Wine Yeast Inhibition by Sulfur Dioxide: A Comparison of Culture-Dependent and Independent Methods

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Culture-independent and culture-dependent methods were used to determine the persistence of *Hanseniaspora uvarum*, *Candida* sp. EJ1, and *Saccharomyces cerevisiae* populations within wine fermentations treated with inhibitory levels of sulfur dioxide. Upon addition, SO_2 completely inhibited growth of *Hanseniaspora* and *Candida* populations on enrichment media; however, PCR and RT-PCR signatures of each species persisted, in some cases, throughout the complete fermentation (as long as 20 days). PCR-DGGE and RT-PCR-DGGE profiles of yeasts present in the fermentations also indicated persistence of *Hanseniaspora* and *Candida* populations after their respective plating populations disappeared. This study demonstrates that molecular signatures of non-*Saccharomyces* yeasts can remain in wine fermentations treated with SO_2 long after those populations become nonculturable.

Key words: PCR, denaturing gradient gel electrophoresis, viable but nonculturable, non-*Saccharomyces* yeast

The fermentation of wine is a complex microbial process involving the transformation of must into wine by the action of different species of yeasts and lactic acid bacteria present on the grapes and winery equipment. Yeasts of the genera *Candida, Hanseniaspora, Pichia, Torulaspora, Metschnikowia, Kluveromyces*, among others, have been shown to grow during the early stages of wine fermentations [14]. Growth of one or more dominant *Saccharomyces cerevisiae* populations, as well as a concurrent increase in ethanol concentration, typically results in a decline of these non-*Saccharomyces* populations [15]. Historically, yeasts present in wine fermentations are differentiated after standard enrichment plating in order to isolate the various microorganisms. Unfortunately, the necessity of strain isolation before taxonomic characterization is known to introduce potential biases. For example, this approach fails to characterize those microorganisms for which enrichment and culturing is problematic or impossible [1]. Alternatively, certain microbial populations in wine may be injured and temporarily unable to grow on media in which they are commonly enriched [18]. Other studies that have employed direct analysis have repeatedly demonstrated a tremendous variance between cultivated and naturally occurring species, thereby altering perceptions of the true microbial diversity present in various habitats [17].

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Several methods have been employed to characterize directly the microbial diversity within wine fermentations without the necessity for enrichment plating. The development of specific stains has advanced tremendously, enabling microscopic observation of active microbial populations [22]. DNA-based approaches, both probe- and PCR-based, have also been developed to discriminate directly the specific microbial populations in wine [3,9,16,21]. While nucleic acid-based methods are often more rapid than enrichment plating, little is known about the persistence of nonculturable yeasts, or their nucleic acids, within wine fermentations.

Many winemakers employ sulfur dioxide, in part, to reduce the microbial populations that may threaten a more dominant *Saccharomyces* population [6]. In this work we compared plating analysis with specific PCR and PCR-DGGE approaches to determine the persistence of non-*Saccharomyces* populations within mixed culture wine fermentations treated with an inhibitory level of SO_2. Direct molecular methods clearly demonstrate persistence of non-*Saccharomyces* RNA and DNA signatures long after additions of SO_2 prevented their growth on plating media. This work suggests that non-*Saccharomyces* yeasts may persist in a nonculturable state within standard wine fermentations and that plating analysis alone is insufficient to monitor the various yeast populations.

Materials and Methods

Yeast strains. *Saccharomyces cerevisiae* S9, *Candida* sp. EJ1, and *H. uvarum* Y1 were isolated from a commercial wine fermentation [23]. The cultures were maintained on YPD agar (glucose 2% [w/v], yeast extract 1% [w/v], peptone 1% [w/v]) and were inoculated in 10 mL of sterile Chardonnay juice 18 to 24 hr before the start of fermentations.

Fermentation experiments. Wine must from Chardonnay grapes (pH 3.0, glucose 113 g/L, and fructose 111 g/L) was sterilized by filtration using a 0.22 µM-pore-size membrane (Millipore, Bedford, MA). Two-liter flasks containing 1 L sterile must were incubated in a water bath at 18°C without shaking. Fifty mg per L of SO_2 was added to one of the juice solutions. Inoculums containing approximately 10^6 cells/mL of *Candida* sp. EJ1 and *H. uvarum* Y1 and 10^3 cells/mL of *S. cerevisiae* S9 were added to the juice (cell number determined with a hemacytometer). All fermentations were carried out in duplicate and followed for a period of 30 to 40 days. One sample was taken daily for the first 10 days of fermentation, after which sampling was performed every three days.

