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A Recombinant *Saccharomyces cerevisiae* Strain Overproducing Mannoproteins Stabilizes Wine against Protein Haze▽

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Stabilization against protein haze was one of the first positive properties attributed to yeast mannoproteins in winemaking. In previous work we demonstrated that deletion of *KNR4* leads to increased mannoprotein release in laboratory *Saccharomyces cerevisiae* strains. We have now constructed strains with *KNR4* deleted in two different industrial wine yeast backgrounds. This required replacement of two and three alleles of *KNR4* for the EC1118 and T73-4 backgrounds, respectively, and the use of three different selection markers for yeast genetic transformation. The actual effect of the genetic modification was dependent on both the genetic background and the culture conditions. The fermentation performance of T73-4 derivatives was clearly impaired, and these derivatives did not contribute to the protein stability of the wine, even though they showed increased mannoprotein release in vitro. In contrast, the EC1118 derivative with both alleles of *KNR4* deleted released increased amounts of mannoproteins both in vitro and during wine fermentation assays, and the resulting wines were consistently less susceptible to protein haze. The fermentation performance of this strain was slightly impaired, but only with must with a very high sugar content. These results pave the way for the development of new commercial strains with the potential to improve several mannoprotein-related quality and technological parameters of wine.

During the alcoholic fermentation of grape must, *Saccharomyces cerevisiae* ferments sugars to ethanol and other metabolites, such as glycerol, acetate, succinate, pyruvate, and several esters, all of which contribute to the sensorial properties of wine. In addition, yeast cells release cell constituents, such as proteins or polysaccharides, which also contribute to the quality of wine (13). Macromolecules derived from the yeast cell wall, particularly mannoproteins, have attracted much attention in the winemaking world for the past 15 years due to their reported contribution to wine quality and chemical stability (5). Chemical stabilization and, more specifically, protection against protein haze in white wines were some of the first enological properties described for mannoproteins. In some white wines, grape proteins aggregate and precipitate due to high temperatures or long storage time. The haziness may be perceived as spoilage by the consumer (49). Wines aged with yeast lees have lower haze potential than wines aged without lees, and this is due to the protective effect of the mannoproteins released from yeast cell walls (30). In fact, addition of some mannoproteins to wine results in higher protein stability, and it has even been possible to identify specific contributions of particular mannoproteins to wine quality (7, 30, 37, 48). Moine-Ledoux and Dubourdieu (37) identified a 32-kDa fragment of *S. cerevisiae* invertase capable of reducing protein haze in white wines, and similar properties were observed for the intact protein (7). Other yeast cell wall proteins have been shown to stabilize wine against protein haze (47, 48). Brown et al. (3) cloned and overexpressed in *S. cerevisiae* laboratory strains YOL155c and YDR055w coding for Hpf1p and Hpf2p (haze-protecting factors), respectively, and showed that the overproduced Hpf2p protein reduced turbidity by up to 40% when it was added to wine. There have been no previous reports of recombinant wine yeast strains engineered for increased mannoprotein release during the fermentation of grape juice.

Other desirable enological properties of mannoproteins include protection against tartaric instability (10), retention of aroma compounds (33, 50), reduction of astringency (8), increased sweetness (18), and increased bodyness and mouthfeel (18, 33, 43, 46), which are especially appreciated in red wines. Furthermore, mannoproteins stimulate the growth of lactic acid bacteria and consequently malolactic fermentation (19, 41), and they improve the foam quality of sparkling wines (9, 11).

The yeast strain is one of the factors that control the amount of mannoproteins released during winemaking. Therefore, there is increasing interest in selection and development of wine yeast strains that release large amounts of mannoproteins. The aim of this work was to take advantage of the current knowledge concerning *S. cerevisiae* cell wall biology, together with our previous results, to genetically engineer wine yeast strains for increased mannoprotein release.

The cell wall of *S. cerevisiae* is a semirigid structure that is required for stabilization of internal osmotic conditions, for protection against physical stress, for maintenance of the cell shape, and as a scaffold for surface glycoproteins (28). It consist of two layers; the inner layer contains β-1,3-glucan (50 to 55% of the cell wall dry weight) and chitin (1 to 2%), and the external layer contains mainly mannoproteins (35 to 40%) and β-1,6-glucan (25). Cell wall mannoproteins are covalently linked to the β-1,3-glucan, either directly or indirectly, through a β-1,6-glucan linker, and most of them are attached to the cell

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wall through a glycosylphosphatidylinositol anchor remnant (27).

The synthesis and organization of the cell wall are highly complex processes that directly or indirectly involve about 1,200 genes (25, 31). In yeast, the PKC1-SLT2 mitogen-activated protein (MAP) kinase module is considered the main signaling pathway essential for proper cell wall construction and for preventing cell lysis. Four protein kinases are involved in this MAP kinase module, and the protein encoded by SLT2/MPK1 is the last element of the module (21, 32). The phosphorylated, active Slt2/Mpk1p kinase enters the nucleus and activates the transcription of genes involved in cell wall remodeling. This induction occurs through activation of the transcription factor Rlm1p and contributes to cell cycle transient arrest via activation of the Swi4p element of the SBF complex factor (20). Knr4p is a protein necessary for correct targeting of the Stl2 MAP kinase to its two known downstream transcriptional targets, Rlm1p and Swi4p (36), and it participates in the coordination of cell wall synthesis with bud emergence (2).

KNR4 (*k*iller *n*ine *r*esistant; also known as *SMI1*) was initially isolated by Hong et al. (22) in a search for mutations affecting β-1,3-glucan biosynthesis. The selection criterion was resistance to k9, a killer toxin that inhibits cell wall synthesis and β-1,3-glucan synthase activity (51, 52). In another study, it was shown that additional copies of *KNR4* can suppress the calcofluor white hypersensitivity of several *cwh* mutants (35). Loss of *KNR4* leads to altered cell wall structure and composition; the amount of chitin is increased fourfold, whereas the β-glucan activity and β-glucan levels are reduced by 50% (23). Δ*knr4* cells are also hypersensitive to caffeine, sodium dodecyl sulfate, Congo red, calcofluor white, caspofungin, and cercosporamide, and growth is arrested with a small bud at temperatures above 37°C (12, 23, 35, 34).

