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Sequential fermentation using non-*Saccharomyces* yeasts for the reduction of alcohol content in wine

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Abstract. Over the last few decades there has been a progressive increase in wine ethanol content due to global climate change and modified wine styles that involved viticulture and oenology practices. Among the different approaches and strategies to reduce alcohol content in wine we propose a sequential fermentation using immobilized non-*Saccharomyces* wine yeasts. Preliminary results showed that sequential fermentations with *Hanseniaspora osmophila*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Starmerella bombicola* and *Saccharomyces cerevisiae* strains showed an ethanol reduction when compared with pure *S. cerevisiae* fermentation trials.

1. Introduction

Over the last few decades, there has been a progressive increase in the ethanol content in wines due to global climate change and to the new wine styles that are associated with increased grape maturity [1,2]. In this context, various lines of research are thus aimed at the reduction in the ethanol content of wines, which have generally focussed on vineyard management and winemaking practices, particularly in the dealcoholisation of wine.

Microbiological approaches for decreasing ethanol concentrations takes advantage of the differences in energy metabolism among the wine yeast species. Several strategies that use genetically modified (GM) yeasts have been proposed for the production low-alcohol wine. Recently, Tilloy and co-workers [3] using evolution-based strategies together with breeding strategy showed that evolved or hybrid strains produced an ethanol reduction of 0.6–1.3% (vol/vol) in comparison with ancestral strain.

Another approach to reduce the production of ethanol might be the use of non-*Saccharomyces* wine yeasts. The use of non-*Saccharomyces* yeasts in combination with *Saccharomyces cerevisiae* has been proposed to improve the quality and enhance the complexity of wine. Following numerous studies on the influence of non-*Saccharomyces* yeast in winemaking, there has been a re-evaluation of the role of these yeasts. Indeed, some non-*Saccharomyces* yeast can enhance the overall profile of the wine, and for this reason the use of controlled multi-starter fermentation using selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeast strains has been encouraged [4]. Indeed, nowadays one of the most recent technological advances in winemaking is the practice of co-inoculation of grape juice with selected culture of non-*Saccharomyces* coupled with *S. cerevisiae* starter strain [5].

In this context, non-*Saccharomyces* wine yeasts in multistarter fermentations could be an interesting way to reduce the ethanol content in wine since this fermentation strategy may affect ethanol yield, alcoholic fermentation efficiency, biomass, by-products formation with a diversion of carbon away from ethanol production. In addition, different respiro-fermentative regulatory mechanisms of some non-*Saccharomyces* yeasts compared to *S. cerevisiae* could be a modality to reduce the ethanol production through partial and controlled aeration of the grape juice. Indeed, in this way sugar is consumed via respiration rather than fermentation. Both these approaches have indicated the promising use of non-*Saccharomyces* wine yeast to limit ethanol production [6,7].

2. Sequential fermentation

Sequential fermentation could be a attractive tool to use non-*Saccharomyces* yeast for the reduction of the ethanol content in wine. This fermentative approach, in which the initial inoculation of non-*Saccharomyces* strain is followed by the inoculation of *S. cerevisiae* starter strain, allows to exert the metabolism of the first inoculated yeast without the influence of the following yeast strain. In this way, the reduction of ethanol content could be obtained depending on the metabolic characteristics of non-*Saccharomyces* strain and the interval between the first and the second inoculation (*S. cerevisiae* starter strain). To exert the metabolic characteristics of non-*Saccharomyces* yeast in sequential fermentation (i.e. low ethanol yield, low fermentation efficiency) two aspects should be take in account: i) the inoculation level; ii) the duration of the interval between first and second inoculation. An enhancement

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Table 1. Reduction in ethanol production observed in sequential fermentations for some non-*Saccharomyces* yeast strains as compared to *S. cerevisiae* control strain.

Species	Wine*	Time of 2nd inoculation (days)	Eth (%vol) reduction	% of reduction	Ref.
M. pulcherrima	W	17	0.90	6.0	[8]
M. pulcherrima	R	9	1.60	11.6	[8]
C. stellata/S. bombicola	S	2	1.60	10.4	[9]
C. stellata/S. bombicola	W	3	0.64	6.0	[10]
L.thermotolerans	R	2	0.68	5.0	[11]

* W = white wine; R = red wine; S = synthetic wine.

of the inoculation level of non-*Saccharomyces* yeast improves the competitiveness towards *S. cerevisiae* and other wild yeasts while the time of second inoculation (time where only non-*Saccharomyces* strain is active) affects the duration of its metabolic activity. In Table 1 are reported the results of sequential fermentations with some non-*Saccharomyces* species and the reduction of ethanol formed in mixed fermentation as compared to *S. cerevisiae* pure fermentation.

Ethanol reduction varies from 0.64 to 1.60 (% vol/vol) with a percentage of reduction from 5 to 11.6 using strains belonging to *Candida stellata* (now reclassified as *Starmerella bombicola*), *Metschnikowia pulcherrima and Lachancea thermotolerans.* Different approaches regarding to the substrates, time of the second inoculation and initial inoculation level of non-*Saccharomyces* strain were used. In fermentation trials with *M. pulcherrima* the ethanol reduction was obtained by delaying the second inoculation with *S. cerevisiae* strain (9 and 17 days using red and white wine respectively), while *C. stellata* and *L. thermotolerans* trials were carried out using high inoculation level of these starter strains (10^7 cell/ml for *K. thermotolerans* and 10^8 cell/ml for *C. stellata* in immobilized form) but using just a delay of the inoculation *S. cerevisiae* strain of two-three day. A long time of delay of second inoculation is difficult to realize in actual winery condition because of a likely contamination of wild microflora even more so when the competitiveness of non-*Saccharomyces* strain is low. On the other hand, the use of a high inoculation level determines an substantial increase in management costs of the fermentation that should be evaluated.

