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Aroma improving in microvinification processes by the use of a recombinant wine yeast strain expressing the *Aspergillus nidulans xln*A gene

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Abstract

A recombinant wine yeast strain has been constructed expressing the gene coding for β-(1,4)-endoxylanase from *Aspergillus nidulans* under the control of the yeast actin gene promoter. The resulting recombinant strain is able to secrete active xylanase enzyme into the culture medium. Wines obtained by microvinification with the control and the recombinant wine yeast strain did not differ in their physicochemical characteristics although an increase in fruity aroma was organoleptically detected in the wine produced by the recombinant yeast. Also, an increase in the concentration of some esters, higher alcohols and terpenes was observed in the case of the recombinant strain. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Recombinant yeasts; Xylanase; Wine; Aroma

1. Introduction

Wine fermentation is a complex microbiological reaction involving the sequential development of various yeast strains and lactic acid bacteria. For many years, wines have been produced by natural fermentation carried out by yeasts that originate from both the grapes and the cellar. Several oxidative and apiculate yeasts are predominant on the surface of grapes, but after 3–4 days of fermentation *Saccharomyces cerevisiae* predominates, playing the major role in alcoholic fermentation (Jackson, 1994). Using mitochondrial DNA restriction analysis it has been possible to find a high diversity of *S. cerevisiae* strains at the beginning of the fermentation although only a few of them dominate the process in the latter stages and produce the final wine (Querol et al., 1994). During the last years many winemakers have used pure *S. cerevisiae* cultures isolated from their own growing regions in order to produce wines of more reproducible quality (Ribereau-Gayon, 1985). Such cultures, in the form of active dry yeasts, are

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supplied to wineries, where they are inoculated to the fresh must in order to perform controlled fermentations. A detailed analysis of the population dynamics of inoculated fermentations have demonstrated that the dry yeast strain competed with the native strains but did not completely suppress their growth until 3–6 days after inoculation. The inoculated strain dominated towards the end of fermentation (Querol et al., 1992a).

This microbiological simplification, together with advances in yeast biotechnology, opens the way for the genetic modification of active dry yeasts and thus for the construction of strains expressing metabolic activities that exert beneficial effects on the organoleptic characteristics of the wine (for a review see Querol and Ramón, 1996). As examples, wine yeast strains expressing both the K1 and K2 killer toxin (Boone et al., 1990) and fungal enzymes that increase fruity aroma have recently been constructed (see below). Also, the construction of a laboratory *S. cerevisiae* strain able to perform a mixed lactic acid–alcoholic fermentation (Dequin and Barre, 1994) or the malolactic fermentation (Volschenk et al., 1997a,b) have been reported. It is also evident that genetic engineering of wine yeast strains requires the knowledge of gene promoters that are specifically activated under fermentation conditions. For this reason, research has been undertaken to understand the regulation of gene expression in *S. cerevisiae* during wine fermentations (Polotnianka et al., 1995; Puig et al., 1996; Riou et al., 1997).

One of the most important characteristics of a wine is its aromatic fragrance. It is now well established that certain monoterpenes contribute significantly to the flavor of wine (Bayonove and Codornnier, 1971). These compounds are present in the must as both free forms and aroma precursors. The latter flavorless glycosidically bound forms consist of β-D-glucopyranosides and diglycosides such as 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranoside, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (Günata et al., 1990). These bounds can be released by enzymatic hydrolysis in two stages: (i) depending on the precursor, the glycosidic linkage is cleaved by either an α-L-arabinofuranosidase, an α-L-rhamnosidase or a β-D-apiosidase, and (ii) a β-glucosidase then liberates the monoterpenols (Günata et al., 1988). We have recently constructed two different recombinant wine yeast strains containing the *Aspergillus niger abf*B gene and the *Candida molischiana bgln* gene coding for an α-L-arabinofuranosidase and a β-glucosidase, respectively (Sánchez-Torres et al., 1996, 1998). Both strains are capable to secrete the corresponding enzymes to the must in sufficient amounts to produce wine with adequate enological properties.

Addition of other enzymes to the must (e.g., endoglucanases, cellobiohydrolases, xylanases or arabinases) to increase wine aroma remains still open. In fact, some of our previous results, such as those obtained with the use of a recombinant wine yeast strain expressing a *Trichoderma longibrachiatum* β-(1,4)-endoglucanase as an enhancer of fruity aroma (Pérez-González et al., 1993), suggest a role for this kind of enzyme, presumably due to the release of aromatic precursors upon enzymatic degradation of cell walls (Montedoro and Bertuccioli, 1976). In this paper we describe the construction of a xylanolytic wine yeast strain expressing the *A. nidulans xln*A gene coding for the major β-(1,4)-endoxylanase (Pérez-González et al., 1996). Fermentations carried out with this recombinant yeast clearly indicate that this enzyme is capable to release wine aroma precursors and to increase the fruity aroma of the final wine.

