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Nitrogen Availability of Grape Juice Limits Killer Yeast Growth and Fermentation Activity during Mixed-Culture Fermentation with Sensitive Commercial Yeast Strains

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The competition between selected or commercial killer strains of type K2 and sensitive commercial strains of *Saccharomyces cerevisiae* was studied under various conditions in sterile grape juice fermentations. The focus of this study was the effect of yeast inoculation levels and the role of assimilable nitrogen nutrition on killer activity. A study of the consumption of free amino nitrogen (FAN) by pure and mixed cultures of killer and sensitive cells showed no differences between the profiles of nitrogen assimilation in all cases, and FAN was practically depleted in the first 2 days of fermentation. The effect of the addition of assimilable nitrogen and the size of inoculum was examined in mixed killer and sensitive strain competitions. Stuck and sluggish wine fermentations were observed to depend on nitrogen availability when the ratio of killer to sensitive cells was low (1:10 to 1:100). A relationship between the initial assimilable nitrogen content of must and the proportion of killer cells during fermentation was shown. An indirect relationship was found between inoculum size and the percentage of killer cells: a smaller inoculum resulted in a higher proportion of killer cells in grape juice fermentations. In all cases, wines obtained with pure-culture fermentations were preferred to mixed-culture fermentations by sensory analysis. The reasons why killer cells do not finish fermentation under competitive conditions with sensitive cells are discussed.

Killer yeast strains (phenotype K^+R^+) produce an extracellular toxin that kills other, sensitive yeast strains (phenotype K^-R^-). There also exist neutral yeasts (phenotype K^-R^+) that are resistant to killer toxin but do not produce it.

The killer phenomenon was discovered in yeast in 1963 by Bevan and Mackower (5). In the genus *Saccharomyces*, killer strains (types K1, K2, and K3) harbor virus-based killer systems. These yeast viruses contain double-stranded RNA genomes (M1, M2, and M3) which encode both immunity and toxin precursors (47). Usually these viruses are advantageous to the cell, although under some special conditions they can be pathogenic (13). In the last decade, genetic and molecular approaches have been applied to elucidate these systems in yeasts (35, 48).

The killer type K2 is common in winery ecosystems (31). The occurrence of this type of killer is now well confirmed in several regions (1, 4, 8, 9, 16, 22, 33, 40, 46). Killer yeast and its relationship to wine making are described in detail by Shimizu (44).

Several researchers have proved the dominance of killer strains in laboratory wine fermentations by mixed cultures at pH 3.0 to 3.5 by indirect methods (2, 15, 39, 43). Direct approaches using differences in colony morphology (16) and colors (9) and genetically marked strains (34) were developed to quantitatively measure the interactions between killer and sensitive yeasts.

Stuck wine fermentation is one of the most important problems in the wine industry (12, 19, 23, 24). Several causes of stuck and sluggish wine fermentation have been described (12, 15, 18, 25–27, 29, 38). As expected, killer toxins can inhibit wine fermentation by sensitive yeasts (9, 10, 36, 46). In our laboratory, we have observed that a certain killer/sensitive yeast (K/S) proportion at the start of fermentation may cause stuck wine fermentation. The effects of the addition of assimilable nitrogen, other nutrients, and adsorbant substances were studied (10), but a more detailed analysis of nitrogen balances during fermentation was necessary to better understand this phenomenon.

In most circumstances, grape juice contains all the nutrients necessary for yeast growth, aroma development, and complete fermentation, but some winemaking practices and events of microbial ecology can vary juice composition within a few hours and affect the nitrogen levels of juice. Consequently, addition of ammoniacal nitrogen to nitrogen-limited musts is widely used by enologists. Therefore, in practice, the cause-and-effect relationships resulting in sluggish fermentation are not usually verified (17). On the other hand, addition of assimilable nitrogen in excess may result in higher concentrations of ethyl carbamate (32), negatively affecting the quality and the aroma character of some varieties of wines (46a).

In this work we examined the competition between selected killer strains (type K2) and sensitive commercial strains under different conditions to study the effect of assimilable nitrogen in grape juice and its relationship to stuck and sluggish wine fermentation. The reasons why killer cells do not finish fermentation under these conditions are discussed.

