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Fermentability of Grape Must after Inhibition with Dimethyl Dicarbonate (DMDC)

CLAUDIO DELFINI,* PIERO GAIA, RAFFAELLA SCHELLINO, MORELA STRANO, ADOLFO PAGLIARA, AND STEFANO AMBRÒ

Istituto Sperimentale per l'Enologia, Sezione di Microbiologia, via P. Micca 35, 14100 Asti, Italy

Dimethyl dicarbonate (DMDC) was added to grape must and to synthetic media and results showed that, at 20 °C, 150 mg·L^{-1} DMDC completely inhibited the fermentation of a grape must that was previously inoculated with 10^6 cells·mL^{-1} *Saccharomyces bayanus* and *Saccharomyces uvarum. Brettanomyces intermedius*, *Candida guilliermondii*, *Hansenula jadinii*, *Hansenula petersonii*, *Kloeckera apiculata*, *Pichia membranaefaciens*, and *Saccharomyces cerevisiae* were inhibited by 250 mg·L^{-1}. *Candida valida* was inhibited in the presence of 350 mg·L^{-1}, whereas *Hanseniaspora osmophila*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, and *Zygosaccharomyces bailii* required 400 mg·L^{-1}. Delay of fermentation (but not inhibition) was noted in the presence of 400 mg·L^{-1} for the following cultures: *Brettanomyces anomalus*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Schizosaccharomyces japonicus*, *Torulaspora delbrueckii*, and *Zygosaccharomyces florentinus*. *Acetobacter aceti* and *Lactobacillus* sp. were completely inhibited using 1000 and 500 mg·L^{-1} DMDC, respectively. The fermentation of a grape must inoculated with 10^6 cells·mL^{-1} of different wine yeasts was delayed for 4 days after the prior addition of 200 mg·L^{-1} of DMDC; 200 mg·L^{-1} DMDC did not show any residual inhibitory effect after 12 h, nor did 300 mg·L^{-1} 24 h after the addition. In cellar experiments, indigenously contaminated grape musts (with and without skins) showed a delay in fermentation of 48 h after the addition of only 50 mg·L^{-1} DMDC. The possibility of using DMDC (as pure grade as commercially available) in grape must as a disinfectant for the decontamination of musts indigenously contaminated with wild yeast should be considered seriously, despite its apparent low solubility in water.

KEYWORDS: DMDC; wine; grape must; wine yeasts; wine bacteria; must decontamination

INTRODUCTION

To reduce the endogenous microbial population in musts, 30−50 mg·L^{-1} sulfur dioxide is usually added to musts, even though, at this level, it may be ineffective against some aerobic yeast species and some lactic and acetic acid bacteria. Furthermore, >50% of the initially added amount ends up as the bound form, which does not have antiseptic and antioxidant activity; unfortunately, it still retains its health-related contraindications. Clarification, deep filtering processes (cloth, diatomaceous earth, fiber filter paper, prefilter cartridges, etc.), cross-flow filtration, and centrifugation have the advantages of not generating toxic residues in wine for the consumer or for the yeast strain used. On the other hand, they result in losses of colloids (which contributes to wine texture and taste), fatty acids, and sterols (*1*−*3*), and they are not applicable when the must contain skins, as in the production of red wine. Finally, pasteurization has been largely abandoned, not only because of its high cost but also because of the cooked taste it imparts to wine.

Although enological processes preclude sterilization, an ideal solution to the problem of decontamination, or disinfection, could be solved by the addition of compounds that, when added, quickly inhibit indigenous microorganisms as it quickly degrades within 12−24 h, without leaving toxic residues. Such a compound could allow for the subsequent addition of a starter yeast inoculum without interfering with the wine-making process.

Several studies (*4*−*14*) have demonstrated the effectiveness of dimethyl dicarbonate (DMDC; also known as dimethyl pyrocarbonate or DMPC) in terms of toxicity (*15*) and germicidal activity in dry, semisweet, and sweet wines ready for bottling. Few data were published about its efficacy in grape juice, according to which (*5*) 40 mg·L^{-1} would be sufficient to kill 278 viable cells·mL^{-1}, without requiring the presence of any ethanol. This result is, in fact, controversial and, according to some authors (*13*, *17*), DMDC should be dispensed as an alcoholic solution to increase the water solubility of DMDC. On the other hand, the water solubility (*16*) declared for the commercial product Velcorin (Bayer Industrial Chemical Division) [3.65 g·(100 g^{-1} of water)] does not appear to be low

* Author to whom correspondence should be addressed (telephone 00390141433818; fax 00390141436829; e-mail sezione.microbiologia@tin.it).

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enough to compromise its sterilizing efficacy, particularly if we consider the low doses of DMDC required in enology (up to 200 mg·L^{-1}). In any case, DMDC should be added to commercial grape musts or wines in as pure a form as possible.