2. Materials and methods

2.1. Strains and culture media

The *S. cerevisiae* industrial wine yeast strain T_{73} (CECT 1894) has been commercialized by Lallemand Inc. (Montreal, Quebec, Canada) and its selection and molecular characterization have been previously reported (Querol et al., 1992b). Yeast cells were grown at 30°C in YPD-rich medium (1% yeast extract, 1.5% peptone, 2% glucose; all components from Sigma, St. Louis, MO, USA). All the recombinant DNA experiments were performed with *E. coli* DH5α which was grown in LB medium (Sambrook et al., 1989).

2.2. DNA manipulation and transformation

E. coli plasmid isolation and general DNA manipulations were carried out using standard protocols

(Sambrook et al., 1989). Yeast DNA isolation was done as described Fujimura and Sakuma (1993). Yeast transformation was performed according to González-Candelas et al. (1995) and the selection and maintenance of the transformants were done on YPD plates (YPD with 2% agar from Pronadisa, Madrid, Spain) containing 1 μg/ml cycloheximide (Merck KGaA, Darmstadt, Germany).

2.3. Construction of the YEPCA1 plasmid

The expression cassette of plasmid pYAL1 (Pérez-González et al., 1996) containing the *S. cerevisiae* actin promotor (pACT1) and the *A. nidulans* endoxylanase X_{22} cDNA was released by digesting the plasmid with the restriction enzymes *Eco*RI and *Hin*dIII. The fragment containing the expression cassette was treated with the Klenow fragment of the *E. coli* DNA polymerase I in the presence of deoxynucleotides to create a filled fragment. Finally, this fragment was cloned into the *Eco*RV site of plasmid YEpCR21 (Navas et al., 1991) to generate plasmid YEpCA1.

2.4. Cellular location

The cellular location of the xylanase X_{22} in the recombinant T_{73} strains containing YEpCA1 was investigated following the protocol described previously (González-Candelas et al., 1995).

2.5. Enzyme determinations

Plate assays were performed as described before (Béguin et al., 1987) using xylan (Sigma) as substrate. Xylanase activity was determined according to Fernández-Espinar et al. (1994), one unit being the amount of enzyme that released 1 μmol of xylose equivalents per minute (IU). Isocyanate dehydrogenase activity was measured as described by González-Candelas et al. (1995).

2.6. Microvinification experiments

Microvinification and enological assays were done in duplicate (Querol et al., 1992b). The fermentation assays were carried out using 1 l of Chenin Blanc must (Tarragona) inoculated up to a final concentration of 2.5×10^5 cells from overnight cultures of T_{73} and T_{73}/YEpCA1. To monitor the progress of the fermentation, reducing sugar concentration was measured using the Nelson–Somogyi method (Somogyi, 1952) with glucose as standard. To determine cell viability, resistance to cycloheximide and xylanase activity, aliquots were taken every day during the fermentation process. At the end of the fermentation, the wine was centrifuged at $4000 \times g$ for 15 min, kept at 4°C and bentonite (Sigma) was added at a final concentration of 0.5 g/l. After shaking overnight, the bentonite was removed by centrifugation at $4000 \times g$ for 15 min. The wine was then treated with 60 ppm of SO_2 and maintained at 4°C prior to analysis. Enological parameters were determined as described previously (Querol et al., 1992b). The organoleptic characteristics of the wine produced were tested by a panel of trained experts.

2.7. Extraction and isolation of volatile compounds

A total of 40 ml of centrifuged wine was used for each assay. The samples were brought to 8% (w/v) NaCl and 1.5 mg/l of 2-octanol (Fluka Chemie AG, Buchs, Switzerland) as internal standard was added. Extraction of volatile compounds from the wine was performed by liquid–liquid extraction with dichloromethane (Fluka). Some anhydrous Na_2SO_4 (Panreac, Madrid, Spain) was added after extraction to remove residual water. The solvent was then evaporated by a N_2 stream to a residual volume of 300 μl. The samples were stored at −20°C until the gas chromatography analyses.

2.8. Extraction and quantification of glycosides compounds in wine

Glycosides compounds in wine were extracted and quantified following the protocol described previously by Williams et al. (1995).