MATERIALS AND METHODS

Yeast strains. *Saccharomyces cerevisiae* wine strains Montrachet 522 (M522), from the University of California, and OC2 and IAM 4274 were used as sensitive strains for mixed cultures. Wine yeasts *S. cerevisiae* KU1, 863 (our laboratory), and KI (ICV, Montpellier, France) were used as type K2 killer strains. These cultures were maintained on yeast extract-peptone-dextrose agar at 4°C, and inoculated strains had previously been grown at 25°C for 18 h in grape juice.

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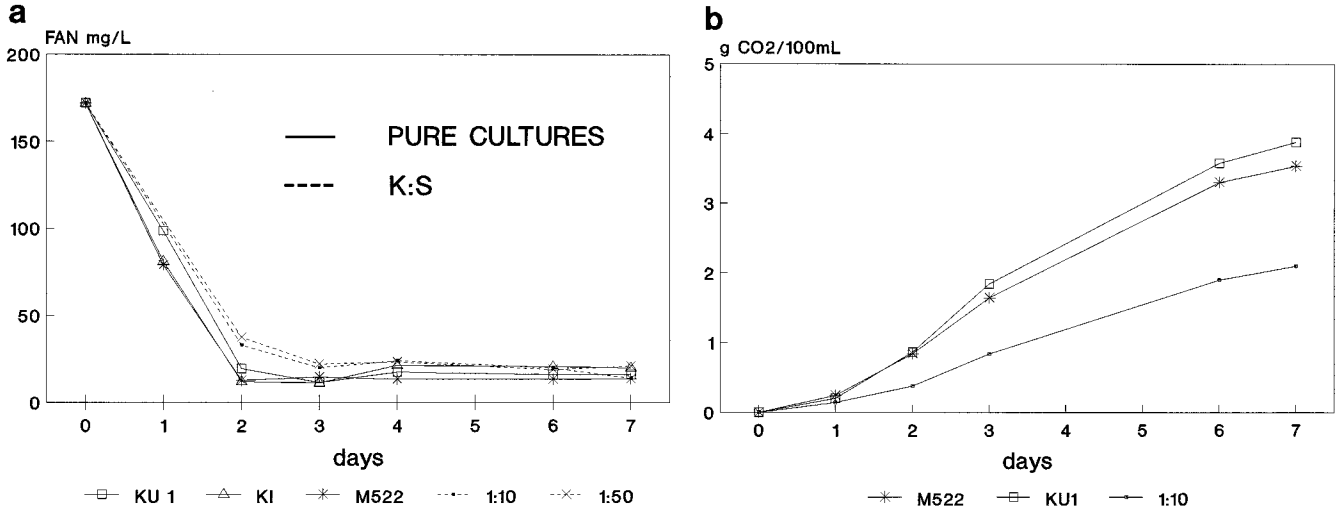

FIG. 1. (a) FAN assimilated during fermentation of grape juice by pure cultures of KU1, KI, and M522 and mixed cultures of strains KU1 and M522 in proportions of 1:10 and 1:50. (b) Fermentation rate measured as evolution of CO_2 in grape juice with pure and mixed cultures.

K/S proportions were confirmed in WL nutrient solid medium (Difco), on which OC2, 863, and KU1 formed white colonies while M522 and KI formed green colonies.

Fermentation conditions. Semillon grape juice (pH 3.3; total reducing sugars, 156 g/liter; free amino nitrogen [FAN], 172 mg/liter) sterilized by filtration through a membrane filter (pore size, 0.45 μm) was used as a fermentation medium.

Static batch fermentations were conducted at 20°C in 50 ml (stopped with Muller valves) of the fermentation medium inoculated with 10^5 cells of killer and/or sensitive strains per ml in exponential phase, except where indicated. The K/S proportions in the inoculated strains varied from 1:1 to 1:100. Single-strain fermentations were also carried out as control tests. Each fermentation was carried out in duplicate, and the results represent the average of the determinations.

Nitrogen addition. K/S proportions 1:10, 1:50, and 1:100 were selected in order to study the effect of addition of 100 mg of $(NH_4)_2HPO_4$ per liter at 0, 48, and 120 h during fermentation.

