Heterologous Expression of a *Candida molischiana* Anthocyanin-β-glucosidase in a Wine Yeast Strain

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A recombinant wine yeast strain expressing the *Candida molischiana bgln* gene encoding a β-glucosidase/anthocyanase under the control of the *Saccharomyces cerevisiae* actin gene promoter has been constructed. The corresponding protein, BGLN, was mainly located on the cell wall. BGLN was purified in a single chromotagraphic step, and different physicochemical and kinetic properties have been determined. BGLN showed maximum activity against the artificial substrate *p*-nitrophenyl β-D-glucopyranoside. It also hydrolyzed salicin, *p*-nitrophenyl β-D-xyloside, cellobiose, and arbutin to a lesser extent. Fructose and SO_2 did not affect enzyme activity, which was activated by ethanol, while glucose was a strong competitive inhibitor. The purified BGLN showed a novel anthocyanase decolorizing capability on red wines. This anthocyanase activity was readily observed during microvinification experiments. However, the physicochemical characteristics of the wines obtained with the recombinant wine yeast strain were indistinguishable from those obtained with the parental strain.

Keywords: *Wine aroma; anthocyanin-β-glucosidase; Candida molischiana; gene expression*

INTRODUCTION

The fermentation of grape juice into wine is a complex microbiological reaction involving the sequential development of various yeast strains and lactic acid bacteria. In an attempt to address the problem of year to year variability on wine quality (Snow, 1983), many winemakers have used pure *Saccharomyces cerevisiae* cultures isolated from their own growing regions (Querol et al., 1992b). Using mitochondrial DNA restriction analysis, recently we have demonstrated the imposition of an inoculated wine yeast in controlled industrial fermentations (Querol et al., 1992a). From a biotechnological point of view, this predominance opens the way for the use of genetic engineering techniques and the construction of recombinant strains expressing metabolic activities that exert beneficial effects on the organoleptical characteristics of the wine. As examples, wine yeast strains expressing both the K1 and K2 killer toxins (Boone et al., 1990), the L-(+)-lactate dehydrogenase from *Lactobacillus casei* (Dequin and Barre, 1994), the malolactic enzyme from *Lactococcus lactis* (Ansanay et al., 1993; Denayrrolles et al., 1995), and different filamentous fungal enzymes (Pérez-González et al., 1993; González-Candelas et al., 1995; Sánchez-Torres et al., 1996) have recently been constructed.

Pigments in red grape-derived musts are mainly anthocyanins. These phenolic molecules are composed of a flavylium ion glycosylated with a β-glycosidic bond. Breakage of this glycosidic bond liberates the corresponding anthocyanidin, which is spontaneously converted to a colorless pseudobase at pH levels within the range of those found in musts (Huang, 1955). This enzymatic process is carried out by an anthocyanin-β-glucosidase (commonly termed as anthocyanase) and involves a decolorization of the juice (Huang, 1955; Ueda, 1991). Anthocyanase has been detected in some fungal macerating enzyme preparations used in fruit juice extraction and has been purified from certain *Aspergillus niger* strains (Blom, 1983). Potential applications of anthocyanase in winemaking include the prevention of sediments in the bottles during storage and, moreover, obtaining free-run juice from red grape varieties in the production of white wines with lower red color (Shoseyov et al., 1988).

Several β-glucosidase-encoding genes have been expressed in laboratory strains of *S. cerevisiae*. In this paper we report the construction of a recombinant wine yeast strain expressing the previously cloned *Candida molischiana bgln* gene encoding a β-glucosidase (Janbon et al., 1995b). The purification and biochemical properties of the *C. molischiana* enzyme produced in *S. cerevisiae* are described, as are the microvinification experiments using the recombinant strain and its effect on wine color.

MATERIALS AND METHODS

Strains and Culture Conditions. *Escherichia coli* DH5α was used as the recipient strain for cloning experiments. The *S. cerevisiae* industrial wine yeast strain T_{73} (CECT1894) is commercially available from Lallemand Inc. (Montreal, Canada). Its selection, molecular characterization, and growth conditions have been published previously (Querol et al., 1992b). *C. molischiana* CBS136 was used as a source of genomic DNA and was grown in Freer medium (Freer, 1985).

