Redox Interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in Mixed Culture under Enological Conditions

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Wine yeast starters that contain a mixture of different industrial yeasts with various properties may soon be introduced to the market. The mechanisms underlying the interactions between the different strains in the starter during alcoholic fermentation have never been investigated. We identified and investigated some of these interactions in a mixed culture containing two yeast strains grown under enological conditions. The inoculum contained the same amount (each) of a strain of *Saccharomyces cerevisiae* and a natural hybrid strain of *S. cerevisiae* and *Saccharomyces uvarum*. We identified interactions that affected biomass, by-product formation, and fermentation kinetics, and compared the redox ratios of monocultures of each strain with that of the mixed culture. The redox status of the mixed culture differed from that of the two monocultures, showing that the interactions between the yeast strains involved the diffusion of metabolite(s) within the mixed culture. Since acetaldehyde is a potential effector of fermentation, we investigated the kinetics of acetaldehyde production by the different cultures. The *S. cerevisiae*-*S. uvarum* hybrid strain produced large amounts of acetaldehyde for which the *S. cerevisiae* strain acted as a receiving strain in the mixed culture. Since yeast response to acetaldehyde involves the same mechanisms that participate in the response to other forms of stress, the acetaldehyde exchange between the two strains could play an important role in inhibiting some yeast strains and allowing the growth of others. Such interactions could be of particular importance in understanding the ecology of the colonization of complex fermentation media by *S. cerevisiae*.

Traditionally, indigenous yeast populations were used in the alcoholic fermentation step of wine making. Due to their strong resistance to ethanol, *Saccharomyces cerevisiae* strains usually predominate until the later stages of fermentation. In the last 20 years, however, winemakers have begun to use pure *S. cerevisiae* strains in the form of active dry yeast (ADY) starters. This process allows better control of fermentation and reduces the risk of organoleptic effects resulting from the growth and metabolism of other indigenous yeasts. In some cases, wine produced with pure yeast monocultures lacks the complexity of taste and other desirable characters that originate from the indigenous yeasts (16, 35, 51). The incorporation of several wine yeast strains with different technological capabilities into the same ADY starter may help overcome these shortcomings.

Metabolic interactions in mixed strain bacterial cultures and between fungi and bacteria have been identified (12, 26, 30, 41, 43, 47), but studies of such interactions in mixed yeast strain cultures are not common. In one study of mixed strain cultures for enological purposes, an exchange of metabolites between strains was observed (20). A mathematical model also is available to simulate growth in a mixed culture of strains of *S. cerevisiae* with and without the KII killer toxin (28, 31), but this model is limited to the interactions due to the toxic effect of the killer toxin.

The impact of cofermentation of *S. cerevisiae* with other yeast species on the final organoleptic balance of the wine also has been studied. Most of these studies focused on the effects of sequential inoculation with these yeasts on the wine produced, since the growth of most indigenous non-*Saccharomyces* species is limited on fermentation media and is rapidly inhibited by ethanol (3, 13, 18, 29, 44, 50). In all of these studies, the analysis focused on the fermentation products and not the fermentation kinetics. Interactions between *Saccharomyces* strains may occur in mixed cultures (7, 20), but the underlying mechanisms have not been investigated in detail.

The objective of the present study is to understand the effect of metabolite diffusion between partners of yeast cocultures, with a special emphasis on the effect on fermentation kinetics, by-product formation, and yeast persistence in the fermentation medium. Our working hypothesis is that metabolite diffusion may occur between two yeast strains, in this case a strain of *S. cerevisiae* and a natural *S. cerevisiae*-*Saccharomyces uvarum* hybrid (24), with different physiological properties during alcoholic fermentations under enological conditions. The knowledge of the underlying mechanism(s) responsible for this symbiotic interaction could be of particular importance in understanding the process through which indigenous *S. cerevisiae* strains colonize complex fermentation media, for explaining the observed persistence of indigenous *S. cerevisiae* strains during fermentations inoculated with pure *Saccharomyces* starter cultures, and for the design of more efficient starter cultures.