2.9. Gas–liquid and gas–mass chromatography spectrometry

The analysis of volatile compounds was carried out using a Hewlett-Packard gas capillary chromatograph (Waldbronn, Germany), model 5890 series II, equipped with a flame ionization detector and a Carbowax 20 M (50 m × 0.2 mm) column (Supelco, Sigma–Aldrich Quimica SA, Madrid, Spain). In-

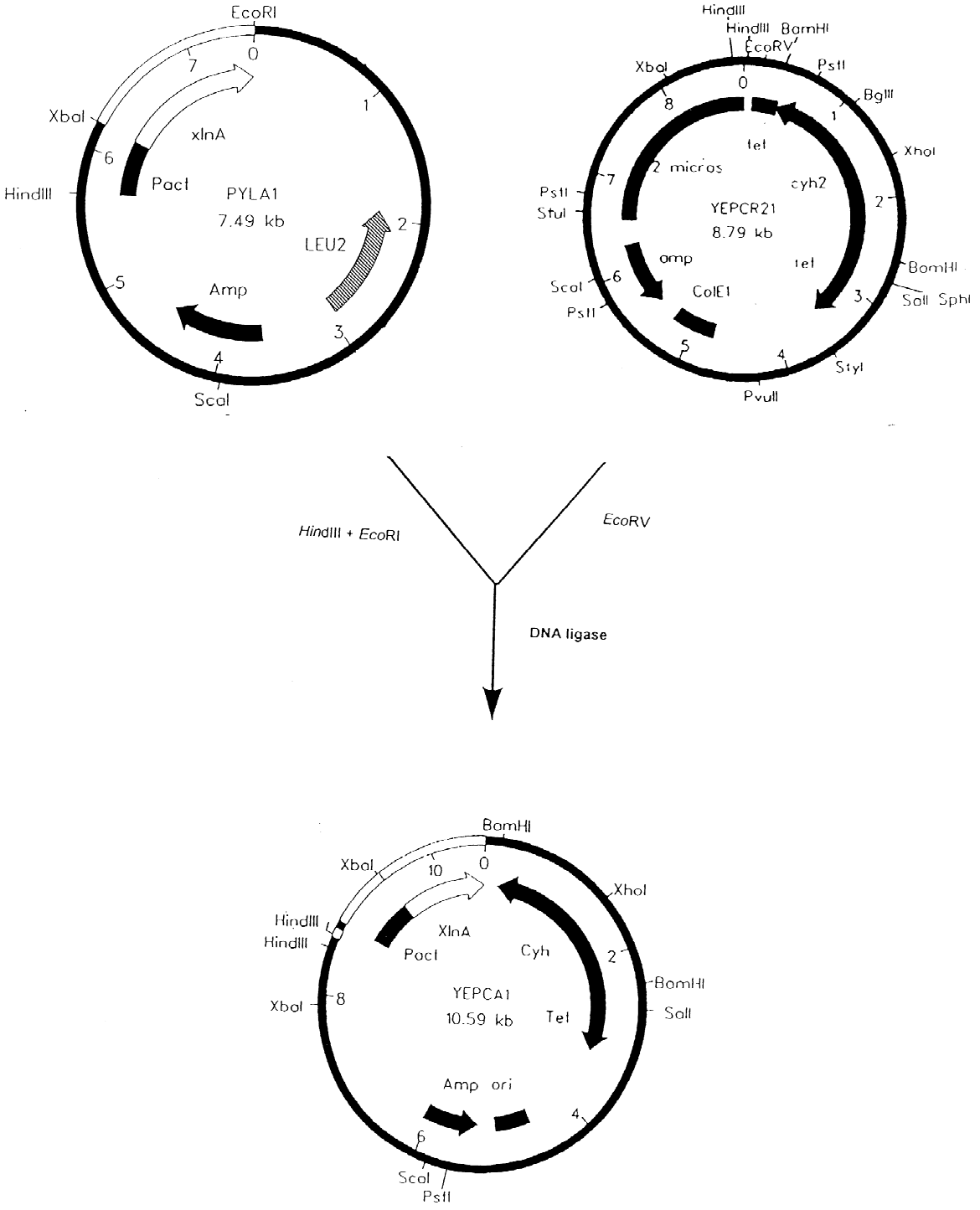

Fig. 1. Strategy used for the construction of plasmid YEpCA1. Details are presented in Section 2.

jections were made in split mode (split ratio, 1:50). Injector temperature was 250°C and the oven was programmed from 60°C isothermal for 10 min and then programmed at 2°C/min until 210°C and held isothermal for 50 min. The carrier gas was He at 1 ml/min. Identification of compounds was determined by comparing retention times with those from pure compounds (all from Fluka) and using a Fisons Trio 1000 mass spectrometer (Fisons Instruments SpA, Rodano, Milan, Italy) under the same chromatographic conditions.