Leuconostoc, *Lactobacillus*, and particularly *Acetobacter* are less sensitive to DMDC than yeasts (*7*). Furthermore, Van Zyl (*18*), Genth (*6*), and Porter et al. (*13*) found that among wine yeast species notable differences exist in their resistance to DMDC, as was demonstrated for diethyl dicarbonate (DEDC) (*19*). *Rhodotorula rubra* and *Schizosaccharomyces pombe* were found to be the species most resistant to DMDC.

Temple (*20*) and Porter et al. (*13*) reported that the antiseptic activity of DMDC is more effective against yeasts than against bacteria, possibly due to the denaturation of the enzymes glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase, resulting in the arrest of cellular growth and the alcoholic fermentation.

In dry, semisweet, or sweet wines the sterilizing effects of DMDC increase with increases in the concentration of hydrogen ions, alcohol, and sulfur dioxide and in the temperature (*10*, *11*, *13*, *17*).

When added to wine, DMDC has the characteristic of hydrolyzing quickly to methanol and carbon dioxide within 1 h at 30 °C and within 5 h at 10 °C. Its cleavage results in the concomitant loss of antiseptic activity and restoration of the must to its prior fermentable status.

The rapid hydrolysis of DMDC identifies it as an ideal antiseptic for enological use, capable of disinfecting a must or wine without leaving significant toxic residues. In fact, unlike DEDC, DMDC is not a precursor of ethyl carbamate in wine and therefore does not appear to have a potential carcinogenic effect. From 200 mg of DMDC, ~96 mg of methanol is formed (48%) together with a few milligrams of methyl carbonate (*14*) and alkyl carbonates (*12*) and a few micrograms of methyl carbamate resulting from reactions with ammonium, amino acids, polyphenols, and organic acids (*9*). Methyl ethyl carbonate is also formed as a stable and proportional derivative of DMDC in hydroalcoholic solutions and can be used as a tracing molecule to determine the amount of DMDC initially added (*21*). Finally, no off-flavors or off-aromas were found in wine after the addition of 200 mg·L^{-1} DMDC (*8*).

The Vine and Wine International Organization (OIV) has recently approved a maximum dose of 200 mg·L^{-1} DMDC in wine but not in must. The United States, South Africa, and New Zealand currently permit the addition to wine of up to 400 mg·L^{-1} DMDC.

The present investigation verified the effectiveness of DMDC against flora of the must and guaranteed the dominance of selected yeast strains when added to must 12 h after DMDC.

MATERIALS AND METHODS

Yeasts and Bacteria. The cultures and strains used were obtained from the Institute of CNLBSV-ISEAT national collection: *Brettanomyces anomalus* strain 371, *Brettanomyces intermedius* strain 373, *Candida guillermondii* strain 310, *Candida valida* strain 317, *Hanseniaspora osmophila* strain 1340, *Hanseniaspora uvarum* strain 1345, *Hansenula jadinii* strain 337, *Hansenula petersonii* strain 339, *Kloeckera apiculata* strain 346, *Metschnikowia pulcherrima* strain 344, *Pichia membranaefaciens* strain 351, *Pichia* sp. strain 304, *Saccharomyces bayanus* strain 196, *Saccharomyces cerevisiae* strains 1 and 41, *Saccharomyces uvarum* strain 270, *Saccharomycodes ludwigii* strain 358, *Schizosaccharomyces pombe* strains 319 and 321, *Schizosaccharomyces japonicus* strain 325, *Torulaspora delbrueckii* strain 286, *Zygosaccharomyces bailii* strain 275, *Zygosaccharomyces florentinus* strain 296, *Acetobacter aceti* strain 1, *Lactobacillus* sp. strain 32, *Leoconostoc oeni*, and *Leuconostoc* sp.

Nutrient Media. The following media were used for fermentation: (a) whole Cortese grape must (Co), with no sulfur dioxide, and containing total extract, 212 g·L^{-1}; glucose + fructose, 190 g·L^{-1}; total acidity, 51.07 mequiv; pH 3.54; (b) Cortese grape must (Co) diluted with water and having (b1) total extract, 93 g·L^{-1}; glucose + fructose, 77 g·L^{-1}; total acidity, 34.70 mequiv; pH 3.21; (b2) total extract, 110 g·L^{-1}; glucose + fructose, 95 g·L^{-1}; total acidity, 56.00 mequiv; pH 2.76; (c) synthetic nutrient media MT and MTB for malolactic bacteria and NSM for yeasts, prepared according to the method of Delfini et al. (*22*, *23*); (d) for large scale cellar experiments, grape must with and without skins prepared from a mixture of white and red grapes.

Reagents. DMDC, 98% pure, was purchased from Sigma or Fluka. The DMDC was weighed out and added directly to a fermentation flask containing only 10% of the uninoculated medium (grape must or synthetic nutrient media) to favor dissolution. Then, the remaining 90% of the inoculated medium was added to restore the desired DMDC and yeast concentrations.