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Yeast strains. S. cerevisiae strain D254 9a2 is a spontaneous mutant of the D254 industrial wine yeast (Lallemand, Montreal, Canada) and is resistant to the mitochondrial inhibitors erythromycin and diuron. This strain is only available on demand for scientific purposes. Strain S6U is a natural hybrid of S. cerevisiae and S. uvarum selected by the Institute of Enology (Velletri, Italy) and available commercially as a dry yeast (Lallemand).

Culture media. All media were heat sterilized (at 110°C for 20 min). The yeast strains were grown in a standard nutrient medium, YPD [10 g of yeast extract (Difco, Detroit, Mich.) per liter, 20 g of Bacto peptone (Difco) per liter, and 50 g of glucose per liter]. A synthetic fermentation medium (SM), pH 3.3, was used for the fermentations. The fermentation was stopped if they were complete when the medium contained 10 g of glucose per liter.

Identification of fermentation by-products. Metabolites were extracted as described by Gonzalez et al. (19): 10 ml of yeast culture was added to 26 ml of an ice-cold solution containing 60% (v/v) methanol and 70 mM HEPES (pH 7.5) and kept at −80°C until further use. The mixture was centrifuged at 5,000 × g for 30 s at −10°C. Cell pellet metabolites were extracted with 5 ml of a solution of 75% (v/v) boiling absolute ethanol containing 0.25 M HEPES (pH 7.5) and incubated for 5 min at 80°C. Extracts were placed on ice for 5 min and then dried for 5 min under vacuum at 70°C in a rotating evaporator (model Laborota 4000; Heidolph Instruments LLC, Cinnaminson, N.J.). The resulting residue was resuspended in a final volume of 1 ml of distilled water and stored at −80°C until use.

Effect of mixed culture on fermentation. Fifty hours after inoculation with a 1:1 mixture of the two strains, D254 9a2 accounted for 70 to 75% of the total population in the mixed culture mixture.
content of NADH and NADPH was lower in strain S6U than in strain D254 9a2 (Fig. 3B and D), resulting in lower NADH to NAD and NADPH to NADP ratios in strain S6U than in strain D254 9a2. The NADH and NADPH content of the mixed culture was intermediate to content values of the S6U and D254 9a2 monocultures, but the NAD content of the mixed culture was similar to that of the D254 9a2 monoculture, particularly during the second half of the fermentation (Fig. 3A). In contrast, NADP content values were very similar throughout the fermentation in all tested cultures (Fig. 3C). Such differences in the redox balance ratios were also recently observed during fermentation of three industrial wine yeast strains (8).

**Effect of fermentation medium renewal on the redox potential of the yeast strains.** Cellular fluorescence (Fig. 4) was used to estimate cellular redox potential during fermentation (4, 23, 36). At the beginning of fermentation, the cellular fluorescence of the mixed culture was similar to that of the S6U monoculture. Removal of the fermentation products did not alter the fluorescence of strain D254 9a2, but the fluorescence of strain S6U increased sharply following medium renewal and then returned to previous levels. Fluorescence emitted by the mixed culture increased sharply following the change of medium and thereafter decreased slowly to a level similar to that of strain D254 9a2. At the end of the fermentation, the fluorescence signal of the mixed culture was higher than that in the presence of 3.5 mM acetaldehyde, which represents the fully oxidized state of intracellular reducing equivalents (Fig. 4). The NAD(P)H content of the mixed culture also decreased, but more gradually, after medium renewal.

**Effect of mixed culture on the acetaldehyde levels during fermentation.** We monitored acetaldehyde levels in S6U and D254 9a2 monocultures and in the mixed culture during fermentation (Fig. 5). Strain S6U produced large amounts of acetaldehyde, particularly during the early phase of fermenta-
The amount of acetaldehyde produced by strain D2549a2 was lower than that produced by strain S6U throughout the fermentation. Even though strain S6U made up ~30% of the total population of the mixed culture, the acetaldehyde production profile of the mixed culture was very similar to that of the D2549a2 culture.