Microbiological analysis. Aliquots (0.1 mL each) of appropriate dilutions in saline-peptone water (8 g/L NaCl, 1 g/L peptone, pH 7) were spread onto plates of Wallerstein Laboratory Nutrient Agar (WL) medium (Difco Laboratories, Sparks, MD), lysine medium (LM) (Difco Laboratories), and WL medium containing 0.1 g/L cycloheximide (cWL) (Sigma, St. Louis, MO). WL medium allowed discrimination between the yeast species by colony morphology and color [7], while LM enabled the enumeration of non-*Saccharomyces* populations. cWL was used to enumerate only the *H. uvarum* populations, as both *S. cerevisiae* and *Candida* sp. EJ1 were eliminated by 0.1 g/L cycloheximide. The use of LM and cWL allowed enumeration of small numbers of non-*Saccharomyces* yeasts even after a dominant population of *S. cerevisiae* (10^7 to 10^8 colony forming units [cfu] per mL) was reached. Plates were incubated at room temperature (~22°C) for 5 days and colonies were counted on the basis of their color and morphology [7].

Nucleic acid extraction. Volumes of must, ranging from 1 to 50 mL, collected on the basis of the optical density in the fermentations, were collected and centrifuged 12,000 rpm for 10 min at 4°C. The cell pellet was washed using saline-peptone water and transferred in a 2-mL tube that was subjected to a second centrifugation. Samples were taken in duplicate, one each for the extraction of DNA and RNA. DNA and RNA were extracted as previously described [23]. DNA and RNA were treated with DNase-free RNase (Roche Diagnostics, Indianapolis, IN) or RNase-free DNase (Roche Diagnostics), respectively. Tubes were incubated at 37°C for 1 hr to overnight. Residual DNA in the RNA preparations was detected by PCR.

PCR primers. Universal primers NL1 (5'-GCATATC AATAAGCGGAGGAAAAG; [19]) and LS2 (5'-ATTCCCAAA CAACTCGACTC; [8]), which amplify the 5' end of the yeast 26S rRNA gene, were used to follow the dynamic changes in yeast populations by DGGE. A GC clamp (5'-CGCCCGCCGCGCC CCGCGCCCGTCCCGCCGCCCCCGCCCG) [8] was added to the NL1 primer when used for DGGE analysis. Primers specific to *Candida* sp. EJ1 (C1; 5'-TACCGCATTTATCTTCCCCC) and *H. uvarum* (K2; 5'-GAGAGGCGAGTGCAT) were generated as part of this study and used to determine the presence of the nucleic acids belonging to these two species by PCR and RT-PCR (Figure 1). These primers were designed to an internal portion of the 26S rRNA D1-D2 region sequence [19] that varied between *S. cerevisiae* and *H. uvarum* or *Candida* sp. EJ1. The primers when paired with the universal primer NL1 resulted in specific amplification of *H. uvarum* (Figure 2A; 429 bp PCR product) or *Candida* sp. EJ1 DNA (Figure 2B; 420 bp product), respectively.

PCR and RT-PCR. The D1 region of the 26S rRNA gene was amplified using the primers $NL1^{GC}$ and the reverse primer LS2. PCRs were carried out using 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 45 sec and extension at 72°C for