Analytical Methods. Fermentation delay, fermentation curves, and ethanol production were determined by measuring the daily loss of weight in the flasks as indicated by the Delfini et al. (*23*) procedure. The total cell number and the percentage of budding cells were assayed microscopically using a counting chamber on samples previously stabilized with 2.5% H_2SO_4. When needed, the dissolution of the suspended solid material in grape must was accomplished by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 4% (w/v). The percentage of viable cells was determined by plating on agar media and reported as colony forming units (CFU·mL^{-1}) or by the Fink and Kühles methylene blue staining procedure (*23*). When very low numbers of cells per milliliter were encountered, the cell samples were concentrated by centrifugation to ~10−20·10^6 cells·mL^{-1} (*23*).

In the wine cellar scale experiments, ethanol was determined by densitometry or by chemical analysis.

Efficacy of 98% Pure Commercial DMDC in Aqueous Nutrient Media. NSM and centrifuged natural Cortese grape musts (obtained from frozen Cortese grapes) were inoculated with 10^6 cells·mL^{-1} of *S. cerevisiae* strain 41. Duplicate samples of the inoculated flasks were prepared to contain 100 or 200 mg·L^{-1} of DMDC. Fermentation delay (measuring the daily weight loss), total cell number, and percentage of dead and/or budding cells were determined daily and compared to control (flasks receiving no DMDC).

Minimum Contact Time Needed for DMDC To Decontaminate a Highly Inoculated Grape Must. The six wine yeast species included in the inoculum were *S. cerevisiae* strain 41, *S. bayanus* strain 196, *S. uvarum* strain 270, *Z. bailii* strain 275, *Schi. pombe* strain 319, and *Brettanomyces* sp.

The three bacterial species included in the inoculum were *Acetobacter aceti* strain 1, *Lactobacillus* sp. strain 32, and *L. oeni*.

All nine microorganisms were mixed in MT broth (10^6 cells·mL^{-1} each). An aliquot of this inoculum was distributed among eight flasks each containing 0.25, 0.5, 1.0, 2.0, 4.0, 5.0, 10.0, or 40.0 g·L^{-1} DMDC.

After 30, 60, and 120 min of contact with DMDC, a 5 mL sample of the inoculated must was filtered through a 0.22 μm filter membrane. The membrane was then placed on MT agar in a Petri plate and incubated overnight at 25 °C, and the total number of colonies formed was recorded.

Inhibitory Effect of DMDC on Different Wine Yeast Species. Twenty different wine yeast species (**Table 2**) were inoculated separately in individual 10 mL (10^6 cells·mL^{-1}) aliquots of diluted Cortese grape must b1 in Einhorn tubes to which were added the following DMDC concentrations: 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 1000, 5000, and 10000 mg·L^{-1}, incubated at 20 °C for 10 days and visually examined for growth (cloudiness and formation of deposit, development of gas bubbles).

Rate of Degradation of DMDC in Grape Must. To flasks each containing five aliquots of Cortese must b was added 0, 50, 100, 200, or 300 mg·L^{-1} DMDC; 150 mL was removed from each DMDC level and transferred to 200 mL flasks. The flasks were closed with Pasteur pipets and incubated at 20 °C. At intervals (0, 0.5, 1, 2, 3, 4, 6, 24, and

Table 1. Effect of DMDC on Cellular Growth of *S. cerevisiae* in Nutrient Synthetic Media (NSM)

nutrient medium	DMDC addition ($mg \cdot L^{-1}$)	fermentation delay (h)	TC[a] $\cdot mL^{-1} \cdot 10^6$ at 48 h	% mortality after 18 h	% mortality after 24 h	% mortality after 48 h	% budding cells after 18 h	% budding cells after 24 h	% budding cells after 48 h
NSM	0[b]	24	73	39	16	13	44	35	16
	0[c]	24	65	32	20	9	42	28	19
	100[b]	64	34	65	42	19	8	17	36
	100[c]	64	15	66	40	22	6	19	32
	200[b]	76	1	88	22	54	2	22	8
	200[c]	*d*	1	99	34	44	0	19	10
grape must	0[b]	18	113	3	2	5	42	27	6
	0[c]	18	134	2	1	3	38	24	10
	100[b]	48	96	44	18	7	12	18	42
	100[c]	48	100	36	19	16	9	15	35
	200[b]	48	53	50	27	18	7	12	39
	200[c]	48	75	46	18	8	6	15	34

[a] Total cell number. [b] Replication I. [c] Replication II. [d] No growth after 6 days.

25 h) after the addition of DMDC, duplicates of each flask containing the five different concentrations of DMDC were inoculated with 10^6 cells$\cdot mL^{-1}$ of *S. cerevisiae* strain 41 and incubated at 25 °C. Cell growth was verified by observing cloudiness and start of fermentation by weighing the flasks daily.

