GENETIC IMPROVEMENT TO REDUCE THE SULPHUROUS PRODUCTION OF HOMOTHALLIC WINE YEAST USING A SIMPLE HYBRIDIZATION METHOD

Rocío Velázquez[1]*, Paolo Antoniali[1], Alberto Martínez[2] and Manuel Ramírez[2].
[1]Dipartamento Scienze Biomediche, Università degli Studi di Padova, 35020 Padua, Italy.
[2]Departamento de Microbiología, Universidad de Extremadura, 06006 Badajoz, Spain.
*Email: rociovelazquez@unex.es.

INTRODUCTION

Despite the intensive use of selected wine yeasts, there is still a margin for their technological improvement. Previous studies on yeast genetics have shown that many of their characters, which determine the quality of wines, have a genetic basis and are susceptible for improving.

Hybridation is the first method to be considered for improvement of diploid industrial yeast strain. For two homothallic wine yeast strains, the method we propose is accomplished by mixing sporulated cultures. Cell fusion can occur between spore germination and diploidization. After easy hybrid identification and selection, a great number of hybrids can be obtained easily. The method takes advantage of the fact that the killer phenotype is very frequent among wine yeasts, which is determined by double-stranded RNA molecules in *Saccharomyces cerevisiae*. By changing the culture conditions, one can make yeasts conjugate or kill each other (1).

On the other hand, sulfites are widely used in oenology. However, there is a tendency to reduce their use and control their levels in the final wine as it can be harmful to health when found in high concentrations. The exogenous contributions of SO_2 are not the only ones involved in the final content of the SO_2 in wines, because wine yeasts can also produce it in variable quantities during alcoholic fermentation as a metabolic intermediate of the sulphate reduction pathway. Certain yeasts can produce quantities of SO_2 greater than their needs or assimilation capacities, and then the excess is excreted in the medium. Its production can vary according to the strains from a few milligrams to more than 100 mg/L (2).

This work addresses the genetic improvement of a high-sulphurous-producing yeast using this method for homothallic wine yeast based on spore hybridization that allows easy hybrid selection and identification.

MATERIAL AND METHODS

Two spontaneous mutants highly homozygous and free from growth-retarding alleles (Mutant-24 and -25), which contains easily detectable genetic markers (cycloheximide-resistant or CYH^R), were obtained from a sensitive (non-killer or K^-) high-sulphurous-producing homothallic yeast (the original yeast). The homozygous Mutant-24 and -25 spores were crossed with killer low-sulphurous-producing wine yeasts to obtain killer (type K2 or Klus) and low-sulphurous producing hybrids. A yeast micromanipulator was used to mix 4 (K^- CYH^R, high-sulphurous-producing) × 4 (K^+ CYH^S, low-sulphurous-producing) spores as previously described (1).

Fermentation trials were carried out in 250 mL Erlenmeyer-flaks, closed with a Muller valve, with 180 mL of synthetic must or fresh grape must (Garganega) at a concentration of 3×10^6 cells/mL. Fermentations were conducted at 20-23ºC. Physicochemical parameters were determined by GC-MS analysis.

Cross	Total of crosses analysed	Selected cross-1[MR1]	Selected cross-2
LM-7D x Mutant-24	24	2	-
LM-7D x Mutant-25	11	5	1
EX229 x Mutant-25	11	8	1
EX88P1A x Mutant-24	20	2	1
EX88P1A x Mutant-25	20	4	-
	86	**21**	**3**

Table 1. Total crossbreeding of K^+ CYH^S, low-sulphurous-producing (LM-7D, EX229 and EX88P1A) with K^- CYH^R, high-sulphurous-producing (Mutant-24 and -25) homozygous homothallic strains to obtain killer K2/Klus cycloheximide-resistant hybrids (K^+ CYH^R).

RESULTS

Initially, as expected many single-cell hybrids colonies K^+ CYH^R were obtained. Many of them were resistant to cycloheximide and presented killer phenotype in low-pH blue plates (Fig. 1). Vinification trials in synthetic must with the 86 hybrids obtained were performed (Table 1). The fermentation kinetics and the sulphurous level of the resulting wines were analysed. In most cases, the hybrids had good fermentative capability in synthetic must with respect to the original yeast (Fig. 2). This result suggests that this method for homothallic yeast hybridization is very useful for industrial yeast improvement.