In previous work we showed that deletion of *KNR4* in laboratory *S. cerevisiae* strains results in the release of increased amounts of mannoproteins (17). This phenotype is recessive and dependent on the genetic background. Supernatants of cultures of laboratory yeast strains lacking all copies of *KNR4* contain more mannoproteins than supernatants of cultures of the control strain, even at the beginning of the stationary phase. In this work we constructed wine yeast strains having deletions of some or all of the copies of *KNR4*. We show here that a wine fermented with a yeast strain lacking *KNR4* is enriched in mannoproteins at the end of the fermentation and more stable against protein haze.

MATERIALS AND METHODS

Strains, media, and culture conditions. EC1118 is a widely used wine yeast strain commercialized by Lallemand Inc. (Montreal, Canada). T73-4 is a uridine auxotroph derived from the winemaking strain T73 (39). *URA3* is defective, but most of the open reading frame is still present in this strain. The yeast strains generated in this work are listed in Table 1. *Escherichia coli* strain DH5α (*supE44* Δ*lacU169* [ϕ80 *lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for construction and amplification of the plasmids employed in this study. Several media were used in this work, as follows: YPD broth (2% glucose, 2% peptone, 1% yeast extract), YPD plates (YPD broth plus 2% agar), GCY (2% glucose, 2% Bacto Casamino Acids [BD, Sparks, MD], 0.67% Difco yeast nitrogen base [BD]), SD-PFP plates (0.67% yeast nitrogen base without amino acids [Difco Laboratories Inc., Detroit, MI], 2% dextrose, 1.67% purified agar, 0.9 g/liter L-tyrosine, 2 g/liter *p*-fluoro-DL-phenylalanine [PFP]), YPD-G418 plates (YPD plates plus 40 μg G418/ml), SD-Ura plates (0.67% Difco yeast nitrogen base [BD], 2% glucose, 1.67% purified agar), YPD-BCIP (YPD plates supplemented with 40 mg/liter 5-bromo-4-chloro-3-indolylphosphate [BCIP]) (Sigma-Aldrich, Spain), synthetic must (20% glucose, 0.6% malic acid, 0.3% tartaric acid, 0.03% citric acid, 0.67% Difco yeast nitrogen base [BD] without amino acids; pH adjusted to 3.5 with KOH), and natural Sauvignon Blanc must (Sauvignon Blanc grape berries were pressed, and potassium metabisulfite was added to the juice to a final concentration of 60 mg/liter).

TABLE 1. Strains constructed in this study

Strain[a]	Genotype
TKD/2-1	T73-4 *knr4*::*ARO4-OFP*/*KNR4*/*KNR4*
TKD-13	T73-4 *knr4*::*ARO4-OFP*/*knr4*::*KanMX4*/*KNR4*
TKD-123	T73-4 *knr4*::*ARO4-OFP*/*knr4*::*KanMX4*/*knr4*::*URA3*
EKD/2-1	EC1118 *knr4*::*ARO4-OFP*/*KNR4*
EKD-13	EC1118 *knr4*::*ARO4-OFP*/*knr4*::*KanMX4*

[a] Strains TKD-123 and EKD-13 do not have any of the original *KNR4* alleles.

For quantification of the mannoprotein released, yeast cells were grown in GCY. Each strain was inoculated from a fresh preculture into the same medium to obtain a starting optical density at 600 nm (OD_{600}) of 0.1 and incubated at 30°C and 150 rpm up to the stationary phase. The media were then recovered by centrifugation for analysis of the polysaccharide content.

For the fermentation experiments, precultures were grown in YPD broth, and must (50 ml) was inoculated to obtain a final concentration of 10^6 cells/ml. Fermentation of Sauvignon Blanc or synthetic must was carried out at 20°C in Erlenmeyer flasks closed with Müller valves. The fermentation time course was monitored by determining the CO_2 production, expressed as weight loss, until the weight was constant. Wines were then recovered, and yeast cells were removed by centrifugation.

Molecular biology techniques. Unless otherwise specified, general molecular biology techniques were used to construct the deletion cassettes (42). The greater-mannoprotein-release phenotype is recessive so it was necessary to delete all the copies of genes present in both strains. Different markers were used to delete each copy, *ARO4-OFP*, and *KanMX4*, and it was also necessary to use *URA3* to replace the third copy of *KNR4* present in strain T73-4. The deletion cassettes consisted of the marker gene flanked by 500 bp corresponding to the promoter and terminator regions of *KNR4.* First, *ARO4-OFP* was isolated from plasmid pEA2 (6) by digestion with SacI and BamHI and inserted by ligation into pUC19 digested with the same restriction enzymes. The resulting plasmid was designated pUCARO. The promoter and terminator regions of *KNR4* were PCR amplified with primer pairs PKARO-f/PKARO-r and TKARO-f/TKARO-r, respectively (Table 2). The two inserts were cloned in pUCARO by using the primer extension technique (15), and the resulting plasmid was designated pDKNR4-1. *URA3* was amplified with primers RURA-f and RURA-r (Table 2) using genomic DNA of EC1118 as the template and cloned in pDKNR4-1, replacing *ARO4-OFP* by the primer extension technique. The resulting plasmid was designated pDKNR4-2. Finally, *KanMX4* was PCR amplified with primers RKAN3-f and RKAN3-r (Table 2) using the pITGPCR3 plasmid as the template (45) and was cloned in pDKNR4-1. The resulting plasmid was designated pDKNR4-3.

For yeast transformation experiments, the deletion cassettes were PCR amplified from the cognate plasmid using primers PKARO-r and TKARO-f (Table 2).

Yeast transformation and analysis of transformants. Transformation of *S. cerevisiae* was carried out by the lithium acetate method described by Ito et al. (24), as modified by Agatep et al. (http://home.cc.umanitoba.ca/~gietz/2HS.html). Briefly, yeast strains were transformed with 20 μl of the appropriate PCR amplification reaction mixture (see above). For the *ARO4-OFP* marker, after the transformation experiment, cells were diluted 10-fold in YPD broth and incubated for 17 h at 30°C and 200 rpm to allow expression of the resistance allele before the selective pressure was applied. Transformants were selected on SD-PFP plates after 5 days of incubation at 30°C (6). For the *KanMX4* marker, cells were diluted twofold in YPD broth and incubated for 1 h at 30°C and 200 rpm to allow expression of the resistance allele. Transformants were selected on YPD-G418 after 2 days of incubation at 30°C (45). Finally, for the *URA3* marker, transformants were directly selected on SD-Ura plates incubated for 2 days at 30°C.