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 by Sherman et al. (1986). *S. cerevisiae* T_{73} transformation and selection procedures were performed as described before (González-Candelas et al., 1995). Restriction enzymes and T4 DNA ligase were purchased from

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Boehringer (Mannheim, Germany), and Dynazyme was obtained from Finnzymes (Espoo, Finland). All of the enzymes were used as recommended by the suppliers.

Construction of Plasmid YCBgln. Construction of the plasmid YCBgln appears in Figure 1 and is summarized as follows. The *C. molischiana bgln* gene was obtained by PCR using the oligonucleotides CMBGL-1 (5′-GGAGTCTAGAC-C***ATG***GAATCAACAATTATC-3′), which contains the translation start codon (shown in bold type and italics), CMBGL-2 (5′-CTGAACAAAGCTTCACTTCTCTCTCGC-3′), CMBGL-3 (5′-GTCCATCAT-GGGATCCTACAACC-3′), and CMBGL-4 (5′-GGTTGTAGGATCCCATGATG-GAC-3′). Two different PCR fragments of 0.75 kb (5′ region of the gene) and 1.85 kb (rest of the gene) were synthesized using 1 μg of genomic DNA, 2 units of Dynazyme, and the oligonucleotides CMBGL-1/CMBGL-4 and CMBGL-2/CMBGL3, respectively. Amplification conditions consisted of 35 cycles of 45 s at 95 °C, 2 min at 52 °C, and 3 min at 72 °C with a final polymerization step of 15 min at 72 °C. The 0.75 and the 1.8 kb fragments were digested with *Nco*I/*Bam*HI and *Bam*HI/*Hin*dIII, respectively. The *S. cerevisiae* actin gene promoter was also obtained by PCR using oligonucleotides ACTSAC1 (5′-GGGACCCGGGTAAGCTGCC-3′) and ACTSAC4 (5′-CATTCTAGAATCC-ATGGTTAATTCAG-3′) and the same amplification conditions described for the *bgln* gene fragments using *S. cerevisiae* chromosomal DNA as template. The amplified fragment was digested with *Sma*I/*Xba*I and ligated to YEplac181 (Gietz and Sugino, 1988) digested with the same enzymes giving the plasmid YEpAct4. This plasmid was digested with *Nco*I and *Hin*dIII and ligated to the previously digested 0.75 and 1.85 kb *C. molischiana* DNA fragments, thus producing plasmid YlBgln, in which the *C. molischiana bgln* gene is under the control of the *S. cerevisiae* actin gene promoter. Digestion of plasmid YlBgln with *Sma*I and *Hin*dIII released the act_p::*bgln* fusion fragment, which was cloned into the *Eco*RV site of plasmid YEpCR21 (Navas et al., 1991), producing plasmid YCBgln.

Cellular Location and Purification of the *C. molischiana* BGLN Protein Produced in *S. cerevisiae*. The cellular location of BGLN in the recombinant T_{73} strains containing YCBgln was investigated following the protocol described previously (González-Candelas et al., 1995). The purification of BGLN was done using the transformant named $YCB3_5$ (deposited in the Spanish Type Culture Collection under accession no. CECT10866) that was grown for 24 h at 30 °C with orbital shaking (200 rpm) in 1 L of selective medium containing 1 μg/mL of cycloheximide (Merck, Darmstadt, Germany). After centrifugation, the supernatant was concentrated to 40 mL in a Minitan ultrafiltration system (Millipore Corp., Bedford, MA) using a 10 000 molecular mass cutoff polysulfone filter. The concentrate was loaded onto a DEAE Bio-Gel A column (4 × 18 cm) equilibrated with 20 mM Bis-Tris buffer (pH 6.8), and the column was washed with 300 mL of the same buffer. Elution of pure BGLN was performed with a linear NaCl concentration gradient (0−0.7 M in Bis-Tris buffer) at a flow rate of 32 mL/h. All steps were carried out at 4 °C.