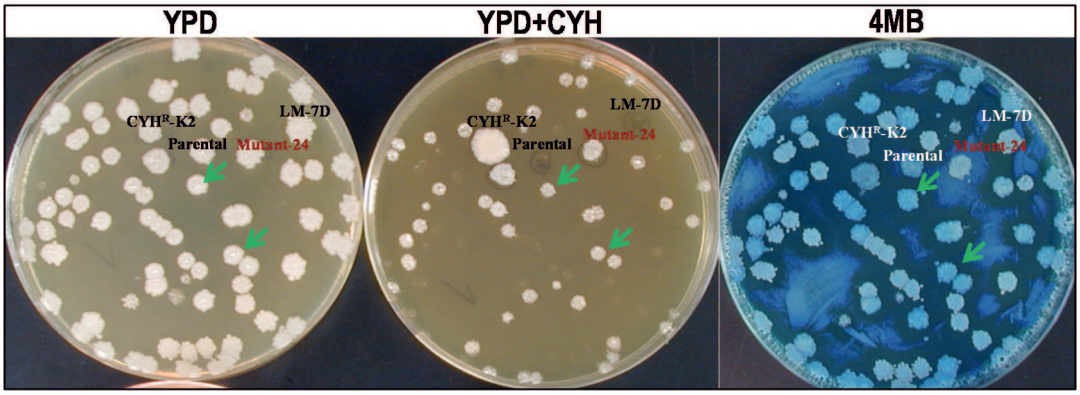

Figure 1. Example of replica plating to obtain CYH^R, killer (type K2) and low-sulphurous producing hybrids. Plate YPD (rich medium), YPD-CYH (supplemented with 2 µg/mL cycloheximide) and 4MB (methylene blue plates) seeded with 100µL of a 48-h grown culture of the sensitive strain. The green arrows indicate two selected hybrid colonies.

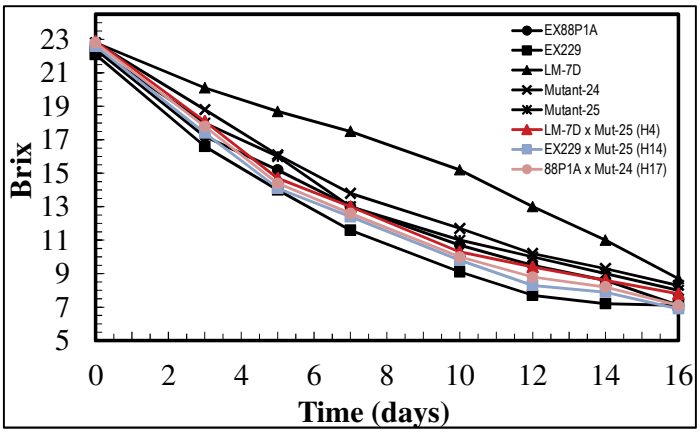

Figure 2. Fermentation kinetics of H4, H14 and H17 in synthetic must.

Thereafter, twenty-one hybrids were preselected, because of their low-sulphurous level respect to the original high-sulphurous producer yeast, to make additional experiments in different working conditions (Selected cross-1, Table 1). Three of them (Hybrid-4, -14 and -17) were selected (Selected cross-2, Table 1), based on the quality of the wines (Table 2) and their molecular polymorphisms (Fig. 3A) with an intermediate pattern between both parental strains, to perform vinification trials with fresh grape must.

Yeast strain	Must	EtOH % V/V	Free SO_2 (mg/L)	Total SO_2 (mg/L)
EX88P1A	synthetic must	13,79	5	16
EX229	synthetic must	13,47	5	13
LM-7D	synthetic must	13,78	5	14
High-SO_2 parental	synthetic must	11,88	5	**30**
H4	synthetic must	13,36	4	**11**
H14	synthetic must	13,52	5	**20**
H17	synthetic must	11,84	3	**17**
EX88P1A	fresh grape must	13,91	6	72
EX229	fresh grape must	13,92	4	32
LM-7D	fresh grape must	13,92	5	50
High-SO_2 parental	fresh grape must	13,87	6	**95**
H4	fresh grape must	13,89	5	**52**
H14	fresh grape must	13,68	4	**49**
H17	fresh grape must	13,96	5	**73**

Table 2. Microvinification analysis of H4, H14, H17 and their parentals with synthetic and fresh grape must.