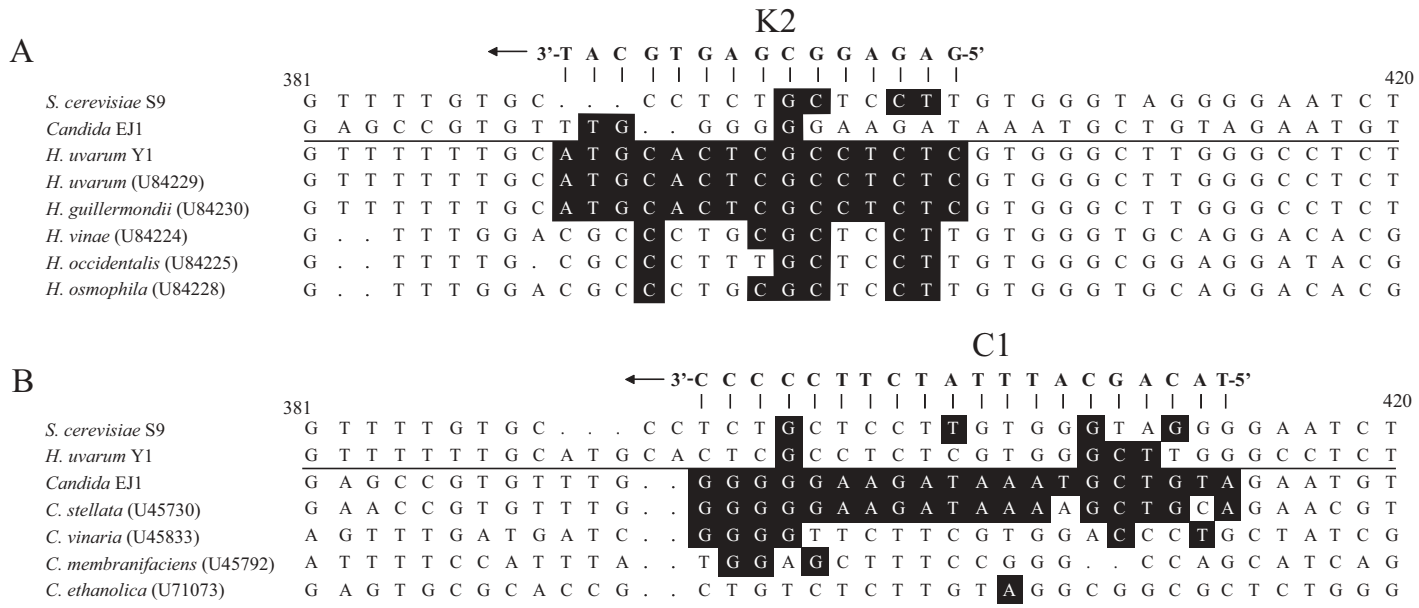

Figure 1 Alignment of partial yeast D1-D2 26S rDNA sequence (5'-3') for the selection of the specific *H. uvarum* (panel **A**) and *Candida* sp. EJ1 (panel **B**) PCR primers. The antisense, 3'-5', sequence of the K2 and C1 primers are shown above the alignments in **A** and **B** (for 5'-3' primer sequences refer to Materials and Methods). Base similarities between the primers and the target sequences are highlighted. GenBank accession numbers are listed in parenthesis after the *Haneniaspora* and *Candida* species. To generate species-specific amplification, either primer was paired with universal yeast primer NL1 [19].