The resistance phenotypes of transformants were confirmed by replica plating on selection media, and positive strains were grown in YPD broth at 30°C and 200 rpm. Genomic DNA was extracted as described by Querol et al. (40). Integration into the *KNR4* locus of the cassette containing the *ARO4-OFP* marker was checked by real-time PCR using the ABI Prism 7500 fast real-time PCR system (Applied Biosystems). The primers used were PromARO4Q and TermKNR4Q (Table 2) targeting the 3′ end of the insertion. Correct insertion

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)
TKARO-f	CCAGTCACGACGTTGTAAAACGACGGCTTGGACCACTGAGCCCTATTTG
TKARO-r	GGTACCGAGCTCGAATTCACTGGAAATATCACAATTAACATTCTACAAC
PKARO-f	GGATCCTCTAGAGTCGACCTGCCTGCCAAGTTGTCGCCTATAGAACG
PKARO-r	GATTACGCCAAGCTTGCATGCCTGCTTCCAAAGCCCTATTGGAGGTCG
RURA3-f	TCTAGAGGATCCCCCATGGCGATTCGGTAATCTCCGAACAGAAG
RURA3-r	CCAGTGAATTCGAGCTCGGTACCGGGTAATAACTGATATAATTAAATTG AAGCTCTAAT
RKAN3-f	TCTAGAGGATCCCCCATGGCTATCACGAGGCCCTTTCGTC
RKAN3-r	CCAGTGAATTCGAGCTCGGTACCTCGATGATAAGCTGTCAAACATGAG
PromARO4Q	CCACGGCTAATTAGGTGATCATG
TermKNR4	CGAAAACCCAATTACCATAAGC
CDKNR-f	ACGTGACATATGTCATTACCCTAGATTAC
CDKNR-r	GGTTCATGCTCTTCAATGTCGTTAC

was additionally confirmed by PCR amplification of the whole locus using primers CDKNR-f and CDKNR-r (Table 2), and the amplicon size was verified by agarose gel electrophoresis. Insertion of the other selection markers was directly analyzed in this way. The expected sizes for the four different amplicons are as follows: wild-type locus, 2,512 bp; DKNR4-1 replaced, 2,974 bp; DNKR4-2′ replaced, 2,070 bp; and DKNR4-3′ replaced, 2,647 bp.

Quantification of total mannoproteins and polysaccharides. Macromolecules in the supernatants of cultures grown in GCY were separated from monosaccharides by gel filtration using Econo-Pac columns (Bio-Rad, Alcobendas, Spain) by following the recommendations of the manufacturer. The concentration of total mannoproteins and polysaccharides in the eluted fraction was determined by using a standard curve for commercial mannan (Sigma, Tres Cantos, Spain) and the phenol-sulfuric acid method described by Segarra et al. (44). Five replicates were performed for each determination, and data were analyzed by using one-way analysis of variance and the Dunett test for comparison of means using the SPSS 13.0 software. The glucose and mannose content of yeast polysaccharides, either released into the medium (after freeze-drying) or in whole cells (dried for 24 h at 50°C), was estimated by acid hydrolysis as described previously (14), followed by high-performance liquid chromatography (HPLC) analysis of the released monosaccharides. A Thermo chromatograph (Thermo Electron Corporation, Waltham, MA) equipped with a P400 SpectraSystem pump, an AS3000 autosampler, and a Thermo SpectraSystem RI150 refraction index detector was used. The column was an HPX-87H column (Bio-Rad). The conditions used in the analysis were as follows: eluent, 1.5 mM H_2SO_4; flux, 0.6 ml/min; and temperature of the oven, 50°C. Samples were filtered through a 0.45-μm polyvinylidene difluoride filter (Teknokroma, Spain).

Concanavalin A detection. The proteins present in the supernatants of cultures in GCY or in Sauvignon Blanc wine were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29). Portions (10 μl) of supernatants from the different cultures were loaded into the wells. The proteins were transferred to a nitrocellulose membrane using the Mini Protean transfer system (Bio-Rad) by following the directions of the manufacturer. The mannoproteins present in the membrane were detected by use of peroxidase-conjugated concanavalin A (Sigma) as described by Klis et al. (26).

Protein haze analysis. The stability of wines was assayed by incubating 5-ml aliquots at 85°C for 30 min and cooling them on ice. The turbidity of wines was determined with a nephelometer (Hach, Loveland, CO), and data from nine replicates were analyzed by using analysis of variance as described above.

HPLC analysis of wines and grape juice. Samples from fermentation experiments were analyzed by HPLC in order to determine the amounts of sugars, glycerol, and ethanol. The chromatographic conditions used were those described above for acid-released monosaccharides. Samples were filtered through a 0.45-μm polyvinylidene difluoride filter, diluted 2- or 10-fold, and injected in duplicate.

Autolytic phenotype. Release of active alkaline phosphatase into the medium (4, 38) was used as an indicator of autolysis. The medium used for this purpose was YPD-BCIP (16). Strains were grown in YPD broth at 30°C and 150 rpm to stationary phase. The cell concentration was determined by microscope counting, and dilution was performed to obtain concentrations of 10^6, 10^5, 10^4, and 10^3 cells/ml. Five-microliter portions of these dilutions were applied onto YPD-BCIP, and the plates were incubated at 20°C for 5 days. Colonies were checked for a blue color during incubation.

RESULTS

Construction of Δ*KNR4* derivatives. For EC1118, the first transformation was performed using the DKNR4-1 deletion cassette (containing the marker gene *ARO4-OFP*), and 15 transformants were analyzed. Seven of these transformants were positive by real-time PCR amplification, as described in Materials and Methods and further confirmed by amplifying the whole locus with primers CDKNR-f and CDKNR-r. Two bands were observed for all of the transformants, one corresponding to the wild-type allele and the other corresponding to the copy replaced by *ARO4-OFP* (Fig. 1, lane 6). One of the strains, EKD/2-1, was selected and transformed with the DKNR4-3 deletion cassette (containing the marker gene *KanMX4*). Eleven transformants were checked for double resistance, and the *KNR4* locus was amplified by performing PCR with primers CDKNR-f and CDKNR-r. All the strains analyzed had lost the original *KNR4* loci, which were replaced by two alleles that were the sizes expected for *ARO4-OFP* and *KanMX4* replacement (Fig. 1, lane 7). One of these strains, EKD-13, was selected for further analysis.

