1})	Surface	*n.d.*	3.30	3.31
	Top	*n.d.*	3.48	3.32
	Middle	*n.d.*	3.54	3.48
	Bottom	*n.d.*	3.53	3.46
Deposit height (cm)		1.80	0.00	3.00

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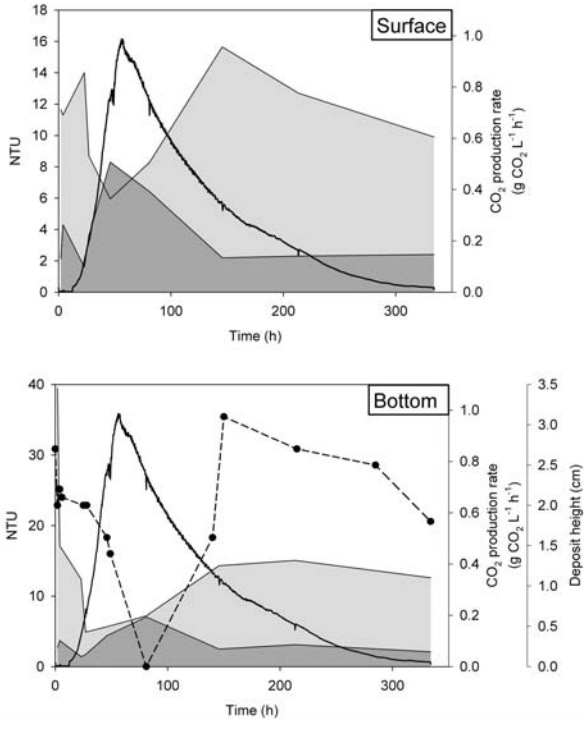


Figure 2 - CO_2 production rate (plain line, g CO_2 L^{-1} h^{-1}), respective proportion of fine particles (dark grey area) and middle and gross particles (dashed light grey area) in the total turbidity (NTU) of wine samples harvested at the surface and at the bottom of the 100 L-tank and height of the solid deposit ((●), cm) measured at the bottom of the tank during fermentation of a Sauvignon blanc must at 18 °C.

Successive differential centrifugation steps allowed us to separate yeast cells from fine and medium/big particles along fermentation. Reconstitution of the different centrifugation residues with water to the initial volume centrifuged allowed separate measurements of turbidity induced by fine or medium/gross particles along fermentation (figure 2). It is noteworthy that, either at the surface or at the bottom of the tank, the medium turbidity never ranges the initial level of 205 NTU even at the onset of fermentation. The maximum turbidity levels due to particles encountered during fermentation at the surface and the bottom of the tank were only 17.8 and 18.1 NTU after 145 h of fermentation, respectively. A more surprising finding is that even if the sludge deposit completely disappeared at the maximum fermentation rate time (at about 70-75 h), the turbidity of the medium due to particles did not increase in correlation (only a turbidity of 14 NTU at the bottom of the tank was observed after 81 h of fermentation) (figure 2). A close examination of the respective influence of fine and medium/gross particles in the turbidity clearly shows that the disappearance of the sludge deposit at the bottom of the tank paralleled a strong decrease of the relative abundance of middle/big particles and a strong raise in the predominance of fine particles in the liquid phase (figure 2). During the stationary phase, once the sludges deposit reappeared at the bottom of the tank, fine particles decreased in proportion, while fine and medium/gross particles reappeared in the liquid phase.

3. Changes in particle size during fermentation

The average particle size and scattering index of particles harvested at the surface and the bottom of the tank were analyzed by Dynamic Light Scattering (DLS) along fermentation (table 2). Before the maximum fermentation rate time, particles in the liquid phase were quite homogenous in size at both the surface and bottom of the tank with an average size of about 170 nm. At the maximum fermentation rate time (81 h), although the sludge deposit has completely disappeared, the mean particle size decreased to about 120 nm with a quite low scattering (range about 69 %). During stationary phase, the average particle size strongly drop up to about 1800-2800 nm in both surface and bottom liquid phases, indicating a strong aggregation of particles in the liquid phase. At the end of the stationary phase, this phenomenon of aggregation decreased in intensity leading to particles with a lower average size about 500-800 nm.