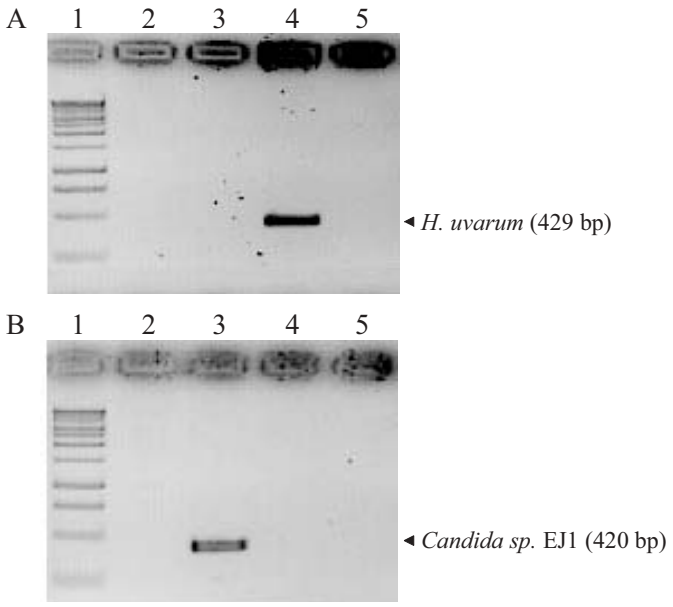

Figure 2 PCR amplicons obtained using the specific primers for (**A**) *H. uvarum* (K2–NL1 primer pair) and (**B**) *Candida* sp. EJ1 (C1–NL1 primer pair) on the three yeast DNA templates (*Saccharomyces, Candida,* and *Hanseniaspora*) used in this study. Designations (for both gels): lane 1, 1 Kb molecular weight ladder (Promega); lane 2, *S. cerevisiae* S9; lane 3, *Candida* sp. EJ1; lane 4, *H. uvarum* Y1; lane 5, no DNA PCR control. Arrows denote the 429 bp PCR amplicon from *H. uvarum* and the 420 bp PCR amplicon from *Candida* sp. EJ1.

1 min. An initial 5 min denaturation at 95°C and a final 7 min extension at 72°C were also used. The reaction mixture, in a 50 µL final volume, contained 10 mM Tris-HCl, pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of the primers, 1.25 UI *Taq*-polymerase (Promega, Madison, WI), and 5 µL of extracted DNA (about 10 to 50 ng total DNA). RT-PCR was performed with the RevertAid™ M-MuLV reverse transcriptase (Promega). One microliter of total RNA (~0.1 µg) was mixed in 10 µL of DNase- and RNase-free sterile water containing 0.5 µg of LS2 primer, and incubated at 70°C for 5 min. Immediately after chilling in ice, a mixture of 25 mM Tris-HCl, pH 8.3, 25 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, 1 mM each dNTPs and 20 UI ribonuclease inhibitor (Roche Diagnostics) was transferred in the reaction tube. After 5 min at 37°C, 1 µL reverse transcriptase was added, followed by incubation at 42°C for 60 min and at 70°C for 10 min to stop the reaction. Three microliters of the synthesized cDNA was used for the PCR as described previously.

For specific amplification of *Candida* sp. EJ1 or *H. uvarum* DNA or RNA, the reverse primers C1 and K2 were used with the forward NL1 primer without the GC clamp. DNA was amplified in a 50 µL final volume using the same PCR reagent conditions described above. Both *Candida* sp. EJ1 and *H. uvarum* DNA were amplified with the same PCR cycle parameters (30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 45 sec and extension at 72°C for 1 min). An initial denaturation at 95°C for 5 min and a final extension at 72°C for 7 min were also used. For the specific RT-PCR protocol, the *Tth* DNA polymerase (Roche Diagnostics) was used. One microgram of total RNA was mixed with 450 nM of each primer and the volume brought to 25 µL with DNase- and RNase-free sterile water. After a denaturation step at 70°C for 5 min, the tubes were placed immediately on ice and 25 µL of a mixture containing 25 mM bicine /KOH, pH 8.2, 57.5 mM potassium acetate, 4% (v/v) glycerol, 2.5 mM $Mn(OAc)_2$, and 5 UI *Tth* DNA polymerase was added. The reactions were incubated for 30 min at 60°C, and after 95°C for 2 min the amplification cycle previously described was carried out. Agarose gel electrophoresis in 1X TAE was used to assess the presence of the PCR products.

DGGE analysis. The Dcode™ Universal Mutation Detection System (BioRad, Hercules, CA) was used for sequence-specific separation of the $NL1^{GC}$-LS2 PCR products. Electrophoresis was performed in a 0.8 mm polyacrylamide gel (8% [wt/v] acrylamide:bisacrylamide 37.5:1) using a denaturant range from 40% to 60% of urea and formamide (100% corresponds to 7M urea and 40% [wt/v] formamide) increasing in the direction of the electrophoresis run. The electrophoresis was performed at 130 V for 4 hr with a constant temperature of 60°C. After the run, the gels were stained for 20 to 30 min in 1X TAE containing 0.5 mg/mL ethidium bromide and photographed under UV illumination by using the Multimage™ Light Cabinet (Alpha Innotech Corporation, San Leandro, CA).

Chemical analysis. Glucose and fructose content was determined by HPLC. Samples and standards were run on a Hewlett-Packard 1100 Series HPLC (Palo Alto, CA) with a cation H guard column, two 30 cm Aminex HPX-87H columns (BioRad), and a HP 1047A refractive index detector. Flow rate was 0.6 mL/min with a 50°C column temperature.