For T73-4, 36 transformants from the first transformation using the DKNR4-1 deletion cassette were analyzed by real-time PCR, and 3 of them showed positive amplification. In the confirmation PCR all of the transformants produced the same bands as EKD/2-1 (Fig. 1, lanes 2 and 6). One of the strains, TKD/2-1, was selected and used for transformation with the DKNR4-2 deletion cassette (containing the *URA3* marker). Forty transformants were checked for insertion of DKNR4-2 by reverse transcription-PCR, and for four of them a positive

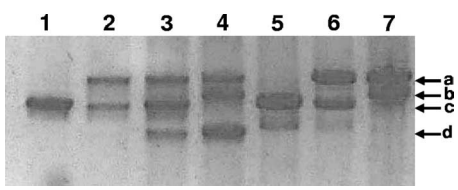

FIG. 1. Amplification of the *KNR4* locus in different strains. Lane 1, T73-4; lane 2, TKD/2-1; lane 3, TKD-12; lane 4, TKD-123; lane 5, EC1118; lane 6, EKD/2-1; lane 7, EKD-13. Arrow a, *knr4*::*ARO4-OFP* (2,974 bp); arrow b, *knr4*::*KanMX4* (2,647 bp); arrow c, *KNR4* (wild type) (2,512 bp); arrow d, *knr4*::*URA3* (2,070 bp).

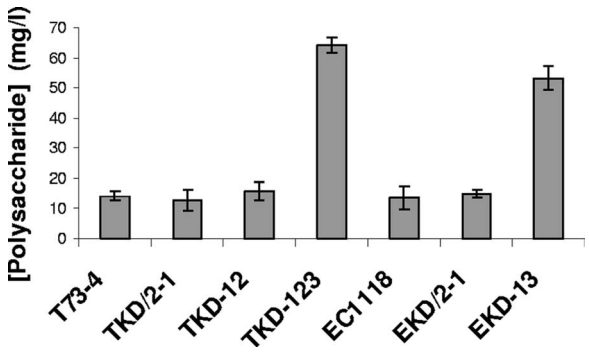

FIG. 2. Final concentrations of the polysaccharides released by different strains in GCY.

result was obtained. PCR amplification of the *KNR4* locus revealed three different alleles, an allele replaced by DKNR4-1, an allele replaced by DKNR4-2, and a wild-type allele (Fig. 1, lane 3). One strain was designated TKD-12 and was transformed with DKNR4-3 containing the *KanMX4* marker. Thirteen transformants were checked for gene deletion; in two of these transformants all the copies of the gene had been deleted, two of the transformants were identical to TKD-12, in four of the transformants the copy of the DKNR4-2 allele was replaced by DKNR4-3, and in four of the transformants the DNKR4-1 allele was replaced by DKNR4-3 (data not shown). One of the strains with all the copies deleted, TKD-123, was selected and used for further analysis (Fig. 1, lane 4).

Release of mannoproteins in laboratory media. Liquid GCY was inoculated with each strain to obtain an OD_{600} of 0.1, and the growth was monitored up to the stationary phase. For strains TKD/2-1 and TKD-12 the growth rate and OD_{600} at the stationary phase were very similar to the growth rate and OD_{600} of T73-4 (data not shown). TKD-123, however, grew more slowly than T73-4; the OD_{600} of this strain at the stationary phase was one-half that of the control strain, and it took twice as long to reach this OD_{600}. Both deletion strains with the EC1118 genetic background grew more slowly than the control, although EKD/2-1 grew faster than EKD-13. In both cases the OD_{600} at the stationary phase was one-half that of EC1118 (data not shown).

The amounts of polysaccharides released during growth in GCY were measured. The amounts of polysaccharides released by the control strains and by the strains that contained at least one functional copy of *KNR4* were very similar (Fig. 2). Nevertheless, the strains lacking the *KNR4* gene in both genetic backgrounds released substantially larger amounts of polysaccharides; TKD-123 released 5 times more polysaccharides than T73-4, while EKD-13 released 3.5 times more polysaccharides than EC1118. In addition, HPLC analysis of acid hydrolysates of EC1118 and EKD-13 cells using the method described by François (14) indicated that the mannoprotein content of the mutant strain was higher. In both cases glucose and mannose accounted for about 30% of the cell dry weight, but the amount of mannose was 1.5 times higher for EKD-13 than for EC1118.

The detection of released mannoproteins with peroxidase-conjugated concanavalin A confirmed the results obtained by quantification of the total polysaccharides; only TKD-123 and EKD-13 showed perceptible qualitative differences from the controls (data not shown).

Finally, polysaccharides released by EC1118 or EKD-13 grown in synthetic must until fermentation was complete (about 3 weeks) were analyzed by using acid hydrolysis combined with HPLC (14). The results indicated that, besides the release of almost three times more polysaccharides by EKD-13 than by EC1118, as estimated by phenol-sulfuric acid analysis (166 mg/liter versus 62 mg/liter), the mannose/glucose ratio of the polysaccharides released by EKD-13 was twice the ratio obtained for the original strain. Altogether, this resulted in the release of roughly 4.5 times more polymeric mannose by EKD-13 than by the original EC1118 strain.

Fermentation performance. Several fermentation assays were performed using different batches of Sauvignon Blanc grape juice. The sugar content of the grape juice harvested in 2006 was high (32% [wt/vol]), whereas the sugar content of the grape juice harvested in 2007 was only 22.5% (wt/vol). Fermentations were performed with the two *KNR4*-defective strains, TKD-123 and EKD-13, and the cognate wild-type strains, T73-4 and EC1118, respectively. The fermentation performance of strains carrying just one copy of *KNR4* was not analyzed, since these strains did not show any increase in mannoprotein release in the previous assays (Fig. 2).

