INFLUENCE OF *ZYGOSACCHAROMYCES* AND *BRETTANOMYCES* ON WINE QUALITY AND THEIR CONTROL DURING VINIFICATION

By

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To the Faculty of Washington State University:

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INFLUENCE OF *ZYGOSACCHAROMYCES* AND *BRETTANOMYCES* ON WINE QUALITY AND THEIR CONTROL DURING VINIFICATION

Abstract

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Yeast contamination during winemaking, particularly by *Brettanomyces bruxellensis* or *Zygosaccharomyces bailii*, can have a detrimental impact on wine quality if growth is not controlled. Nevertheless, speculation exists that some *Z. bailii* strains could be beneficial during vinification, particularly for stuck fermentations. Therefore red wines adjusted to 13, 15, or 17% v v^{-1} ethanol and containing 40 or 60 g l^{-1} fructose were inoculated with *Z. bailii*. However, a *Saccharomyces* wine strain was more effective at removing the residual sugar and produced less volatile acidity. Consequently, antimicrobial technologies to limit undesirable yeast growth were also evaluated. The efficacy of 200 mg l^{-1} dimethyl dicarbonate (DMDC) was determined against yeasts originally isolated from regional vineyards, including *Candida oleophila*, *Candida californica*, *Metschnikowia pulcherrima*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, and *Wickerhamomyces anomalus,* inoculated into grape must. Following treatment to 10^4 or 10^6 cfu ml^{-1} of each yeast, populations initially declined several logs but within several days increased to $>10^6$ cfu ml^{-1}, regardless of inocula or strain. When DMDC was added to grape must with *Z. bailii*, 10^3 cfu ml^{-1} populations were not detected following treatment, although 10^6 cfu ml^{-1} did grow. Conversely, when DMDC was added to wines with $\leq 10^6$ cfu ml^{-1} *Z. bailii*, growth was limited for ≥85 days. Growth of *B. bruxellensis* in wines at populations $\leq 10^4$ cfu ml^{-1} was

generally limited by DMDC, although sensitivity was dependent upon strain. However, 4-ethylphenol and 4-ethylguiacol production was limited in wine for several years. Alternatively, a factorial experiment using wines inoculated with *B. bruxellensis* evaluated storage temperature (22°, 18°, 15°, or 10°C) and molecular SO_2 (mSO_2) addition (≈0.0, 0.2, 0.5, or 1.1 mg l^{-1}) and determined that while some strains could grow at either 10°C or with ≈0.5 mg l^{-1} mSO_2; conditions of ≤15°C and ≥0.40 mg l^{-1} mSO_2 synergistically limited growth. The cumulative results demonstrated that while commercial application of *Z. bailii* during vinification may not be practical, reliance on DMDC or the interactive impacts of SO_2 and temperature could limit a variety yeasts associated with wine spoilage including *Z. bailii* and *B. bruxellensis.*

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CHAPTER I

ADVANCES IN THE CONTROL OF WINE SPOILAGE BY *ZYGOSACCHAROMYCES* AND *DEKKERA*/*BRETTANOMYCES*

Introduction

Microbiological spoilage is a major concern throughout the wine industry. Two of the most significant threats come from *Zygosaccharomyces* and *Brettanomyces* yeasts that have been the subjects of reviews by Thomas & Davenport (1985), Oelofse et al. (2008), Wedral et al. (2010), and others. Because of their impacts on wine quality and potential large economic losses, advances in characterization, detection, and control of these yeasts continue to be important fields of study (Stratford et al. 2006). This review serves to update the distribution, detection, and control of *Zygosaccharomyces* and *Brettanomyces* by the wine industry.

Physiology and biochemistry

The genus *Zygosaccharomyces* was initially described by Barker (1901) for *Saccharomyces*-like yeasts that exhibited conjugation. Currently, the genus consists of six species, *Zygosaccharomyces kombuchaenis, Zygosaccharomyces lentus, Zygosaccharomyces mellis, Zygosaccharomyces bailii, Zygosaccharomyces bisporous,* and *Zygosaccharomyces rouxii* (James & Stratford 2011). The latter three species, *Z. bailii, Z. bisporous,* and *Z. rouxii*, have been associated with the spoilage of grape must, grape juice concentrate, and wine (Fugelsang & Edwards 2007).

Relationships between species of *Zygosaccharomyces* and other yeast genera found in wines have been investigated. Research by James et al. (1994) regarding comparative analysis of 18S ribosomal RNA (Rrna) revealed that the majority of the genus was more closely related to *Saccharomyces* and *Torulaspora delbrueckii* than to other non-*Saccharomyces* yeasts. More

recently, Novo et al. (2009) isolated a large DNA region from several strains of *Saccharomyces cerevisiae* used in wine production that were thought to be unique to *Z. bailii*, suggesting that exogenous transfer events in a yeast's genome may be more common than previously believed.

Although some diversity exists, *Zygosaccharomyces* species share several phenotypic and physiological traits. Cells occur singly or in pairs and can appear microscopically as spherical, cylindrical, or ellipsoidal, with dimensions of 3 to 9 µm by 3 to 13 µm (Edwards 2005, James & Stratford 2011; Thomas & Davenport 1985). Typical colony morphology has been described as smooth, convex, and white to cream in color (Edwards 2005). Vegetative reproduction includes multilateral budding with occasional pseudohyphae (James & Stratford 2011). *Z. bailii* is haploid and heterothallic, so sporulation occurs only with conjugation. Here, a dumbbell shape of two connected cells may be observed of cells grown in solidified media but not in grape juice nor wine (Figure 1).

Like *Zygosaccharomyces*, *Brettanomyces* has historically been identified with food and beverage spoilage. Although it was first isolated from beer in the late 1920s, wine infections were not reported until the 1950s in France, South Africa, and Italy (Sponholz 1993). Five individual species of *Brettanomyces* (teleomorph *Dekkera*) are now recognized: *Brettanomyces bruxellensis*, *Brettanomyces anomala*, *Brettanomyces custersiana*, *Brettanomyces naardensis*, and *Brettanomyces nanus* (Smith 2011). Currently, only *B. bruxellensis* is thought to be associated with grape and wine contamination (Egli & Henick-Kling 2001; Mitrakul et al. 1999).

Physiological traits of *B. bruxellensis* vary depending on strain, growth phase, and environmental conditions. As illustrated in Figure 2, cell shape may be ovoid, ellipsoidal, cylindrical, or boat-shaped, and dimensions are typically 2 to 7 µm in length (Edwards 2005; Smith 2011). Singular cells, along with pairing, clustering, or short chains, have been observed

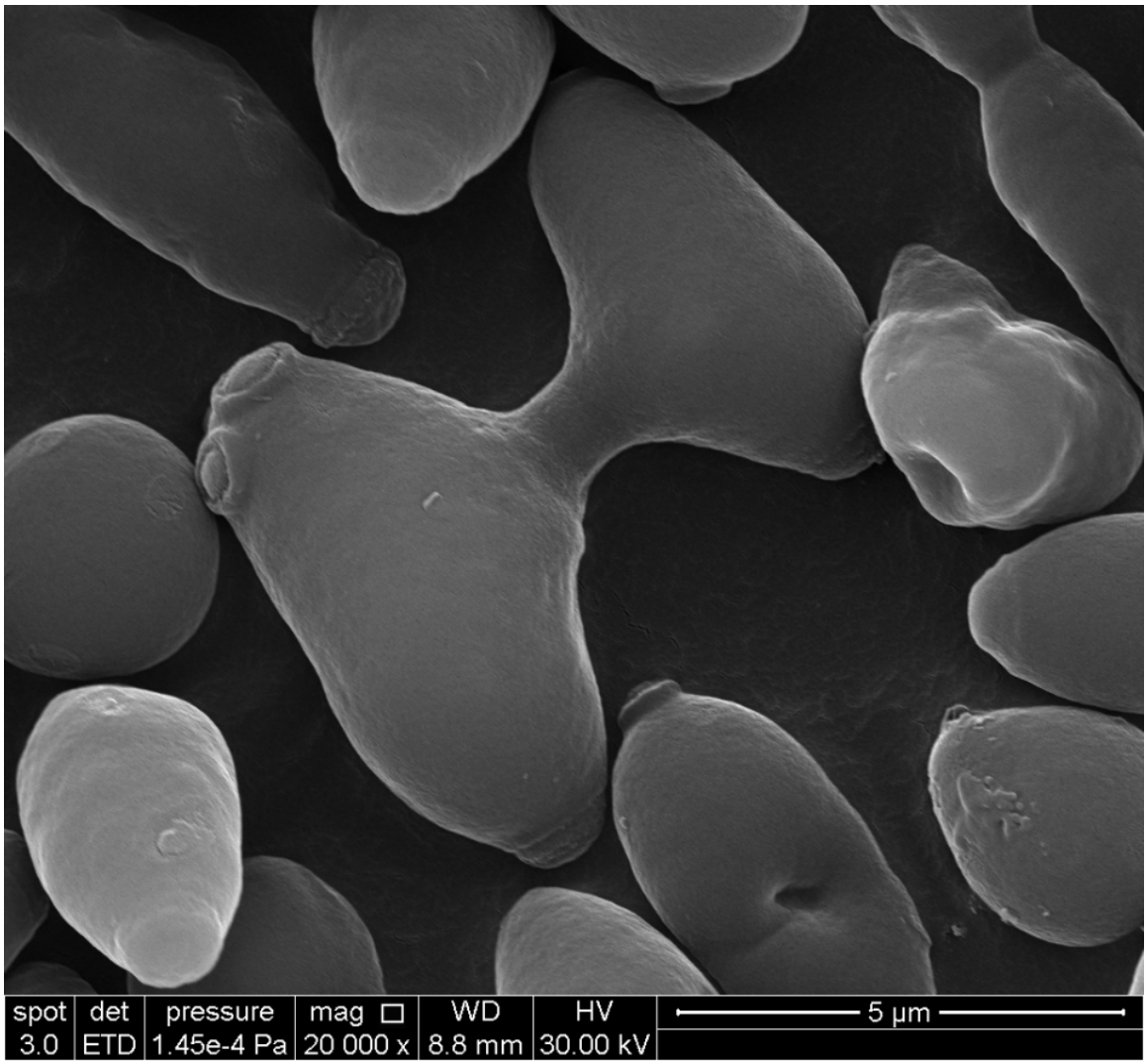

Figure 1. Scanning electron micrograph showing conjugation by *Z. bailii* grown on solid Yeast Maintenance Medium for 14 days

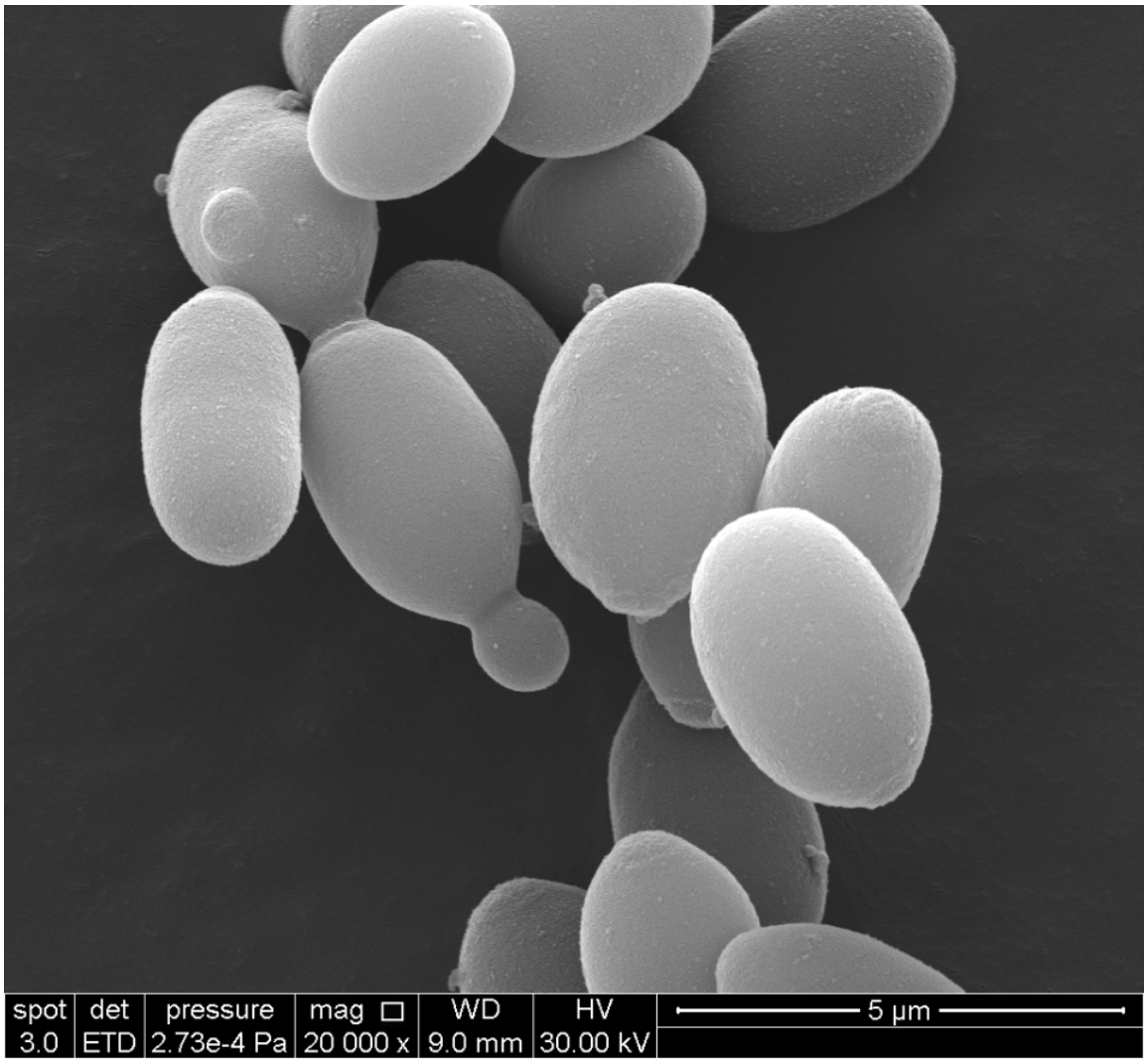

Figure 2. Scanning electron micrograph of *B. bruxellensis* grown in an enrichment medium

microscopically (Edwards 2005) occasionally having pseudomycelium (Aguilar-Uscanga et al. 2000; Smith 2011). Typical colony morphology has been described as matte to glossy, convex, light to cream in color, with smooth or mycelium-fringed margins (Edwards 2005; Smith 2011). Reproduction occurs asexually through multilateral budding or through sporulation, the latter of which has been demonstrated in certain media but not in wine (Smith 2011).

Recent investigation of the genome of *B. bruxellensis* has confirmed *B. bruxellensis* was separate from the Saccharomycetaceae or CTG groups defined by Dujon (2010). When compared with twenty other fungi, *B. bruxellensis* was most similar to several *Pichia* species (Curtin et al. 2012). Vigentini et al. (2012) noted that the species could be genetically divided into two primary clusters, with frequent occurrences of genetic polymorphism, but observed poor correlation among the clusters based on the geographic location of isolation. In contrast, earlier work by Conterno et al. (2006) suggested the species could be separated into six clusters with good correlation with the geographic location of isolation.

Isolation and identification

Distribution

Z. bailii and *B. bruxellensis* are renowned as two of the most detrimental spoilage-causing yeasts (Deak 2008; Sponholz 1993). Thought by some to be the most dangerous yeast to food processors (James & Stratford 2003), the yeast has infected such products as juices, sauces, soft drinks, salad dressings, ketchup, candied fruits, fruit juice concentrates, and syrups (Martorell 2007; Rossi et al. 2010). The first documented case of wine spoilage by *Z. bailii* occurred in 1973 when contamination was isolated from a pressure gauge and corking machine used to process sweetened wines (Rankine & Pilone 1973). Currently, both yeasts have been

isolated from wine-producing regions worldwide and throughout the winemaking process (Loureiro & Malfeito-Ferreira 2003, Conterno et al. 2006).

Although *Zygosaccharomyces* has been isolated in vineyard and winery equipment, it is most commonly found in grape juice concentrate or sweetened wines (Deak 2008; Loureiro & Malfeito-Ferreira 2003). If detected in the vineyard, isolation of *Zygosaccharomyces* species has been almost exclusively limited to sour rot damaged fruit (Costa et al. 2008; Loureiro & Malfeito-Ferreira 2003). In the winery, *Z. bailii* is the most frequently isolated species, followed by *Z. rouxii*, and *Z. bisporous* (James & Stratford 2003). Loureiro & Malfeito-Ferreira (2003) suggested detection rates from wine may be less than actual incidences, potentially because of inadequate methods of detection or a slow proliferation until environmental conditions eliminate competitive organisms. Control and early detection are challenging, as one cell in a bottle may eventually cause a spoilage event (Deak & Reichart 1986).

More frequently isolated than *Z. bailii*, the distribution of *B. bruxellensis* includes the vineyard (Renouf & Lonvaud-Funel 2007a), grape musts (Mateo et al. 1991), and bottled products, whether residual sugar is present or not (Renouf et al. 2008). As populations are likely to be small if present on grapes, reported isolations are fewer and less frequent (Renouf & Lonvaud-Funel 2007a). However, some strains have been reported to survive throughout alcoholic fermentation (Renouf et al. 2008).

As *B. bruxellensis* is frequently associated with barrel-aged red wines (Chatonnet et al. 1992; Ciani & Ferraro 1997), wood cooperage used in storage and aging may be a common vector for the introduction of *B. bruxellensis* into wines (Chatonnet et al. 1992). In fact, certain strains of *Brettanomyces* have been known to consume cellobiose (Blondin et al. 1982), a sugar often associated with barrels. The yeast has also been isolated from white and sparkling wine as

well as wine-processing equipment, including pumps, presses, transfer lines, tank valves, bottling lines, floor drains, and even air samples obtained from wineries (Conterno et al. 2006). As a consequence, the transport of contaminated equipment or bulk wine between separate facilities without proper care may spread *B. bruxellensis* to various wineries.

Selective and Differential Media

In addition to growing well in most enrichment media, several selective and differential options for isolation of *Zygosaccharomyces* exist. Although capitalizing on the ability of the organism to grow under stressful conditions is feasible, incubation times of several days to a week may be required (Erickson 1993; Makdesi & Beuchat 1996; Schuller et al. 2000). Currently, other media options exist for the isolation of *Z. bailii* and other wine yeasts (Table 1).

Acetic acid continues to be a frequent selective agent used for identification of *Z. bailii* (Erickson 1993; Fugelsang & Edwards 2007). The acid was first used by Dakin & Day (1958) in a fortified malt extract agar to isolate these spoilage yeasts from pickled products and dressings. At a concentration of 1.0%, acetic acid has been employed in a glucose enrichment medium for a simple and robust method for *Zygosaccharomyces* isolation (Fugelsang & Edwards 2007). Previously, Erickson (1993) described the *Z. bailii* selective medium (ZBA) in which selectivity was achieved with a lower concentration of acetic acid (0.5%) but also with 2.5% NaCl and 0.01% potassium sorbate. Used for yeast contamination in acidified food products, incorporation of 3.0% (w v^{-1}) fructose was noted by the authors to stimulate growth. The required incubation time of five days for ZBA can be reduced by the addition of a hydrophobic grid membrane filtration (HGMF) plating procedure. Here, HGMF allowed for greater aeration, improved agar structure, and easier colony color observation to differentiate *Zygosaccharomyces* from

Table 1. Common media for isolation / identification of wine yeasts.

	Selective agent	Differential agent	General selectivity
Zygosaccharomyces medium	1.0% acetic acid	--	*Zygosaccharomyces* spp.
Z. bailii selective medium (ZBA)	0.5% acetic acid	Hydrophobic grid membrane filtration	*Zygosaccharomyces* spp. and acid tolerant yeasts
Z. bailii differential medium (ZDM)	0.4% formic acid	Hydrophobic grid membrane filtration	*Z. bailii*
Dekkara bruxellensis differential medium (DBDM)	20 to 100 mg l^{-1} cycloheximide	*p*-coumaric acid	*Brettanomyces bruxellensis*

Hansenula or *Pichia*, which can also grow in the medium. Because of poorer selectivity, ZBA was determined to be less useful with nonacidified foods (Loureiro & Malfeito-Ferreira 2003).