Enzymatic Assays. β-Glucosidase activity was assayed using 5 mM *p*-nitrophenyl β-D-glucopyranoside (pNPG) as substrate in McIlvaine buffer (pH 5.0) containing appropiate amounts of the sample in a final volume of 250 μL. Incubation was done at 30 °C for 20 min, and the reaction was terminated by adding 0.5 mL of 0.2 mM Na_2CO_3 (pH 10.2). The absorbance was measured at 400 nm (*p*-nitrophenol extinction coefficient = 18 300 M^{-1} cm^{-1}). β-Glucosidase assays during microvinifications were carried out at 37 °C with 20 μL of must, including cells and must, employing conditions described above. Similar conditions were used with other *p*-nitrophenyl derivative substrates. With additional substrates, reactions were terminated by boiling for 10 min, and activity was measured by quantification of glucose using a glucose oxidase kit (Sigma Chemical Co., St. Louis, MO). All of the enzymatic measurements were done in triplicate.

Protein concentration was measured by using the Lowry method with bovine serum albumin as standard (Lowry et al., 1951). Deglycosylation of the pure enzyme was carried out as described by Coligan et al. (1995) using *N*-glycosidase purchased from Boehringer. Protein electrophoresis, determination of kinetic constants, pH and temperature relationships, and the effect of different compounds on the enzymatic activity were performed as described previously (Sánchez-Torres et al., 1996).

Table 1. Location of β-Glucosidase Activity in Transformant $YCB3_5$ Grown in Selective Medium

time (h)	OD_{600}	total activity (nkat/mL)	% of activity		
			extracellular	cell wall	intracellular
16	0.73	4.30	15.0	80.5	4.5
24	3.55	8.69	17.1	69.2	13.7
40	3.71	8.58	18.7	69.1	12.2
48	3.19	10.88	15.4	50.9	33.7
64	3.97	10.43	13.2	64.9	21.9
72	5.71	12.12	14.9	60.2	24.9
88	4.16	10.57	17.2	57.3	25.4

Microvinification Experiments. Microvinifications and enological assays were done in duplicate following the protocols described before (Sánchez-Torres et al., 1996). Microvinifications were done using the T_{73} strain or its derivatives (YCA_1 and $YCB3_5$). The YCA_1 strain was used as a control because it contains the same vector used for the construction of the $YCB3_5$ strain. Novoferm 12G (Novo Nordisk Ferment Ltd., Switzerland) was a gift from A. Cortell (Vinival, Spain). Wine color experiments were done by incubation of 2.05 nkat of pure BGLN or Novoferm 12G with 14 mL of Bobal rosé wine for 6 days at 20 °C. As a control, enzymes were denatured by heating at 100 °C for 15 min. Wine color was determined by measuring absorbance at 520 nm.

RESULTS

Construction of a Recombinant Wine Yeast Strain Expressing the *C. molischiana bgln* Gene. Plasmid YCBgln was constructed as described under Materials and Methods (see Figure 1). In this plasmid the *C. molischiana bgln* gene is under the control of the *S. cerevisiae* actin gene promoter. Cells of the industrial wine yeast strain T_{73} were transformed with YCBgln and, after 48 h of growth, several cycloheximide-resistant transformants arose on selective plates. Twenty-four randomly selected transformants and one YEpCR21 transformant (negative control) were grown in liquid selective medium for 24 h, and β-glucosidase activity was determined in the culture filtrates. Activity was only detected in YCBgln transformants and 10 of them, named $YCB3_1$ to $YCB3_{10}$, were chosen for further studies. The presence of intact YCBgln molecules in all of these transformants was confirmed by retransformation in *E. coli* and plasmid restriction map with the exception of the $YCB3_6$ transformant, which gave a complex pattern of reorganization and was discarded for further work.

The cellular location of the BGLN protein (extracellular, cell wall, or intracellular) was studied in the nine selected transformants. β-Glucosidase activity was mainly associated with cell wall (50−80%), but a minor fraction was detected in the culture filtrate (15%) or intracellular (5−35%). The transformant $YCB3_5$ was selected for a further time course experiment of BGLN cellular location (Table 1) and, independent of the growth stage, most of the β-glucosidase activity was cell wall bound.