The fermentation profiles of the 2007 must were similar for all the strains tested (examples are shown in Fig. 3B and 3D). The residual sugar and glycerol contents were also similar for strains with the same genetic background (Table 3). The only noticeable difference was in the amount of ethanol at the end of the fermentation for EC1118, which was more than 1° lower than the amount for EKD-13. Therefore, under these conditions the fermentation performance of EKD-13 was not impaired.

Fermentation of the 2006 must, which contained half as much sugar as the 2007 must, was more difficult for the recombinant *KNR4*-defective strains, indicating that there was some impairment of the fermentation performance, which was much more evident in the T73-4 background than in the EC1118 background (examples are shown in Fig. 3A and 3C). EKD-13 fermented slightly more slowly than EC1118, but at the end of the fermentation the amounts of CO_2 released were virtually identical for these two strains (Fig. 3A). The analysis of the wines showed that there were small differences in the concentrations of sugars and ethanol at the end of the fermentation (Table 3). Despite the high concentration of sugars in the must, EKD-13 and the control strain consumed almost all of the carbon source, and there was a small difference in the residual sugar concentration (0.63% [wt/vol] for EKD-13 versus 0.3% [wt/vol] for EC1118). The ethanol concentration of the wine was very high in both cases, in accordance with the large amount of sugar metabolized, but EKD-13 produced slightly less ethanol than EC1118 produced. The differences between TKD-123 and T73-4 were greater, and the fermentation rate for TKD-123 was clearly lower (Fig. 3C). This was also reflected in the amount of residual sugar (2.17% [wt/vol] for TKD-123 versus 0.12% [wt/vol] for T73-4). The final ethanol concentration was also consistently lower for TKD-123 than for T73-4.

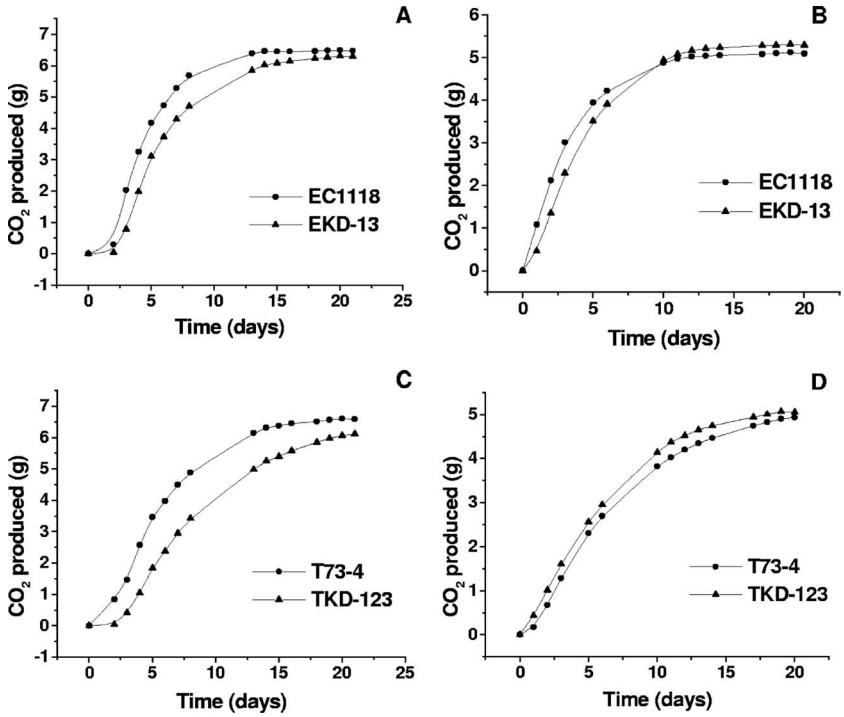

FIG. 3. Time courses of fermentation of Sauvignon Blanc musts. (A and C) Must from 2006. (B and D) Must from 2007.

Protein stability of wines and mannoprotein release. The protein stability of the fermented wines was also tested (Fig. 4). The wines from the 2006 season were always less stable than the wines from 2007. The differences must be attributed to intrinsic differences in must composition; thus, only experiments with must from the same year should be compared. In view of the results shown in Fig. 2, the wines made with the recombinant strains were expected to be more stable than the wines made with the cognate wild-type strains. This was indeed the case for EKD-13. The turbidity of the wines made with this strain was always significantly lower ($P < 0.05$) than that of the wines made with EC1118. The values were 18% lower for the wine from 2006 and 26% lower for the wine from 2007. The results were similar in three additional experiments, and the reductions in the turbidity values ranged from 18 to 35% (data not shown).

Surprisingly, this was not the case for TKD-123 (Fig. 4). The wines made with TKD-123 were even slightly less stable than the wines made with T73-4 in some of the experiments performed (data not shown).

In order to find an explanation for this unexpected behavior, we analyzed the mannoprotein content of wines fermented with the four different strains using peroxidase-conjugated concanavalin A detection, as described in Materials and Methods. A clear increase in the mannoprotein content was de-

TABLE 3. Residual sugar (glucose plus fructose), glycerol, and ethanol contents of wines fermented with strains T73-4, TKD-123, EC1118, and EKD-13

Wine	Strain	% Sugar	% Glycerol	% Ethanol
2006	T73-4	0.45	0.7	16.85
	TKD-123	2.17	0.82	15.61
	EC1118	0.3	0.76	17.16
	EKD-13	0.63	0.85	16.17
2007	T73-4	0.12	0.63	13.28
	TKD-123	0.16	0.63	13.48
	EC1118	0.34	0.61	12.21
	EKD-13	0.38	0.57	13.6

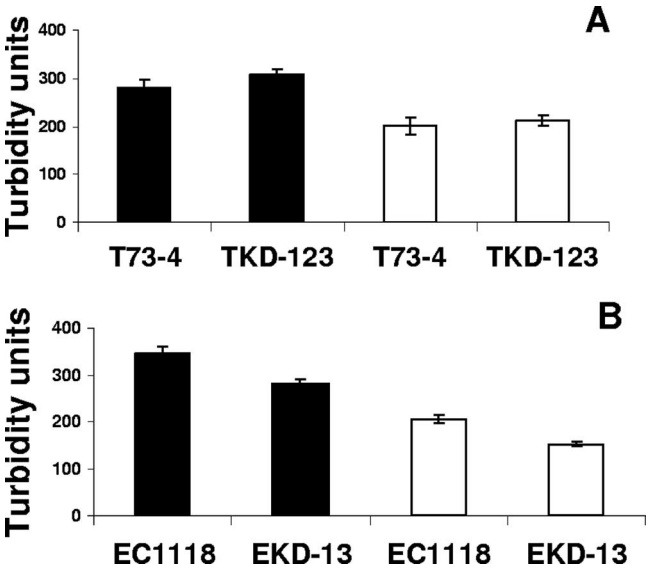

FIG. 4. Protein stability assays for Sauvignon Blanc wines fermented with T73-4 and TKD-123 (A) or with EC1118 and EKD-13 (B). Filled bars, wine from 2006; open bars, wine from 2007.