Inhibitory Effect of DMDC on Wine Bacteria. To each of five aliquots of MT broth (pH 5) was added 0, 200, 300, 500, or 1000 $mg \cdot L^{-1}$ DMDC, and each was inoculated with 5×10^6 CFU$\cdot mL^{-1}$ *A. aceti* and 5×10^6 cells$\cdot mL^{-1}$ *Lactobacillus* sp. After 24 h of incubation at 25 °C, the percent survival in the presence or absence of DMDC was determined after plating on MT agar.

Decontaminating Effect of DMDC on a Grape Must Inoculated with *S. cerevisiae*. Flasks containing 400 mL of Cortese grape must, nonsterile and not treated with sulfur dioxide, were inoculated with 10^6 cells$\cdot mL^{-1}$ *S. cerevisiae* strain 41. The inoculated must was divided in two 200 mL aliquots and placed in 300 mL flasks; one had 40 mg of DMDC to achieve a final concentration of 200 $mg \cdot L^{-1}$. The flasks were closed using Pasteur pipets and incubated at 20 °C. The flasks were weighed and the number of CFUs determined at zero time and thereafter up to 80 h of incubation.

Effect of DMDC on the Decontamination of a Grape Must Inoculated with a Mixture of Yeasts and Bacteria. Flasks containing 200 mL of nonsterile Cortese grape must (not treated with sulfur dioxide) were inoculated with 5×10^3 cells$\cdot mL^{-1}$ from each of the following microorganisms: *S. cerevisiae* strain 1, *Schi. pombe* strain 321, *Candida guilliermondii* strain 310, *Pichia* sp. strain 304, *Kloeckera apiculata* strain 346, and *Leuconostoc* sp. The inoculated must was divided into two 100 mL aliquots and placed in 200 mL flasks, one of which had 20 mg of DMDC to yield a final concentration of 200 $mg \cdot L^{-1}$. The flasks were then closed using Pasteur micropipets, weighed, and incubated at 25 °C. The number of surviving cells (CFU$\cdot L^{-1}$) was determined periodically for both the control samples and the DMDC samples. Each microorganism was identified microscopically by its cellular morphology.

Fermentation of Musts Treated with 200 $mg \cdot L^{-1}$ DMDC. One liter of Cortese grape must a was pasteurized (heating at 70 °C for 30 min), divided into two aliquots, and placed in two flasks, one of which contained 100 mg of DMDC to yield a final concentration of 200 $mg \cdot L^{-1}$. The flasks were sealed with Pasteur pipets and incubated at 25 °C. After 12, 24, and 48 h, 100 mL samples were removed from both control and DMDC-treated flasks, placed into 200 mL flasks, and inoculated with 10^4 cells$\cdot mL^{-1}$ of *S. cerevisiae* strain 41 in its exponential phase of growth (2 days of incubation). After the addition of DMDC, changes in growth, weight loss, and alcohol production were determined at intervals to evaluate the length of time required for the must to recover its ability to ferment.

Assessing Restoration of Fermentation Ability at a Wine Cellar Scale. The day preceding the experiment 1000 kg of normal grapes d were harvested by hand, placed in small containers, and stored in the cellar at 20 °C. After random separation of this harvest into two aliquots, one was crushed using a crusher-stemmer and then placed in a horizontal press to separate juice from skins (aliquots M). The juice was subdivided in five parts (60 kg each) and placed in five stainless steel 100 L containers.

The second grape aliquot was arbitrarily subdivided into five parts and crushed individually, without removing the skins (fractions MSk). Each complete (must + skins) aliquot (MSk) was placed in stainless steel 100 L tanks, the same as the first five M parts and receiving no yeast or bacterium.

While the tanks were being filled, each part received the following measured amounts of DMDC expressed as $mg \cdot kg^{-1}$ (a commercial 98% pure product from Fluka): 0 (control), 27.43, 48.68, 99.05, and 217.41 $mg \cdot kg^{-1}$ for parts M; 0 (control), 25.78, 51,78, 101.40, and 209.62 $mg \cdot kg^{-1}$ for parts MSk. The required amount of DMDC was weighed out and placed in test tubes, which remained sealed until utilized. The contents of each tube were transferred all at once to the corresponding tank, and the must was gently stirred to facilitate dissolution and distribution of the DMDC, particularly in the samples containing skins (MSk). The concentrations were considered as w/w because of the presence of the skins in the Msk aliquots. In any event, it is possible to calculate a w/v concentration by taking into account a liquid must percentage yield of ~70% v/w and its densities ($d^{20/20} = 1.0895$).

All fractions were sampled once before treatment and daily thereafter to determine the following: the start of fermentation as detected visually, microscopic examination, density ($d^{20/20}$), and percent alcohol production.