Purification and Biochemical Characterization of the *C. molischiana* BGLN Produced in *S. cerevisiae*. BGLN was purified from transformant $YCB3_5$ as described under Materials and Methods. The single

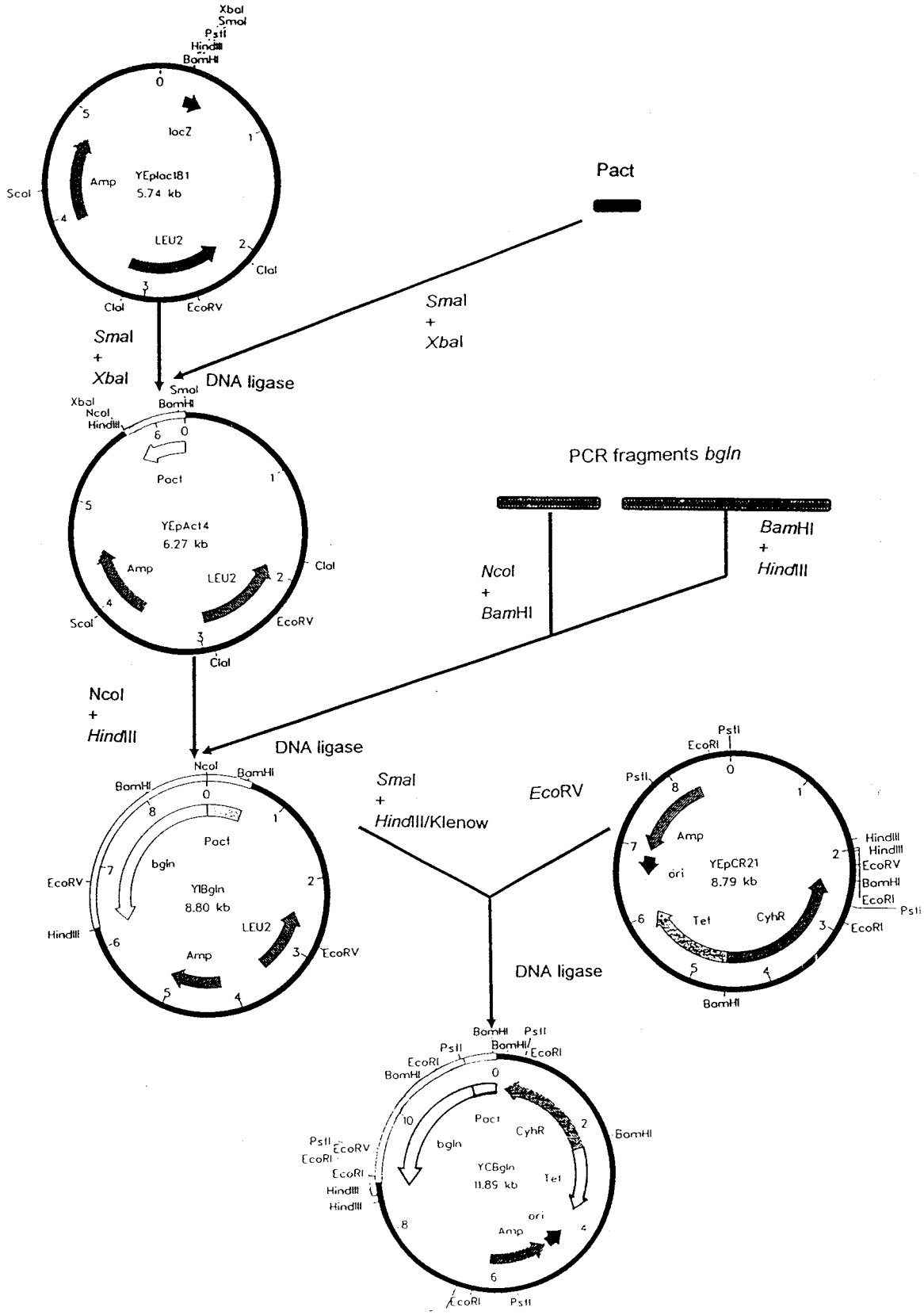

Figure 1. Strategy used for the construction of plasmid YCBgln. Details are given under Materials and Methods.