Results and Discussion

Plating analysis of wine fermentations. Two separate fermentations were carried out using mixed cultures of *S. cerevisiae, H. uvarum,* and *Candida* sp. EJ1 with (fermentation 2) and without (fermentation 1) an addition of sulfur dioxide at 50 mg per L. Initial inoculum sizes were ~10^3 cells per mL for *S. cerevisiae* and ~10^6 cells/mL for *H. uvarum* and *Candida* sp. EJ1. These inoculum concentrations were specifically chosen to mimic a commercial must containing high levels of non-*Saccharomyces* yeasts (one that might benefit from added SO_2). WL, LM, and cWL media were used to track the yeast cfu populations during the fermentations. All fermentations were characterized by a dominance of *S. cerevisiae,* resulting in the complete consumption of the glucose and fructose originally present in the juice. Colony forming unit trends and glucose/fructose consumption profiles for fermentations 1 and 2 are shown in Figures 3 and 4, respectively. In fermentation 1 (Figure 3), which contained no added SO_2, the non-*Saccharomyces* yeasts reached 10^7 to 10^8 cfu per mL after only a single day, while the *S. cerevisiae* population reached 10^7 to 10^8 cfu/mL on day 9. The *H. uvarum* cfu population became undetectable after 17 days (<10 cfu/mL on cWL plates), whereas *Candida* sp. EJ1 slowly decreased after 14 days and was undetectable at the last sampling point (day 38). Persistence of the same *Candida* sp. EJ1 strain in the face of a superior *Saccharomyces* population was previously observed

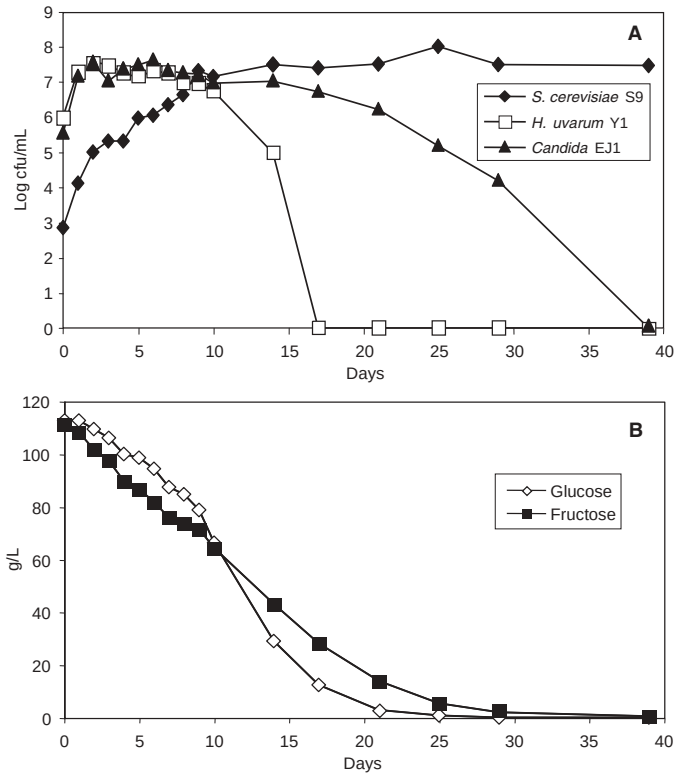

Figure 3 Colony forming unit analysis (**A**) and glucose and fructose concentration profile (**B**) for fermentation 1 containing no added SO_2. Replicate fermentations were performed; however, a single representative is shown.