RESULTS AND DISCUSSION

Efficacy of 98% Pure Commercial DMDC in Aqueous Nutrient Media. The results indicate that DMDC was efficacious as a decontaminant when added directly to the inoculated medium. As anticipated, must samples treated in this manner displayed a delay in fermentation lasting 30 h longer than control samples. Similarly, fermentation in DMDC-treated NSM samples was delayed 40 and 52 h compared to controls. Moreover, no growth was observed in one of the two 200 $mg \cdot L^{-1}$ DMDC samples (**Table 1**). The significance of this observation was reinforced by comparing the percent mortality, budding cells percent, and the total cell number obtained in the control samples with those for the treated samples (**Table 1**). Thus, the assumed lack of efficacy of DMDC due to its apparent low solubility in water (*13*, *17*) cannot be substantiated with the two types of nutrient media used in this study. Furthermore, the sterilizing efficacy of DMDC by the direct addition of a pure commercial product was already reported by Daut et al. (*5*) in grape juice and by Bizri (*24*) in tomato juice.

Minimum Contact Time Needed for DMDC To Decontaminate a Highly Inoculated Grape Must. The results (**Figure 1**) showed that 1 h of contact with 10 $g \cdot L^{-1}$ DMDC or 2 h of contact with 5 $g \cdot L^{-1}$ DMDC was sufficient to sterilize the inoculated must. Considering that natural grape musts are

Table 2. Minimum Inhibitory Concentration of DMDC Expressed as Delayed Fermentation (See Text) for Several Yeast Strains

	level of DMDC									
yeast species	0 mg·L^{-1}	50 mg·L^{-1}	75 mg·L^{-1}	100 mg·L^{-1}	150 mg·L^{-1}	200 mg·L^{-1}	250 mg·L^{-1}	300 mg·L^{-1}	350 mg·L^{-1}	400 mg·L^{-1}
Brettanomyces anomalus 371	2	3	3	4	5	6	6	6	6	7
B. intermedius 373	7	7	7	7	7	8	–[a]	–	–	–
Candida guilliermondii 310	5	5	5	6	11	12	–	–	–	–
C. valida 317	1	1	2	3	4	5	7	8	–	–
Hanseniaspora osmophila 1340	1	2	2	3	4	6	6	7	9	–
H. uvarum 1345	1	1	2	2	3	4	6	7	8	10
Hansenula jadinii 337	10	11	13	15	17	18	–	–	–	–
H. petersonii 339	8	8	8	8	8	9	–	–	–	–
Kloeckera apiculata 346	1	2	2	2	4	6	–	–	–	–
Metschnikowia pulcherrima 344	3	3	3	4	5	6	8	9	10	10
Pichia membranaefaciens 351	3	3	3	4	5	5	–	–	–	–
Saccharomyces bayanus 196	1	2	4	8	–	–	–	–	–	–
S. cerevisiae 41	1	2	3	6	10	15	–	–	–	–
S. uvarum 270	1	2	4	8	–	–	–	–	–	–
Saccharomycodes ludwigii 358	2	3	3	5	6	7	7	9	10	–
Schizosaccharomyces pombe 319	3	6	7	9	9	9	10	11	11	–
S. japonicus 325	3	3	4	5	5	5	5	5	6	8
Torulaspora delbrueckii 286	1	2	3	4	5	6	7	8	9	10
Zygosaccharomyces bailii 275	2	3	3	4	5	6	5	6	7	–
Z. florentinus 296	2	2	2	3	3	4	6	7	8	9

[a] –, no fermentation or cell growth after 22 days.

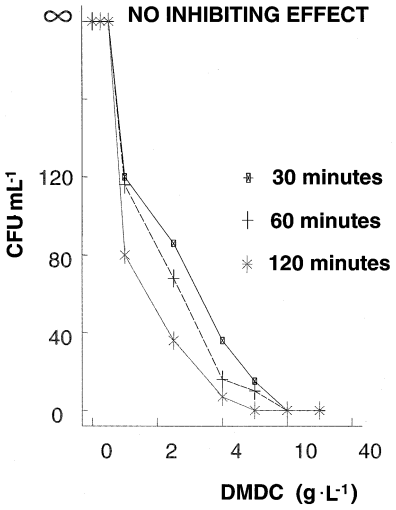

Figure 1. Effect of DMDC on the viability of wine microorganisms in grape must. A mixture of six wine yeasts and three wine bacteria (see text) was added to grape must containing increasing concentrations of DMDC. After 30, 60, and 120 min, the musts were assessed for viable cells, reported as CFU·mL^{-1}.