DEAE Bio-Gel A purification step yielded a pure BGLN fraction representing 52% of the total activity present in the culture filtrate. Protein degradation was detected during the purification procedure. As can be seen in Figure 2, the purified BGLN runs as a diffused band with an apparent molecular mass higher the 94 kDa described for the protein purified from *C. molischiana* 35 (Vasserot et al., 1991). Nevertheless, treatment with endo-H of the recombinant BGLN resulted in a reduction in apparent molecular mass to a size of 94 kDa. The optimal pH and temperature determined for the BGLN produced by *S. cerevisiae* were 4.5 and 60 °C,

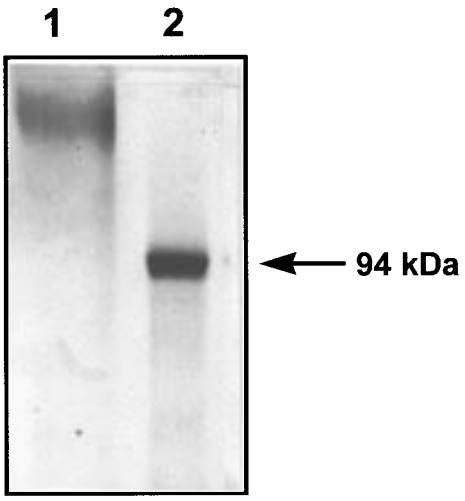

Figure 2. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of purified BGLN protein from $YCB3_5$ strain: lane 1, 20 μg of BGLN; lane 2, 20 μg of BGLN treated with endo-H.

Table 2. Activity of BGLN Protein Purified from $YCB3_5$ Transformant against Different Substrates[a]

substrate	type of bond	concn (mM)	% of activity
pNPG	β-(1,4)	5	100.00 ± 2.25
salicin	β-(1,4)	20	19.98 ± 0.55
arbutin	β-(1,4)	20	1.81 ± 0.14
cellobiose	β-(1,4)	50	9.11 ± 1.30
sophorose	β-(1,2)	50	ND
gentibiose	β-(1,6)	50	ND
laminaribiose	β-(1,3)	10	ND
maltose	α-(1,4)	50	ND
lactose	β-(1,4)	50	ND
sucrose	β-(1,2)	50	ND
pNPX	β-(1,4)	2	16.60 ± 1.00
pNPA	α-(1,6)	2	ND

[a] The percentage of activity refers to the value obtained using pNPG as substrate. Results represent the average and standard deviation of three different experiments. ND, not detected.

respectively. Using pNPG as substrate, the K_m and V_{max} values were 0.25 ± 0.01 mM and 25.89 ± 0.29 nkat/mg, respectively.

BGLN substrate specificity against different compounds was investigated. BGLN showed activity only toward arbutin, cellobiose, salicin, pNPG, and pNPX (Table 2). The influence of divalent metal cations (Co^{2+}, Cu^{2+}, Hg^{2+}, Mo^{2+}, and Zn^{2+}) and putative inhibitors such as DTT or EDTA at 10 mM on β-glucosidase activity was also assayed. Only EDTA (34%) and Hg^{2+} (91.5%) inhibited the enzyme. Certain factors such as fructose, glucose, ethanol, or SO_2 concentration that may affect enzyme activity under enological conditions were also investigated (Figure 3). Ethanol was found to be an activator up to 20% (w/v). Fructose up to 0.5 M or SO_2 to a level of 150 ppm, concentrations that can be found at different stages during wine fermentations, did not significantly affect the activity. However, glucose concentration strongly affected β-glucosidase activity. Glucose effect was studied showing a competitive type inhibition with a K_i of 0.63 ± 0.04 mM.

Microvinification Experiments. Duplicate experiments were carried out using the untransformed T_{73} strain and the transformant $YCB3_5$. Reducing sugar concentration was followed as a marker of the fermentation progress and, as can be seen in Figure 4A, no significant differences were found between the wines