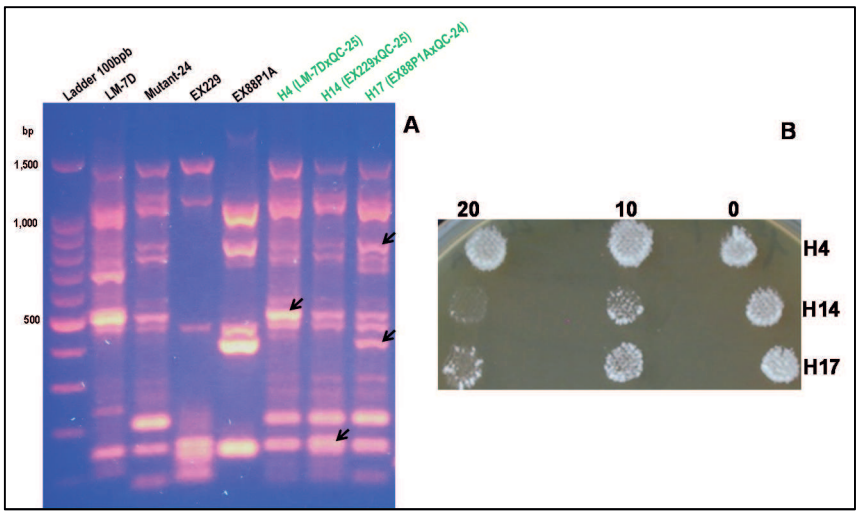

Figure 3. Verification of hybrids by molecular polymorphisms: INTERDELTA PROFILE (A). Analysis of hybrid markers CYH^R of H4, H14 and H17 after 0, 10, and 20 transfers (100 doubling) (B).

All hybrids showed good fermentation kinetics in fresh grape must, very similar to that of the original yeast. Hybrid-4 and -14 reduced the sulfurous level down to 50%, while Hybrid-17 only down to 20% with respect to the original high-sulphurous producer yeast (Table 2).

Finally, Hybrid-14 and Hybrid-17 showed genetic instability, because they loss the CYH^R marker after 100 doubling. Hybrid-4 was instead very stable (Fig. 3B) so it can be ready for commercial vinification trials. Alternatively, for additional improvement, new spore-clones (K^+, CYH^S, low-sulphurous-producing) from this hybrid can be backcrossed with Mutant-24 or Mutant-25 to get an aromatic profile closer to the original yeast.

CONCLUSIONS

The method used for homothallic yeast hybridization was very useful for industrial yeast improvement as previously described (1). The Hybrid-4, which seems genetically stabilized, could be used as low-sulphurous producer in winemaking. Alternatively, Hybrid-4 genetically stabilized could be used as low-sulphurous producer after backcrossing with Mutant-24 or -25 to get an aromatic profile closer to the original yeast strain.

REFERENCES

(1) Ramírez, M., Pérez, F., and Regodón, J. (1998). A simple and reliable method for hybridization of homothallic wine strains of *Saccharomyces cerevisiae*. Applied Environmental Microbiology 64, 5039-5041.
(2) Noble, J., Sanchez, I., and Blondin, B. (2015). Identification of new *Saccharomyces cerevisiae* variants of the MET2 and SKP2 genes controlling the sulfur assimilation pathway and the production of undesirable sulfur compounds during alcoholic fermentation. Microbial cell factories 14, 68.

ABSTRACT

This work addresses the genetic improvement of a high-sulphurous-producing yeast using a method for homothallic wine yeast based on spore hybridization that allows easy hybrid selection and identification. Many low-sulphurous-producing hybrids K^+ CYH^R were obtained, all of them with a good fermentative capacity in synthetic must with respect to the original yeast. Thereafter, twenty-one hybrids were preselected to make additional experiments in different working conditions. Three of them (H4, H14 and H17) were selected, based on the quality of the wines, to perform vinification trials with fresh grape must. All hybrids showed good fermentation kinetics, very similar to that of the original yeasts. The Hybrid-4 and -14 reduced sulfurous levels up to 50%, while Hybrid-17 only down to 20% with respect to the original yeast. H14 and H17 showed genetic instability, due to loss of the CYH^R marker after 100 doubling, H4 was instead very stable. Consequently, Hybrid-4 genetically stabilized could be used as low-sulphurous producer once backcrossed with the Mutant-24 or -25 to get an aromatic profile closer to the original yeast.

ACKNOWLEDGMENT

This research was funded by grants GR18117 of the Extremadura Regional Government and AGL2017-87635-R of the Spanish Ministry of Economy, Industry and Competitiveness (co-financed with FEDER funds). Rocío Velázquez acknowledges support by a postdoctoral fellowship from the Extremadura Regional Government (file number PO17004, co-financed with FEDER funds).