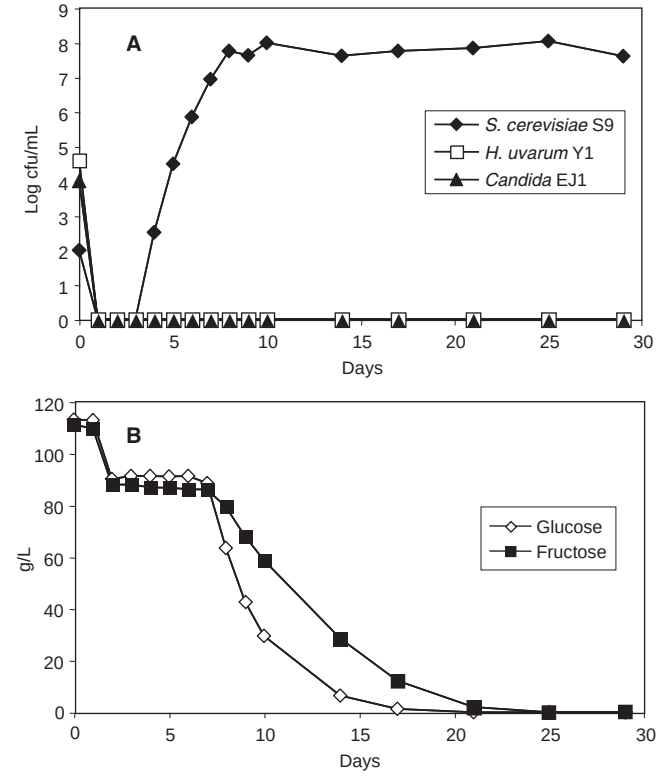

Figure 4 Colony forming unit analysis (**A**) and glucose and fructose concentration profile (**B**) for fermentation 2 containing 50 mg/L added SO_2. Replicate fermentations were performed; however, a single representative is shown.

in a commercial fermentation [23]. Glucose and fructose concentrations progressively decreased from day 1, becoming depleted by day 30 (Figure 3B). The average maximum rate of sugar consumption achieved in fermentation 1 was 12.4 g/L/day for glucose and 7.2 g/L/day for fructose.

As expected, the pattern of yeast persistence was dramatically different in the fermentation treated with SO_2 (fermentation 2; Figure 4). Plating analysis of fermentation 2 revealed an immediate drop in all yeast cfu populations such that no yeast cfu were observed from samples taken on day 1 (Figure 4A). The *S. cerevisiae* population was able to recover after three days and by day 10 reached a population level of $10^7$to 10^8 cfu/mL, which remained stable throughout the remainder of the fermentation. However, no cfu of *H. uvarum* or *Candida* sp. EJ1 were observed throughout the fermentation. The sugar utilization pattern also reflected this dramatic shift in microbial populations (Figure 4B). Fermentation 2 was characterized by an initial drop in the sugar content between day 2 and day 3, followed by a transition period where the concentration of glucose and fructose remained stable for 6 days. On day 8, glucose and fructose again began to decrease, becoming undetectable within 21 days. The average maximum rate of sugar consumption in fermentation 2 during this second phase was 25.2 g/L/day for glucose and 11.3 g/L/day for fructose, significantly higher than the maximum rates obtained in fermentation 1. Also during this latter phase, the ratio of glucose to fructose in fermentation 2 was lower than that observed in fermentation 1, which was likely due to the longer persistence of the fructophilic *Candida* sp. EJ1 strain in fermentation 1 [23].

PCR and RT-PCR DGGE profiles. The DGGE patterns obtained from both DNA and RNA templates in fermentations 1 and 2 are shown in Figures 5 and 6, respectively. In fermentation 1 (Figure 5A) both PCR-DGGE (DNA) and RT-PCR-DGGE (RNA) patterns were characterized by the presence of two strong bands at the beginning of the fermentation, belonging to *H. uvarum* and *Candida* sp. EJ1. The band specific for *S. cerevisiae* was not visible until day 3 once the corresponding *S. cerevisiae* population had become greater than 10^5 cfu/mL. After this point, DGGE bands corresponding to *S. cerevisiae*, *H. uvarum*, and *Candida* sp. EJ1 were clearly visible at the end of the fermentation, even though the *H. uvarum* cfu population was eliminated by day 20. The primer set $NL1^{GC}$/LS2, used to amplify rDNAs or rRNAs before DGGE, produced faint amplicons from RNA samples obtained on days 1, 2, 17, and 21, such that little or no banding patterns were observed by DGGE.