Analytical methods. Residual sugars were analyzed (42) throughout the fermentation periods, and the final values were determined 5 days after fermentation had stopped. Loss of carbon dioxide was measured by weight loss, and the numbers of dead cells were counted by the methylene blue-staining technique. The total cell population was counted in an improved Neubauer chamber. FAN was analyzed spectrophotometrically by a ninhydrin method (14).

RESULTS AND DISCUSSION

Consumption of FAN by pure and mixed K/S cultures with proportions of 1:10 and 1:50 is shown in Fig. 1a. Behaviors in all cases studied were similar: assimilable nitrogen was practically depleted in the first 2 days of static batch fermentations. This is commonly observed at the industrial level. At this time point, CO_2 started to evolve in an appreciable way (Fig. 1b). These results are in agreement with the results of studies of nitrogen assimilation of amino acids by pure cultures of yeasts measured by other techniques (6, 20, 45). No differences were found between the profiles of nitrogen assimilation in fermentations with pure strains and those with mixed K/S strains. Results obtained with strains 863 and OC2 in pure and mixed cultures were also similar (data not shown). The effects of a single strain of killer or sensitive yeast and a mixed-strain fermentation with an initial K/S proportion of 1:10 on the fermentation rate are shown in Fig. 1b. While fermentation with a killer or sensitive strain used as the sole inoculum progressed to completion (Table 1), the mixed-strain fermentation became stuck.

Rapid uptake and storage of all available nitrogen, for use when needed, in the cytoplasmic vacuoles is a typical strategy of *Saccharomyces* strains. This regulatory mechanism has probably evolved in response to its natural habitat, such as fermenting grape juice (6), because the ethanol which accumulates in the medium is a strong inhibitor of amino acid transport. Ethanol has been shown to induce an excessive proton influx for the cell (30).

The direct relationship between residual sugars and the percentage of dead cells at the end of fermentation is shown in Table 1. These results were obtained with K/S proportions of 1:1, 1:10, and 1:100, with three different mixed cultures giving qualitatively similar data, i.e., the most severe stuck fermentations occurred principally with a K/S proportion of 1:10. In all cases studied, sugar consumption in the pure-strain control fermentations was practically completed (Table 1). The aroma quality of the obtained white wines was analyzed by three tasters, and in all cases the wines resulting from fermentations

TABLE 1. Residual sugar concentration of produced wine and percentage of dead cells at the end of fermentation periods with selected K/S strains[a]

Strains and K/S proportion	Residual sugar concn (g/liter)	% Dead cells
KI and OC2		
1:0	1.3	3.0
1:1	4.1	4.1
1:10	11.2	17.0
1:100	2.5	7.1
0:1	1.9	2.0
863 and M522		
1:0	2.1	4.3
1:1	3.1	2.7
1:10	6.4	17.0
1:100	2.1	3.2
0:1	2.3	2.5
KU1 and M522		
1:0	1.1	2.0
1:1	3.5	2.2
1:10	8.8	8.1
1:100	6.6	8.5
0:1	2.3	2.5

[a] Fermentations were carried out at 20°C.