3. Results and discussion

3.1. Construction of a recombinant wine yeast strain

Plasmid YEpCA1 (Fig. 1) was constructed as described in Section 2. In this plasmid the cDNA of the *A. nidulans* endoxylanase X_{22} is under the control of the *S. cerevisiae* actin gene promoter. Cells of the industrial wine yeast strain T_{73} were transformed with YEpCA1 and, after 48 h of growth, several cycloheximide-resistant transformants arose on the selection plates. In a preliminary screening, randomly selected transformants and one T_{73}/YEpCR21 transformant as a negative control were grown in liquid selective medium for 24 h. Xylanase activity was only detected in T_{73}/YEpCA1 transformants, indicating that the xylanase of the *A. nidulans* signal peptide was efficiently processed by the yeast secretory machinery. The presence of structurally unaltered YEpCA1 was confirmed by plasmid rescue in *E. coli*, followed by a restriction map analysis (data not shown).

One randomly selected transformant named T_{73}/YEpCA1-A was used to study the cellular location of the fungal enzyme in *S. cerevisiae*. The recombinant strain was cultured in liquid selective medium for 72 h and aliquots were taken after various times. The cellular location of X_{22} (extracellular, cell wall or intracellular) was determined in each sample. As can be seen in Table 1, about 90% of the total activity was secreted by the recombinant strain.

3.2. Microvinification experiments

Duplicate microvinification experiments were carried out using the T_{73}/YEpCA1-A strain and the untransformed T_{73} as a control. Samples were periodically collected and reducing sugar concentration and yeast cell counts were measured as a control of the fermentation. As can be seen in Fig. 2A, no difference was observed in yeast viability between the T_{73}/YEpCA1-A strain and the untransformed T_{73} strain. However, a slight difference in the rate of sugar consumption was found between fermentation carried out by T_{73}/YEpCA1-A and that performed by the T_{73} strain (Fig. 2B). The stability was defined as the percentage of colonies appearing in selective medium versus nonselective medium. Only between 5 and 8% of the yeast population lost the xylanase phenotype indicating a high level of mitotic stability of the transformant plasmid.

Fig. 2C shows the xylanase activity during the fermentation carried out with the T_{73}/YEpCA1-A strain. The enzyme was detected from the beginning of the process reaching the greatest increase in the second day, which corresponds to the final of the exponential growing phase. During the stationary phase the xylanase activity decreased to a level of 40% of its maximum, probably due to the proteolytic degradation of the X_{22} in the must.

As can be seen in Table 2, the physicochemical parameters analyzed in the two wines were very similar, demonstrating the feasibility of the technological use of recombinant yeast strains. Similar results were obtained by our group using other recombinant wine yeasts (Pérez-González et al.,

Table 1
Intracellular versus extracellular isocitrate dehydrogenase and xylanase activities in the transformant T_{73}/YEpCA1-A

Time (h)	Isocitrate dehydrogenase		Xylanase	
	Intracellular	Extracellular	Intracellular	Extracellular
24	100	0	10	90
48	100	0	5	95
72	100	0	11	89