**DISCUSSION**

Several previous studies have shown that growth or metabolism increases in *S. cerevisiae* and bacterial cocultures (11, 12, 26, 30, 47). This increase could result from competition between the yeast and bacteria for nutrients, particularly sugars and vitamins (3, 11, 43, 47). However, these effects also could result from the diffusion of metabolites between the two species (11, 20, 30, 47). To our knowledge, no work was initiated...
to identify the effect of metabolite diffusion between the partners of yeast cocultures. In order to define an experimental preliminary model allowing such a study, we examined the interactions between two yeast strains (one a strain of \textit{S. cerevisiae} and the other a natural \textit{S. cerevisiae-S. uvarum} hybrid) known to exhibit different physiological properties during alcoholic fermentations under enological conditions (24). Fermentation with \textit{S. uvarum} is known to produce much more acetaldehyde in the resulting wines than fermentation with \textit{S. cerevisiae} (9, 14). We found that the \textit{S. cerevisiae-S. uvarum} hybrid (S6U) produces during fermentation large quantities of acetaldehyde that \textit{S. cerevisiae} strain D254 9a2 can utilize in mixed cultures. This process results in a shift toward lower concentrations of ethanol (45). In contrast to this inhibitory effect, low levels of acetaldehyde utilizes the same mechanisms that participate in the response to other forms of stress. Thus, acetaldehyde exchange between strains could inhibit the growth of some yeast strains while encouraging the growth of others. This phenomenon could be particularly important for understanding the ecology of the colonization of complex fermentation media by \textit{S. cerevisiae} after the elimination of non-\textit{Saccharomyces} yeasts. During spontaneous fermentations, a succession of different indigenous \textit{S. cerevisiae} yeasts could be observed throughout the fermentation stationary phase, although no arguments were found to explain such behavior (16, 48). When fermentations are inoculated with pure \textit{Saccharomyces} starter cultures, the persistence of several indigenous \textit{S. cerevisiae} strains during fermentation may also be observed (16). These two examples could represent interesting models for determining the role of acetaldehyde during fermentation.

There is great variation in metabolic capability among isolates of naturally occurring \textit{S. cerevisiae}. The variation includes significant heterogeneity among strains in the production of ethanol, acetic acid, sulfite, and other products of metabolism (10). To our knowledge, there are very few data on the production of acetaldehyde during the colonization of complex fermentation media. The initial acetaldehyde level in a fermentation medium could encourage the growth of \textit{S. cerevisiae} yeasts, e.g., D254 9a2, that can function as receptor strains and utilize the acetaldehyde produced. To test this hypothesis, effort should be made to develop a bioreactor in which differential growth of a yeast strain in a homogenous fermentation medium can be established. We are now testing a two-reservoir, hollow-fiber bioreactor (28) for use in the study of the dynamics of such mixed populations.

Over 200 different wine yeast strains are currently available for use in ADY starters; however, their physiological differences have not been systematically evaluated (9, 14, 15, 24, 32, 42). Empirical experiments have been conducted on both the laboratory and commercial scale on the effect of changes in the yeast population on the reliability of mixed fermentations and on the quality of the wines produced (13, 29, 44, 50). From a technological point of view, more ADY starters containing different mixtures of wine yeast strains are likely to be introduced to the market. Thus, the potential interactions between the yeast strains used and the potential effects (detrimental or otherwise) on the final product need to be evaluated. Including strains with opposite acetaldehyde-producing properties in the same starter could bloom the wine yeast, resulting in a higher fermentation rate or improved organoleptic properties. A general survey of the acetaldehyde-producing properties of and the physiological response to acetaldehyde of all of the commercially available wine yeast strains would therefore be valuable.

\section*{REFERENCES}


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