Other media proposed to culture *Z. bailii* have been described. As examples, Makdesi & Beuchat (1996) suggested a tryptone, glucose, yeast extract agar (TGYA) with 0.5% acetic acid in which the authors reported improved recovery rates for *Z. bailii* in comparison with ZBA. Selectivity, however, remained a challenge as other spoilage yeasts (e.g., *Schizosaccharomyces pombe* and *Pichia membranefaciens*) could also be grown on TGYA (Hocking 1996). More recently, Schuller et al. (2000) described the *Z. bailii* differential medium (ZDM), which included 0.4% formic acid and 0.1% glucose as the sole carbon sources. Here, application of HGMF was also recommended to improve low growth recoveries of *Z. bailii* (60%), which could limit commercial application. Recovery rates of ZDM could be improved using a lower concentration of formic acid (0.3%), albeit with decreased selectivity against *Z. rouxii* and *Z. bisporous*.

Similarly to the selectivity of acetic acid for *Zygosaccharomyces*, most media used for isolation of *B. bruxellensis* rely on inclusion of cycloheximide. Although the compound inhibits protein biosynthesis in many eukaryotes, including *Saccharomyces* (Leach et al. 1947), *B. bruxellensis* is resistant (Smith 2011). Cycloheximide, also known as actidione, is frequently applied in media at concentrations ranging from 20 to 100 mg l^{-1} for isolation of *B. bruxellensis* or lactic acid bacteria (Fugelsang & Edwards 2007). However, Morneau et al. (2011) reported that other wine spoilage yeasts, including *Hanseniaspora uvarum*, *Pichia guillermondii*, and *Schizosaccharomyces pombe*, actively grew in 50 mg l^{-1} cycloheximide.

For greater specificity among non-*Saccharomyces* yeasts, Rodrigues et al. (2001) introduced *Dekkera bruxellensis* differential media (DBDM). The medium utilized 6.0% ethanol

as the sole carbon source and 10 mg l^{-1} cycloheximide as the selective agent. A differential agent was also included as 100 mg l^{-1} *p*-coumaric acid, a precurser in 4-ethylphenol production. The user was to identify 4-ethylphenol by the unique odor and confirm growth as *B. bruxellensis* (described below).

A major limitation of DBDM is the capability of all strains of *B. bruxellensis* to utilize ethanol as a sole carbon source. Conterno et al. (2006) reported that among 35 strains of *B. bruxellensis* studied, only 26% could utilize ethanol as a sole carbon source. In agreement, Morneau et al. (2011) isolated several strains that grew poorly or not at all on DBDM. In contrast, Jensen et al. (2009) successfully used DBDM media for the presumptive identification of nine *B. bruxellensis* isolates from Washington state wineries.

Molecular detection

Although plating on selective media is the traditional method for microorganism identification (Fugelsang & Edwards 2007), technologies specific to nucleic acid sequence amplification are gaining popularity because of advantages in time and specificity (Martorell et al. 2005; McKillip & Drake 2004). Here, sequences of DNA or RNA from specific microorganisms are targeted with unique probes and amplified by polymerase chain reaction (PCR) to positively identify a sample. Quantitative real-time PCR (qRT-PCR) utilizes a real-time thermocycler and fluorescent tags to both identify and quantify the population (Arya et al. 2005; McKillip & Drake 2004). Recently, qRT-PCR assays have been developed for several wine yeasts because they are faster, more specific, and more sensitive than traditional methods (Martorell et al. 2005; Phister & Mills 2003; Rawsthorne & Phister 2006).

To quantify *Z. bailii* populations in grape juice or wine, Rawsthorne & Phister (2006) targeted a sequence of the D1/D2 loop in the 26S rRNA subunit to measure the microorganism

by qRT-PCR. With total assay time of three hours, sensitivity was 22 cells ml^{-1} in grape juice and 6 cells ml^{-1} in wine also containing 10^7 cells *S. cerevisiae*. However, a limitation of the process was a type-I error due to quantification of nonviable cells, an issue that can be addressed through the use of ethidium monoazide (EMA) dye (Rawsthorne & Phister 2009). Selective for viable cells by permeating the cell membrane of dead cells and interfering with DNA (Soejima et al. 2007), qRT-PCR with EMA yielded results that were equivalent to traditional plating techniques. In fact, populations of only 12.7 viable cells ml^{-1} were detected among 10^5 heat-killed cells ml^{-1} in grape juice, thereby demonstrating the potential application of qRT-PCR with EMA for identification of *Z. bailii* in the wine industry.

Similar applications of PCR have been used for successful identification of *Brettanomyces*. Egli & Henick-Kling (2001) found the one and two internal transcribed spacer (ITS) regions of the *B. bruxellensis* rRNA gene an effective target for species, but not strain, identification using RFLP. More recently, qRT-PCR with primers for 26S rRNA was applied by Phister & Mills (2003) to detect populations ranging from 1 cell ml^{-1} to 10^6 cells ml^{-1} in wine. The authors reported no interferences from other wine yeast or bacteria. With a total assay time of three hours, this method demonstrates potential application in the wine industry for *Brettanomyces* identification.

Wine spoilage

Conditions affecting growth

Wine is a hostile environment for most microorganisms given its high concentrations of ethanol and acidic pH as well as the presence of SO_2 [sulfur dioxide (sulfites)] and cool temperatures during cellar storage. Additionally, the lack of usable carbon sources, nutrients, and oxygen can limit infections and growth. Thus, only a few microorganisms are capable of

tolerating these conditions, including *Zygosaccharomyces* and *Brettanomyces* (Loureiro & Malfeito-Ferreira 2003).

Zygosaccharomyces species have carbon and nitrogen requirements similar to *Saccharomyces* (James & Stratford 2011). One important difference is that whereas *S. cerevisiae* preferentially utilizes glucose rather than fructose, *Z. bailii* is the opposite. This fructophillic nature is due to a separate, specific, high-capacity transport system along with a glucose transport that additionally accepts fructose (Emmerich & Radler 1983; Sousa-Dias et al. 1996). Some additional carbon compounds fermented by *Z. bailii* include D-mannitol, D-glucitol, and sometimes ethanol, galactose, L-sorbose, sucrose, trehalose, glycerol, and ribitol (James & Stratford 2011). Ethylamine, L-lysine, and cadaverine can serve as nitrogen sources, whereas nitrate and nitrite are not assimilated. Additionally, one or more B-vitamins are typically required for growth (James & Stratford 2011; Thomas & Davenport 1985).

Exceptional tolerances to osmotic stress, ethanol, and a variety of common preservatives and organic acids characterize the ability of *Z. bailii* to contaminate and spoil wine. Martorell et al. (2007) found *Z. bailii* able to grow in up to 72% glucose (w v^{-1}) or, following an adaptation period, 90% glucose (w v^{-1}). Although ethanol tolerance of *Z. bailii* is strain dependent, growth in 20% has been recorded (Thomas & Davenport 1985). Kalathenos et al. (1995) suggested that *Z. bailii* may be more sensitive to ethanol than *S. cerevisiae*, indicating that variation between strains may be large. Strain variation was also documented by Betts et al. (1999), who reported the ideal pH for *Z. bailii* ranges from 2.5 to 7.0 at 22°C or 3.5 to 4.5 at 8°C, dependent on the strain. Growth at pH 2.2 was observed while documenting a range of sensitivity to propionic, sorbic, benzoic, or acetic acid (Martorell et al. 2007).

Similar to *Z. bailii*, the tolerance of *B. bruxellensis* to increasing ethanol concentrations varies by strain. Although ethanol exceeding 9.0% slows growth, some populations have been observed in wine with greater than 12.0% alcohol (Barata et al. 2008a, Rodrigues et al. 2001). Time for adaptation to ethanol is strain dependent as well, potentially causing an either enhanced or reduced sensitivity (Vigentini et al. 2008). Additionally, greater ethanol concentrations may repress the organism's ability to use ethanol as a sole carbon source (Silva et al. 2004).

Growth characteristics of the *B. bruxellensis* were further impacted by temperature, pH, and oxygen. The optimal temperature range for *B. bruxellensis* was 25 to 28°C (Smith 2011), although Barata et al. (2008b) demonstrated spoilage of red wine at 15°, 20°, and 25°C. However, the authors reported that 12 hours in 36°C wine resulted in a complete loss of viability in the population. Additionally, Couto et al. (2005) further suggest ethanol and ferulic acid increase sensitivity to thermal inactivation. In relation to pH, the effect on *B. bruxellensis* has not been thoroughly investigated in wine; however, growth in enrichment media was observed at pH 2.0 (Conterno et al. 2006).

Oxygen is not required for growth of *B. bruxellensis*, a yeast that is considered to be a facultative anaerobe (Smith 2011). Under certain conditions, however, *Brettanomyces* spp. may metabolize carbohydrates to acetic acid rather than ethanol in glycolysis (Ciani & Ferraro 1997). Through this pathway, the accumulated NADH may be reoxidized more quickly. Known as Custer's Effect, alcoholic fermentation is temporarily repressed under anaerobic conditions (Wijsman et al. 1984). Semiaerobic environments increase growth rates, alcoholic fermentation, and acetic acid production (Ciani & Ferraro 1997).

Commonly reported in bacteria, there is growing evidence that environmental stress can induce a viable but nonculturable (VBNC) state in both *Zygosaccharomyces* and *Brettanomyces*

(Fleet 1999; Serpaggi et al. 2012). In this dormant physiological state, cells remain alive but cannot be cultured using synthetic media without some form of resuscitation (Palkova & Vachova 2006). In wine, both *Z. bailii* and *B. bruxellensis* have been reported to enter VBNC states following sulfur dioxide additions (Agnolucci et al. 2010; Divol & Lonvaud-Funel 2005; du Toit et al. 2005). Although Divol & Lonvaud-Funel (2005) reported that removal of free SO_2 was not sufficient to revive VBNC yeast populations in wine, the opposite effect was reported by Serpaggi et al. (2012).

Impact on wine quality

Spoilage activity manifested as carbon dioxide production within sealed containers of grape juice concentrate or sweetened wine is a major concern with *Zygosaccharomyces* contamination. Excessive gas formation carbonates still wines or even causes containers to burst or explode (Deak 2008; Stratford 2006). In a recent survey on the use of grape juice concentrate, *Z. rouxii* represented 75% of total isolated yeast (Combina et al. 2008). Additionally, the authors identified *Z. rouxii* in each case of spoilage in concentrate. Risks of *Zygosaccharomyces* infections are exacerbated if room temperature storage is used for grape juice concentrate rather than cooler, cellar conditions (Boulton et al. 1996). Besides carbon dioxide production, additional impacts of *Zygosaccharomyces* on grape juice or wine quality include taste modifications, hazes, surface films, or sediments (James & Stratford 2003; Fleet 2003; Pretorius 2000). For example, *Zygosaccharomyces* can produce a variety of sensorial compounds, such as acetic acid, fruity esters, acetoin, and higher-order alcohols (Romano & Suzzi 1993; Thomas & Davenport 1985).

Although these and other metabolites cause spoilage, some evidence suggests that some benign, nonspoilage strains of *Zygosaccharomyces* may not be as detrimental to wine quality as

others (Romano & Suzzi 1993; Thomas & Davenport 1985). When compared with other non-*Saccharomyces* wine yeasts, most *Zygosaccharomyces* produced less ethyl acetate, an off-aroma in wine (Domizio et al. 2011). Additionally, *Zygosaccharomyces* species may produce 150 to 250 mg l^{-1} of polysaccharides during grape juice fermentation, thereby positively contributing to mouthfeel (Domizio et al. 2011; Romani et al. 2009). Finally, although malolactic fermentations using lactic acid bacteria are common in the wine industry, *Z. bailii*, like *S. cerevisiae*, is also capable of malic acid metabolism (Kuczynski & Radler 1982).

Typically more identifiable and distinct than *Zygosaccharomyces*, metabolites produced by *B. bruxellensis* result in a unique sensory impact known as the Brett character. The wide array of descriptive terms include leather, clove, spice, smoky, animal, stable, and medicinal (Chatonnet et al. 1992; Romano et al. 2009). Compounds responsible for the Brett character primarily include volatile phenols, although acetic acid and some fatty acids are produced as well (Ciani & Ferraro 1997; Ciani et al. 2003). The impact on wine quality becomes obvious as the volatile phenol concentration exceeds the sensory threshold of 700 mg l^{-1} (Loureiro & Querol 1999).

Volatile phenols produced by *B. bruxellensis* include 4-ethylphenol and 4-ethylguaiacol (Chatonnet et al. 1992). Synthesis occurs through decarboxylation of hydroxycinnamic acid precursors to intermediates 4-vinylphenol and 4-vinylguaiacol followed by an enzyme-catalyzed reduction to the final products (Figure 3). Although production of 4-ethylphenol and 4-ethylguaicol is most frequently attributed to *B. bruxellensis* contamination, lactic acid bacteria, *Pichia guilliermondii*, and several *Candida* species also produce the compounds (Barata et al. 2006; Chatonnet et al. 1992, 1997; Couto et al. 2006; Dias et al. 2003). However, *B. bruxellensis*

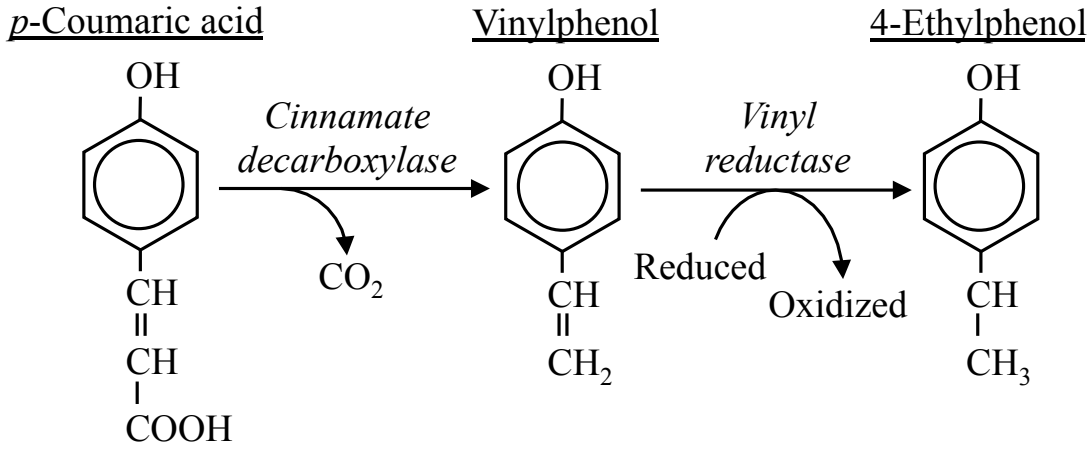

Figure 3. Pathway for volatile phenol synthesis by *B. bruxellensis*

is the only microorganism isolated producing these volatile phenols in wine at great enough concentrations for a sensory impact (Chatonnet et al. 1997; Dias et al. 2003).

Methods of control

To reduce microbial spoilage during wine processing, adherence to proper sanitation protocols throughout the winery are essential (Fugelsang & Edwards 2007; Marriot & Gravani 2006). Good manufacturing practices (GMPs) and standard operating procedures for wineries include washing and sanitizing tanks, lines, pumps, and equipment between each use (Loureiro & Malfeito-Ferreira 2003; Storm 1997). Specifically, Martorell et al. (2007) emphasized the importance of proper cleaning and biocidal agents because of the extensive tolerance of *Z. bailii* and *Z. rouxii* to common food preservatives. Sanitation practices are also essential to mitigate formation of persistent biofilms, which *Z. bailii*, *Z. rouxii*, and *B. bruxellensis* can form in the winery (Joseph et al. 2007; Tomita et al. 1997; Tristezza et al. 2010).

Although sanitation protocols are critical, the winemaker must also balance the application of antimicrobial technologies with maintaining wine style and quality. While storage conditions such as temperature or exposure to oxygen as well as ethanol concentration and pH can limit microbial growth, of concern is that adjustment of any of these attributes will impact the sensory attributes of the wine. To minimize any detrimental impact on quality, a variety of traditional and emerging nonthermal antimicrobial technologies exist (Table 2).

Sulfites (SO_2)

SO_2 has been extensively used in food and wine processing as an antioxidant and antimicrobial agent (Ough 1993). The compound is often added as the potassium metabisulfite salt, although gaseous SO_2 is sometimes used (Fugelsang & Edwards 2007). In the United States, the maximum legal limit of total SO_2 in wine is 350 mg l^{-1}, with labeling required for

Table 2. Technologies to limit yeast spoilage of wine.

	Primary application	Current use in winemaking	Relative lethality	
			Z. bailii	*B. bruxellensis*
Sulfur dioxide	Pre/post-fermentation	Frequent	Low	Moderate
Dimethyl dicarbonate	Sterile bottling	Moderate	Moderate	Moderate
Chitosan	Finished wine	Emerging	Moderate	Moderate
Pulsed electric field	Grape must treatment	Emerging	High	High
Low electric current	Pre/post-fermentation	Emerging	Unknown	Moderate
Ultrasonics	Barrel sanitation	Emerging	High	High
Ozone	Winery sanitation	Moderate	High	High
Filtration	Finished wine	Frequent	Low to high	Low to high

concentrations exceeding 10 mg l^{-1} (Code of Federal Regulations 2011b). More recently, the International Organization of Vine and Wine (OIV) and others have advocated less use because of health risks posed to sulfite-sensitive or asthmatic individuals (Santos et al. 2012; Threlfall & Morris 2006).

The chemical and antimicrobial effect of SO_2 is related to the availability of the molecule and the pH of the wine. In solution, SO_2 exists in either free or bound forms, the latter created through reaction with carbonyl-containing compounds, such as acetaldehyde. Within the free forms, a pH-dependent equilibrium exists between molecular ($SO_2 \bullet H_2O$), bisulfite (HSO_3^-), and sulfite (SO_3^{2-}) as follows:

$$SO_2 + H_2O \leftrightarrow SO_2 \bullet H_2O \quad pK_a = 1.8$$

$$SO_2 \bullet H_2O \leftrightarrow HSO_3^- + H^+$$

$$HSO_3^- \leftrightarrow SO_3^{2-} + H^+ \quad pK_a = 7.2$$

Of forms of sulfites found in grape juices or wines, molecular SO_2 (mSO_2) is believed to be the most important for control of wine microorganisms (Fugelsang & Edwards 2007). Although bisulfite is the most abundant form at normal wine pH, the neutral charge of mSO_2 allows for diffusion across cell membranes. Once in the cell, the less acidic cytoplasm promotes an increase in the relative amount of bisulfite and sulfite from mSO_2, which, in turn, reduces its internal concentration to allow more to enter the cell. This equilibrium activity induces a concentration gradient that ultimately reduces intercellular pH. Microbial inhibition by sulfites is caused by its interactions with ATP, NAD^+, and FAD; induction of mutations in genetic material through deamination of cytosine and uracil; and disruption of disulfide bridges in proteins (Hinze & Holzer 1986; Pagano et al. 1990; Schimz 1980). In winemaking, mSO_2 is a calculated value

based on free SO_2 and pH in which 0.8 mg l^{-1} is often recommended to control most spoilage microorganisms (Fugelsang & Edwards 2007).

Application of SO_2 has little impact on *Z. bailii* populations because of various resistance mechanisms. Similar to many other yeasts, the cellular response includes sulfur reduction, acetaldehyde production, or even an active molecular transport to remove SO_2 from the cell (Park & Bakalinsky 2000; Stratford et al. 1987). In addition, *Z. bailii* is unique with a less permeable cellular membrane and a much greater ability to increase acetaldehyde production rates (Pilkington & Rose 1988, Warth 1984). As a result, growth of *Z. bailii* in excess of 3.0 mg l^{-1} mSO_2 is possible (Thomas & Davenport 1985).

Most wine microorganisms such as *B. bruxellensis* are typically less resistant to SO_2 than *Z. bailii*. Although growth in 1.79 mg l^{-1} mSO_2 was observed with a single strain of *B. bruxellensis* in an enrichment medium (Jensen et al. 2009), concentrations of 0.2 to 0.5 mg l^{-1} mSO_2 typically inhibit growth in wines (Barata et al. 2008a; Conterno et al. 2006; du Toit et al. 2005). This increased sensitivity to mSO_2 in wine is caused by increasing ethanol concentrations as well as less available oxygen (Barata et al. 2008a; Duckett 2012; du Toit et al. 2005). Additionally, some evidence suggests that individual cell size may shrink when exposed to sulfur dioxide (Agnolucci et al. 2010).