3. Sequential fermentation and immobilized non-*Saccharomyces* yeasts

To carry out the fermentation in a non sterile environment, the yeast starter must possess a suitable fermentation rate. This limitation could be overcome by using immobilized yeasts. Among the different advantages in the use of immobilized yeasts, one of the most important is the possibility of using high cell concentrations to obtain a high reaction rate, avoiding problems of competition

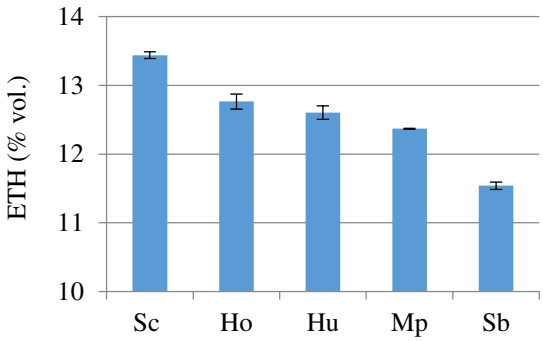

Figure 1. Ethanol production by *S. cerevisiae* in pure and mixed fermentation with various non-*Saccharomyces* yeasts.

with wild microflora. Here, we investigated on the effect of sequential fermentation using immobilized cells of some non-*Saccharomyces* yeast strains belonging to *Hanseniaspora*, *Candida*, *Metschnikowia* genera and selected for its use in mixed fermentation with *S. cerevisiae* starter strain. Fermentation trials were carried out using *H. osmophila*, *H. uvarum*, *M. pulcherrima*, *S. bombicola* immobilized cells. After 48 h the immobilized cells were removed and the substrate was inoculated with *S. cerevisiae* strain free cells. Control trial was carried out by *S. cerevisiae* free cells. Cells for immobilization were grown in YPD at 25 °C in a rotary shaker (150 rpm) harvested by centrifugation, washed three times with sterile distilled water, and added to 2.5% Na-alginate (Carlo Erba, Milan, Italy) at a ratio of 5% (wet weight/vol) (biomass moisture, 70%; final concentration, 1–2× 10^9 cells per g of beads). The inoculum for immobilized cells was 2–4 × 10^8 cells per ml (10% [wt/vol] of the amount of beads in the medium). Fermentations were carried out in duplicate at 25 °C in synthetic grape juice (SGJ) [4] containing 220 g/L sugar. All fermentation trials completed alcoholic fermentation with less than 2 g/L residual sugar. Several phenotypic traits were measured: fermentation kinetics, yeast population, fermentation products.

The analytical results for ethanol production during fermentation are showed in Fig. 1. All sequential fermentations carried out by immobilized non-*Saccharomyces* yeasts during the first 48 h showed significant reduction in ethanol content in comparison with control fermentation trial (*S. cerevisiae* free cells).

Indeed, the ethanol reduction of sequential fermentation trials using non-*Saccharomyces* immobilized cells varied from 5.1 to 14.0% of final content. In particular, *H. osmophila* (Ho) and *H. uvarum* (Hu) fermentation trials exhibited a reduction of final alcohol content of 5.1 and 6.3% respectively.

Sequential fermentation trials with *M. pulcherrima* immobilized cells showed a reduction in ethanol content of 8% compared to *S. cerevisiae* control trial. *S. bombicola* immobilized cells exhibited, in the condition tested, the lowest alcohol content showing a reduction in ethanol of 14% when compared to *S. cerevisiae* pure fermentation.

These results are confirmed by the evaluation of ethanol yield. In Fig. 2 are showed the results of ethanol yield of the sequential fermentation trials inoculated with

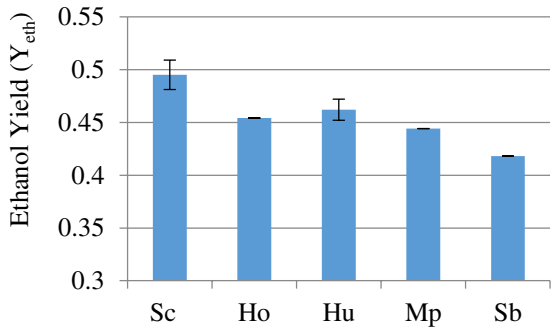

Figure 2. Ethanol yield (Y_{eth}) by *S. cerevisiae* in pure and mixed fermentation with various non-*Saccharomyces* yeasts.

non-*Saccharomyces* immobilized cells. In the condition tested (beads in contact with the substrate for 48 h) where a significant reduction in ethanol content was showed. The Evaluation the fermentation efficiency [($Y_{Eth}/Y_{Eth\ Theor}$) × 100] of the trials were: *S. cerevisiae* 96.9%, *H. osmophila* 89.0%, *H. uvarum* 90.4%, *M. pulcherrima* 86.9% and *S. bombicola* 81.8%.

4. Conclusion

These results are encouraging to use non-*Saccharomyces* in controlled mixed fermentation to reduce the ethanol content in wine. The aim to reduce the ethanol concentration of about 2% is attainable. The use of sequential fermentation in conjunction with immobilized cells seems an effective fermentation strategy to enable the metabolic activity of non-*Saccharomyces* strain with low fermentation efficiency. However, in view of its possible application, the modalities and the duration to exert the metabolic activities of non-*Saccharomyces* strain in sequential fermentation should be further defined, evaluating times and modalities of inoculation of starter strains.

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