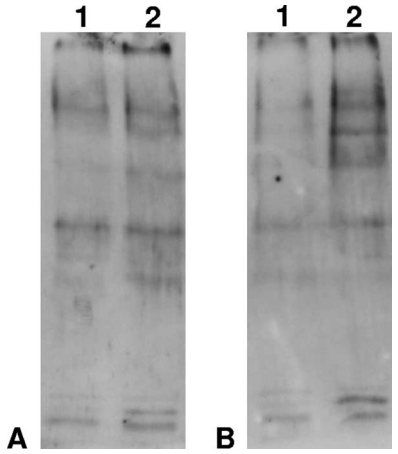

FIG. 5. Mannoproteins released during fermentation of Sauvignon Blanc wine from 2006 by strains T73-4 (panel A, lane 1), TKD-123 (panel A, lane 2), EC1118 (panel B, lane 1), and EKD-13 (panel B, lane 2).

tected for EKD-13 compared to EC1118 (Fig. 5B). The increased intensity was general and not concentrated in a small number of protein bands, in accordance with the nature of the genetic modification, which is expected to result in a general release of cell wall mannoproteins. In contrast to this and to the previous results obtained with laboratory medium, no differences between T73-4 and TKD-123 were observed (Fig. 5A). This would explain the lack of stabilizing effect observed in the protein haze assays for the TKD-123 strain. Additionally, we considered the possibility that the genetic modification resulted in increased cell autolysis with a concomitant release of intracellular material, which would explain the increased turbidity observed in some cases, as well as the impaired fermentation properties of TKD-123. The autolytic phenotype, analyzed as described in Materials and Methods, was clearly more pronounced for TKD-123 than for T73-4 (data not shown). Smaller differences between EC1118 and EKD-13 were observed.

DISCUSSION

One of the conclusions of previous work with laboratory strains was that *KNR4* inactivation was recessive and the phenotype was dependent on the genetic background (17). This prompted us to use two different industrial genetic backgrounds and to target all the *KNR4* alleles present in each of them in order to obtain a wine yeast derivative that released increased amounts of mannoproteins. Additional constraints were due to the nature of the selection markers available. Two of the markers that we used were yeast genes that had long regions of homology in the yeast genome. To compensate for this, we decided to use long flanking regions (about 500 bp) to increase the frequency of homologous recombination at the *KNR4* locus rather than gene conversion at the markers' loci. A high frequency of homologous recombination was also desirable to counteract the low frequency of spontaneous mutation in *ARO4* that could also lead to the selection of false positives (6). The use of three different selection markers and long homologous flanking regions turned out to be very convenient, especially in the case of the T73-4 strain carrying three alleles of *KNR4*.

Preliminary phenotypic analysis of the recombinant strains in GCY also confirmed the predictions of the previous study performed with laboratory strains; only replacement of all of the *KNR4* alleles resulted in increased mannoprotein release in GCY. The influence of the genetic background was not apparent in this medium but was striking in grape juice fermentation experiments, where EKD-13 (EC1118 background) outperformed TKD-123 (T73-4 background) in terms of fermentation kinetics, fermentation completion, and protein haze stabilization. Indeed, no stabilization was observed for TKD-123.

As mentioned in the Introduction, *KNR4* is involved in the yeast cell integrity pathway (2, 36). It activates two transcriptional factors, Rlm1p, which is implicated in the expression of cell wall-related genes (36), and Swi4p, which participates in cell cycle regulation (20). Because of this important role in cell integrity and also based on previous results obtained with laboratory strains, it could be anticipated that *KNR4*-deficient strains would have impaired growth and fermentation performance, at least to some extent. From a practical point of view this was probably one of the most important hypotheses tested in this work. In spite of the difficulties inherent in batch-to-batch variations, we performed this analysis using natural musts in order to better stimulate the industrial process and also as a way to directly test the protein haze stability of the wines produced. The use of different batches of must also permitted us to show that the usefulness of the recombinant strains might be context dependent. This was striking in the case of TKD-123 (Fig. 3A and 3D), but it was also apparent for the other recombinant strain. EKD-13 might experience some difficulty in attempting to completely ferment the must to dryness in cases in which the grape juice sugar content is very high, like in the 2006 must. However, the residual sugar content of the wine obtained was not really high (wine is considered to be dry if the residual sugar content is less than 5 g/liter, and the wine obtained contained 6.3 g/liter), and these limitations were not observed with lower-sugar-content must. Noticeably, in all of the wine fermentation assays, the use of EKD-13 resulted in wines that were more stable against protein haze than the wines produced with EC1118 (two examples are shown in Fig. 4).

One surprising observation was the contradiction between the results obtained with TKD-123 grown in GCY (Fig. 2) and the lack of stabilization in natural must fermentation. This finding was addressed in two ways, assessing the autolytic behavior of the strain and confirming the release of mannoproteins during wine fermentation. It was concluded that the mannoprotein-releasing phenotype observed in GCY was not expressed in wine fermentation assays with TKD-123. In addition, TKD-123 showed an autolytic phenotype that would result in the release of intracellular material during wine fermentation. This released material would eventually contribute to wine instability.

Somehow, the positive correlation between mannoprotein enrichment and protein stability observed for strains EKD-13 and TKD-123 (Fig. 4 and 5) provided indirect additional support for the stabilizing effect of mannoproteins; only the wine whose mannoprotein content was enriched was more stable than the control.