Dimethyl dicarbonate

Similar to SO_2, dimethyl dicarbonate (DMDC) is added to fruit juice, must, and wine to inactivate spoilage microorganisms (Costa et al. 2008). Sold under the trade name Velcorin®, the compound has been approved since 1988 in the United States for use in product contaminated with fewer than 500 cells ml^{-1} of yeast, bacteria, or molds. Technically a processing aid in both the United States (Code of Federal Regulations 2011a) and European Union (Official Journal of

the European Union 2006), maximum legal concentrations of 200 mg l^{-1} are allowed. Although treatment of grape must is legal, the more common application of DMDC is to finished wine immediately prior to the filling bowl before bottling (Renouf et al. 2008). In wineries, the primary challenges of DMDC treatment include the cost of dosing equipment, proper safety and operator training, and maintaining a constant rate of addition (Boulton et al. 1996).

The antimicrobial activity of DMDC is largely related to enzyme inhibition. Methoxycarbonylation of imidazoles, amines, and thiols results in the disruption of metabolic enzyme (e.g, alcohol-dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase) function in glycolysis (Ough 1993; Renouf et al. 2008). The inhibitory effect of a 200 mg l^{-1} treatment lasts for less than 12 hours (Delfini et al. 2002), as DMDC rapidly hydrolyzes to negligible amounts of carbon dioxide and methanol (Figure 4), the latter of which should be below legal concentration limits.

Recent evidence suggests that DMDC may be less effective at controlling contamination with *Zygosaccharomyces* than with *Brettanomyces* spp. For example, treatment of 200 mg l^{-1} did not inhibit growth of 10^6 cfu ml^{-1} populations of *Z. bailii* in grape must or red wine (Delfini et al. 2002; Martorell et al. 2007). Even when DMDC treatment was used in wine already containing free mSO_2, growth of *Z. bailii* was only temporarily prevented (Divol et al. 2005). However, small populations of *Z. bailii* (500 cfu ml^{-1}) were reportedly among the most sensitive of wine yeasts, requiring treatment with only 25 mg l^{-1} DMDC (Costa et al. 2008). In contrast, populations of *B. bruxellensis* exceeding 10^4 cfu ml^{-1} were removed in wine with a 200 mg l^{-1} treatment (Costa et al. 2008; Renouf et al. 2008). Here, populations less than 500 cfu ml^{-1} were eliminated using only 100 mg l^{-1} DMDC (Costa et al. 2008).

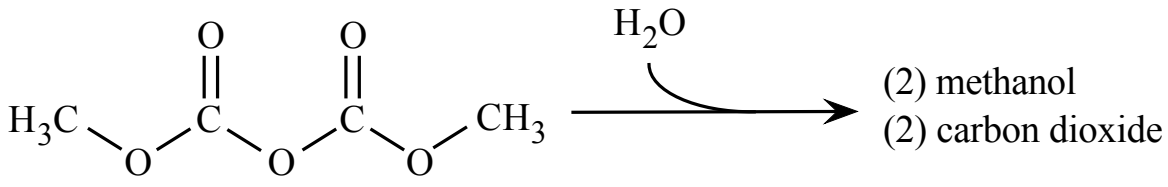

Figure 4. Hydrolyzation of dimethyl dicarbonate

Chitosan

Being the N-deacetylated derivative of chitin, chitosan represents a broad group of cationic polymeric molecules (Kong et al. 2010; Rinaudo 2006). Unique among most polysaccharides, chitosan is highly basic and thus most soluble in weak organic acid solutions (Kumar 2000). These molecules are biodegradable, environmentally nontoxic, and antimicrobial to many yeasts, bacteria, and filamentous fungi (Kong et al. 2010; Kumar 2000). Recently, chitosan has been used in a variety of applications throughout food, beverage, and medical fields, including as chelating or clarifying agents (Kong et al. 2010).

The antimicrobial effect of chitosan is related to an interaction between the cationic polymer and the cell membrane (Kong et al. 2010). Cell death is due to the compromised integrity of the cell membrane and cell wall causing the leakage of cellular components along with interference to membrane-bound energy generation pathways (Eaton et al. 2008, Kong et al. 2010; Zakrzewska et al. 2005). Additionally, chitosan can act as a fining agent, causing agglutination and precipitation due to electrostatic interaction with the cell membrane (Sudarshan et al. 1992; Savard et al. 2002). Antimicrobial efficacy is dependent on the molecular weight and degree of deacetylation of the compound as well as pH, Pk_a, specific microorganism targeted, and the presence of metal cations (Rabea et al. 2003; Zivanovic et al. 2004; Kong et al. 2010).

Although the application of chitosan as an antimicrobial technology in the wine industry is relatively recent, treatment to limit growth of both *Z. bailii* and *B. bruxellensis* has been effective (Gomez-Rivas et al. 2004; Zakrzewska et al. 2005). Application of 0.1 to 6.0 g l^{-1} slowed the lag growth phase of *B. bruxellensis* and completely inhibited growth of *Z. bailii* (Gomez-Rivas et al. 2004; Roller & Covill 1999). Furthermore, when a mixed culture of *S.*

cerevisiae and either *Z. bailii* or *B. bruxellensis* was treated with chitosan, growth of the spoilage yeast was inhibited, but *S. cerevisiae* growth continued unabated (Gomez-Rivas et al. 2004; Zakrzewska et al. 2005).

Pulsed electric field

Pulsed electric field (PEF) processing is an emerging, nonthermal technology for pasteurization or sterilization of homogeneous liquids (Santos et al. 2012). Strong electric fields ranging from 26 to 35 Kv cm^{-1} are produced in 1 to 4 μs pulses between two electrodes contacting the product (Garde-Cerdan et al. 2007; Marselles-Fontanet et al. 2009; Puertolas et al. 2009; Raso et al. 1998). Cell death is achieved as dielectric breakdown increases permeability of the cytoplasmic and nuclear membranes, leading to cell lysis (Puertolas et al. 2010). Although PEF processing is effective against most microorganisms, yeasts tend to be more sensitive than bacteria (Marselles-Fontanet et al. 2009).

PEF has been investigated as a means to reduce microbial contamination in wines (Santos et al. 2012). Applied to both grape musts and finished wines, treatment is generally more effective when used after completion of fermentation (Puertolas et al. 2009). Although a five log reduction of a *Z. bailii* population was recorded in grape must, similar populations were completely eradicated in finished Sauvignon Blanc wine using between 2 and 30 pulses (Zhong et al. 2007). In contrast, Puertolas et al. (2009) reported that PEF treatment reduced populations of *B. bruxellensis* by 99.9% in both grape must and wine.

In addition to microbial control, PEF treatment has been shown to inactivate enzymes in grape must. Specifically, structural alterations of polyphenol oxidase and peroxidase reduced the abilities of the enzymes to brown grape musts (Zhong et al. 2007). In fact, Garde-Cerdan et al. (2008a,b) demonstrated that a reduced concentration of SO_2 was required when applied along

with PEF treatment for an organoleptically equivalent wine. Additionally, prefermentation PEF treatment had no measurable impact on free amino acid or fatty acid content, important components for yeast nutrition (Garde-Cerdan et al. 2007).

Low electric current

In contrast to PEF, treatment with low electric current (LEC) uses a low-power, constant electric charge to prevent microbial growth (Palaniappan et al. 1990). Current is generally applied to the product at less than 200 Ma over the period of several days to several months (Lustrato et al. 2010; Palaniappan et al. 1992). Microbial inactivation is caused by electrical breakdown in the membrane's lipid bilayer (Lustrato et al. 2003). Increasing this current resulted in a direct relationship with reduced membrane integrity and metabolic activity (Lustrato et al. 2003; Ranalli et al. 2002).

LEC can be applied throughout alcoholic fermentation or to finished bulk wine to prevent growth of spoilage microorganisms. When used during the fermentation in place of SO_2, LEC generally was lethal to non-*Saccharomyces* yeasts, whereas inoculations of *Saccharomyces* were able to complete fermentation (Lustrato et al. 2003; 2006). Applied to bulk finished wine, Lustrato et al. (2010) concluded that a 200 Ma treatment over a 60-day interval reduced *B. bruxellensis* populations by over six logs.

The impact on extended LEC on wine sensory quality is unclear. Lustrato et al. (2006) reported that wine fermented using LEC was not organoleptically different from wine fermented using SO_2. In contrast, Nakanishi et al. (1997) observed that wine fermented using a 100 Ma treatment had overall greater final concentrations of higher alcohols, esters, some organics acids, and acetaldehyde.

Ultrasonics

High-power ultrasonic technologies have been used throughout the past decade for pasteurization, sterilization, and enzyme inactivation of foods (O'Donnell et al. 2010; Piyasena et al. 2003). A wand or probe is submerged in liquid and used to create rapid pulses of energy between 20 and 100 kHz. Consequently, pressure differences cause cavitations with rapid creation and destruction of microscopic bubbles. By creating brief and highly localized regions of pressure and temperature that can exceed 5,500°C and 50 Mpa, microbes and some enzymes are inactivated from heat and stress, although the overall product temperature is unaffected (Butz & Tauscher 2002; Knorr et al. 2004; O'Donnell et al. 2010; Piyasena et al. 2003).

In the wine industry, ultrasonic technologies have been used for cleaning tartrate deposits from barrels and, more recently, inactivation of spoilage microbes (Jiranek et al. 2008, Schmid et al. 2011). For example; Luo et al. (2012) reported that treatment for 20 minutes reduced *Zygosaccharomyces* populations by 15% in wine and 50% in juice. In contrast, a 10-minute treatment of oak barrels contaminated with *B. bruxellensis* effectively eliminated the entire population (Schmid et al. 2011). In fact, the authors reported that ultrasonic treatment was more effective at barrel sanitation than high-pressure hot water washes. Furthermore, no organoleptic differences between wines aged in ultrasonic treated barrels or ones washed with hot water were found.

Ozone

Rather than an antimicrobial treatment to grape must or wine, ozone is used in a gas or water carrier to sanitize processing equipment and surfaces (Kim et al. 2003). Declared GRAS (generally recognized as safe) for processing in the United States in 1997, ozone is produced by passage of oxygen gas through a high-voltage electric field (Green et al. 1993; Kim et al. 2003).

Once in aqueous solution, ozone (O_3) decomposes into oxygen (O_2). Half-life may be several minutes to several hours depending on temperature, pH, and purity of the water (Khadre et al. 2001; Wickramanayake et al. 1984; Wynn et al. 1973).

The antimicrobial effect of ozone is caused by oxidation reactions with a variety of components of the cell (Hampson 2000; Kim et al. 2003). Both molecular ozone and hydroxyl radical by-products can react with proteins, respiratory enzymes, nucleic acids, and unsaturated lipids within the cell membrane, typically through oxidative radical reaction (Dubeau & Chung 1982; Khadre et al. 2001). The antimicrobial effects of ozone have been demonstrated with yeasts, bacteria, and viruses (Hampson 2000; Khadre et al. 2001; Kim et al. 2003).

In the wine industry, ozone and ozonated water have been used for sanitation of stainless steel tanks, surfaces, oak barrels, and clean-in-place (CIP) systems (Hampson 2000; Khadre et al. 2001). Guillen et al. (2010) demonstrated that a 10-minute treatment with ozonated water was a more effective antimicrobial agent in winery CIP systems than either peracetic acid or caustic soda cleaning agents. Similarly, gaseous ozone effectively reduced *B. bruxellensis* populations in oak cubes meant to simulate wine barrels (Cantacuzene 2004). Furthermore, barrels treated with room-temperature ozonated water lost fewer oak volatiles than barrels cleaned with scalding water (Marko et al. 2005).

Filtration

Filtration technology is used to stabilize many commercial wines. In addition to reducing browning (Goodwin & Morris 1991) and removing colloids to minimize haziness (Manninger et al. 1998; Peri et al. 1988), specific porosity membranes are commonly used to filter yeast and bacteria from the wine (Fugelsang & Edwards 2007). If wines are not filtered prior to bottling,

there is potential for spoilage by a variety of microorganisms (Rayess et al. 2011; Ubeda & Briones 1999).

Selection of a membrane pore size in wine filtration depends on requirements for throughput, economy, and effectiveness at removing microorganisms. Although some winemakers elect to use larger porosities to improve flow rates, 0.45 μm pore sizes are conventionally recommended to remove yeast and bacterial populations from wine (Fugelsang & Edwards 2007). Renouf et al. (2007b) reported that a 1.0-μm filter is sufficient to remove either *Z. bailii* or *B. bruxellensis* from wine, but this study utilized nominal filtration. Rather than absolute membranes, the more economical nominal filters are constructed to only remove a majority of particles of the indicated micron rating. Using absolute membrane filters, several authors have demonstrated that cell size of *B. bruxellensis* may shrink when exposed to sulfur dioxide (Agnolucci et al. 2010; Millet & Lonvaud-Funel 2000). In fact, Millet & Lonvaud-Funel (2000) reported that 0.45-μm filtration was insufficient to remove *B. bruxellensis* from wine after sulfur dioxide treatment.

Although microbiological stabilization is a priority for some winemakers, there is debate regarding the impact of sterile filtration on wine quality. For example, Cabernet Sauvignon filtered through a 0.65-μm membrane reduced color intensity and the concentration of approximately 10% of aroma compounds measured (Arriagada-Carrazana et al. 2005). Additionally, total ester concentration was reduced after diatomaceous earth filtration, and amounts of colloids and phenolics decreased after ultrafiltration (Cattaruzza et al. 1987; Moreno & Azpilicueta 2006). However, filtration did not reduce the organoleptic character of wine in reports by Gergely et al. (2003). Furthermore, red wine flavor along with white wine color and

clarity both improved following ultrafiltration treatment (Peri et al. 1988; Spassov & Dinkov 2002).

Conclusions

Control of wine spoilage by *Z. bailii* and *B. bruxellensis* requires rapid detection methods and a balance between inhibiting yeast growth and maintaining wine quality. Limiting wine spoilage may best be accomplished by avoiding cross contamination through adherence to proper sanitation procedures. Additionally, reduced cellar temperatures may limit growth of some *B. bruxellensis* strains but is less effective against *Z. bailii*. Similarly, sulfur dioxide is more likely to limit growth of *B. bruxellensis* than *Z. bailii*. Research continues to evaluate the effectiveness of DMDC, chitosan, PEF, LEC, ultrasonics, ozone, and filtration to provide winemakers additional technologies to combat yeast spoilage of wine.

CHAPTER II

APPLICATION OF *ZYGOSACCHAROMYCES BAILII* TO REMOVE RESIDUAL SUGAR FROM STUCK FERMENTATIONS

Abstract

An alternative approach to remove residual sugar from partially-fermented wines using yeasts other than *Saccharomyces* was studied. To simulate stuck fermentations, dry red wines were adjusted to 13, 15, or 17% v v^{-1} ethanol and 40 or 60 g l^{-1} fructose prior to inoculation with *Zygosaccharomyces bailii* (strains W3, ZB2, or ZB6) or S*accharomyces cerevisiae* (strain EC1118) at ≈10^8 cfu ml^{-1}. Most strains maintained culturable populations ≥10^6 cfu ml^{-1}, the exceptions being W3 and EC1118 which declined to undetectable levels past day 75 in 17% ethanol wines. However, these same strains consumed >90% of the 40 g l^{-1} or 33% of the 60 g l^{-1} fructose present in the 15% ethanol wines. None of the wines containing 15 or 17% ethanol and inoculated with *Zygosaccharomyces* reached dryness (<2 g l^{-1}), although wines inoculated with W3 achieved 3 g l^{-1} fructose (40 g l^{-1} fructose/13% ethanol). Few differences in sugar utilization were noted in wines containing 17% ethanol where metabolic activities ceased at approximately 50% of the original amount of fructose present. In general, volatile acidities were higher in wines inoculated with *Zygosaccharomyces* compared to *Saccharomyces*. While *Z. bailii* metabolized some residual fructose in wines of varying alcohol content, the use of *S. cerevisiae* was generally more effective and did not produce as much acetic acid.

Introduction

Stuck or sluggish fermentations result in wines of lower than expected ethanol amounts and concentrations of residual sugars in excess of 2 g l^{-1} (Bisson & Butzke 2000). While a number of causative factors have been implicated such as enological practices, nutrient

deficiencies, or inhibition by substances such as ethanol or acetic acid (Alexandre & Charpentier 1998; Bisson 1999), rectifying strategies are rather limited. To restart fermentation, wines can be racked, supplemented with nutrients, and re-inoculated with different strains of *Saccharomyces* or incrementally added to vigorous fermentations (Bisson & Butzke 2000; Cavazza et al. 2004; Fugelsang & Edwards 2007). Under these conditions, fermentative success can be limited due to inhibitory amounts of ethanol (Cavazza et al. 2004). Furthermore, *Saccharomyces* prefers glucose rather than fructose, however the latter sugar is commonly present in higher relative amounts in sluggish/stuck alcoholic fermentations (Berthels et al. 2006; 2008).

As alternative methods to remove residual sugar, Santos et al. (2008) suggested the use of non-*Saccharomyces* yeasts such as *Zygosaccharomyces bailii*. Even though *Z. bailii* is traditionally associated with spoilage (Rankine & Pilone 1973), some strains may not adversely impact wine quality as previously thought (Romani et al. 2009; Domizio et al. 2011). In fact, many are fructophilic and able to grow in ≥18% v v^{-1} ethanol which would be advantageous to restart stuck fermentations (Emmerich & Radler 1983; Santos et al. 2008). In support, Santos et al. (2008) demonstrated that *Z. bailii* could metabolize 25 g l^{-1} fructose within six days in a medium that simulated a stuck fermentation (12% v v^{-1} ethanol). However, the authors did not examine ethanol concentrations higher than 12% or fructose concentrations exceeding 25 g l^{-1}, which are frequently encountered by the industry.

In support of the hypothesis that some *Z. bailii* strains may utilize sugar from high alcohol environments without detrimental sensory affects, two strains, ZB2 and ZB6, were recently isolated from a large commercial winery without a history of associated spoilage issues. As these strains did not cause obvious spoilage, their abilities to metabolize residual fructose present in a red wine simulating stuck fermentations was evaluated.

Materials and methods

Wines

A commercially produced 2009 Cabernet Sauvignon (pH 3.92, 13.4% v v^{-1} ethanol) was filtered through 1 μm nominal pads (Gusmer Enterprises, Fresno, CA) and total SO_2 was removed using hydrogen peroxide. The pH was adjusted to 3.7 using tartaric acid (500 g l^{-1}) and 0.1 g l^{-1} yeast extract was aseptically added. The wine was sterile filtered through Nylon® 0.2 μm cartridge membranes in sanitary filter housings (Pall, Port Washington, NY) before addition of 0.1 g l^{-1} cellulose (20 μm). Wines were then adjusted to contain 40 or 60 g l^{-1} fructose and 13, 15, or 17% v v^{-1} ethanol before transfer into sterile dilution bottles (100 ml) and incubation at 18°C.

Yeast cultures

Zygosaccharomyces bailii ZB2 and ZB6 were originally isolated from a commercial winery while *Z. bailii* W3 and *S. cerevisiae* EC1118 were obtained from Lallemand Inc. (Quebec, Canada). Inoculums were prepared from a single colony in 10 ml yeast/mold (YM) broth (pH 4.5), which was subsequently added into 100 ml YM broth containing 5% v v^{-1} ethanol to acclimate cells to wine conditions. Cells grown to late exponential growth phase were then harvested by centrifugation (2000 *g*) for 20 min and washed twice in 0.2 M Na_2HPO_4 (pH 7.0) buffer before inoculation into wines at 10^8 cfu ml^{-1}.

Analyses

Yeast culturability was determined by plating on Wallenstein Laboratory agar (WL) using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD) while fructose concentration was determined by enzymatic assay (r-biopharm, Darmstadt, Germany). Volatile acidity was measured using a Cash still (Research & Development Glass, Berkeley, CA). All treatments (fructose x ethanol x strain) were conducted in triplicate and reported as mean

populations. Two-way analysis of variance (ANOVA) and Fisher's LSD were applied for mean separation using XLSTAT software (Addinsoft, New York, NY) for significance ($p \leq 0.05$).

Results

In wines with 13% ethanol, culturable populations remained close to inoculation levels of approximately 10^8 cfu ml^{-1} for 100 days (Figure 5). With higher amounts of ethanol, culturabilities quickly declined approximately one log but remained between 10^6 and 10^8 cfu ml^{-1}. The exceptions to this observation were *Z. bailii* W3 and *S. cerevisiae* EC1118 which slowly declined in 17% v v^{-1} ethanol wines to 10^5 cfu ml^{-1} by day 65 and were less than limits of detection by day 100 (<30 cfu ml^{-1}).