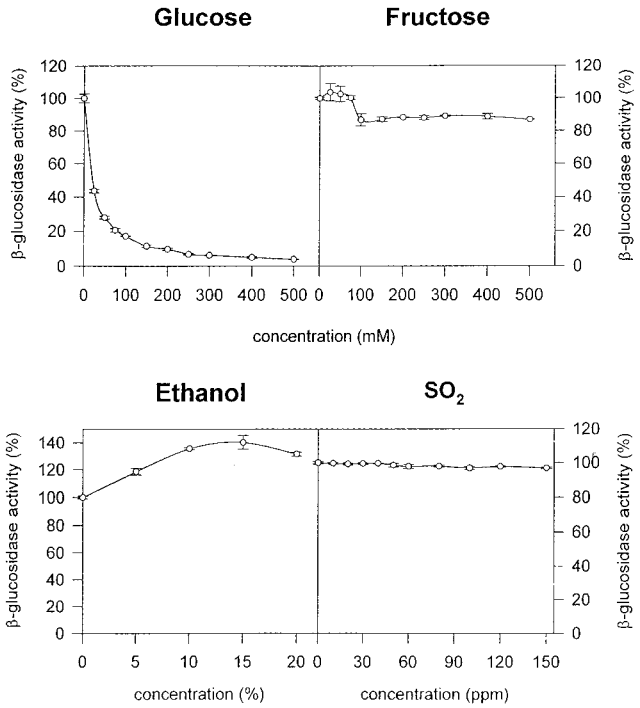

Figure 3. Effect of different enological parameters on β-glucosidase activity using pure BGLN protein.

produced by the $YCB3_5$ transformant and by the T_{73} strain. Population dynamics of the inoculated yeast were similar for both strains (Figure 4B), although the percentage of cycloheximide-resistant cells (determined in $YCB3_5$ microvinifications) declined throughout the fermentation due to plasmid loss and/or decreased cell viability on selective medium. In this sense, experiments to integrate the β-glucosidase expression cassette into the genome of the T_{73} strain are now in progress. In the case of the must inoculated with the $YCB3_5$, strain it was possible to detect β-glucosidase activity during all stages of the process, but the greatest increase in enzyme activity took place during the first 2 days of fermentation (Figure 4C). A residual β-glucosidase activity was detected only at the third day of fermentation on musts inoculated with the T_{73} strain. As can be seen in Table 3, the physicochemical parameters analyzed in the four wines were very similar.

Wine Color Decrease by BGLN. Parallel experiments were carried out to study the effect on wine color of the addition of pure BGLN to rosé wines in comparison to Novoferm 12G (Figure 5). Only in the case of the wine treated with nondenatured Bgln was it possible to detect a reduction on wine color around 30% (Figure 5B). Prompted by these results, microvinification experiments on Bobal red musts were done with T_{73}, $YCB3_5$, and YCA_1 [a strain isogenic to $YCB3_5$ but containing the *Aspergillus niger abfB* gene instead of *bgln*; for details see Sánchez-Torres et al. (1996)] strains. As can be seen in Figure 6, a strong decolorization was observed in the wine inoculated with the $YCB3_5$ strain.

DISCUSSION

A wine yeast strain producing an active *C. molischiana* β-glucosidase activity has been constructed. The recombinant strain, $YCB3_5$, produces wines with the same physicochemical parameters as those produced with the recipient strain, demonstrating the feasibility of using recombinant yeast strains from a technological

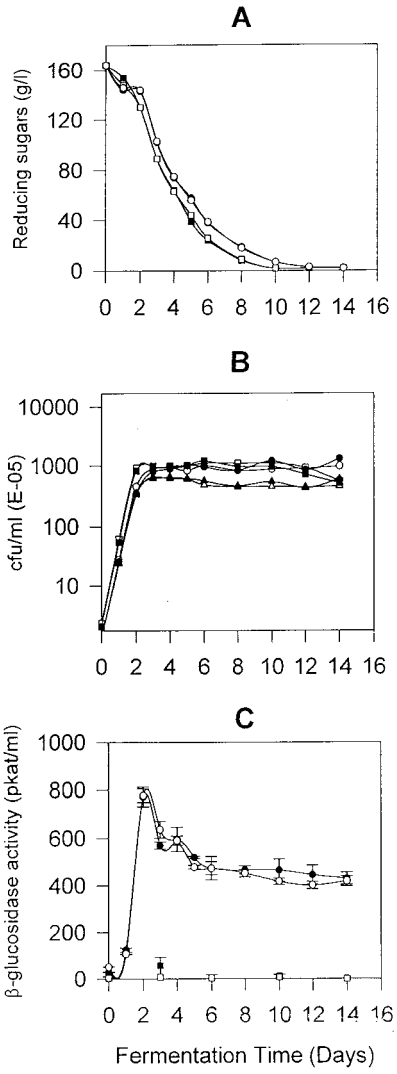

Figure 4. Microvinification experiments using strains T_{73} and $YCB3_5$: (A) consumption of reducing sugars; (B) growth curve of inoculated strains; (C) β-glucosidase activity throughout microvinifications. Open and solid symbols correspond to duplicate microvinifications. Squares and circles represent T_{73} and $YCB3_5$ microvinifications, respectively. Triangles represent cycloheximide resistance cell number for $YCB3_5$ microvinifications. Bars represent standard deviation of triplicate measurements.