Summarizing the positive aspects of our analysis, we showed for the first time that increases in mannoprotein content, as well as protein haze stabilization of wine, can be achieved by use of genetically engineered wine yeast strains. Wines fermented with EKD-13 showed reductions in turbidity values ranging from 18 to 35% in protein stability assays. The impairment of fermentation performance in this strain was minor and was detected only for fermentation of high-sugar-content must.

Previously, other groups addressed protein haze stabilization of white wines by use of genetic engineering techniques also targeting mannoprotein production (3). Brown et al. (3) constructed recombinant laboratory strains overexpressing Hpf2p, a haze-protecting factor previously identified by Waters et al. (47). The purified mannoprotein was able to reduce turbidity values by up to 40% upon addition to wine. The approach used in the present work was somewhat less focused, since EKD-13 released increased amounts of many different mannoproteins. We agree that not all of these mannoproteins would contribute to protein haze stability, but it has also been shown that mannoproteins other than Hpf1p and Hpf2p, such as invertase, may play a role in this process (7). Probably, the concentrations of most of these compounds would be increased in wines made with the recombinant strain. In addition, a general increase in the mannoprotein content, such as that seen for EKD-13 (Fig. 5), would eventually make this strain useful for improving other technological and sensory properties of wine also related to mannoprotein content. Finally, a recombinant wine yeast strain overproducing mannoproteins would have the advantage of direct application in wine fermentation.

Possible future developments in this line of research include the construction of strains generally regarded as safe with *KNR4* deleted in order to perform pilot-scale assays and to test other mannoprotein-related properties of the wines produced; testing the effect of deletion of some other genes involved in cell wall metabolism; and overproduction in industrial strains of specific mannoproteins, like Hpf2 or other mannoproteins for which interesting properties could be determined.

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REFERENCES

1. Reference deleted.
2. **Basmaji, F., H. Martin-Yken, F. Durand, A. Dagkessamanskaia, C. Pichereaux, M. Rossignol, and J. François.** 2006. The interactome of the Knr4/Smi1, a protein implicated in coordinating cell wall with bug emergence in *Saccharomyces cerevisiae*. Mol. Gen. Genomics **275:**217–230.
3. **Brown, S. L., V. J. Stockdale, F. Pettolino, K. F. Pocock, M. De Barros Lopes, P. J. Williams, A. Bacic, G. B. Fincher, P. B. Høj, and E. J. Waters.** 2007. Reducing haziness in white wine by overexpression of Saccharomyces cerevisiae genes YOL155c and YDR055w. Appl. Microbiol. Biotechnol. **73:**1363–1376.
4. **Cabib, E., and A. Duran.** 1975. Simple and sensitive procedure for screening yeast mutants that lyse at nonpermissive temperatures. J. Bacteriol. **124:** 1604–1606.
5. **Caridi, A.** 2006. Enological functions of parietal yeast mannoproteins. Antonie van Leeuwenhoek **89:**417–422.
6. **Cebollero, E., and R. Gonzalez.** 2004. Comparison of two alternative dominant selectable markers for wine yeast transformation. Appl. Environ. Microbiol. **70:**7018–7023.
7. **Dupin, I. V., B. M. McKinnon, C. Ryan, M. Boulay, A. J. Markides, G. P. Jones, P. J. Williams, and E. J. Waters.** 2000. Saccharomyces cerevisiae mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. J. Agric. Food Chem. **48:**3098–3105.
8. **Escot, S., M. Feuillat, L. Dulau, and C. Charpentier.** 2001. Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. Aust. J. Grape Wine Res. **7:**153–159.
9. **Feuillat, M.** 1987. Preparation d'autolysats de levures et addition dans les vins effervescents elaborés selon la methode champenoise. Rev. Fr. Oenolog. **109:**17–27.
10. **Feuillat, M., C. Charpentier, and T. Nguyen Van Long.** 1998. Yeast's mannoproteins: a new possible enological adjuvant. Bull. O. I. V. **71:**945–967.
11. **Feuillat, M.** 2003. Yeast macromolecules: origin, composition and enological interest. Am. J. Enol. Vitic. **54:**211–213.
12. **Fishel, B. R., A. O. Sperry, and W. T. Garrard.** 1993. Yeast calmodulin and a conserved nuclear protein participate in the in vivo binding of a matrix association region. Proc. Natl. Acad. Sci. USA **90:**5623–5627.
13. **Fleet, G. H.** 1993. Yeast growth during fermentation, p. 27–55. *In* G. H. Fleet (ed.), Wine microbiology and biotechnology. Harwood Academic Publishers, Basingstoke, United Kingdom.
14. **François, J. M.** 2006. A simple method for quantitative determination of polysaccharides in fungal cell wall. Nat. Protocols **1:**2995–3000.
15. **Geiser, M., R. Cebe, D. Drewello, and R. Schmitz.** 2001. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. BioTechniques **31:**88–92.
16. **Gonzalez, R., A. Martinez-Rodriguez, and A. V. Carrascosa.** 2003. Yeast autolytic mutants potentially useful for sparkling wine production. Int. J. Food Microbiol. **84:**21–26.
17. **Gonzalez-Ramos, D., and R. Gonzalez.** 2006. Genetic determinants of the release of mannoproteins of enological interest by *Saccharomyces cerevisiae*. J. Agric. Food Chem. **54:**9411–9416.
18. **Guadalupe, Z., A. Palacios, and B. Ayestarán.** 2007. Maceration enzymes and mannoproteins: a possible strategy to increase colloidal stability and color extraction in red wines. J. Agric. Food. Chem. **55:**4854–4862.
19. **Guilloux-Benattier, M., J. Guerreau, and M. Feuillat.** 1995. Influence of initial colloid content on yeast macromolecule production and on the metabolism of wine microorganisms. Am. J. Enol. Vitic. **46:**486–492.
20. **Harrison, J. C., E. S. Bardes, Y. Ohya, and D. J. Lew.** 2001. A role for the Pkc1p/Mpk1p kinase cascade in the morphogenesis checkpoint. Nat. Cell Biol. **3:**417–420.
21. **Heinisch, J. J., A. Lorberg, H. P. Schmitz, and J. J. Jacoby.** 1999. The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. Mol. Microbiol. **32:**671–680.
22. **Hong, Z., P. Mann, N. H. Brown, L. E. Tran, K. J. Shaw, R. S. Hare, and B. Didomenico.** 1994. Cloning and characterization of *KNR4*, a yeast gene involved in (1,3)-β-glucan synthesis. Mol. Cell. Biol. **14:**1017.
23. **Hong, Z., P. Mann, K. J. Shaw, and B. Didomenico.** 1994. Analysis of β-glucans and chitin in a *Saccharomyces cerevisiae* cell wall mutant using high-performance liquid chromatography. Yeast **10:**1083–1092.
24. **Ito, H., Y. Fukada, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
25. **Klis, F. M.** 1994. Cell wall assembly in yeast. Yeast **10:**851–869.
26. **Klis, F. M., A. F. J. Ram, R. C. Montijn, J. C. Kapteyn, L. H. P. Caro, J. H. Vossen, M. A. A. Van Berkel, S. S. C. Brekelmans, and H. Van den Ende.** 1998. Posttranslational modifications of secretory proteins. Methods Microbiol. **26:**223–238.
27. **Klis, F. M., M. Pieternella, K. Hellingwerf, and S. Brul.** 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **26:** 239–256.
28. **Klis, F. M., A. Boorsma, and P. W. J. De Groot.** 2006. Cell wall construction in *Saccharomyces cerevisiae*. Yeast **23:**185–202, 2006.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
30. **Ledoux, V., L. Dulau, and D. Dubourdieu.** 1992. Interprètation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies. J. Int. Sci. Vigne Vin **26:**239–251.
31. **Lesage, G., A. M. Sdicu, P. Menard, J. Shapiro, S. Hussein, and H. Bussey.** 2004. Analysis of beta-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. Genetics **167:**35–49.
32. **Levin, D. E.** 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **69:**262–291.
33. **Lubbers, S., A. Voilley, M. Feuillat, and C. Charpentier.** 1994. Influence of mannoproteins from yeast on the aroma intensity of a model wine. Lebensm.-Wiss. Technol. **27:**108–114.
34. **Markovich, S., A. Yekutiel, I. Shalit, Y. Shadkchan, and N. Osherov.** 2004. Genomic approach to identification of mutations affecting caspofungin sus-