Metabolic utilization of fructose depended not only on species and strain but also the amount of ethanol present (Figure 6). With the exception of *Z. bailli* ZB6, all species entirely consumed 40 g l^{-1} fructose in wines containing 13% ethanol. Despite the loss of culturability, *Z. bailii* W3 and *S. cerevisiae* EC1118 consumed >90% of the 40 g l^{-1} or 33% of the 60 g l^{-1} fructose present in the 15% ethanol wines. However, none of the wines containing 15 or 17% ethanol reached dryness (<2 g l^{-1}) when inoculated with *Zygosaccharomyces*, although wines inoculated with W3 achieved 3 g l^{-1} fructose (40 g l^{-1} fructose/13% ethanol). Few differences in sugar utilization were noted in wines containing 17% ethanol where metabolic activities ceased at approximately 50% of the original amount of fructose present.

As illustrated in Table 3, wines inoculated with *S. cerevisiae* contained less volatile acidity (VA) than those inoculated with *Z. bailii* in 15% ethanol wines. While VA values of wines with *Saccharomyces* were 0.71 to 0.77 g l^{-1}, those inoculated with *Zygosaccharomyces* ranged between 0.8 g l^{-1} up to 1.0 g l^{-1}. This affect was only attributable to strain and no significant differences were noted with wines containing 17% ethanol (Table 4).

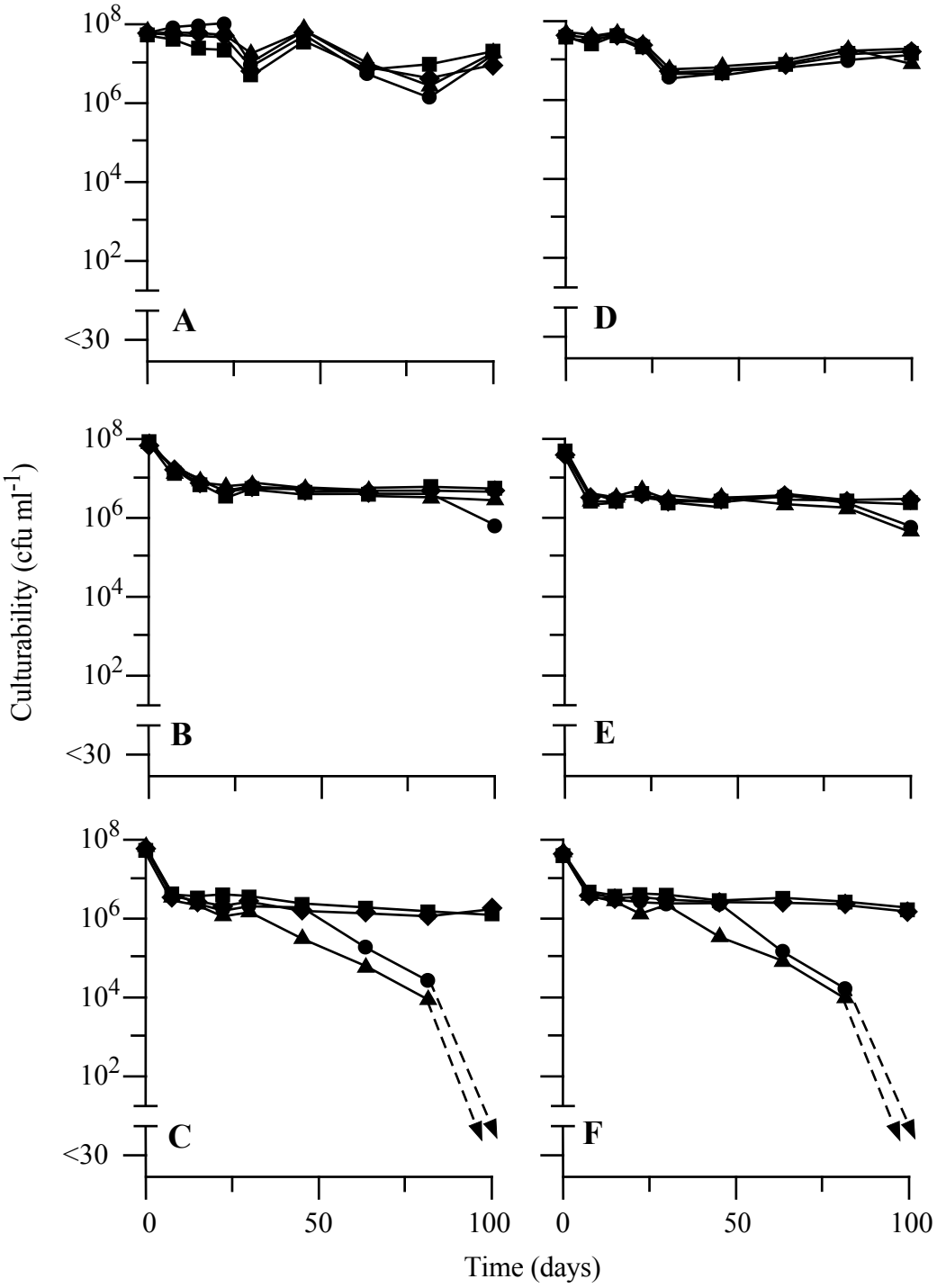

Figure 5. Culturability of *Z. bailii* ZB2 (◆), *Z. bailii* ZB6 (■), *Z. bailii* W3 (▲), or *S. cerevisiae* EC1118 (●) inoculated into red wines containing 40 (A, B, C) or 60 (D, E, F) g l^{-1} fructose and 13% (A, D), 15% (B, E), or 17% (C, F) v v^{-1} ethanol.

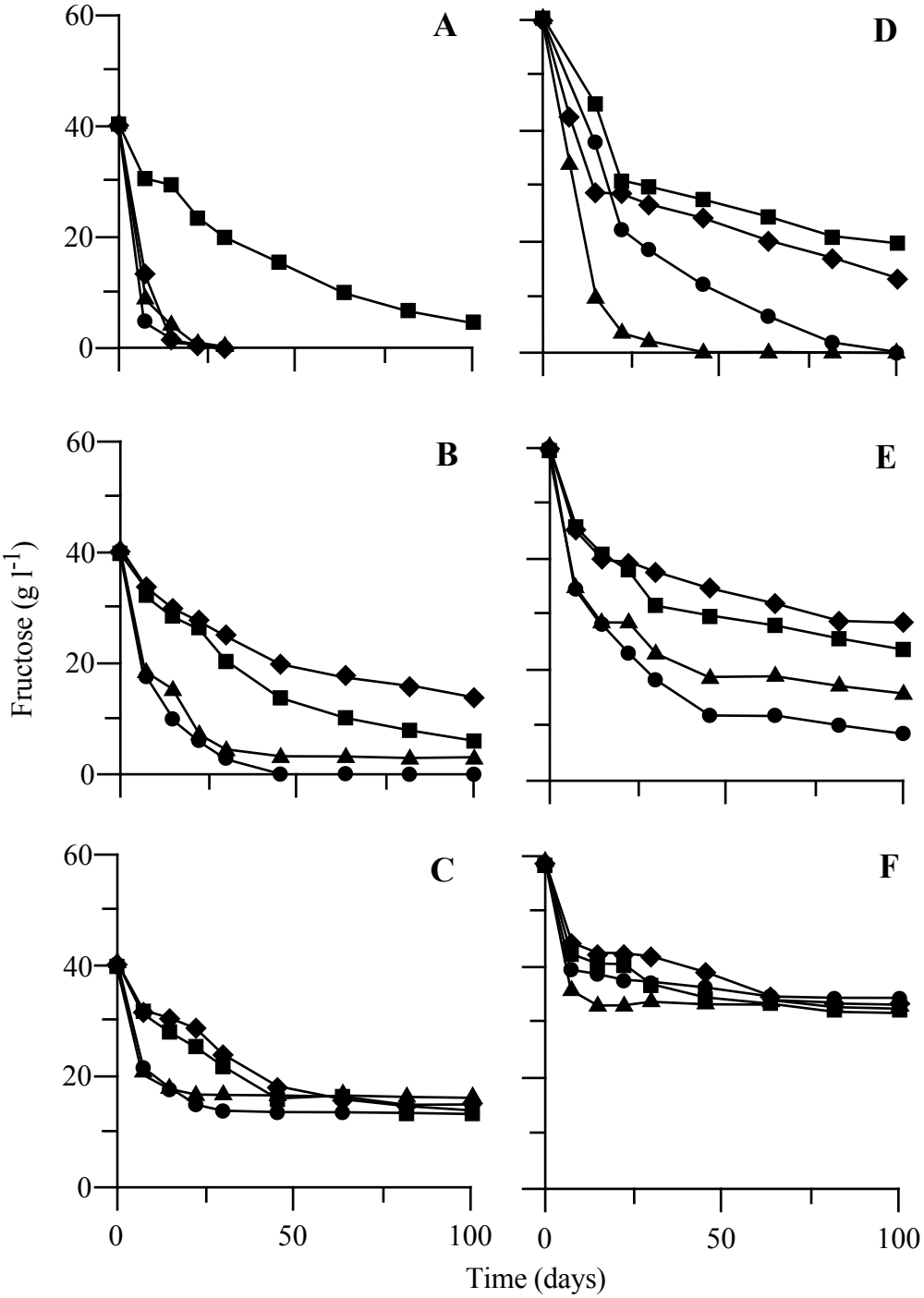

Figure 6. Declines in fructose concentrations of red wines initially containing 40 (A, B, C) or 60 (D, E, F) g l^{-1} fructose and 13% (A, D), 15% (B, E), or 17% (C, F) v v^{-1} ethanol inoculated with *Z. bailii* ZB2 (◆), *Z. bailii* ZB6 (■), *Z. bailii* W3 (▲), or *S. cerevisiae* EC1118 (●).

Table 3. Volatile acidities (g l^{-1}) measured at day 100 in red wines containing 15% or 17% ethanol and 40 or 60 g l^{-1} fructose.

Ethanol (v v^{-1})	Fructose (g l^{-1})	*Z. bailii* ZB2	*Z. bailii* ZB6	*Z. bailii* W3	*S. cerevisiae* EC1118
15%	40	0.98 [a]	0.94 [ab]	0.86 [abc]	0.77 [c]
	60	1.00 [a]	0.89 [ab]	0.94 [abc]	0.79 [bc]
17%	40	0.82 [a]	0.92 [a]	0.80 [a]	0.71 [a]
	60	0.82 [a]	0.80 [a]	nd	nd

Means within each ethanol concentration with different superscripts are significant at $p \leq 0.05$.
nd: not determined

Table 4. Calculated f-values and significant interaction of measured volatile acidity in wines containing 15% or 17% ethanol.

Source of variation	df	15% wines	17% wines
Fructose (F)	1	0.003	3.240
Strain (S)	3	14.746*	4.578
F*S	3	0.417	0.360

Significance is denoted as * $p \leq 0.05$

Discussion

Wines were inoculated with 10^8 cfu ml^{-1} of each yeast to ensure survival of the populations. Due to ethanol sensitivity, this practice is often recommended for stuck fermentations (Fugelsang & Edwards 2007) and has been used in previous studies as well (Cavazza et al. 2004). Ethanol concentration is often considered to be the primary stress associated with wine yeasts (Alexandre & Charpentier 1998; Cavazza et al. 2004). Although the mechanism for growth inhibition is multifaceted, in general ethanol causes interference with various transport systems, damage to mitochondrial DNA, and modification of plasma membrane fluidity and permeability (Thomas et al. 1978; Leao & Van Uden 1982; Leao & Van Uden 1984; Jones & Greenfield 1987; Ingram & Buttke 1984; Bisson 1999).

Although all populations were unaffected by ≤15% ethanol, only two strains (ZB2, ZB6) of *Z. bailii* persisted $\geq 10^6$ cfu ml^{-1} in 17% wines while neither W3 nor *S. cerevisiae* were detectable by day 100. In agreement, additional studies have described the survival of *Z. bailii* in 18% (Santos et al. 2008) or even 20% ethanol (Thomas & Davenport 1985). However, other authors have proposed that *S. cerevisiae* is actually the more ethanol tolerant yeast (Rankine & Pilone 1973; Kalathenos et al. 1995; Fernandes et al. 1997), suggesting that ethanol tolerance in *Z. bailii* may be a function of strain. This is substantiated by the poor survival of strain W3 in 17% wines. The greater ethanol tolerance observed in some *Z. bailii* strains may related to a diminished degree of unsaturation in cellular membrane fatty acids (Couto & Veld 1995).

Despite the better survival of two *Z. bailii* strains in 17% ethanol wines, fructose utilization was no more effective than for *S. cerevisiae*. In fact, although *S. cerevisiae* populations declined in 17% ethanol wines, fructose concentrations at day 100 were similar regardless of the yeast. Conversely, Fernandes et al. (1997) reported that in ethanol

concentrations found in wines *Z. bailii* utilized glucose better than *S. cerevisiae*. However, while hexose transport in most yeasts is a function of carrier mediated facilitated diffusion and active proton symporters (Boles & Hollenberg 1997; Berthels et al. 2008), the fructophillic nature of *Z. bailii* is due to an additional specific high-capacity transport system for fructose (Emmerich & Radler 1983; Sousa-Dias et al. 1996). Santos et al. (2008) suggested that this fructose transport system in *Z. bailii* could be more sensitive to ethanol than the overall organism. Thus, although *Z. bailii* can survive in high ethanol environments, it may not be able to efficiently utilize fructose. This evidence supports the observation that strain W3 fully consumed 60 g l^{-1} fructose more effectively than *S. cerevisiae* but only in the lowest ethanol wines.

When compared to *S. cerevisiae* EC1118, commercial application of *Z. bailii* to rectify stuck fermentations appears limited. However, this strain of *S. cerevisiae* has been specifically recommended to restart stuck fermentations (Henschke 1997) and demonstrated particularly effective ethanol production in high sugar ferments (Malacrino et al. 2005). Despite this, in one condition in the present study (60 g l^{-1} fructose, 13% v v^{-1} ethanol) *Z. bailii* W3 did outperform *S. cerevisiae* EC1118. Taking into account the vast strain variability observed with both *S. cerevisiae* and *Z. bailii*, situations may exist in which different *Z. bailii* strains or inoculation techniques could be an appropriate choice in restarting stuck fermentations.

When *Z. bailii* is used in fermentations, recent evidence has suggested that some strains are not as detrimental to wine quality as previously believed (Thomas & Davenport 1985; Romano & Suzzi 1993). In support, only one strain of *Z. bailii* produced more volatile acidity than *S. cerevisiaiae* in 15% wines and no differences were determined in 17% wines. Similarly, *Zygosaccharomyces* strains also produced less ethyl acetate than other non-*Saccharomyces* wine yeasts (Domizio et al. 2011). Furthermore, beneficial impacts on wine quailty could be imparted

as well. For example, *Zygosaccharomyces* species can produce 150 to 250 mg l^{-1} of polysaccharides during grape juice fermentation, benefiting wine mouthfeel by increasing texture and body (Romani et al. 2009; Domizio et al. 2011). Additionally, although malolactic fermentations using lactic acid bacteria are common in the wine industry, *Z. bailii* is also capable of malic acid metabolism (Kuczynski & Radler 1982).

Despite the limited success of *Z. bailii* in removing residual sugar from simulated stuck wines, this study conveys important findings. Wines with 13% ethanol and ≤60 g l^{-1} fructose or 15% and ≤40 g l^{-1} did achieve dryness with a high inoculation of *S. cerevisiae* EC1118. These data support the commercial use of this strain in stuck fermentations. However, in one wine *Z. bailii* W3 was more effective than *S. cerevisiae* in utilizing the residual sugar. With the documented strain variability found in *Z. bailii*, examination of additional strains may isolate ones even more applicable in stuck fermetnations. Furthermore, no difference in volatile acidities was measured in wines with strain W3 or *S. cerevisiae*, supporting evidence that some strains of *Z. bailii* may not be particulally dangerous wine spoilage yeasts.

CHAPTER III

EFFICACY OF DIMETHYL DICARBONATE AGAINST YEASTS INOCULATED IN GRAPE MUST OR WINE

Abstract

The efficacies of dimethyl dicarbonate (DMDC) against different species and populations of yeasts associated with grape musts and wines were evaluated. Species studied were *Brettanomyces bruxellensis*, *Candida oleophila*, *Candida californica*, *Metschnikowia pulcherrima*, *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Wickerhamomyces anomalus,* and *Zygosaccharomyces bailii*, all originally isolated from regional vineyards and wineries. Yeasts were inoculated into grape musts or wines at 10^2 or 10^4 cfu ml^{-1} and achieved culturable populations of 10^4 or 10^6 cfu ml^{-1}, respectively, prior to addition of 200 mg l^{-1} DMDC. After DMDC treatment, culturability of *C. oleophila*, *C. californica*, *Mt. pulcherrima*, *My. caribbica*, *My. guilliermondii*, and *W. anomalus* declined several logs, at times to levels below the limit of detection (<30 cfu ml^{-1}). However, populations of these yeasts soon increased to >10^6 cfu ml^{-1} regardless of the initial inoculum. In contrast, *Z. bailii* was less resistant to DMDC as culturability never recovered when inoculated into grape musts at low initial populations or into wines at high initial populations. While the population of *B. bruxellensis* strain I1a was not observed after DMDC in wines containing <10^5 cfu ml^{-1}, culturability quickly returned for strain F3 no matter the initial inoculum. Treatment of wines with DMDC using commercial equipment resulted in a loss of culturability but cells remained viable as determined using real-time PCR even though concentrations of 4-ethylphenol or 4-ethylguiacol were unchanged after several years of storage. While low populations (≤10^4 cfu ml^{-1}) of *B. bruxellensis* are inhibited

depending upon strain, DMDC should not be relied upon to eradicate all yeasts from grape musts or wines.

Introduction

Yeasts found in vineyards and in wines encompass a vast number of species of *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula, Hansenulaspora, Issatchenkia*, *Kluyveromyces, Metschnikowia*, *Pichia, Rhodotorula*, *Saccharomyces*, *Zygosaccharomyces*, and several others (Barata et al. 2012; Martins et al. 2012; Settanni et al. 2012). Recently, Bourret (2012) isolated a number of yeasts from Chardonnay and Riesling vineyards located in central Washington state including *Hanseniaspora uvarum, Metschnikowia pulcherrima,* and *Pichia membranifaciens*, species known to occur on grapes and/or be present in wines (Barata et al. 2012). However, of the total 55 species of yeasts characterized, 16 were not previously reported from wine grapes and 17 were not previously isolated within North America (Bourret 2012; Bourret et al. 2012).

If allowed to proliferate in grape musts or wines, many of these yeasts, sometimes collectively call "non-*Saccharomyces*" yeasts, will produce a range of volatile and non-volatile compounds that adversely affect wine quality. For example, *Candida*, *Hansenula*, *Pichia*, and *Saccharomyces* all synthesize various amounts of higher alcohols such as isobutanol, *n*-propanol, isoamyl alcohol, and active amyl alcohol (Rankine 1967; Edwards et al. 1990; Holloway and Subden 1991; Lambrechts and Pretorius 2000), compounds that impart sensory descriptors as 'fusel' (butanol), 'alcoholic' (isobutyl alcohol), 'marzipan' (active amyl alcohol and isoamyl alcohols), or 'rose' (phenethyl alcohol). Besides alcohols, Lambrechts and Pretorius (2000) and Verstrepen et al. (2003) noted that yeast-synthesized esters possess aromas such as 'solvent-like' or 'nail polish' (ethyl acetate), 'fruity,' 'pear,' or 'banana' (isoamyl acetate), 'floral' or 'fruity'

(ethyl butanoate), 'sour apple' (ethyl caproate and ethyl caprylate), and 'flowery,' 'roses,' or 'honey' (phenyl ethyl acetate). Another yeast, *Brettanomyces bruxellensis*, uniquely synthesizes volatile phenols (*e.g.*, 4-ethylphenyl and 4-ethylguaiacol) and imparts aromas described as being 'animal', 'stable', 'medicinal', and others (Chatonnet et al. 1992; Romano et al. 2009).