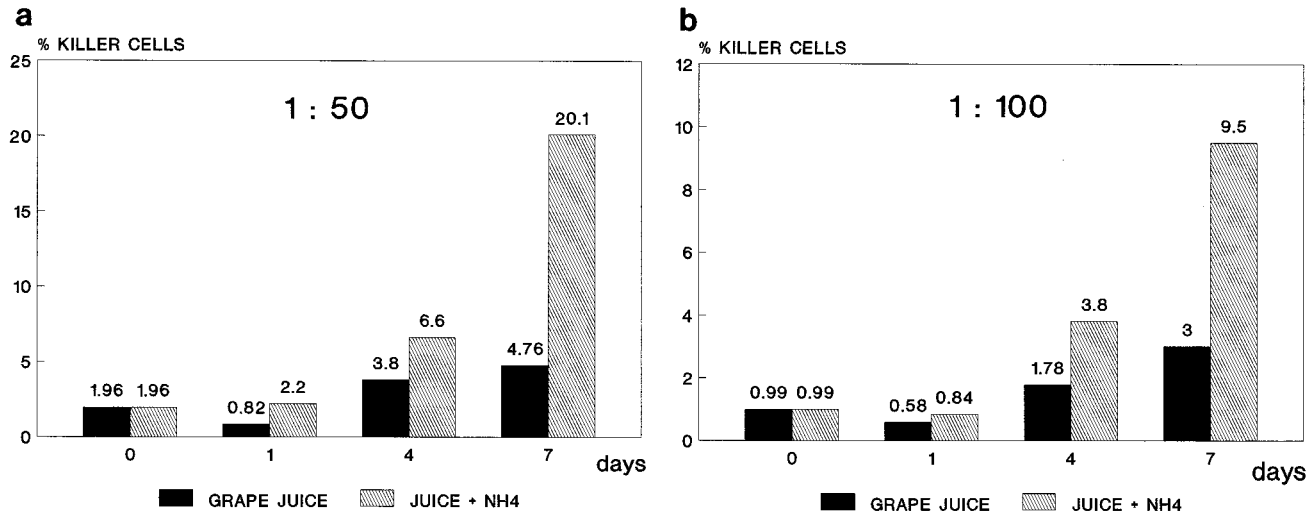

FIG. 2. Effects of diammonium phosphate (100 mg/liter) on the percentage of killer cells in grape juice inoculated at zero time with mixed cultures of strains 863 and M522 at proportions of 1:50 (a) and 1:100 (b) fermented at 20°C.

of pure cultures and 1:1 proportions of mixed cultures were preferred (data not shown). These results suggest that a must nitrogen limitation which reduced the quality of aroma in mixed-culture fermentations in proportions of 1:10 and 1:100 existed (21, 37).

Differential WL nutrient medium (Difco) was used to study the effects of the addition of assimilable nitrogen on the kinetics of the two populations in mixed-culture fermentations (growing with different colony colors). The effect of ammonium salt addition at zero time was studied with strains 863 and M522 in a proportion of 1:50. Figure 2a shows a significant increase in the killer population in grape juice supplemented with ammonium salts, up to four times that seen in grape juice alone, by the 7th day. Similar results were obtained with a K/S proportion of 1:100, resulting in a killer population in grape juice supplemented with ammonium salts three times that of the control in grape juice alone (Fig. 2b). Similar behavior was observed with the other K/S strain pairs studied (KU1 and M522 and KI and OC2) in mixed-culture fermentations (data not shown).

The results obtained in these experiments suggest the existence of a limitation of assimilable nitrogen. In cases of small killer cell populations, this situation does not permit the development of the killer yeast competitive potential: the sensitive cells probably have sufficient time to accumulate assimilable nitrogen from the medium before high killer toxin activity has been produced. Killer toxin activity has been observed 24 h after the start of fermentation, with maximum activity by the third day (44).

Addition of diammonium phosphate to a final concentration of 100 mg/liter did not affect the pH of the grape juice (less than 0.05 pH unit increase), seemingly ruling out a favorable effect for killer toxin activity in the medium.

Relatively sluggish wine fermentations were caused by K/S yeast proportions of 1:50, compared with the pure-culture fermentations. Figure 3 shows the effect of ammonium salt addition at different times on the fermentation rate. The highest fermentation rates were obtained when the ammonium was added at 48 h under these conditions. This result may be explained by the fact that favorable conditions for the killer population existed after the toxin had already been produced in the medium. Figure 3 also shows that in the case of ammonia addition at 120 h of fermentation, the fermentation rate was not significantly higher than that of the control. This result may be due to the toxic effect of ethanol on the nitrogen transport systems and is in agreement with a previous report (3).

Effect of inoculum size. The inoculum size used in the experiments described above (10^5 cells/ml) is smaller than that typically used by industry when selected starter cultures of dry yeast are used; usually an initial cell number of 1×10^6 to 5×10^6 cells/ml is obtained at inoculation. In order to study different initial inoculum levels in the wine industry, where the initial yeast cell number may vary between 2×10^4 and 2×10^6 cells/ml, the effect of inoculum size on the proportion of killer cells evolved during mixed-culture fermentations was studied.