ceptibility in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **48:** 3871–3876.
35. **Martin, H., A. Dagkessamanskaia, G. Satchanska, N. Dallies, and J. Francois.** 1999. KNR4, a suppressor of Saccharomyces cerevisiae cwh mutants, is involved in the transcriptional control of chitin synthase genes. Microbiology **145:**249–258.
36. **Martin-Yken, H., A. Dagkessamanskaia, F. Basmaji, A. Lagorce, and J. Francois.** 2003. The interaction of Slt2 MAP kinase with Knr4 is necessary for signaling through the cell wall integrity pathway in *Saccharomyces cerevisiae*. Mol. Microbiol. **49:**23–35.
37. **Moine-Ledoux, V., and D. Dubourdieu.** 1999. An invertase fragment responsible for improving the protein stability of dry white wines. J. Sci. Food Agric. **79:**537–543.
38. **Molero, G., M. Yuste-Rojas, A. Montesi, A. Vazquez, Nombela, and C. M. Sanchez.** 1993. A *cdc*-like autolytic *Saccharomyces cerevisiae* mutant altered in budding site election is complemented by SPO12, a sporulation gene. J. Bacteriol. **175:**6562–6570.
39. **Puig, S., D. Ramon, and J. E. Perez-Ortin.** 1998. Optimized method to obtain stable food-safe recombinant wine yeast strains. J. Agric. Food Chem. **46:**1689–1693.
40. **Querol, A., E. Barrio, and D. Ramon.** 1992. A comparative study of different methods of yeast strain characterization. Syst. Appl. Microbiol. **15:**439–446.
41. **Rosi, I., A. Gheri, P. Domizio, and G. Fia.** 1999. Formation of cell wall macromolecules by Saccharomyces cerevisiae during fermentation and their influence on malolactic fermentation. Rev. Oenolog. Tech. Vitivinicol. Oenolog. **94:**18–20.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1998. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
43. **Saucier, C., Y. Glories, and D. Roux.** 1999. Tannin-colloid interactions: new advances concerning the concept of 'good' and 'bad' tannins. Rev. Oenolog. Tech. Vitivinicol. Oenolog. **94:**9–10.
44. **Segarra, I., C. Lao, E. López-Tamames, and M. C. de la Torre-Boronat.** 1995. Spectrophotometric methods for the analysis of polysaccharide levels in winemaking products. Am. J. Enol. Vitic. **46:**564–570.
45. **Tabera, L., R. Muñoz, and R. Gonzalez.** 2006. Deletion of *BCY1* from the *Saccharomyces cerevisiae* genome is semidominant and induces autolytic phenotypes suitable for improvement of sparkling wines. Appl. Environ. Microbiol. **72:**2351–2358.
46. **Vidal, S., L. Francis, P. Williams, M. Kwiatkowski, R. Gawel, V. Cheynier, and E. Waters.** 2004. The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. Food Chem. **85:**519–525.
47. **Waters, E., I. Dupin, and V. Stockdale.** 2000. A review of current knowledge on polysaccharides that protect against protein haze in white wine. Aust. Grapegrower Winemaker **438a:**13–16.
48. **Waters, E. J., W. Wallace, M. E. Tate, and P. J. Williams.** 1993. Isolation and partial characterization of a natural haze protective factor from wine. J. Agric. Food Chem. **41:**724–730.
49. **Waters, E. J., P. Pellerin, and J. M. Brillouet.** 1994. A *Saccharomyces* mannoprotein that protects wine from protein haze. Carbohydr. Polym. **23:**185–191.
50. **Wolz, S.** 2005. Extraction of mannoproteins and polysaccharides and their effect on aroma. Improvement of mouthfeel. Dtsch. Weinmagazin **22:**21–25.
51. **Yamamoto, T., T. Hiratani, H. Hirata, M. Imai, and H. Yamaguchi.** 1986. Killer toxin from *Hansenula mrakii* selectively inhibits cell wall synthesis in a sensitive yeast. FEBS Lett. **197:**50–54.
52. **Yamamoto, T., K. Uchida, T. Hiratani, T. Miyazaki, J. Jaqiu, and H. Yamaguchi.** 1988. In vitro activity of the killer toxin from the yeast *Hansenula mrakii* against yeasts and molds. J. Antibiot. **41:**398–403.