Controlling the growth of these yeasts during the winemaking process can be difficult. For instance, reliance on SO_2 is generally ineffective because many are resistant to molecular levels of more than 3 mg l^{-1} (Fugelsang & Edwards 2007). Furthermore, one of the major metabolites of these yeasts is commonly acetaldehyde, a compound which can effectively bind SO_2 and decrease its antimicrobial properties. Additional methods of control such as elevated alcohol concentrations coupled to low temperature storage can be effective as noted by Dittrich (1977). The author reported no growth of some of these yeasts in wines of 10 to 12% v v^{-1} alcohol when stored at 8° to 12ºC whereas growth was observed in wines containing 14% v v^{-1} alcohol but held at warmer temperatures.

As an alternative, dimethyl dicarbonate (DMDC or Velcorin™) has been used in research and commercial applications to limit yeasts. On a laboratory scale, several authors have used DMDC as a means to sterilize grape musts or wines (Jacobs & van Vuuren 1991; Arnink & Henick-Kling 2005; Cavazza et al. 2011). Commercially, DMDC is most commonly added just prior to bottling using specialized dosing equipment as a deterrent to *Brettanomyces* and other spoilage yeasts (Renouf et al. 2008). In the United States, DMDC is legally restricted to ≤200 mg l^{-1} in products microbially contaminated with ≤500 cfu ml^{-1} (Code of Federal Regulations 2011a) and functions by disruption of metabolic enzyme function through methoxycarbonylation of imidazoles, amines, and thiols in the cell (Ough 1993; Renouf et al. 2008). However, the

compound hydrolyzes to negligible concentrations of carbon dioxide and methanol within 12 to 24 hours, leaving no discernable impact on composition (Delfini et al. 2002).

While DMDC can inhibit *B. bruxellensis* in wine at populations of 500 cfu ml^{-1} (Costa et al. 2008; Renouf et al. 2008), its efficacy against greater populations of this and other yeasts is not clear. Furthermore, little is known regarding its impact on many of the yeasts isolated by Bourret (2012), which would be present in grape musts. Thus, the objectives of this study were to evaluate the efficacy of DMDC against several non-*Saccharomyces* yeasts isolated from regional vineyards (*C. californica*, *C. oleophila*, *Mt. pulcherrima*, *My. caribbica*, *My. guilliermondii*, and *W. anomalus*), and wineries (*B. bruxellensis* and *Z. bailii*).

Materials and methods

Yeast cultures

Candida californica P01C003, *Candida oleophila* P40C006, *Metschnikowia pulcherrima* P01A016, *Meyerozyma caribbica* P46A001, *Meyerozyma guilliermondii* P40D002, and *Wickerhamomyces anomalus* P01A017 were recently isolated from vineyards in the Washington State University Irrigated Agriculture Research and Extension Center as described by Bourret (2012). *Saccharomyces cerevisiae* EC1118 was obtained from Lallemand Inc. (Quebec, Canada) while *Zygosaccharomyces bailii* ZB2 and ZB6 were isolated from commercial wines. *Brettanomyces bruxellensis* B3, E1, F3, and I1a were from commercial red wines from Washington State as described by Jensen et al. (2009).

Inocula were prepared from a single colony in 10 ml yeast/mold (YM) broth (pH 4.5) and then transferred to 100 ml broth. YM used for *B. bruxellensis* included either 5% v v^{-1} (I1a, E1, and F3) or 12.6% v v^{-1} (B3) ethanol to better acclimate the yeast to wine conditions. Cultures

were harvested by centrifugation at 2000 *g* for 20 min and washed in 0.2 M Na_2HPO_4 (pH 7.0) buffer (2x) prior to inoculation at different populations.

Laboratory-scale experiments

A 2012 Chardonnay grape must (pH 3.34; 22.3 °Brix; 4.4 g l^{-1} titratable acidity), a 2009 Cabernet Sauvignon (pH 3.92, 13.4% v v^{-1} alcohol; 6.5 g l^{-1} titratable acidity), and a 2009 Pinot noir wine (pH 3.90; 12.5% v v^{-1} alcohol; 5.5 g l^{-1} titratable acidity) were obtained from commercial sources. Total SO_2 was removed using hydrogen peroxide prior to aseptically adding a blend of nutrients to grape must (0.183 g l^{-1} diammonium phosphate) or wines (0.5 g l^{-1} fructose, 0.5 g l^{-1} glucose, 0.1 g l^{-1} yeast extract, and 0.1 g l^{-1} 20 µm cellulose). The grape must and wines were separately sterile filtered through Nylon® 0.2 µm cartridge membranes in sanitary filter housings (Pall, Port Washington, NY) and transferred into sterile dilution bottles (100 ml).

After yeast inoculations, DMDC (Scott Laboratories, Healdsburg, CA) was dissolved in 95% v v^{-1} ethanol and added to samples on day two (grape must) or day fourteen or thirty (wines) to yield 200 mg l^{-1}. All treatments were kept at 18°C for monitoring culturabilities and conducted in triplicate with the population means determined.

Commercial-scale experiments

A commercial 2007 Cabernet Sauvignon wine (pH 3.83; 14.3% v v^{-1} alcohol; 5.5 g l^{-1} titratable acidity) was transferred into stainless steel tanks in 100 gallon lots. Starter cultures of *B. bruxellensis* strains B3 were then transferred into the wines to yield low (3×10^2 cfu ml^{-1}), medium (3×10^3 cfu ml^{-1}), or high (3×10^4 cfu ml^{-1}) initial populations. After inoculation and acclimation for one to three hours, wines were passed through a portable commercial unit which added DMDC at 200 mg l^{-1}. After treatment, wines were bottled and stored at 22° to 24°C for

200 days followed by additional storage at 7°C for 875 days. All treatments were conducted in triplicate and the population means determined.

Microbiological and chemical analyses

Culturability was monitored by plating samples onto Wallenstein Laboratory agar (WL, Difco, Detroit, MI) using an Autoplate 4000 (Spiral Biotech, Bethesda, MD) while viability was determined by real-time PCR with Scorpion™ probes (Scott Laboratories, Petaluma, CA). General wine analysis was conducted based on the methods of Edwards and Watson (2013). Volatile acidity in wines was measured by Cash still (Research & Development Glass, Berkely, CA) while acetic acid in grape must was determined using High Performance Liquid Chromatography as described by Semon et al. (2001).

Concentrations of 4-ethylphenol and 4-ethylguiacol were determined in Cabernet Sauvignon after 1075 days of storage using a headspace-solid phase microextraction method. Here, a 85 μm polyacrylate fiber (Supelco, Bellefonte, PA) was thermally desorbed at 280°C for 3 min in a GC-MS/MS (Varian 4000, Walnut Creek, CA) while separation was accomplished using a 0.18 mm x 20 m DB-5MS capillary column (0.18 μm film thickness, J&W/Agilent Technologies, Wilmington, DE) and helium as the carrier gas (0.8 ml min^{-1}). The oven temperatures increased after 2.0 min from 40°C to 160°C at a rate of 20°C min^{-1} and then increased to 300°C at 50°C min^{-1} and held for 0.2 min. Retention time as well as fragmentation patterns were used to identify the volatile phenols.

Statistical analyses

A two-way analysis of variance (ANOVA) and Fisher's LSD were applied for mean separation with XLSTAT software (Addinsoft, New York, NY) used to determine significance at $p \leq 0.05$. Means of populations (cfu ml^{-1}) were calculated from on Log_{10} values.

Results

Regardless of initial inoculum (10^2 vs. 10^5 cfu ml^{-1}) and in the absence of DMDC, *C. californica*, *C. oleophila*, and *Mt. pulcherrima* (Figure 7) as well as *My. caribbica*, *My. guilliermondii*, and *W. anomalus* (Figure 8) all achieved and maintained culturabilities $\geq 10^6$ cfu ml^{-1} within about five days of being inoculated into the grape musts. In all cases, populations remained approximately 10^7 cfu ml^{-1} before slight declines for some yeasts by day 25.

Addition of DMDC 48 hours after inoculation resulted in a loss of culturability, the extent dependent on yeast species and population. On the one hand, *Mt. pulcherrima* (Figure 10) decreased approximately three logs, from $\approx 10^7$ down to 10^4 cfu ml^{-1}. On the other hand, culturability of *C. californica*, *My. caribbica*, and *My. guilliermondii* declined from $\geq 10^6$ to ≤ 30 cfu ml^{-1} within two days (Figures 7, 8). However, even with a complete loss of culturability, populations quickly increased to $\geq 10^5$ cfu ml^{-1} by day 8. The only exception was *My. guilliermondii* which reached this population between day 15 and 20. Despite the growth of each yeast, in grape musts with high inocula of *C. californica* or low inocula of *My. guilliermondii*, less acetic acid was present with DMDC addition (Table 5).

In the absence of DMDC, *S. cerevisiae* and *Z. bailii* grew well in grape must and achieved populations similar to the other yeasts studied (Figure 9). However, *Z. bailii* was more sensitive to DMDC than the other yeasts as illustrated by an inability to regain culturability by day 25 when inoculums were initially 10^2 to 10^3 cfu ml^{-1}. Furthermore, these grape musts contained less than half of the acetic acid concentration as musts without DMDC (Table 5). Conversely, culturability was regained for both *Z. bailii* ZB2 and ZB6 initially inoculated with high populations (10^5 cfu ml^{-1}). The strain of *S. cerevisiae* studied behaved similarly to *W. anomalus* where DMDC reduced populations a few logs before rebounding to $\geq 10^6$ cfu ml^{-1}.

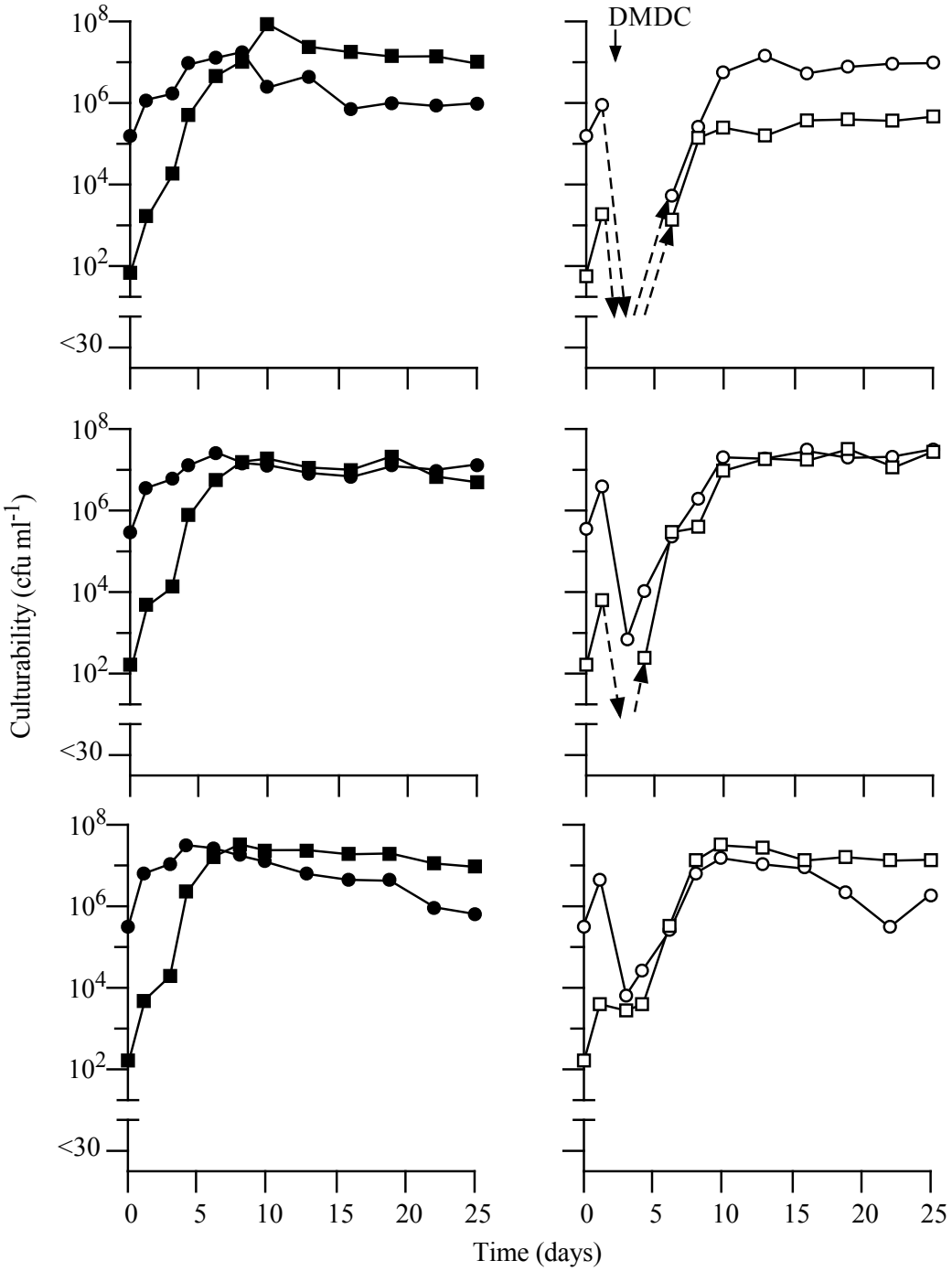

Figure 7. Culturability of two different initial inoculums of *C. californica* P01C003 (top), *C. oleophila* P40C006 (middle), or *Mt. pulcherrima* P01A016 (bottom) in Chardonnay grape must with (○,□) or without (●,■) 200 mg l^{-1} DMDC added after 48 hours.

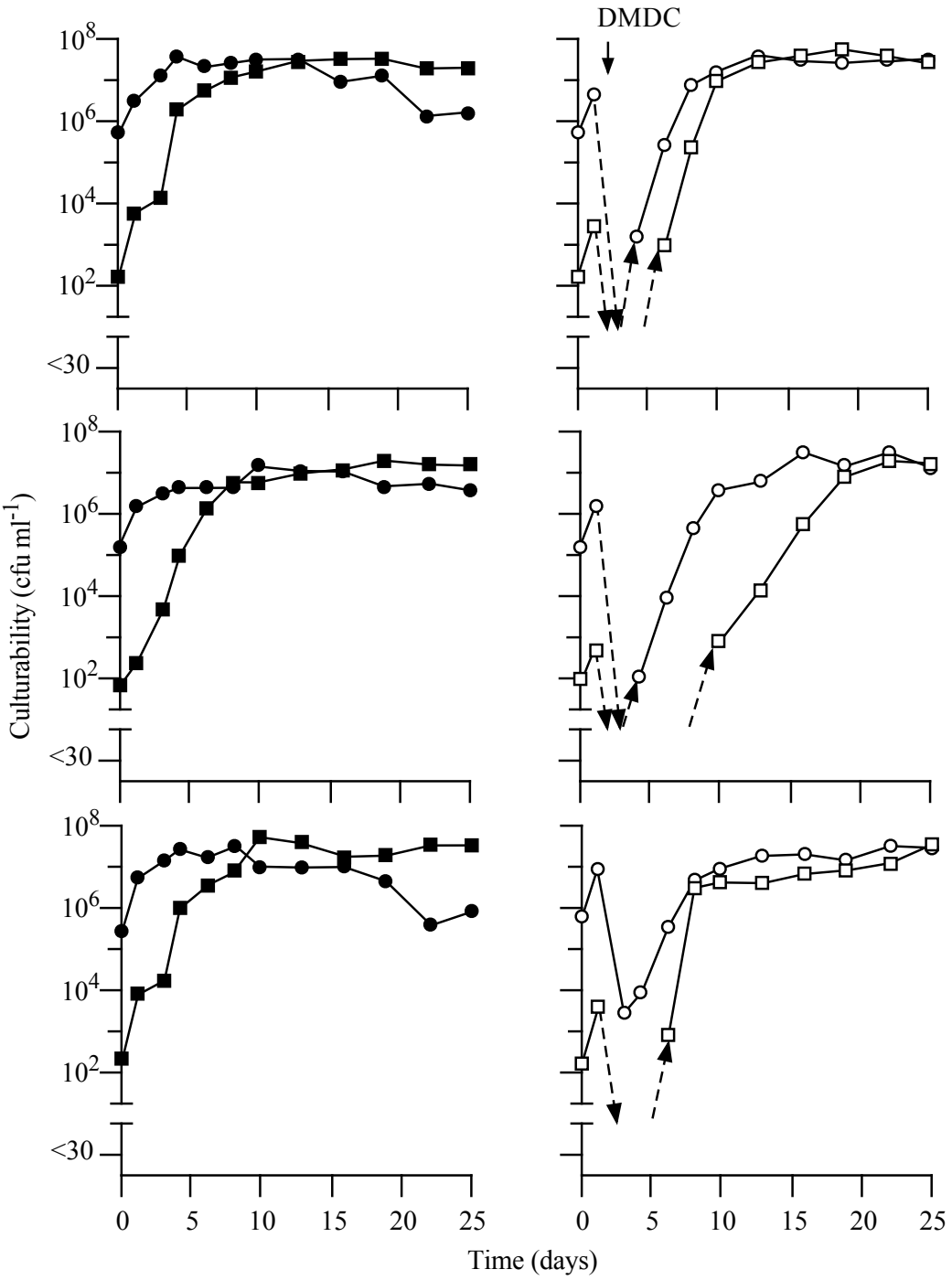

Figure 8. Culturability of two different initial inoculums of *My. caribbica* P46A001 (top)*, My. guilliermondii* P40D002 (middle), or *W. anomalus* P01A017 (bottom) in Chardonnay grape must with (○,□) or without (●,■) 200 mg l^{-1} DMDC added after 48 hours.

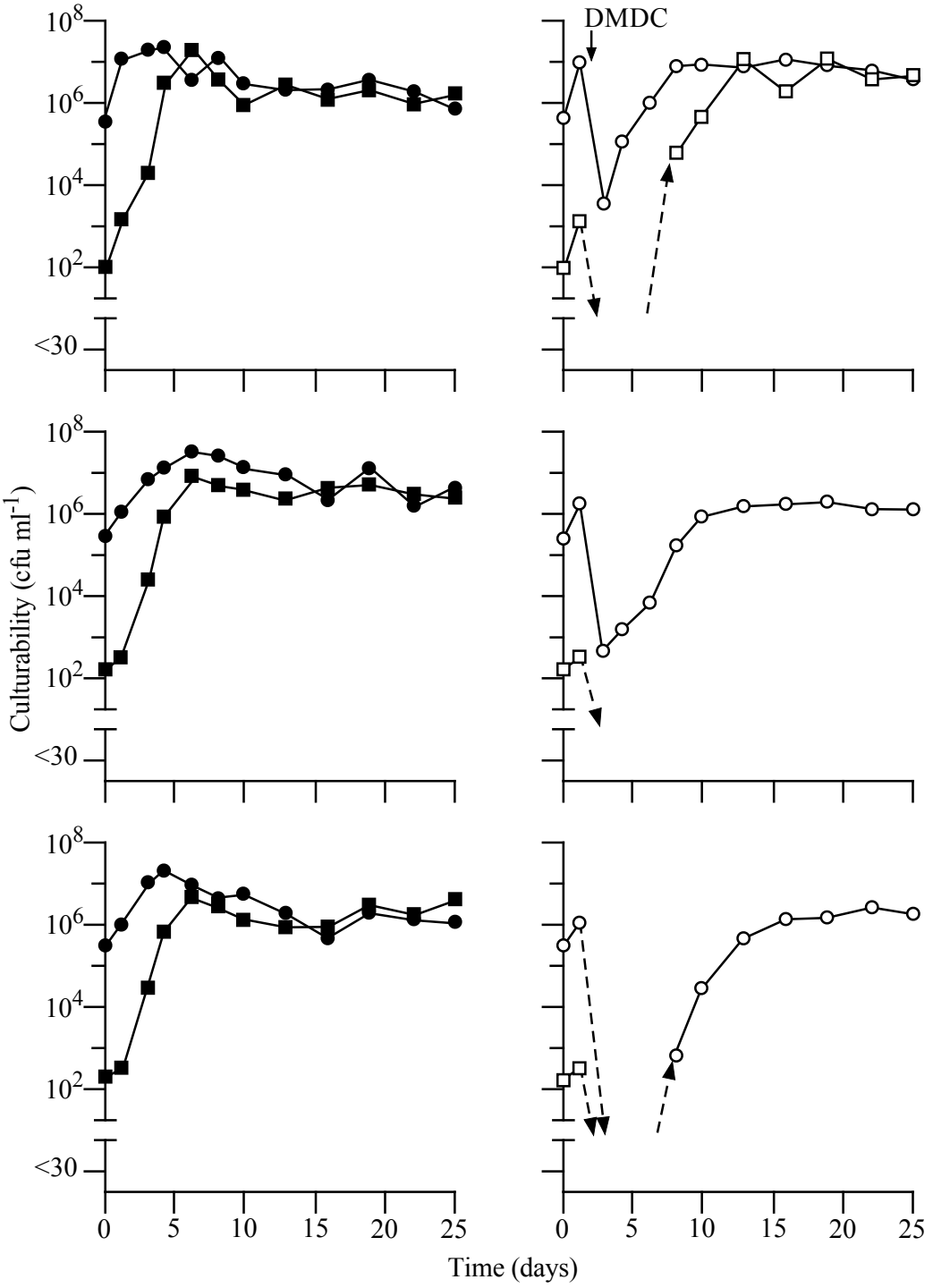

Figure 9. Culturability of two different initial inoculums of *S. cerevisiae* EC1118 (top), *Z. bailii* ZB2 (middle), or *Z. bailii* ZB6 (bottom) in Chardonnay grape must with (○,□) or without (●,■) 200 mg l^{-1} DMDC added after 48 hours.