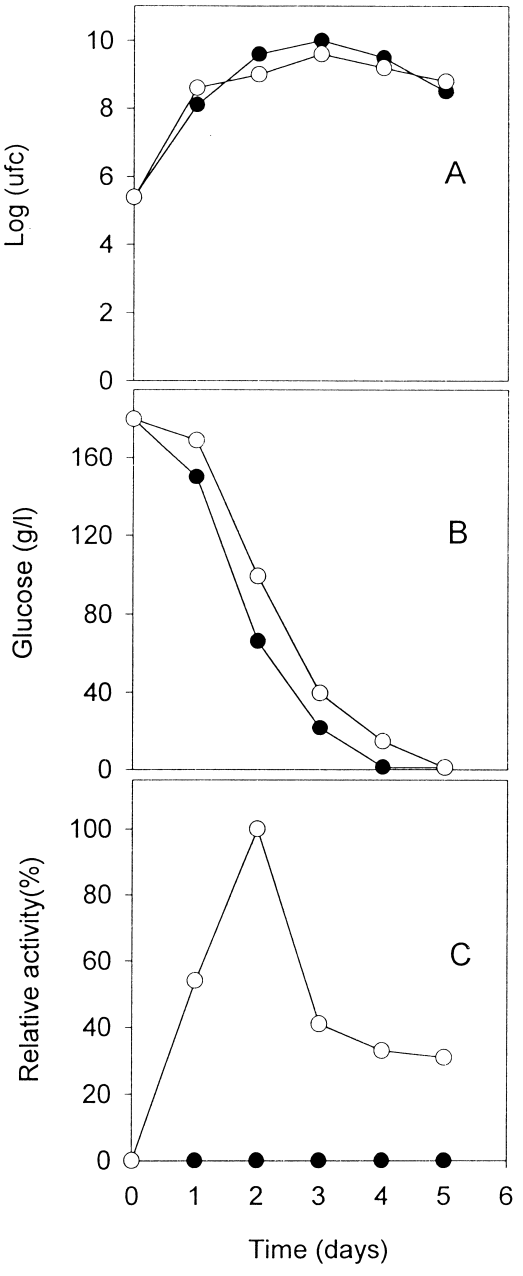

Fig. 2. Time course of *S. cerevisiae* strain T73 growth (A), glucose consumption (B) and xylanase production (C) (—•— T_{73}; —○— T_{73}/YEpCA1-A).

Table 2
Physicochemical analysis of wines obtained by fermentation with T_{73} and T_{73}/YEpCA1-A strains

Parameters	T_{73}	T_{73}/YEpCA1-A
pH	3.30	3.33
Ethanol (vol.%)	11.00	11.00
Total acidity (g/l)	7.31	7.45
Volatile acidity (g/l)	0.12	0.10
Free SO_2 (ppm)	7.00	7.00
Total SO_2 (ppm)	18.00	22.00
Reducing sugars (g/l)	1.00	1.00
Malic acid (g/l)	4.30	4.40
Lactic acid (g/l)	0.013	0.014
Color intensity (A_{420})[a]	0.275	0.240

Reported values represent the average of triplicate analyses.
[a]Optical density at 420 nm of diluted (1:50) wine.

Table 3
Concentration (ppm) of some volatile compounds present in wines produced by the T_{73} and T_{73}/YEpCA1-A strains

Compounds	T_{73}	T_{73}/YEpCA1-A
Acids		
Capric acid	0.80	1.14
Caproic acid	0.34	0.60
Caprylic acid	2.82	2.80
Geranic acid	0.55	1.60
Esters		
Ethyl acetate	20.56	65.42
Ethyl caproate	0.15	0.18
Geranyl acetate	0.04	Not detected
Hexyl acetate	0.06	0.14
Linalyl acetate	0.11	0.44
Neryl acetate	0.72	1.32
Phenylethyl acetate	0.08	0.18
Alcohols		
1-Butanol	0.40	0.50
1-Hexanol	1.26	1.60
3-Methyl-1-butanol	1.74	38.40
1-Pentanol	0.56	0.66
Phenylethanol	22.40	40.80
Phenylmethanol	0.11	1.13
Terpenes		
Citronellol	0.03	0.03
Geraniol	0.02	0.03
Linalool	0.60	2.46
Linalool oxide	1.02	0.90
Terpineol	0.07	0.08

Reported values represent the average of duplicate analyses.

1993; González-Candelas et al., 1995; Sánchez-Torres et al., 1996, 1998). However, the panel of experts detected an increase in fruity aroma in the wine produced by the T_{73}/YEpCA1-A strain. Table 3 shows the concentration of some volatile compounds detected in the wines obtained by the T_{73} or T_{73}/YEpCA1-A strains. The results clearly showed an increase in the concentration for some esters, higher alcohols and terpenes. Similar results were obtained

with the T_{73} strain transformed with the gene encoding the *T. longibrachiatum* β-(1,4)-endoglucanase (Pérez-González et al., 1993). In general, an increase in the content of some alcohols was observed, among them special mention deserves the rise undergone by the 2-phenylethanol. This alcohol has an important role in rose aroma (Rapp, 1986). The presence of a non-volatile conjugated form of the 2-phenylethanol have been also described in the skin and pulp of the grape (Voirin et al., 1990).

The use of cellulases or hemicellulases could be involved in the degradation of the cell wall contributing to the release of this precursors. The esters are other significant components in wine aroma composition. Numerous esters have a fairly low detection odor threshold and they contribute favorably to wine aroma as fruit and flower notes (Jackson, 1994). We observed a significant increase in the case of linalyl acetate, phenylethyl acetate and hexyl acetate in the wines obtained with the T_{73}/YEpCA1-A. Finally, a significant rise in linalol, terpineol and geraniol was observed in the case of T_{73}/YEpCA1-A contributing to enhance the typical floral aroma of wine. In conclusion, xylanolytic recombinant wine yeast is a tool to modify the aromatic profile of a wine.

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