Dramatically different DGGE profiles were observed for fermentation 2 that contained SO_2 (Figure 6A). DGGE bands belonging to *H. uvarum* and *Candida* sp. EJ1 remained visible until day 6 or 7 of fermentation from either DNA or RNA templates (respectively) even though the corresponding cfu populations were eliminated immediately. A band corresponding to *S. cerevisiae* appeared in the DGGE pattern on day 6, as the corresponding *S. cerevisiae* cfu population became greater than 10^4 per mL. Differences between the RNA and DNA templates were also noted in the DGGE gels (Figure 4). In the RT-PCR-DGGE, the *Candida* sp. EJ1 band became faint on day 2 (but was still observed until day 6), while in the PCR-DGGE, both the *H. uvarum*

and *Candida* sp. EJ1 bands were of the same intensity. That may reflect the impact of differential RNA extraction or rRNA copy number as compared to DNA templates. Once the *S. cerevisiae* reached 10^6 to 10^7 cfu/mL, it was the only species visible on DGGE regardless of the nucleic acid template.

Specific amplification of *H. uvarum* and *Candida* sp. EJ1. Previous DGGE analysis of yeasts in wine demonstrated that, as populations fell below ~10^4 cfu/mL, the cognate DGGE bands became faint or disappeared [8]. This threshold is the result of a larger quantity of *Saccharomyces* DNA in these samples outcompeting the lower amounts of template from the non-*Saccharomyces* yeasts for amplification of the rDNA [11]. Thus, the presence of non-*Saccharomyces* bands in the previous DGGE profiles depends on DNA (or RNA) template ratios within the PCR reaction that change within samples taken over the course of a fermentation.

To eliminate the competition from *Saccharomyces* DNA or RNA, we developed specific primers for amplification of *H. uvarum* and *Candida* sp. EJ1. The specific primers were used to detect *H. uvarum* and *Candida* sp. EJ1 DNA or RNA within fermentation 1 or 2 samples (Figures 5B and 6B). In fermentation 1, specific *H. uvarum* and *Candida* sp. EJ1 amplicons were detected throughout the fermentation, in agreement with the corresponding DGGE analysis. While no *H. uvarum* RT-PCR amplicon was detected on day 17, amplicons were detected on days 21, 25, and 29. In fermentation 2, PCR detection of *H. uvarum* from RNA templates persisted until day 17, after which detection was only observed from DNA templates until day 25. As previously witnessed in the fermentation 2 DGGE profiles, the *Candida* sp. EJ1 population became undetectable earlier than the *H. uvarum* population, with faint amplicons produced until day 14 from either RNA or DNA templates. Since neither *H. uvarum* nor *Candida* EJ1 was detected on plates immediately after inoculation, the persistence yeast rRNA from washed cell pellets taken from these fermentations suggests that injury by SO_2 may have resulted in a prolonged nonculturable state.

Previously, RT-PCR has been used to determine nonculturable microorganisms [5,20,24,25]. RNA has been proposed as a more representative target for assessing bacteria viability [4]. Messenger RNA (mRNA) targets have also been chosen for viability studies; however, variable half-lives and expression patterns can make mRNA less attractive [2,24]. Because the number of intact ribosomes approximately reflects the rates of protein synthesis, others have used rRNA as a marker for general metabolic activity [12,26]. Moreover, rRNA has been suggested as an indicator of viability in *Mycobacterium smegmatis* [25], and a correlation between viability and cellular rRNA content was also observed in *Escherichia coli* cells undergoing starvation [10]. Conversely, Sheridan et al. [24] was not able to use rRNA as a useful indicator of *E. coli* viability evaluated over a relatively short time frame (16 hr) after heat or ethanol treatment.

Little is known about the persistence of nonculturable yeasts or yeast nucleic acid in various food or beverage environments. The fact that cellular rRNA of nonculturable yeasts can persist long into wine fermentations demonstrates the need for detailed examination of the size and potential metabolic activity of these yeasts [23]. In the context of wine production, it remains to be determined if these populations are metabolically active in a fashion that would alter the finished product. Given that indigenous yeasts are potential producers of extracellular enzymes, such as pectinases, proteases, and glycosidases, the persistence of such yeasts (and potential enzymatic activities within) may impact wine processing and aroma formation [13]. Alternatively, regrowth of injured or viable but nonculturable (VBNC) yeast populations might be a source of wine spoilage at a later stage in production or bottling.