Table 5. Acetic acid concentrations (g l^{-1}) of grape musts inoculated with non-*Saccharomyces* yeasts and treated with 200 mg l^{-1} DMDM.

	10^2 inocula		10^5 inocula		f- values		
Species	Control	DMDC	Control	DMDC	Inocula (I)	DMDC (D)	I x D
C. californica	nd	nd	1.074 [a]	0.661 [b]	325**	19.4*	19.4*
C. oleophilia	0.994 [a]	0.969 [a]	1.027 [a]	0.770 [a]	0.871	2.54	0.320
Mt. pulcherrima	0.732 [ab]	0.706 [b]	0.824 [ab]	0.998 [a]	18.2	2.73	4.90
My. caribbica	0.712 [b]	0.703 [b]	1.073 [a]	0.712 [b]	52.2*	52.1*	47.1*
My. guilliermondi	0.695 [a]	0.300 [a]	0.613 [a]	0.444 [a]	0.064	5.40	0.862
W. anomalus	1.031 [a]	0.958 [a]	0.894 [a]	3.309 [a]	0.855	0.956	1.07
Z. bailii ZB2	1.556 [a]	0.749 [b]	1.477 [a]	1.576 [a]	40.1*	35.8*	58.9*
Z. bailii ZB6	1.531 [a]	0.599 [b]	1.452 [a]	1.529 [a]	154*	156**	216**

Means within each strain were determined to be significant at $p \leq 0.05$

f-values were significant at $p \leq 0.05$ (*) or $p \leq 0.01$ (**)

nd: not determined

When *Z. bailii* ZB2 and ZB6 were grown in Cabernet Sauvignon wine, inoculations of $\approx 10^3$ or $\approx 10^5$ cfu ml^{-1} persisted for 100 days, although populations declined by about one log during that time (Figure 10). When DMDC was added to the lower inocula, no growth was detected in either strain for ≥85 days. Conversely, after DMDC the higher inocula gradually declined for 35 days, from $\approx 10^5$ cfu ml^{-1} to below the limits of detection (≤30 cfu ml^{-1}). At this point no growth was detected in any of the wines regardless of strain for ≥50 days.

Upon inoculation into the Pinot noir wine, *B. bruxellensis* strains E1, F3, and I1a, achieved $\geq 10^6$ cfu ml^{-1}, the time required being dependent on the initial inoculum. In general, higher inoculums reached 10^6 cfu ml^{-1} within about ten days, while if present at a lower initial inoculum, upwards of 50 were required (Figure 11). All three strains maintained populations in excess of 10^6 cfu ml^{-1} up to day 100.

At day 30 when DMDC was added, the populations were $\approx 10^4$ or $\approx 10^7$ cfu ml^{-1}. Immediately following DMDC none of the strains were detected in the wine regardless of initial population. However, within five days each high inocula was observed and grew to stationary phase $\geq 10^6$ cfu ml^{-1} within 50 days. Much like with *Z. bailii*, low inocula of strains I1a and E1 were not detected for ≥70 days after DMDC. Conversely, low inocula of strain F3 did rebound within five days of DMDC and achieved $\geq 10^6$ cfu ml^{-1} by day 60. Here, strain F3 also produced >1.0 g l^{-1} of volatile acidity while I1a and E1 did not (data not shown).

After application of 200 mg l^{-1} DMDC to a Cabernet Sauvignon wine using commercial dosing equipment, strain B3 survived up to 107 days but only in the wines containing the high initial population (Figure 12). These populations gradually decreased from day 23 until day 142 where none were detected. Cells were never observed in wines with low to medium initial populations using WL agar and standard plate counting methods. On day 200, real-time PCR

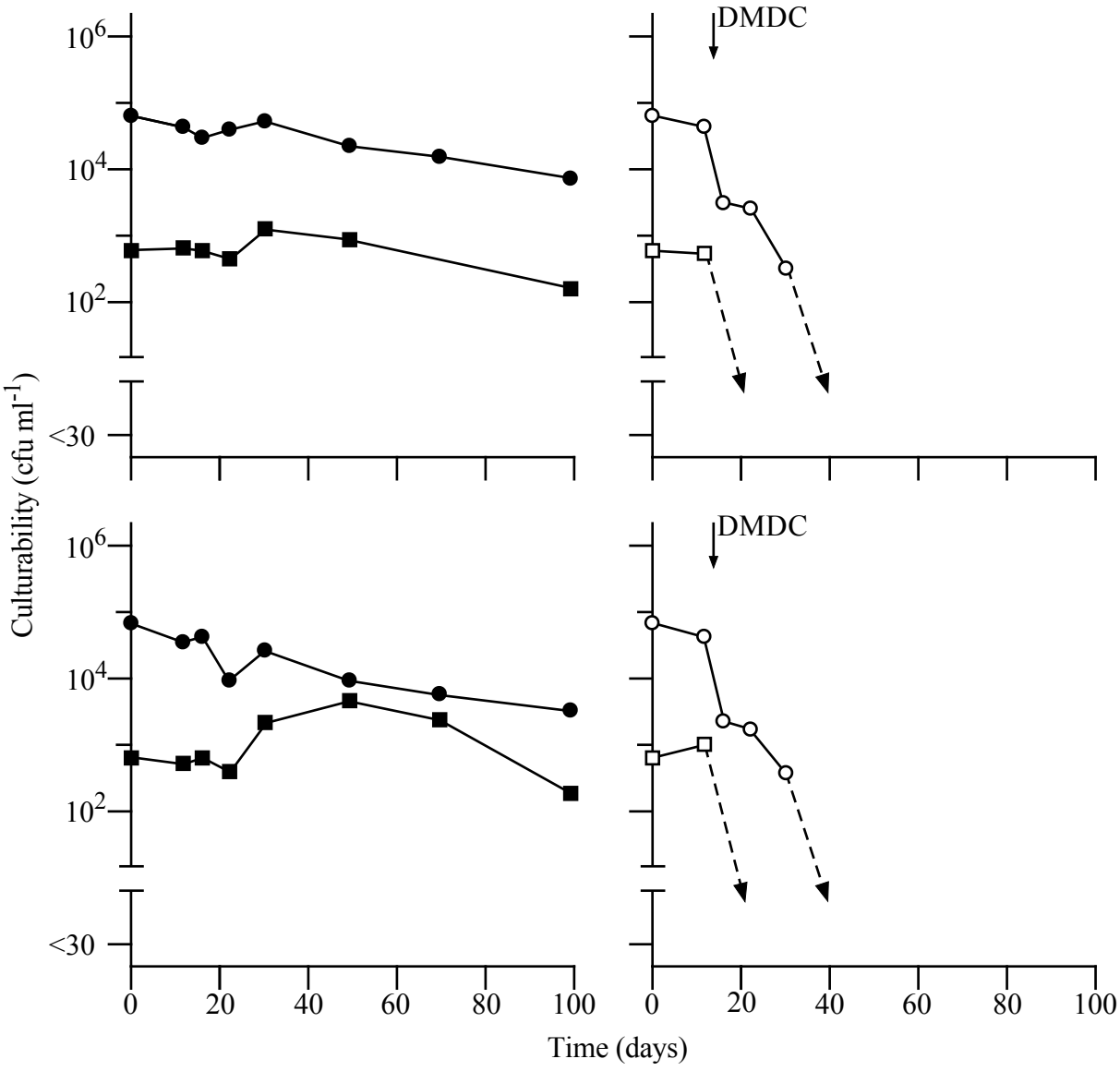

Figure 10. Culturability of *Z. bailii* ZB2 (top) or ZB6 (bottom) in Cabernet Sauvignon wines stored at 20°C. Addition of 200 mg l^{-1} DMDC occurred on day 14 to yeast populations inoculated at 500 (□) or 50,000 (○) cfu ml^{-1}. Culturability of untreated populations inoculated at 500 (■) or 50,000 (●) cfu ml^{-1} was measured concurrently.

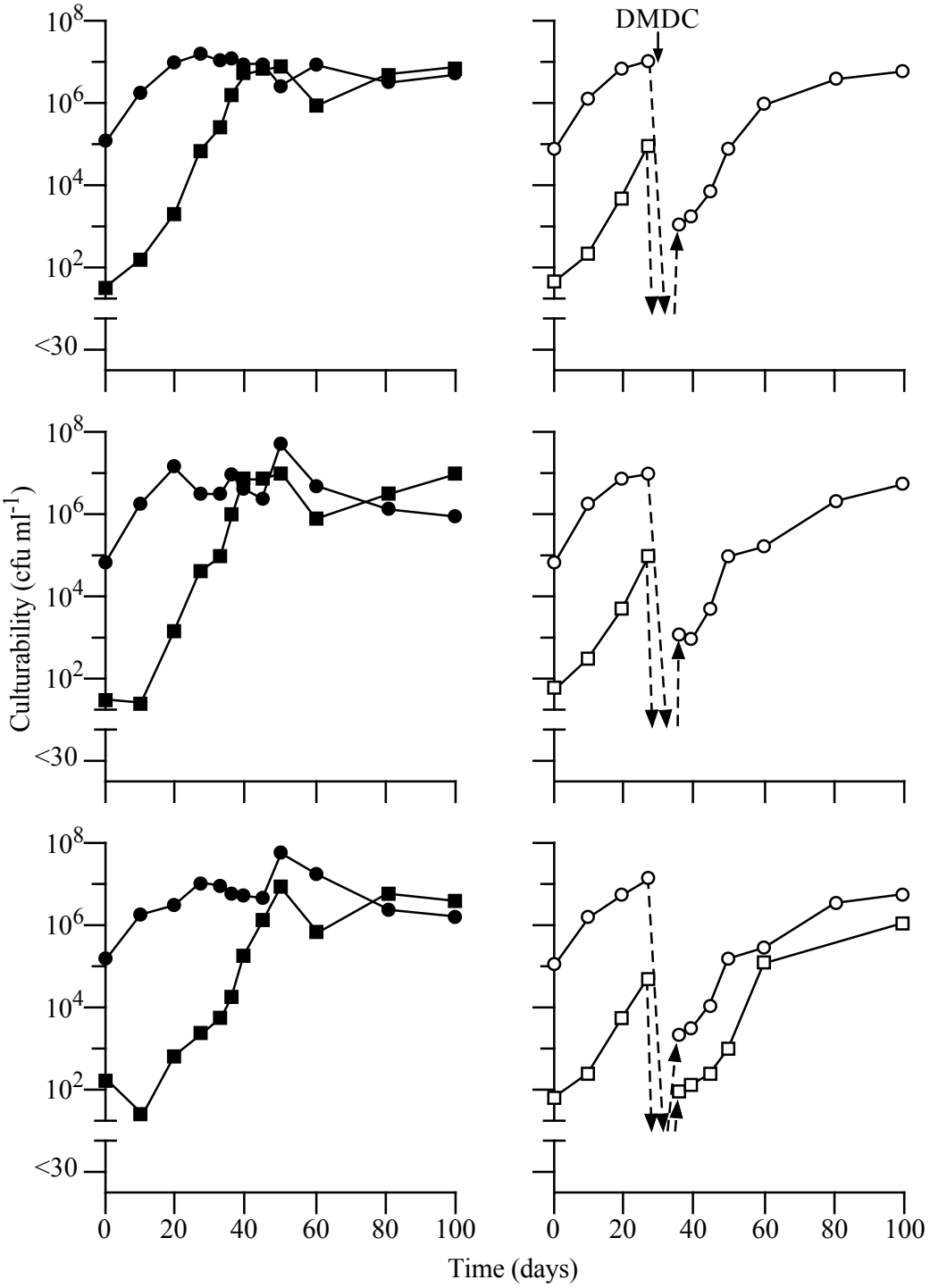

Figure 11. Culturability of two different initial inoculums of *B. bruxellensis* I1a (top), E1 (middle), or F3 (bottom) in Pinot noir wines with (○,□) or without (●,■) 200 mg l^{-1} DMDC added on day 30.

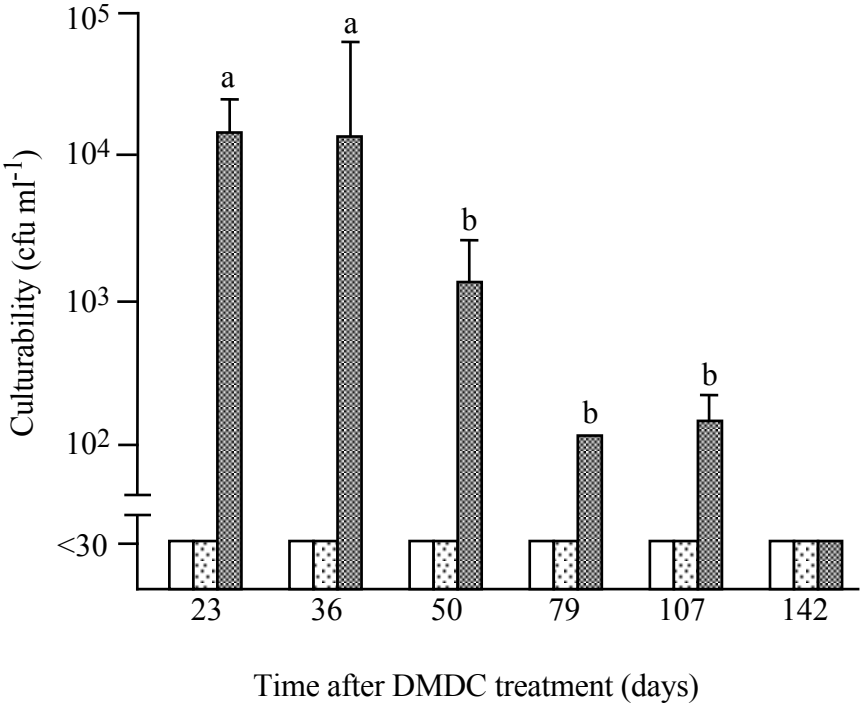

Figure 12. Culturability of Cabernet Sauvignon wines inoculated with *B. bruxellensis* B3 at 3 x 10^2 (□), 3 x 10^3 (▨), or 3 x 10^4 cfu ml^{-1} (▩) and following addition of 200 mg l^{-1} DMDC. Mean values with different letters are significantly different at $p \leq 0.05$.

analysis revealed viable populations of 3.8×10^2 and 4.9×10^4 cells ml^{-1} in wines initially containing medium or high inocula, respectively (data not shown). However, chemical analysis of the wines 1275 days after DMDC treatment revealed no significant increase in 4-ethylphenol and greater volatile acidity only in wines with the high inocula (Table 6).

Discussion

The efficacy of DMDC was found to vary widely depending on the yeast species studied. DMDC was very effective against some yeasts such as *Z. bailii* where culturability of either strain disappeared when present at low populations ($<10^3$ cfu ml^{-1}). While high populations of the yeast at the time of application resulted in a reduction in culturability of three to four logs, populations slowly rebounded to eventually achieve approximately 10^6 cfu ml^{-1}. In agreement, Siricururatana et al. (2013) did not observe growth of *Z. bailii* inoculated at 10^2 cfu ml^{-1} in Niagara grape juice for ≥153 days after addition of 250 mg l^{-1} DMDC. Higher populations (10^6 cfu ml^{-1}) reportedly required at least 400 mg l^{-1} DMDC to limit growth in grape must (Delfini et al. 2002).

However, several of the yeasts originally isolated from vineyards (*C. californica*, *C. oleophila*, *Mt. pulcherrima*, *My. guilliermondii*, *My. caribbica*, and *W. anomalus*) were much more resistant than *Z. bailli*. In general, populations declined after treatment but then quickly increased regardless of the population at time of application. Furthermore, the strain of *S. cerevisiae* studied (EC1118) behaved similarly as *W. anomalus* with population reductions followed by rapid growth.

Application of 200 mg l^{-1} DMDC as a means to limit growth of ≤500 cfu ml^{-1} of various non-*Saccharomyces* yeasts has been well documented (Daudt & Ough 1980; Threfall & Morris 2002). However, populations on grapes often exceed these populations as evidenced by

Table 6. Chemical analysis of commercial wines inoculated with *B. bruxellensis* B3 and treated with DMDC after storage for 1275 days.

Initial Population†	Volatile Acidity ($g\ l^{-1}$)	Alcohol (% v/v)	4-Ethylphenol ($\mu g\ l^{-1}$)	4-Ethylguaiacol ($\mu g\ l^{-1}$)
None	0.65 [a]	14.2 [ab]	13.7 [a]	<4.0
Low	0.75 [ab]	14.2 [ab]	13.3 [a]	<4.0
Medium	0.72 [a]	14.6 [a]	14.7 [a]	<4.0
High	0.86 [b]	13.8 [b]	14.7 [a]	<4.0

[a,b]Means within a column with different superscripts are significant at $p \leq 0.05$.

†Corresponds to 3×10^2 (low), 3×10^3 (medium), or 3×10^4 (high) cfu ml^{-1}.

Prakitchaiwattana et al. (2004) who documented non-*Saccharomyces* yeasts on harvested grapes at 10^3 to 10^6 cfu g^{-1}. Given that growth was observed after DMDC in grape musts with 10^3 and 10^5 cfu ml^{-1} populations of the six species evaluated, 200 mg l^{-1} of the compound may not be sufficient to eradicate moderate or greater yeast contamination in grape musts. In fact, Ough (1993) noted that many yeasts required higher concentrations of DMDC than 200 mg l^{-1} for 100% kill, even with low populations, in agreement with Costa et al. (2008). Although Cavazza et al. (2011) did not observe fermentation in a Pinot gris must with 10^4 cfu ml^{-1} indigenous grape yeasts after addition of 200 mg l^{-1} DMDC, Divol et al. (2005) reported the same treatment was insufficient to limit $\geq 10^6$ cfu ml^{-1}. However, neither author reported which non-*Saccharomyces* yeasts were actually present.

Non-*Saccharomyces* yeasts can create noticeable sensory effects with less than two days of growth in a grape must (Gil et al. 1996; Romano et al. 2003; Clemente-Jimenez et al. 2005). At this time, little is known regarding the sensory impacts of a number of the yeasts examined in the current study. *W. anomalus* has been reported to produce glucose and ethanol tolerant glycosidases, which hydrolyze the glycoside from sugar molecules to enhance wine aroma (Madrigal et al. 2013). Conversely, *My. guilliermondii* can produce the spoilage compound 4-ethylphenol from phenolic precursors in grape juice, similar to *B. bruxellensis* (Barata et al. 2006). Fermentation ecology can also be affected as *Mt. pulcherrima* and *C. oleophila* have demonstrated antifungal and inhibitory yeast-yeast activity (Schutz & Gafner 1993; Droby et al. 1998). The impacts, if any, of *My. caribbica*, *C. californica*, or *C. oleophila* on wine quality is not known.

Given the non-*Saccharomyces* yeast populations potentially found on harvested grapes (Fleet et al. 2002; Prakitchaiwattana et al. 2004), DMDC cannot necessarily be relied upon to

completely eradicate these populations. Although the yeasts isolated from grapes in vineyards (*C. californica*, *C. oleophila*, *Mt. pulcherrima*, *My. caribbica*, *My. guilliermondii*, and *W. anomalus*) eventually grew in grape must, DMDC delayed the onset of growth for several days, particularly of lower populations. Thus, the delay in growth that was observed following DMDC addition may provide opportunity for growth of inoculated *S. cerevisiae* outcompete the indigenous non-*Saccharomyces* populations and limit potential spoilage (Maro et al. 2007).