Table 3. Physicochemical Analysis of Duplicate Microvinifications, Labeled 1 and 2, Carried out with Strains T_{73} and $YCB3_5$

	strain			
	T_{73}-1	T_{73}-2	$YCB3_5$-1	$YCB3_5$-2
density at 20 °C	0.9912	0.9912	0.9918	0.9917
ethanol (% vol)	12.39	12.44	12.36	12.33
total acidity (g/L)	4.47	4.23	4.07	3.64
volatile acidity (g/L)	0.20	0.21	0.21	0.27
free SO_2 (mg/L)	13	10	17	17
total SO_2 (mg/L)	47	47	56	57
pH	3.76	3.79	3.83	3.83
reducing sugars (g/L)	1.20	1.10	1.60	1.50
acetaldehyde (mg/L)	43	43	42	40

point of view. This confirms previous findings of our group using cellulolytic and hemicellulolytic recombinant yeasts strains (Pérez-González et al., 1993; González-Candelas et al., 1995; Sánchez-Torres et al., 1996), as well as the results of other groups using other genetically modified yeast strains [for a review see Querol and Ramón (1996)].

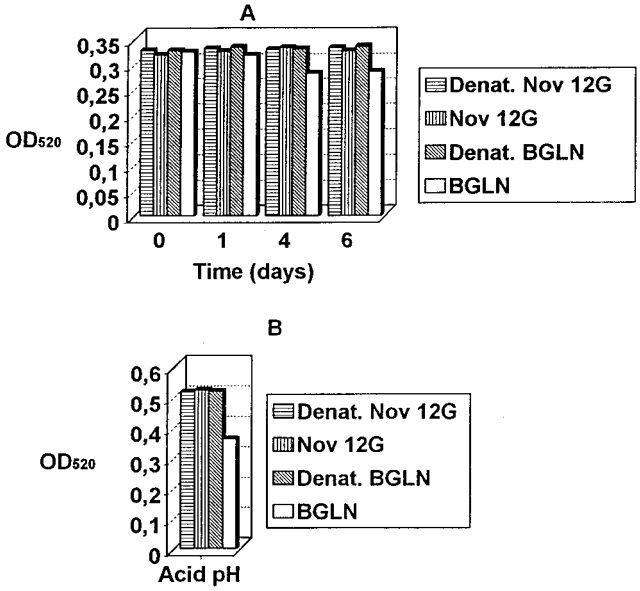

Figure 5. β-Glucosidase effect on wine color: (A) absorbance at 520 nm of rosé wine aliquots incubated with pure BGLN and Novoferm 12G; (B) absorbance at 520 nm of wine samples incubated with pure BGLN and Novoferm 12G for 6 days and acidified to pH 1.5 with HCl.

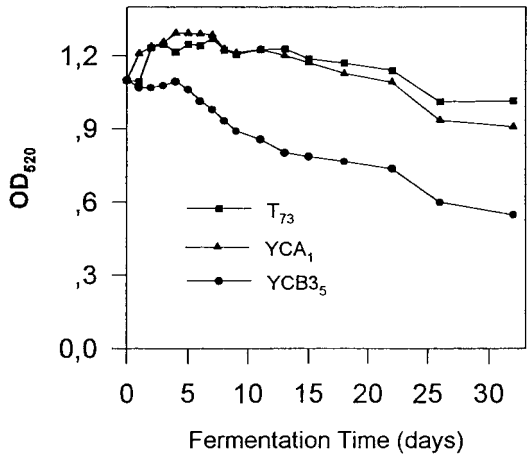

Figure 6. Evolution of red wine color throughout microvinifications carried out with different strains.