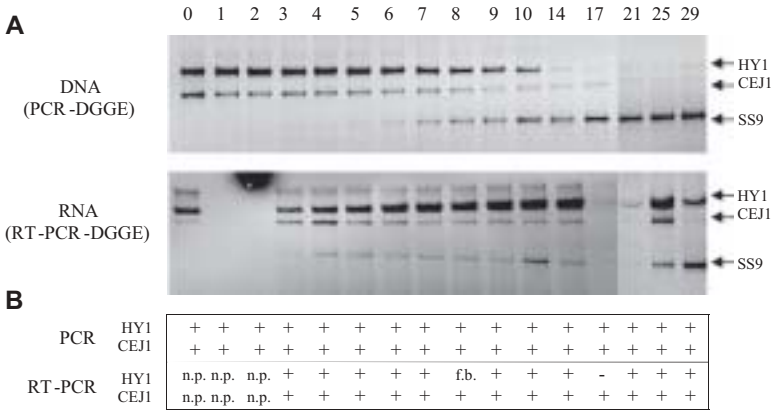

Figure 5 PCR analysis of samples from fermentation 1. Panel **A**: DGGE analysis from DNA (PCR-DGGE) or RNA (RT-PCR-DGGE) templates. Top lane labels indicate day of sampling. Panel **B**: Specific PCR and RT-PCR of *H. uvarum* and *Candida* sp. EJ1 populations. Sampling points for specific PCR correspond with day reported in panel A DGGE profile. Abbreviations: SS9, *S. cerevisiae* S9; HY1, *H. uvarum* Y1; CEJ1, *Candida* sp. EJ1; +, PCR product observed; -, no PCR product observed; f.b., faint band observed; n.p., not performed.

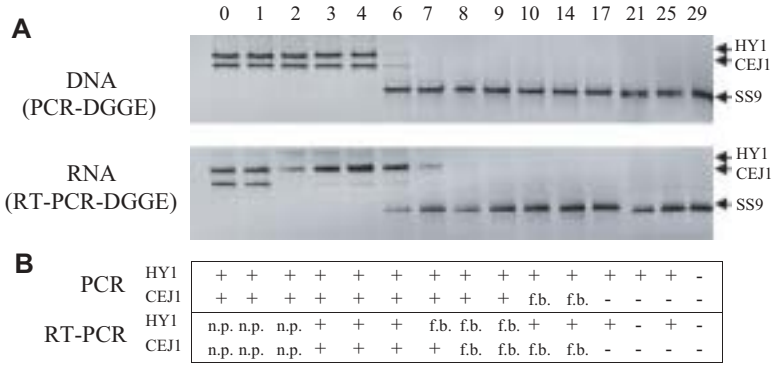

Figure 6 PCR analysis of samples from fermentation 2. Panel **A**: DGGE analysis from DNA (PCR-DGGE) or RNA (RT-PCR-DGGE) templates. Top lane labels indicate day of sampling. Panel **B**: Specific PCR and RT-PCR of *H. uvarum* and *Candida* sp. EJ1 populations. Sampling points for specific PCR correspond with day reported in panel A DGGE profile. Abbreviations: SS9, *S. cerevisiae* S9; HY1, *H. uvarum* Y1; CEJ1, *Candida* sp. EJ1; +, PCR product observed; -, no PCR product observed; f.b., faint band observed; n.p., not performed.

Conclusions

Direct molecular methods for characterizing microbial constituents often reveal different populations than observed by standard plating analysis. This is due to multiple factors, including VBNC populations, injured populations, and/or dead populations in which the cellular DNA or RNA is protected from degradation. This work demonstrates that molecular signatures of non-*Saccharomyces* yeasts can persist in wine fermentations long after the plating population has been inhibited by sulfur dioxide. The persistence of such signatures must be taken into account in direct microbial identification schemes that employ DNA- or RNA-based detection, and future work is needed to discriminate VBNC, injured, and dead populations within wine. Regardless, this study exemplifies the utility of polyphasic approaches to characterize the microbial flora in wine fermentations, as a single approach by itself may only provide a restricted view of the true microbial complexity present.

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