As opposed to supposed sterilization of grape musts, a more common commercial application of DMDC is to wines containing low ($\leq$500 cfu ml^{-1}) microbial populations just prior to bottling (Fugelsang & Edwards 2007; Renouf et al. 2008). In fact, the compound is generally more effective in wine rather than grape must due to the synergistic relationship between DMDC and ethanol concentration (Porter & Ough 1982). This was demonstrated in the present study as DMDC limited growth of 10^5 cfu ml^{-1} *Z. bailii* in wines but not grape must. Costa et al. (2008) also reported that 200 mg l^{-1} DMDC limited growth of 10^6 cfu ml^{-1} *Z. bailii* in wines for at least five days, although the gradual population decline in the in the present study was not observed. Other studies have reported that when DMDC limited the growth of high populations of *Z. bailii* the yeast sometimes entered a viable-but-not-culturable state (Divol et al. 2005), although long term observations for a recurrence of growth was not conducted.

In the present study, responses by *B. bruxellensis* to DMDC added to red wines depended on the strain and population. In laboratory scale experiments, culturabilities of strains E1 and I1a disappeared and never returned after application when populations were approximately 10^5 cfu ml^{-1} in contrast to strain F3 which regained culturabilities within a few days. However, commercial application of DMDC resulted in a disappearance of culturability where populations were $<10^3$ cfu ml^{-1} but a slow decline if populations were originally $>10^4$ cfu ml^{-1}. Even though

cells were not detected by standard plating using a non-selective medium nor was any 4-ethylphenol or 4-ethyl guaiacol produced, a real-time PCR method confirmed the presence of viable cells on day 200.

The efficacy of DMDC against *Brettanomyces* depends on many variables. For instance, while Costa et al. (2008) reported that 100 mg l^{-1} DMDC was necessary to inhibit 500 cfu ml^{-1} *B. bruxellensis,* Renouf et al. (2008) noted that a much higher population (10^4 cfu ml^{-1}) was inhibited in wine for at least six months after 200 mg l^{-1} DMDC. In fact, Renouf et al. (2008) reported that the minimum inhibitory concentration of DMDC among ten strains of *B. bruxellensis* was identical, in contrast to the current findings of diversity amongst various strains of *Brettanomyces*.

In summary, in grape must a variety of non-*Saccharomyces* yeasts native to vineyards grew without exception after DMDC. Conversely, 10^3 cfu ml^{-1} populations of *Z. bailii* were not observed following treatment. Although DMDC may not effectively act as a sterilant for moderate to heavy yeast contamination in grape must it can reduce the microbial load for a short period of time. In wines, *Z. bailii* was more sensitive to DMDC, and $\leq 10^5$ cfu ml^{-1} did not grow after treatment. Similarly, growth of 10^4 cfu ml^{-1} populations of *B. bruxellensis* was generally limited by DMDC, although one strain was more tolerant. When greater populations (10^7 cfu ml^{-1}) were treated, each strain grew following DMDC. Furthermore, when commercial dosing equipment was evaluated wine spoilage by 10^4 cfu ml^{-1} populations of strain B3 was limited for several years.

CHAPTER IV

IMPACT OF TEMPERATURE x SULFUR DIOXIDE ON *BRETTANOMYCES BRUXELLENSIS* IN WINE

Abstract

The interaction between temperature and sulfur dioxide on the culturability of three strains of *B. bruxellensis* was studied in red wine. A 4 x 4 factorial experimental design with storage temperature (22°, 18°, 15°, or 10°C) and molecular SO_2 concentration (approximately 0.0, 0.2, 0.5, or 1.1 mg l^{-1}) was used. Compared to strains B5 and B1b, F3 was less sensitive to cool temperatures (10°C), remaining culturable in the absence of SO_2 at 10°C for up to 95 days after inoculation. In contrast, while B5 grew poorly at 15° or 10°C, it was the only strain to eventually regain culturability following addition of approximately 0.5 mg l^{-1} molecular SO_2 at 18°C. Combining a reduced temperature and molecular SO_2 treatment was more effective at limiting growth of B. *bruxellensis* than either factor alone, although each strain responded differently to temperature and SO_2. Conditions to limit growth of *B. bruxellensis* during wine aging appear to be temperatures ≤15°C with ≥0.4 mg l^{-1} molecular SO_2.

Introduction

Microbiological contamination and spoilage by the yeast *Brettanomyces bruxellensis* is a major concern with red wines. Growth can result in undesirable aromas with descriptors such as 'animal', 'stable', 'medicinal', and others (Chatonnet et al. 1992; Romano et al. 2009). *B. bruxellensis* spoilage is commonly correlated with aged wines (Chatonnet et al. 1992; Ciani & Ferraro 1997), potentially due to the yeast's ability to utilize cellubiose from barrels or ethanol as sole carbon sources (Blondin et al. 1982; Silva et al. 2004).

One approach to inhibit *B. bruxellensis* during aging is to reduce the cellar temperature below 13°C (Fugelsang & Edwards 2007). While optimal growth temperature is 25° to 28°C (Smith 2011), many strains grow well outside this range as noted by Barata et al. (2008b) who demonstrated growth and volatile phenol production in red wine maintained as low as 15°C. Brandam et al. (2008) suggested that the rate of spoilage is slower if temperatures are 15°C or less.

Because temperature alone is often not sufficient to limit spoilage, winemakers also rely upon the addition of SO_2. The molecular form diffuses across the yeast cell membrane, creating a concentration gradient and reducing intercellular pH (Warth 1984; Pilkington & Rose 1988). The antimicrobial effect is due to interactions with ATP, NAD^+, and FAD; induction of mutations by deamination of cytosine and uracil; and disruption of disulfide bridges in proteins (Schimz 1980; Hinze & Holzer 1986; Pagano et al. 1990). Maintaining a concentration range of 0.4 to 0.6 mg l^{-1} molecular SO_2 (mSO_2) is recommended during wine storage when *B. bruxellensis* spoilage presents a concern (Fugelsang & Edwards 2007).

While both temperature control and SO_2 addition are common practices in wineries, synergistic antimicrobial effects have not been evaluated. Both factors have, however, been incorporated into hurdle technologies used elsewhere in the food industry. Hurdle technology involves combining multiple antimicrobial approaches to mitigate risk of microbial growth or spoilage (Leistner 2000). For example, Sinha and Chandra (2012) successfully used reduced temperature and SO_2, among other factors, to stabilize fresh cauliflower against yeast and mold spoilage. While the interaction of temperature and SO_2 on anthocyanin and color quality of red wines has been studied (Sims & Morris 1984; Ivanova et al. 2009), their effect on growth of *B. bruxellensis* has not.

Based on the hypothesis that reduced temperatures would alleviate mSO_2 requirements for controlling *B. bruxellensis* growth, the objective of this study was to evaluate the impact of the interactions between wine temperature and mSO_2 concentrations on three strains of *B. bruxellensis* in Cabernet Sauvignon.

Materials and methods

Yeast cultures

Three strains of *B. bruxellensis*, F3, B1b, and B5, were obtained from the Washington State University culture collection. Each had been isolated from commercial red wine produced in Washington State (Jensen et al. 2009). Inoculums were prepared by transferring a single colony into 10 ml YM broth (pH 4.5, Difco, Detroit, MI) which was then inoculated into 100 ml YM broth (pH 4.5, ethanol 5.0% v v^{-1}). Cells were harvested by centrifugation at 2000 *g* x 20 min and washed twice in 0.2 M Na_2HPO_4 (pH 7.0) buffer. Wines were inoculated to contain around 10^5 cfu ml^{-1} of culture in late exponential growth phase.

Wine preparation

A 2009 commercially produced Cabernet Sauvignon (pH 3.92, ethanol 13.4%) was filtered through pads (1 μm nominal pore size, Gusmer Enterprises, Fresno, CA) using a 20 cm square pad filter (Zambelli, Italy). Total SO_2 was removed by addition of equal molar amounts of hydrogen peroxide and the pH was adjusted to 3.7 using tartaric acid stock solution (500 g l^{-1}). To limit the potential for nutritional deficiencies, 0.5 g l^{-1} fructose, 0.5 g l^{-1} glucose, 0.1 g l^{-1} yeast extract, and 0.1 g l^{-1} cellulose (20 μm) were aseptically added to all wines. The wine was then filter sterilized through a pleated, Nylon absolute cartridge membrane (0.2 μm) installed in a stainless steel sanitary filter housing (Pall, Port Washington, NY) into sterile bottles (100 ml).

Treatments

Wines were incubated at 22°, 18°, 15°, or 10°C prior to inoculation with *B. bruxellensis* at 10^5 cfu ml^{-1}. After incubation of 13 days, enough potassium metabisulfite solution (20,000 mg l^{-1}) was added to yield concentrations of 0, 60, 100, or 180 mg l^{-1} total SO_2. All treatments were replicated in triplicate. mSO_2 was calculated from free SO_2 measured by the aeration-oxidation method (Buechsenstein and Ough 1978). Culturability was determined by plating on Wallenstein Laboratory medium (Difco, Detroit, MI) incubated at 27°C using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD).

In a second experiment, the effect of temperature on the concentration of free SO_2 in sterile wines was monitored over a 50 day period. Cabernet Sauvignon wine (150 ml) was sealed in bottles with < 5% headspace and incubated at 22°, 18°, 15°, or 10°C. Potassium metabisulfite was added to provide an initial concentration of 100 mg l^{-1} total SO_2. Treatments were conducted in triplicate and mSO_2 was observed over 50 days.

Statistical analyses

Statistical analysis was conducted by analysis of variance (ANOVA) and Fisher's LSD to assess sources of variation between means. Significance was determined at probability $p \leq 0.05$ using XLSTAT software (Addinsoft, New York, NY).

Results

Temperature affected the culturability of the individual *B. bruxellensis* strains. Strain F3 was impacted the least, growing well in the absence of added SO_2 at 22°, 18°, or 15°C, while remaining at 10^4 cfu ml^{-1} for 90 days at 10°C (Figure 13). In contrast, strain B1b grew at 22°, 18°, or 15°C but not at 10°C (Figure 14). At 10°C, the population of strain B1b cells decreased from $>10^5$ cfu ml^{-1} to undetectable levels (<30 cfu ml^{-1}) within 40 days and did not recover

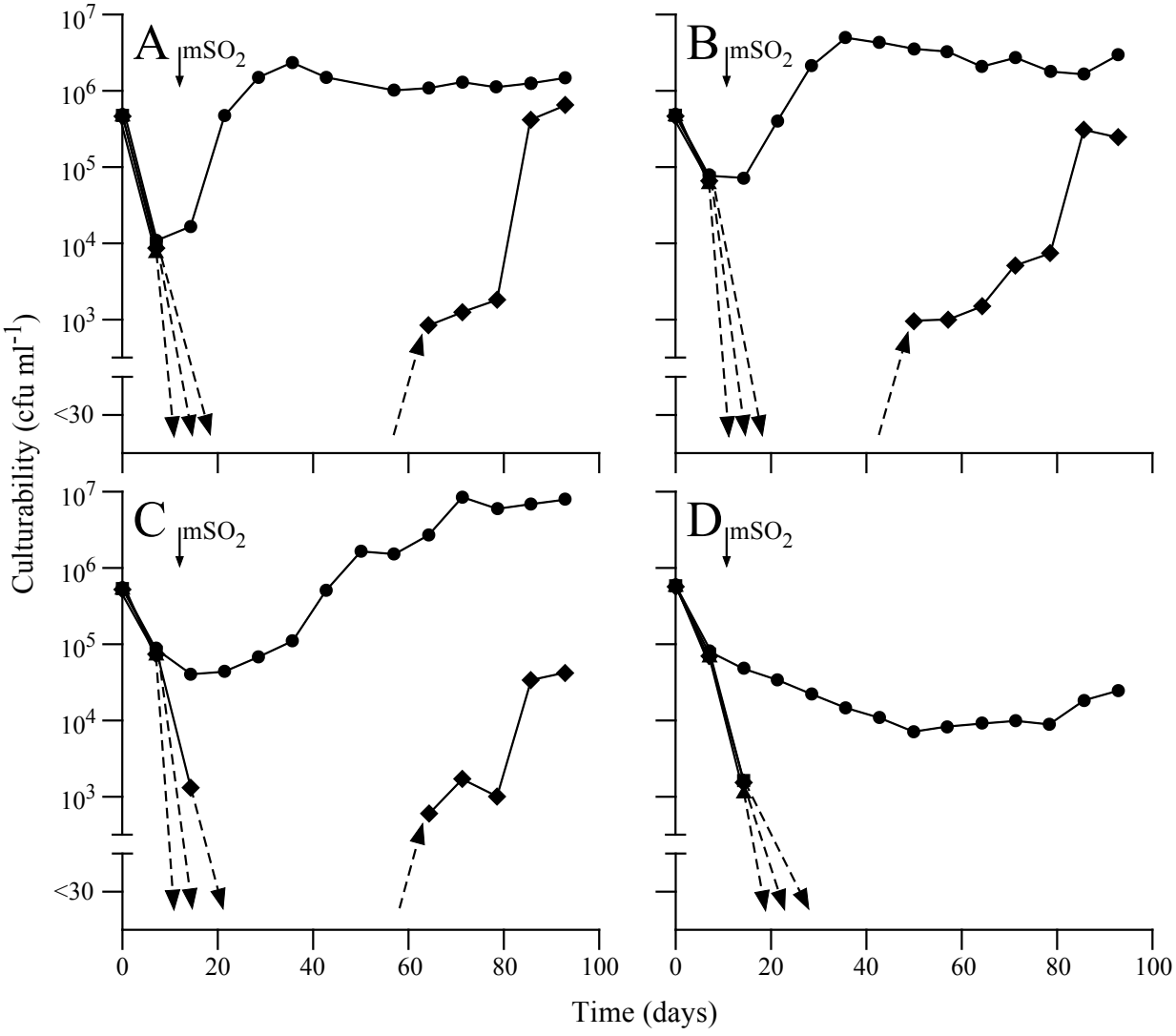

Figure 13. Culturability of *B. bruxellensis* F3 in wine maintained at 22° (A), 18° (B), 15° (C), or 10°C (D). Addition of approximately 0.0 (●), 0.2 (◆), 0.5 (■), or 1.1 (▲) mg l^{-1} molecular sulfur dioxide occurred on day 14.

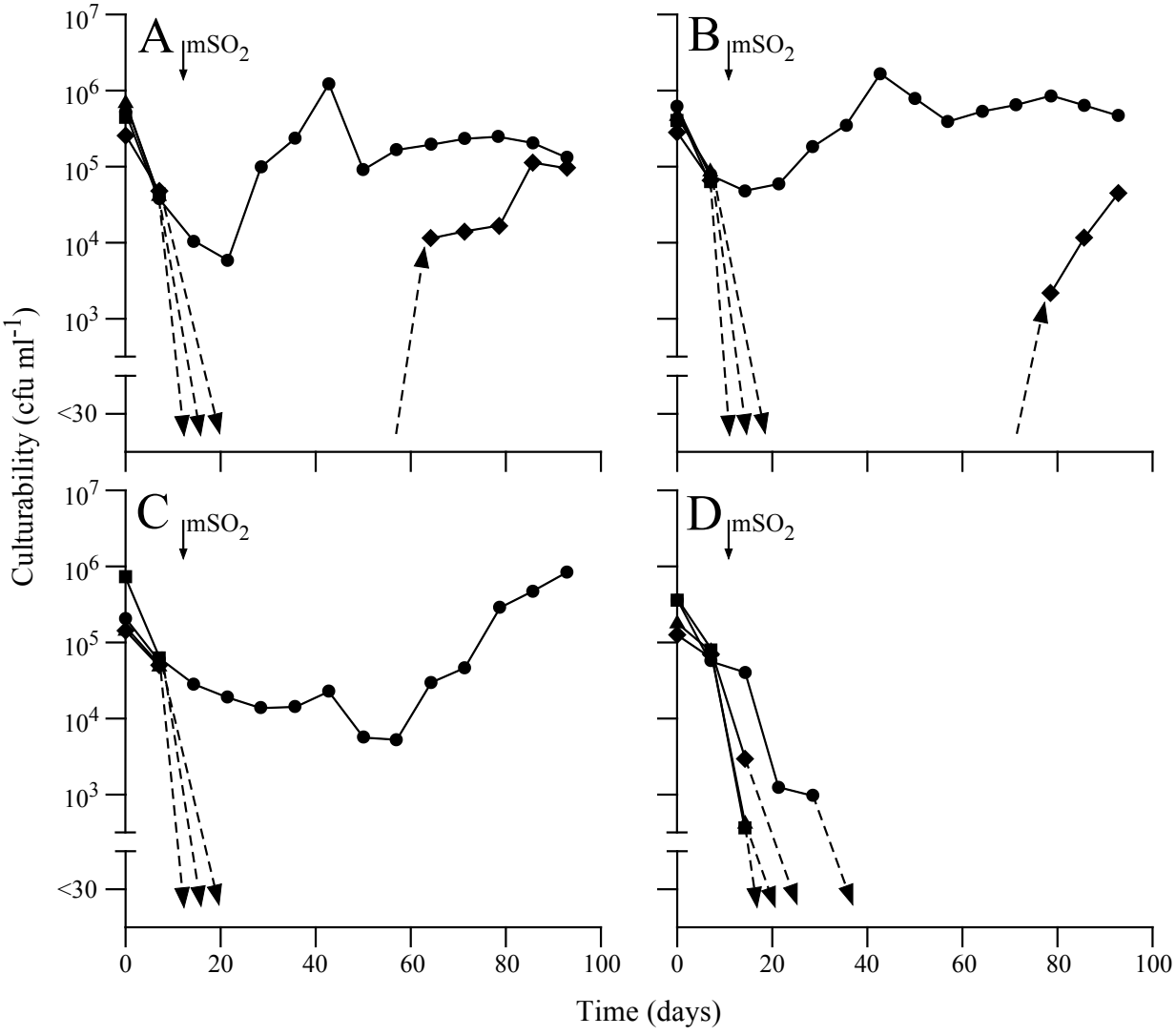

Figure 14. Culturability of *B. bruxellensis* B1b in wine maintained at 22° (A), 18° (B), 15° (C), or 10°C (D). Addition of approximately 0.0 (●), 0.2 (◆), 0.5 (■), or 1.1 (▲) mg l^{-1} molecular sulfur dioxide occurred on day 14.