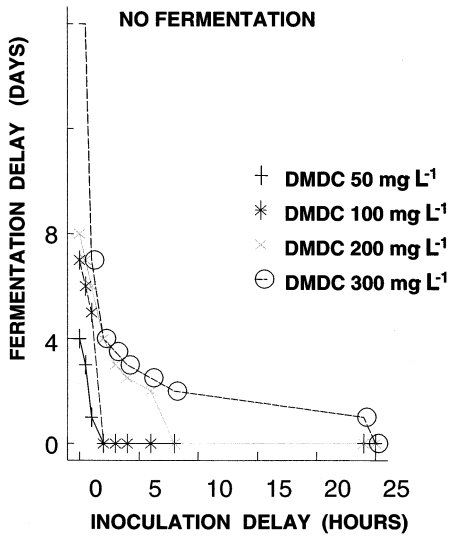

Figure 2. Rate of degradation of DMDC in grape must inoculated with *S. cerevisiae* at various intervals (from 0 to 25 h). (The delay in the initiation of fermentation was the parameter used to measure loss of inhibitory activity resulting from the spontaneous degradation of DMDC.)

rarely as heavily contaminated as the hypothetical typical must reinforces the possibility of obtaining effective decontamination at lower concentrations of DMDC (<200 mg·L^{-1}) by simply using longer exposure times. This appears to be a reasonable premise considering that realistically musts are indigenously contaminated to the extent of only 10^3-10^5 cells·mL^{-1}.

Inhibitory Effect of DMDC on Different Wine Yeast Species. The results summarized in **Table 2** indicate that the most resistant species were *Brettanomyces anomalus*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Schizosaccharomyces japonicus*, *Torulaspora delbrueckii*, and *Zygosaccharomyces florentinus* because they survived exposure to 400 mg·L^{-1} but not 500 mg·L^{-1} DMDC. *S. cerevisiae* was resistant to 200 mg·L^{-1}, whereas the species *S. bayanus* and *S. uvarum* were inhibited at 150 mg·L^{-1}. The most common yeasts generally found in grape must, *Brettanomyces*, *Kloeckera*, *Candida*, and *Pichia*, could be controlled with 200−250 mg·L^{-1} DMDC.

Rate of Degradation of DMDC in Grape Must. Figure 2 shows that the 100 mg·L^{-1} DMDC grape must sample strongly reduced its inhibitory effect on yeasts after 7 h of incubation at 20 °C, the 200 mg·L^{-1} sample after 10 h, and the 300 mg·L^{-1} sample after 25 h. These observations clearly suggested that a grape must treated with 200 mg·L^{-1} DMDC should not be inoculated with a selected yeast strain for at least 12 h.

Inhibitory Effect of DMDC on Wine Bacteria. The results showed that 200 mg·L^{-1} was not sufficient for inhibiting either all of the *Acetobacter aceti* or all of the *Lactobacillus* and that 300 mg·L^{-1} killed only 70% of the *Lactobacillus* cells; however, at 500 mg·L^{-1} 50% of the *Acetobacter aceti* and 100% of the

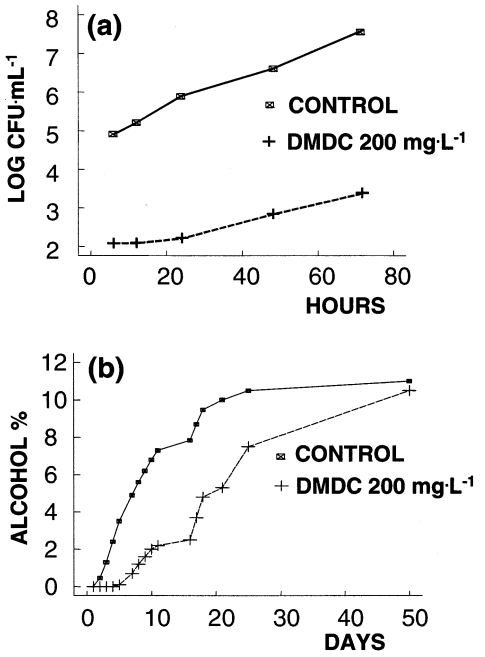

Figure 3. Recovery of indigenously contaminated grape must, supplemented with additional wine yeasts, to support alcohol fermentation: viability (a) and alcohol production (b) in the presence of DMDC.

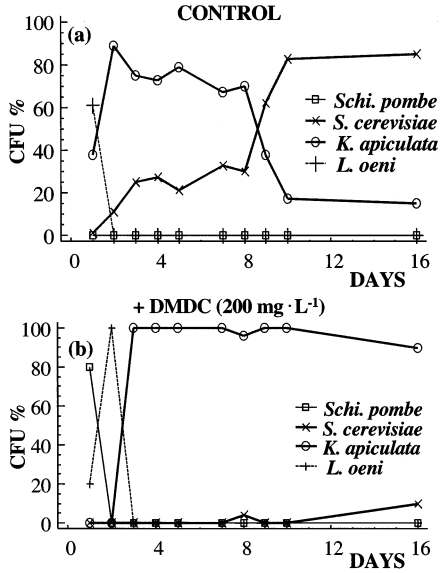

Figure 4. Survival dynamics of various wine yeasts and bacteria in nonsterile grape must with (b) and without (a) DMDC.

Lactobacillus were killed. The complete sterilization of the composite bacterial suspension was achieved with 1 g·L^{-1} DMDC.