In microvinification experiments conducted with the transformant $YCB3_5$, β-glucosidase activity was detected from the beginning of the fermentation in agreement with the constitutive expression of the *bgln* gene driven by the *S. cerevisiae* actin gene promoter. In the $YCB3_5$ transformant a high percentage of BGLN was located in the cell wall. This poor secretion of the heterologous protein contrasts with the previously reported efficient secretion of the *A. niger abfB* (Sánchez-Torres et al., 1996) or the *Fusarium solani* f. sp. *pisi pel*A (González-Candelas et al., 1995) gene products in the same wine yeast. Some authors are of the opinion that *C. molischiana* BGLN is a highly glycosylated protein (Vasserot et al., 1991). As can be seen in Figure 2, this hyperglycosylation was also detected in the case of the protein produced by the recombinant T_{73} strain and could be an explanation for the lack of its efficient secretion. Nevertheless, the amount of active BGLN in the musts was sufficient for technological purposes (see Figure 6).

The $YCB3_5$ strain can be used as a heterologous host to specifically produce BGLN. Using supernatant cultures of this strain, >50% of the β-glucosidase activity was purified in only one step. The purified protein showed enological properties such as its optimal pH value, activation by ethanol, and lack of inhibition by fructose or SO_2 at the common concentrations found on wine fermentations. On the contrary, the purified activity was strongly inhibited in vitro by glucose (Figure 3). Surprisingly, this inhibition was not detected during the technological process because color reduction was observed from the first days of $YCB3_5$ vinification (when glucose concentration was high). Moreover, the fact that β-glucosidase activity was detected throughout the fermentation, even when reducing sugar concentration reached a basal level at the end of the process, ensured the technological effect of the enzyme.

Extracellular β-glucosidases from *C. molischiana* strains CBS136, 35, and 35M5N have been purified and characterized. The former showed quite different properties with respect to the other two β-glucosidases, possibly indicating the capability of *C. molischiana* to produce different β-glucosidases (Gondé et al., 1985; Janbon et al., 1995a; Vasserot et al., 1991). BGLN protein purified from strain $YCB3_5$ showed different properties, such as molecular mass and substrate specificity against arbutin and salicin, when compared with the protein purified from *C. molischiana* CBS136 (Gondé et al., 1985). These differences could reflect the existence of at least two different β-glucosidase-encoding genes. However, the kinetic characteristics of the *C. molischiana* 35 protein (Vasserot et al., 1991), the amino acid sequence of which was the basis for the cloning of the *bgln* gene (Janbon et al., 1995a), showed also striking differences with BGLN from $YCB3_5$, when in theory both proteins are encoded by the same *bgln* gene. To detect possible polymerization mistakes introduced by the *Taq* polymerase during the PCR reactions, ≈1700 nucleotides (nt) *bgln* gene were sequenced, including 1450 nt from the coding region (results not shown). Only one nucleotide substitution was detected involving a change of glutamine for lysine at residue 261. Whether this change was due to a polymerase error was not clear, since a glutamine residue was also found in the β-glucosidases from *Kluyveromyces fragilis* and *Candida pelliculosa* in the equivalent position (Rojas and Romeu, 1996). Using the information previously published by Janbon et al. (1995b), a detailed inspection of the *bgln* encoded protein showed differences with the sequences obtained by the same group using direct sequence of the purified protein. A logical explanation to these contradictory results could be the presence of more than one β-glucosidase encoding gene in the *C. molischiana*. To test this possibility, a Southern analysis of *C. molischiana* was carried out using a conserved region of the *bgln* gene with respect to the homologous gene from *C. pelliculosa* as a probe. Several bands which probably correspond to different β-glucosidase encoding genes were detected (results not shown). At present we are cloning some of these putative genes. Nevertheless, the possible difference in glycosylation of both proteins could also account for the observed differences.

The availability of BGLN protein and the recombinant $YCB3_5$ strain to decrease wine color is of industrial relevance, mainly in the production of white wines with lower red color such as in the production of blush wines from red grape varieties. In these wines, maceration could be prolonged and the excess color removed by enzymatic treatment instead of using activated charcoal. Other possible technological applications of this recombinant yeast such as obtaining wines with higher levels of resveratrol and terpenols (obtained through the hydrolysis of their glycosidic precursors) are under investigation. Industrial pilot plant vinifications need to be carried out to confirm these observations and to further elucidate the role of BGLN in the fermentation processes.

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