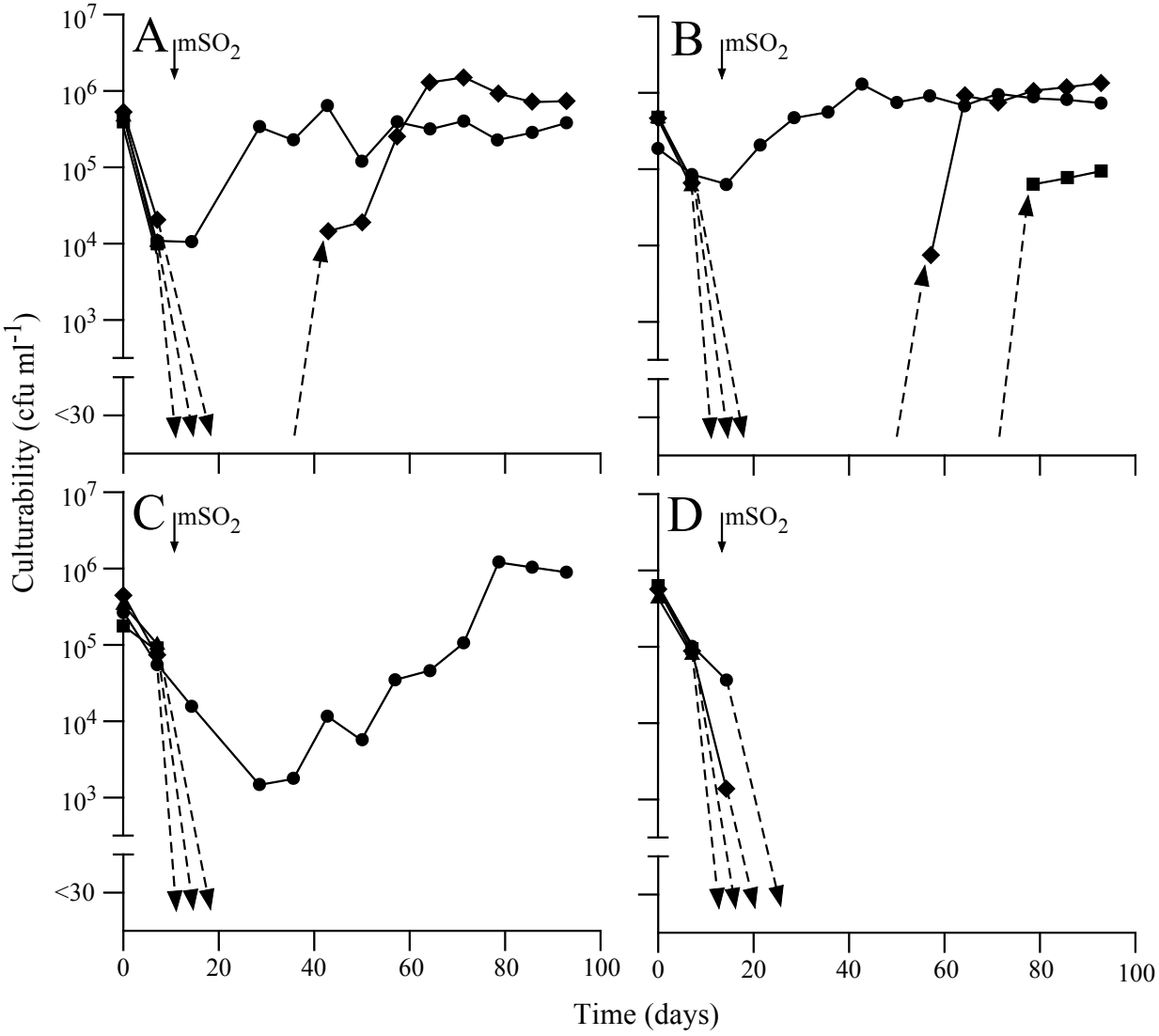

Figure 15. Culturability of *B. bruxellensis* B5 in wine maintained at 22° (A), 18° (B), 15° (C), or 10°C (D). Addition of approximately 0.0 (●), 0.2 (◆), 0.5 (■), or 1.1 (▲) mg l^{-1} molecular sulfur dioxide occurred on day 14.

within 90 days. Similarly, stain B5 was not detected 30 days after inoculation at 10°C although growth was observed at 15°C (Figure 15).

Individual strains also demonstrated a variable response to mSO_2. In wines inoculated with *B. bruxellensis*, the mean initial mSO_2 concentrations were 0.0, 0.2, 0.5, and 1.1 mg l^{-1} respectively for each of the four total SO_2 additions (Table 7). Measured mSO_2 was affected by both the total SO_2 added, as well as incubation temperature and yeast strain (Table 8). This interaction was further illustrated by Principal Component Analysis where total SO_2 addition represented the most significant factor (Figure 16). Strain F3 was most sensitive to mSO_2, with growth delayed at ≈0.2 mg l^{-1} mSO_2 for >45 days at 22°, 18°, or 15°C (Figure 13). Similarly, growth of B1b was delayed for 60 days at 22°C and 75 days at 18°C (Figure 14). At 18°C, only strain B5 grew at 100 mg l^{-1} total SO_2 (0.30 mg l^{-1} initial mSO_2), although this growth was delayed 75 days (Figure 15). In the presence of 60 mg l^{-1} total SO_2, growth of strain B5 was delayed 40 days (22°C) or 55 days (18°C). No growth was observed in the other wines containing SO_2.

Reduced temperatures and mSO_2 impeded growth of *B. bruxellensis*, but a combination of these was more effective. For example, while strain F3 grew at 15°C in 0.24 mg l^{-1} mSO_2, no growth was observed at 10°C following the same SO_2 treatment. Similarly, strain B1b grew at 18°C with 0.12 mg l^{-1} mSO_2 but not 15°C. Finally, strain B5 grew with 0.08 or 0.30 mg l^{-1} mSO_2 at 18°C but not at 15°C.

Molecular SO_2 concentrations declined over time during the storage of wine, with the rate dependant on temperature (Figure 17). In sterile wines initially containing >0.6 mg l^{-1} mSO_2, the concentration declined to <0.05 mg l^{-1} at 22°C after 50 days (97% loss). Meanwhile, at 10°C >0.40 mg l^{-1} remained after the same amount of time (55% loss).

Table 7. Initial concentrations of mSO_2 (mg l^{-1}) in the Cabernet Sauvignon inoculated with *B. bruxellensis* B1b, B5, or F3 following total SO_2 addition of 0, 60, 100, or 180 mg l^{-1}.

	Strain											
	F3				B1b				B5			
Total SO_2	22°C	18°C	15°C	10°C	22°C	18°C	15°C	10°C	22°C	18°C	15°C	10°C
0 mg l^{-1}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
60	0.14^{a}	0.22^{a}	0.24^{a}	0.25^{a}	0.10^{a}	0.12^{a}	0.18^{a}	0.28^{a}	0.06^{a}	0.08^{a}	0.10^{a}	0.18^{a}
100	0.51^{b}	0.55^{b}	0.58^{b}	0.61^{b}	0.30^{b}	0.34^{b}	0.36^{b}	0.52^{b}	0.28^{b}	0.30^{b}	0.40^{b}	0.50^{b}
180	1.23^{c}	1.39^{c}	1.39^{c}	1.39^{c}	0.80^{c}	0.92^{c}	0.99^{c}	1.11^{c}	0.56^{c}	0.88^{c}	0.93^{c}	1.05^{c}

nd: not detected ($mSO_2 \leq 0.02$ mg l^{-1})

Means within strain for mSO_2 in wine with different superscripts are significant at $p \leq 0.05$.

Table 8. Calculated f-values and significant interaction of total SO_2 and temperature in wines containing *B. bruxellensis* B1b, B5, or F3.

F-value Table

Source of variation	df	B1b	B5	F3
Temperature (T)	1	24.02***	1.788	5.957*
total SO_2 (S)	3	1280***	184.2***	1222***
T*S	3	3.921*	0.3970	1.081

Significance is denoted as * $p \leq 0.05$; ** $p \leq 0.001$; *** $p \leq 0.0001$

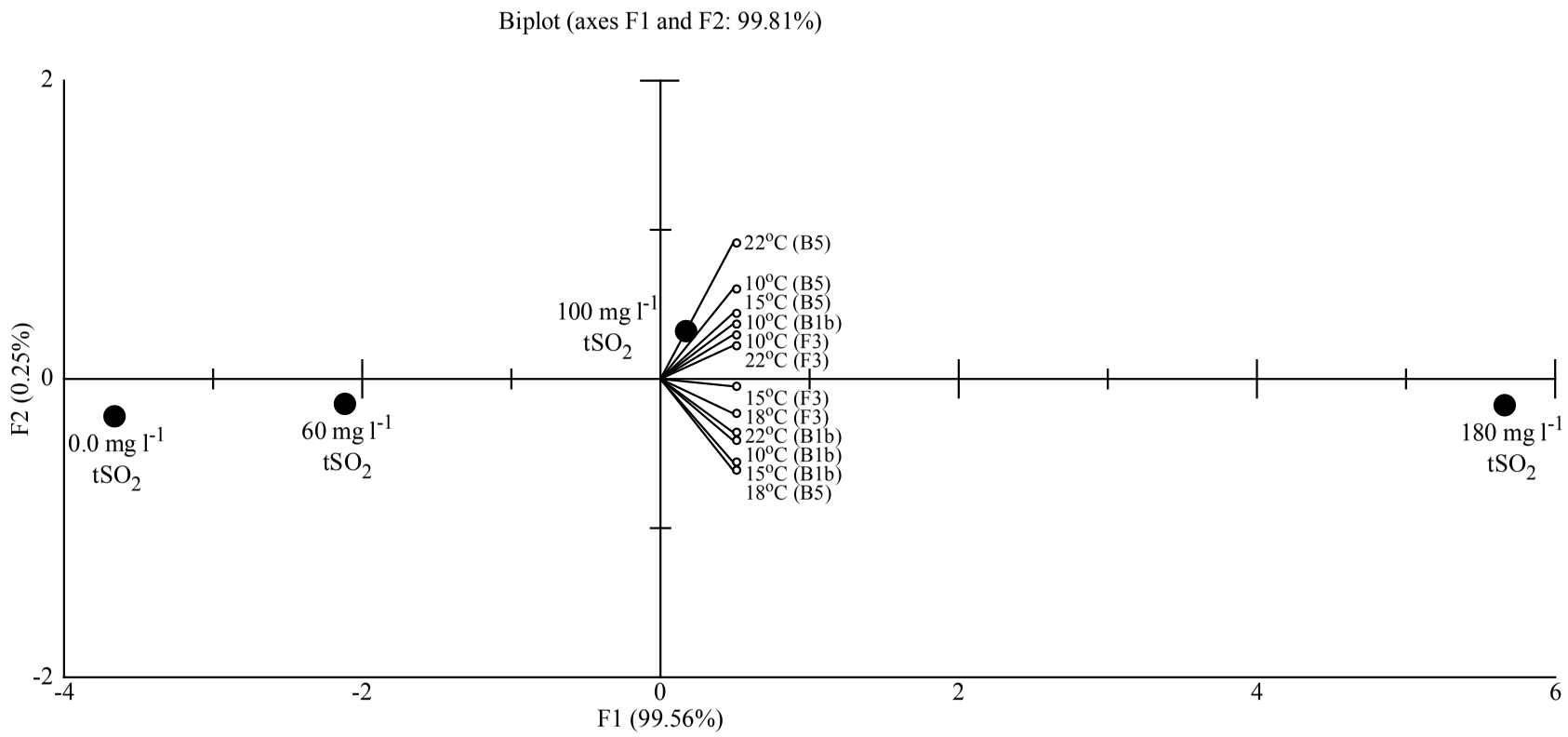

Figure 16. Principal Component Analysis of total SO_2 addition (●) and wine storage temperature by *B. bruxellensis* strain (○).

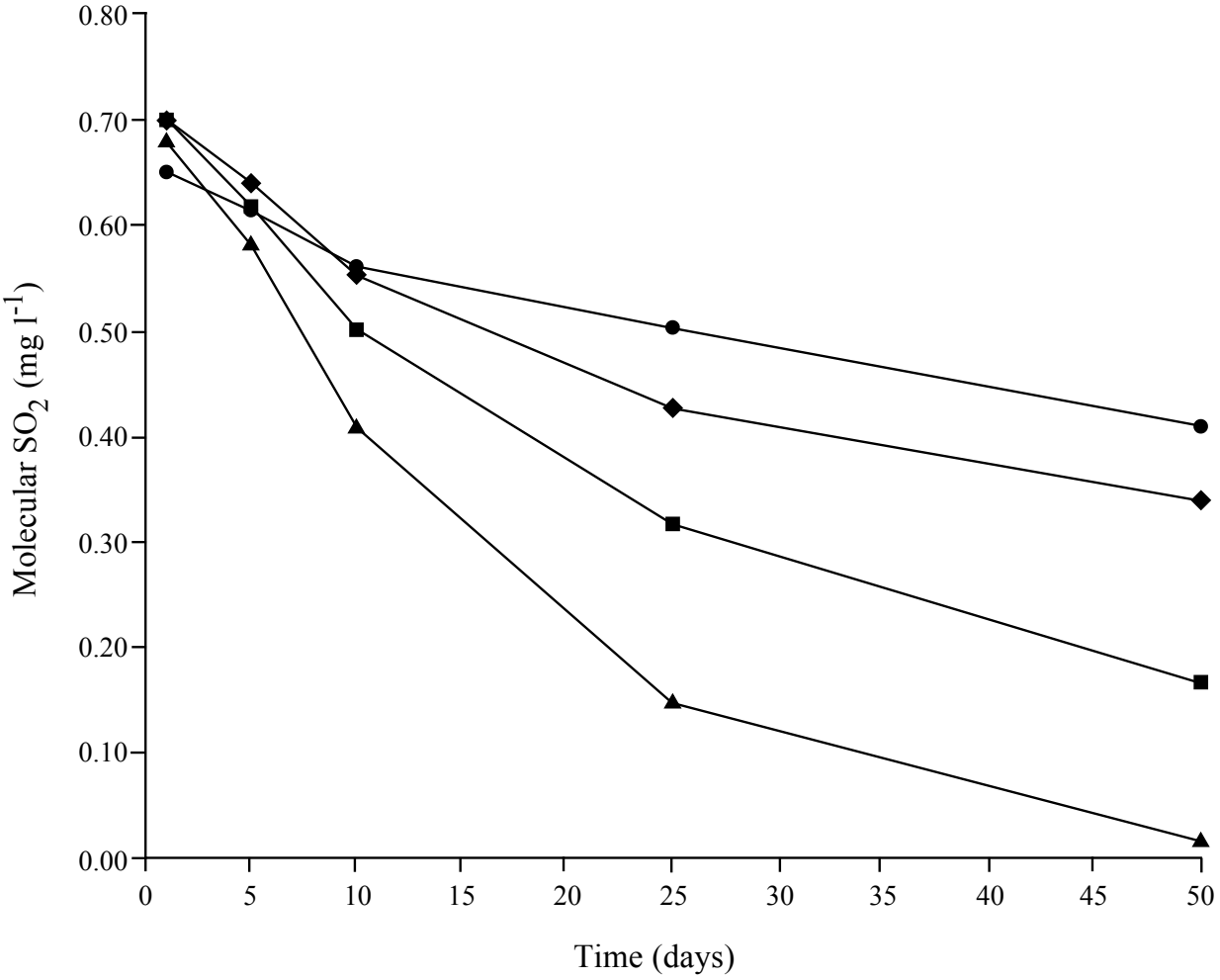

Figure 17. Changes in mSO_2 concentration in red wines incubated at 22° (▲), 18° (■), 15° (◆), or 10°C (●) following addition of 100 mg l^{-1} total SO_2.

Discussion

While reduced cellar temperatures are used to limit the growth of *B. bruxellensis* during wine storage, individual strains are affected differently (Sponholz 1993; Loureiro & Malfeito-Ferreira 2003). At the lowest storage temperature (10°C) in this study, two of the three strains studied did not multiply, even in the absence of SO_2. Variation between *B. bruxellensis* strains has been documented before. For example, Conterno et al. (2006) reported that 15 of 35 strains studied were capable of growth in a synthetic wine medium below 10°C. Furthermore, Barata et al (2008b) reported growth and spoilage by *B. bruxellensis* in wines cellared at 25°, 20°, and 15°C. However, according to Brandam et al. (2008) growth rates and glucose consumption were slower at 15°C than at warmer temperatures.

While reduced temperature limited some *B. bruxellensis* growth, enough SO_2 limited all culturability. In fact, among the three strains evaluated, no growth was detected when the initial mSO_2 exceeded 0.30 mg l^{-1}. These findings agree with recent reports that concentrations of 0.2 to 0.5 mg l^{-1} mSO_2 typically inhibits growth in wines (du Toit et al. 2005; Conterno et al. 2006; Barata et al. 2008a; Agnolucci et al. 2010). However, the International Organization of Vine and Wine (OIV) and others have advocated less SO_2 use due to health risks posed to sulfite-sensitive or asthmatic individuals (Threlfall & Morris 2006; Santos et al. 2012) so wineries need additional strategies to control growth.

In general, when wines were incubated at lower temperatures (≤15°C) less SO_2 was required to prevent or delay *B. bruxellensis* growth. Other previously studied microbiological hurdles such as ethanol concentration, oxygen availability, sorbic acid, and dimethyl dicarbonate have also been employed to reduce SO_2 use while preventing wine spoilage by *B. bruxellensis* (Terrell et al. 1996; du Toit et al. 2005; Barata et al. 2008a; Duckett 2012). Although appropriate

use of individual hurdles will depend on the situation, temperature regulation and SO_2 addition are already common winery practices making them good candidates to limit *B. bruxellensis* growth.

One of the challenges associated with the use of SO_2 to preserve wine quality is that mSO_2 concentration declines over time. One cause is the reaction of mSO_2 with carbonyl compounds in wine such as acetaldehyde, as well as anthocyanins, pyruvate, or α-ketoglutarate (Ough 1993, Fugelsang & Edwards 2007). mSO_2 is also lost through reactions with dissolved oxygen and hydrogen peroxide (Brajkovich et al. 2005; Danilewicz et al. 2008). Therefore, winemakers must monitor mSO_2 concentrations in bulk wines, and higher storage temperatures may require additional vigilance to ensure that mSO_2 concentrations are sufficient to limit spoilage.

A second challenge to wine quality is posed by the ability of some *B. bruxellensis* strains to persist in a viable but not culturable (VBNC) state when exposed to environmental stress (Divol 2012; Serpaggie et al. 2012). This phenomenon has been observed in *B. bruxellensis* populations in wine following SO_2 addition (Millet & Lonvaud-Funel 2000; du Toit et al. 2005; Agnolucci et al. 2010). In the VBNC state, cells are unable to grow in a culture medium but remain metabolically active in the wine, however, the extent of these activities are not yet fully understood (Divol 2012). For example, although Agnolucci et al. (2010) did not observe an increase in concentration of 4-ethylphenol or 4-ethylguaiacol, Serpaggie et al. (2012) suggested that these off-flavors can be produced by *B. bruxellensis* even in a VBNC state. While research to understand the VBNC state of *B. bruxellensis* is important, simply limiting culturability of the yeast is a priority for winemakers.

The present study demonstrates that the application of temperature and mSO_2 can be used to control the growth of *B. bruxellensis*. With the documented strain variability in *B. bruxellensis*, capitalizing on the use of multifaceted approaches to synergistically limit growth is important. Future research may increase in complexity by including additional hurdles to evaluate in concert with temperature and mSO_2 concentrations, preferably alternatives that can reduce the levels of mSO_2 necessary for *B. bruxellensis* control.

CONCLUSIONS

Although limited potential for *Z. bailii* to reduce residual sugars in stuck wine fermentations was discovered, growth of this and other non-*Saccharomyces* yeasts in grape must or wine was susceptible to several antimicrobial strategies. When DMDC was added to grape musts containing non-*Saccharomyces* yeasts isolated from vineyards, populations of 10^3 or 10^5 cfu ml^{-1} declined immediately after treatment but eventually grew to $\geq 10^6$ cfu ml^{-1}. Conversely 10^3 cfu ml^{-1} populations of *Z. bailii* did not grow in grape musts with DMDC whereas 10^5 cfu ml^{-1} populations did. However, DMDC addition to wines containing *Z. bailii* did limit the growth of 10^3 and 10^6 cfu ml^{-1} populations. Similarly, addition of DMDC to wines limited growth of $\leq 10^4$ cfu ml^{-1} *B. bruxellensis* but efficacy against greater populations depended upon strain. Furthermore, although *B. bruxellensis* grew in wines either stored at 10°C or containing ≈0.5 mg l^{-1} mSO_2, capitalizing on the interactive impact of these factors limited growth at ≤15°C with ≥0.4 mg l^{-1} mSO_2. The consequences of these studies serve to better characterize methods of limiting spoilage yeast growth throughout vinification.

FUTURE RESEARCH

Although the research presented here adds to the volume of literature concerning the growth of spoilage yeasts during winemaking, much remains unknown. In particular, several topics applicable to both *Z. bailii* and *B. bruxellensis* are apparent as the logical continuation of the present work. Specifically, additional factorial studies to better characterize yeast growth and control methods along with further exploration of the VBNC state are of interest.

The interactive impacts of temperature x SO_2 demonstrated the well-studied food microbiology concept of "hurdle technology" in wines. However, a multitude of factors remain to be evaluated with both *B. bruxellensis* and *Z. bailii*. Here, factors intrinsic to wines such as pH, ethanol, or nutrient or oxygen availability, along with extrinsic aspects such as temperature, antimicrobials, or growth of other yeast or bacteria could all be compared in factorial experiments. Although factorial design can cause projects to rapidly grow in size and workload by adding variables, use of easier to achieve metrics such as turbidity in preliminary studies would make the undertaking more feasible.

When *B. bruxellensis* and *Z. bailii* were measured using methods for culturability (plate count) and viability (real-time PCR), evidence for VBNC populations was found. Better characterization of VBNC yeasts using these and addition methods such as fluorescence would be of interest to both the research and winemaking communities. Specifically, what types and degrees of stress that cause injury, VBNC, or cell death are unknown, along with potential of VBNC populations to cause spoilage or eventually exit the VBNC state. Ultimately, protocols to ensure that the growth of spoilage yeasts can be prevented from the time of wine production through consumption should be established.

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