Decontaminating Effect of DMDC on a Grape Must Inoculated with *S. cerevisiae*. The number of CFUs recorded during the first 80 h (**Figure 3a**) demonstrated that DMDC became ineffective for 10−20 h of exposure, after which time the added selected yeast initiated growth. In contrast, in the control sample the same selected yeast started to grow soon after inoculation. This response agreed with those of a previous experiment in which the inhibition by DMDC disappeared completely within 10 h. On the other hand, the alcohol production curves of the control and DMDC samples (**Figure 3b**) were quite different from each other even though they both achieved the same percentage of alcohol after 70 days. Although both behaved like a very slow stuck fermentation, it was more pronounced in the DMDC samples (**Figure 3b** shows only data for 50 days). After 50 days, however, we should consider that only the resistant cells that survived the DMDC treatment multiplied and completed the fermentation.

Effect of DMDC on the Decontamination of a Grape Must Inoculated with a Mixture of Yeasts and Bacteria. The results of the control sample (**Figure 4a**) showed that the growth behavior of each wine yeast was a consequence of their specific competition capability and can be explained by the presence of the strongly competitive facultative anaerobic yeast, *S. cerevisiae*: initially both *S. cerevisiae* and *Schi. pombe* grew, but after 8 days of active competition *S. cerevisiae* overcame *K. apiculata*. *L. oeni* was able to compete only on the first day, but after that it was completely overcome by competitors. *Schi.* showed no growth. For *C. guilliermondii* and *Pichia* sp., no additional CFUs were detected above the initial number added (10^3·mL^{-1}) for both the control and the DMDC samples (data not shown).

In the presence of DMDC (**Figure 4b**), growth of the more resistant *K. apiculata* was totally impeded during the first 24 h; however, the surviving cells were able to rapidly grow during the second day when two favorable concomitant facts occurred: a complete degradation of DMDC and a reduced number of *S. cerevisiae* and *Schi. pombe*. In contrast, *L. oeni*, which is less sensitive to DMDC than yeasts, could grow during the first day at a time when there were only a few viable yeast cells among the survivors. However, by the second day the growth of *K. apiculata* completely stopped the growth of *L. oeni*.

Although *S. cerevisiae* showed a strong sensitivity to DMDC, after only 10 days the cells that survived were able to overcome the dominant *K. apiculata* (**Figure 4b**). This dominance was completed after 50 days when only *S. cerevisiae* was detected (data not shown).

The results of this experiment suggested that (1) in a grape must, 200 mg·L^{-1} of DMDC is sufficient to effectively decrease the population of contaminating *S. cerevisiae*, *Schi. pombe*, and *K. apiculata* during the first 24 h; after 24 h, only the more aggressively competitive yeast cells will grow; and (2) a sufficiently large inoculum of a competitive selected yeast strain, when added within 24 h of the DMDC addition, could probably dominate and grow to purity and ferment more rapidly than if it were added to untreated samples. This hypothesis was previously verified in an earlier experiment.

Fermentation of Musts Treated with 200 mg·L^{-1} DMDC. The results are shown in **Figure 5**. Individual flasks were inoculated with 10^4 cells·mL^{-1} 12, 24, or 48 h after the addition of DMDC. That size inoculum was sufficient to show that fermentation in the samples treated with DMDC was virtually identical to control samples. In all cases the alcohol production curves in control and DMDC samples were very close to each other. However, the final cell count and ethanol concentration in the control samples were always greater than in the DMDC samples (**Table 3**). The viable cell counts in the DMDC samples were 35, 23, and 5% less in the 12, 24, and 48 h flasks, respectively, than in equivalent control flasks. However, ethanol production was off by only 0.1−0.2%. This unexpected disparity is difficult to explain at this time and certainly suggests further investigations to determine what modifications, if any, DMDC imposes upon the nutrient medium that could affect the yeast's ability to carry out a normal fermentation. In fact, Bizri et al. (*24*) found that the DMDC treatment of tomato juice significantly diminished the content of important growth factors such

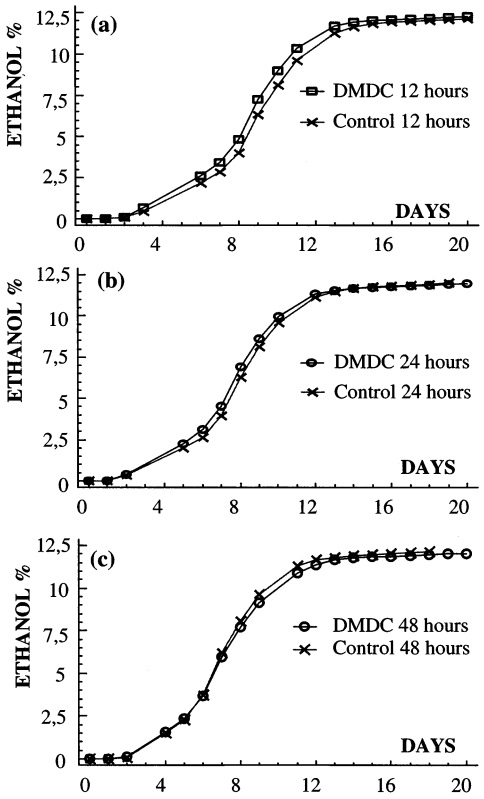

Figure 5. Restoration of fermentation ability (ethanol production) of grape must treated with (O) and without (×) DMDC after 12 (a), 24 (b), and 48 h (c) of exposure to DMDC (200 mg·L^{-1}).

as ascorbic acid, total amino acid, fructose, glucose, lycopene, and β-carotene. Conceivably, a similar effect on must could compromise a yeast's ability to carry out a fermentation efficiently.