DISCUSSION

Supplementation with sludges of white musts exhibiting very low turbidity is often recommended in order to avoid sluggish and stuck fermentations (Feuillat, 1995). The role of sludges as a fermentation activator can be explained by the impact on two levels : i) nutritional level, as solid particles contain lipids (unsaturated fatty acids and sterols), ii) hydrodynamic level, providing nucleation sites for CO_2, and therefore allowing a better homogeneity throughout the tank. In the present study, blank experiments performed on the same Sauvignon must in the absence of added sludges led to sluggish fermentations (maximum fermentation rate : 0.47 g CO_2 L^{-1} h^{-1}, and total fermentation time : 600 h, data not shown). Supplementation of the must with sludges (1.8 %, v/v) led to a clear improvement of fermentation kinetics with a higher maximum fermentation rate (1.0 g CO_2 L^{-1} h^{-1}) and a shorter total fermentation time (300 h).

In this last condition, turbidity and deposit height measurements showed that the sludge physical behavior during wine fermentation in liquid phase, could be divided in three phases. The first phase occurred during the lag phase and at the early beginning of the growth phase. Sludges quickly deposited at the bottom of the fermentor, leading to a strong decrease of turbidity. The second phase took place during the main part of the growth phase.

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Table 2 - Average particle size (nm) and scattering index (%) of particles (determined by Dynamic Light Scattering) harvested at the surface and the bottom of the 100 L-tank as a function of fermentation progress and duration. Means +/- standard deviation (number of experiments).

Fermentation progress		0.01	0.11	0.40	0.75	0.90
Fermentation time (h)		23	46	81	146	213
Surface	Z average (nm)	137.2 +/- 68.5 (4)	170.2 +/- 22.5 (2)	*n.d.*	1820.8 +/- 956.6 (3)	*n.d.*
	scattering index (%)	83.9 +/- 3.1	85 +/- 1.7		87.2 +/- 5.1	
Bottom	Z average (nm)	173.8 +/- 56.5 (3)	*n.d.*	119.9 +/- 55.2 (3)	*n.d.*	807.1 +/- 124.6
	scattering index (%)	76.7 +/- 5.6		69.3 +/- 3.5		49.0 +/- 7.9

Medium and gross-sized sludge particles were broken into more little particles, as revealed by both differential particle centrifugations and Dynamic Light Scattering experiments, phenomenon likely related to the strong agitation provided by CO_2 sparkling. In fact, medium and gross sludge particles are composed of several aggregated little particles (data not shown). At this time of the fermentation progress, sludges were dispersed in the medium and the deposit quickly disappeared. It could be noticed that maximum turbidity of the fermenting must is always lower that the turbidity measured at the time of must supplementation with sludges (about 18 NTU instead of 205 NTU in our case). The third phase is achieved during the stationary phase. Part of the little particles re-aggregated, leading to the appearance of new medium and gross particles, which then deposited with the lees at the bottom of the tank. At the end of this last phase, the deposit height decreased a little, due to the packing down of the sludges at the bottom of the tank, and the size of the aggregates decrease a little.

These results evidenced dynamics in both sludges location (sedimentation and dispersion in the medium) and in sludge particle sizing (aggregation and disruption) during alcoholic fermentation. These changes clearly depended on the yeast CO_2 production rate and certainly impacted the yeast metabolism. For example, during the growth phase, sludges were totally dispersed in the medium and as their main particle size decreased, yeasts could more easily mobilized sludge nutriments, such as fatty acids or sterols. In the same time, their size decreased allowing a better CO_2 nucleation, likely decreasing the dissolved CO_2 contents in the liquid phase, which was then less toxic to yeasts (Jones and Greenfield, 1982). Although a complete dispersion of the sludges occurred during the fermentation, it could be observed that either at the surface or at the bottom of the tank, the medium turbidity never ranges the initial turbidity value (205 NTU) classically evaluated on serial dilutions with the same must at the laboratory level. This surprising result raises new questions about the adequate level of turbidity required for white wine production : 1) are the 50-150 NTU levels of turbidity recommended for the musts before fermentation really kept during most of the performed fermentations, with regards to the sedimentation of the sludges ?, 2) what is therefore the relationship between initial must turbidity and nutrient availability in trouble musts, since sludges are only dispersed in the fermenting must during a very short phase after the initiation of cell growth ? From the results of the present work, it appears that the evolution of must turbidity during fermentation is complex, and that it is therefore not easy to recommend an exact value of adequate turbidity during fermentation. Further research works are needed to study the factors which can impact sludge particle size modification and consequently sludges nutrient accessibility for yeast growth.

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