In Fig. 4, an indirect relationship between the size of the inoculum, in which the K/S proportion was 1:10, and the percentage of killer cells during fermentation is shown. A higher

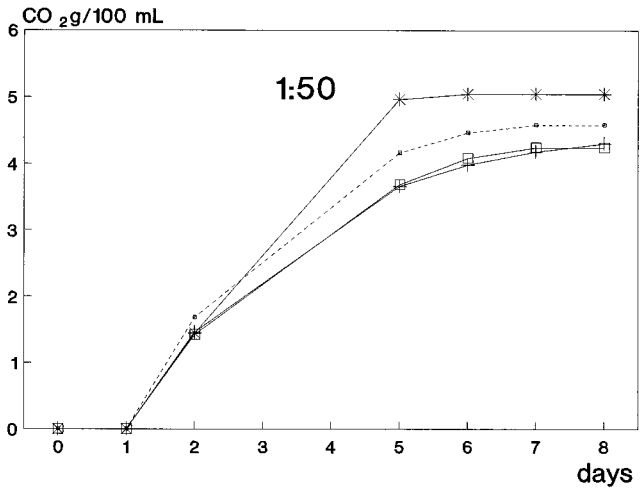

FIG. 3. Effects of diammonium phosphate (100 mg/liter) added at days 0 (○), 2 (*), and 5 (□) on the fermentation rate, as measured by weight loss of CO_2 (g/100 ml of grape juice), in grape juice inoculated with a K/S (strains 863 and M522) proportion of 1:50 at 20°C. +, fermentation rate in grape juice not supplemented with diammonium phosphate.

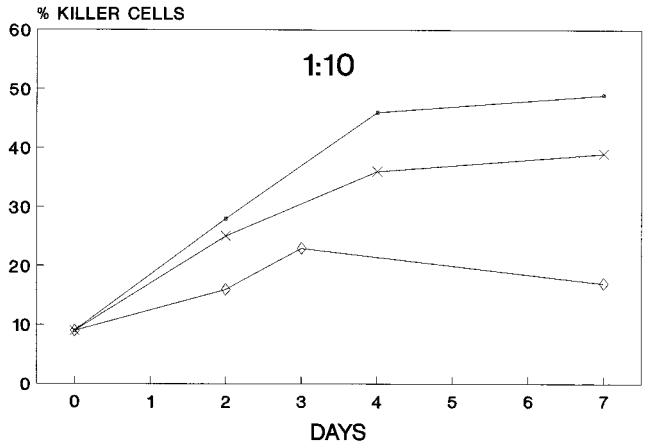

FIG. 4. Effects of the size of the inoculum of KI and OC_2 at a proportion of 1:10 on the percentage of killer cells during fermentation of grape juice at 20°C. Inoculum sizes of 2×10^4 (□), 2×10^5 (×), and 2×10^6 (◇) cells are shown.

inoculum size resulted in a lower percentage of killer cells during fermentation. These results may be in agreement with the hypothesis that a higher number of cells at the beginning of fermentation remove more of the assimilable nitrogen. This situation may limit the available nitrogen of the medium for the reproduction and fermentation of the killer cells, resulting in a stuck fermentation. These results also may explain published results of another study (34), in which killer strains did not dominate during mixed-culture fermentations inoculated with more than 10^6 cells/ml.

It was seen that the percentage of dead cells in these experiments was similar in all cases studied, i.e., between 11 and 14% (data not shown). This observation suggests that the conditions in these experiments were similar in relation to the effects of the toxin on the sensitive cells, in which only the initial population of total cells and the consequential competition for the available nitrogen source was changed.

Conclusions. The observations shown in this work may support our hypothesis (10) that dead cells may not release critical growth-stimulating nutrients into the medium and that early nitrogen source depletion in the medium may not permit killer cells to complete fermentation. After several hours of nitrogen starvation, the killer cells may lose the ability to maintain high rates of turnover of sugar transport proteins (28, 41). Although this conclusion is speculative, it may also be supported by the fact that the sensitive cells killed by killer toxin did not release macromolecules (7) and that the lysis process in wine takes several months to commence (11).

We can conclude that stuck and sluggish wine fermentations due to K/S strain interaction depend, in addition to K/S cell proportions, on (i) the size of the inoculum; (ii) the nitrogen level in the must at the beginning of fermentation and probably the nitrogen demand of the strains in competition; (iii) the time at which nitrogen is added during fermentation; and (iv) the level of killer activity of the strain in competition.

These results support the idea that the use of neutral yeast would be useful at the industrial level. Although little is known about neutral yeasts and their character stability in grape must fermentation, these strains would avoid stuck fermentations, thus improving the aroma quality of wine with less nitrogen demand during vinification.

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