Assessing Restoration of Fermentation Ability at a Wine Cellar Scale. Soon after the grapes were crushed and before DMDC was added, the M and MSk parts were found to contain 4.9×10^3 and 9.6×10^3 CFU·mL^{-1}, respectively, of indigenous yeasts. The daily visual inspection and microscopic examination of the control and treated samples revealed yeast growth and a fermentation delay almost proportional to the concentrations of the DMDC initially added. The addition of 25 mg·kg^{-1} of DMDC appeared to be sufficient in the M aliquots to guarantee a fermentation delay of at least 24 h as compared to the control samples. In the aliquots containing skins (MSk), a 24 h delay was obtained with 50 mg·kg^{-1} of DMDC (**Figure 6**). Thus, a low indigenous contamination of healthy grape that can occur during careful hand harvesting and 24 h of storage at 20 °C can be significantly reduced by adding small amounts of DMDC, causing a fermentation delay of several hours.

This result has technological interest because the addition of 50 mg·kg^{-1} DMDC would release into the must only 24 mg·kg^{-1} methanol, without significantly affecting the possibility of a further addition of DMDC during bottling within legal limits of the methanol content.

In samples containing DMDC, an increase in color intensity was observed as an increase in OD at 420 and 520 nm. The proportional increase in the OD 420/520 nm ratio during the first 24 h diminished progressively from the second day until it reached the initial value (data not shown). This observation suggested a possible interaction between DMDC and substances responsible for color, which probably stops when DMDC begins to degrade. The kinds of reactions and technological consequences that occur between DMDC and polyphenols are still unknown and are worthy of further investigation.

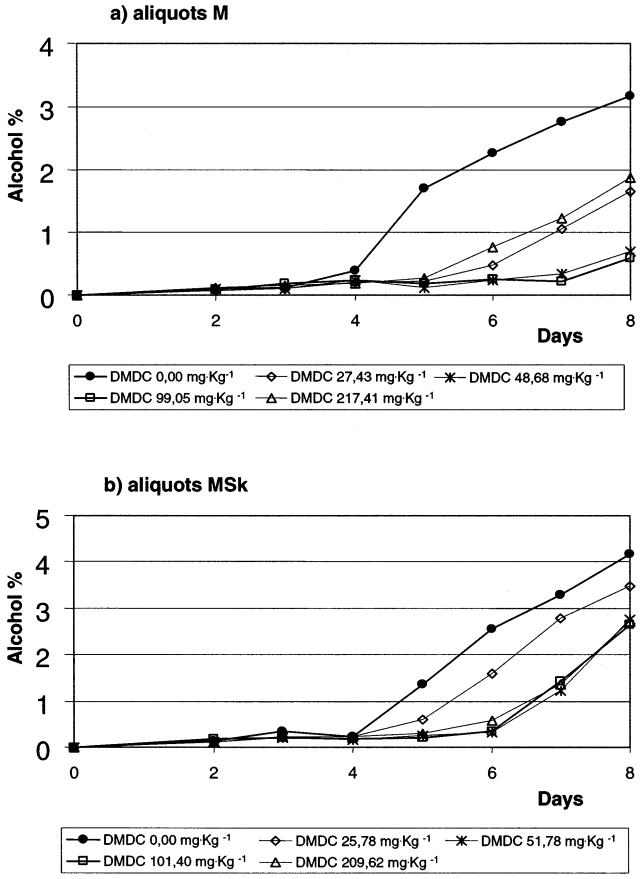

Figure 6. Restoration of fermentation ability (alcohol production) on a wine cellar scale. Freshly prepared must with (b) and without (a) skins was treated with increasing levels of DMDC (0–200 mg·kg^{-1}) and allowed to undergo a natural spontaneous fermentation.

Table 3. Ability of the Must To Alcohol Ferment after Exposure to 200 mg·mL^{-1} DMDC at 25 °C Expressed as Growth and Ethanol Production

sample	exposure (h)	final ethanol % (v/v)	final total cell no.·mL^{-1} (× 10^6)
control	12	12.12	143
+ DMDC	12	11.96	94
control	24	12.12	129
+ DMDC	24	11.96	99
control	12	12.04	103
+ DMDC	48	11.96	98

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