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Short communication

Evaluation of the inhibitory effect of dimethyl dicarbonate (DMDC) against wine microorganisms

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Abstract

Several microbial species associated with wine were challenged against increasing concentrations of dimethyl dicarbonate (DMDC). The concentration inducing complete cell death upon addition to red wine was regarded as the minimum inhibitory concentration (MIC). In dry red wines with 12% (v/v) ethanol and pH 3.50, the inactivation depended on the initial cell concentration. For an initial inoculum of 500 CFU/ml, the MIC of the yeasts species *Schizosaccharomyces pombe, Dekkera bruxellensis*, *Saccharomyces cerevisiae* and *Pichia guilliermondii* was 100 mg/l. The most sensitive strains belong to *Zygosaccharomyces bailii*, *Zygoascus hellenicus* and *Lachancea thermotolerans*, with MIC of 25 mg/l DMDC. For inoculation rates of about 10^6 CFU/ml, the maximum dose of DMDC legally authorised (200 mg/l) was not effective against the most resistant species. The addition of 100 mg/l potassium metabisulphite (PMB), equivalent to 1 mg/l molecular sulphur dioxide, increased the inactivation effect of 100 mg/l DMDC over initial yeast populations of 10^6 CFU/ml but did not fully kill *S. pombe* and *S. cerevisiae*.

Lactic acid and acetic acid bacteria were not killed by the addition of 300 mg/l of DMDC. Trials performed in wines before bottling showed that in most samples indigenous bacterial populations were not affected by 200 mg/l DMDC. Therefore, under winery practice, DMDC at the maximum dose legally permitted may be regarded as an efficient preservative to control low contamination rates of yeasts but ineffective against lactic acid and acetic acid bacteria.
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Keywords: Wine; DMDC; Spoilage yeasts; Lactic acid and acetic acid bacteria; *Dekkera/Brettanomyces bruxellensis*; Cell death

1. Introduction

The microbiological stability of wines may be achieved by the use of chemical, physical and thermal treatments (Loureiro and Malfeito-Ferreira, 2003). The most common chemical preservative is sulphur dioxide but nowadays there is a trend to reduce its levels in wines. Another preservative is dimethyl dicarbonate (DMDC), authorised in the USA up to the cumulative amount of 200 ppm (Anonymous, 2002) and in Australia up to 200 mg/kg (Anonymous, 2004). In Europe, DMDC has just been authorised with the maximum limit of 200 mg/l (Anonymous, 2006a). The effect of DMDC has been evaluated in wines against several yeast and bacterial species. Its efficiency depends on the strain, initial cell concentration, temperature, ethanol content and pH (Daudt and Ough, 1980; Ough et al., 1978, 1988; Porter and Ough, 1982; Threlfall and Morris, 2002). Appropriate usage of this product requires a dosing apparatus to ensure product homogenisation and consequent inactivation efficiency (Anonymous, 2006b). Although the efficiency of DMDC has already been studied, this work is justified by the recent authorisation of this product in Europe, where it may be added to wine with a sugar content of not less than 5 g/l only a short time prior to bottling (Anonymous, 2006a). The purpose of this work was to provide an update of the knowledge of the action of DMDC against wine microorganisms in order to advise winemakers on its practical utilisation.

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2. Material and methods

2.1. Yeast strains and maintenance conditions

The yeast and bacterial strains used were obtained from culture collections or isolated in our laboratory (Table 1). Yeast strains were maintained on GYP slants (20 g/l glucose (Merck, Darmstadt, Germany), 5 g/l yeast extract (Difco Laboratories, Detroit, USA), 10 g/l peptone (Difco) and 20 g/l agar, pH 6.0). *Dekkera bruxellensis* was kept in GYP plus 5 g/l of calcium carbonate (Merck). Lactic acid bacteria were used as advised by the supplier (CHR Hansen, Hørsholm, Denmark). Acetic acid bacteria were maintained on GY slants (50 g/l glucose (Merck), 10 g/l yeast extract (Difco), and 13 g/l agar, pH 4.5), at 4 °C.

2.2. Effect of DMDC in wines

A loopful of fresh yeast culture (24–48 h) was used to inoculate 50 ml of modified YNB broth (6.7 g/l YNB with amino-acids (Difco), 20 g/l glucose, 10% (v/v) ethanol), adjusted to pH 3.50 ± 0.01 and sterilised by filtration through membrane of 0.22 μm pore size. Incubation was performed at 25 °C with occasional hand shaking and growth was followed by measuring the absorbance at 640 nm. When OD was about 0.5 units, diluted red wine with 2% (v/v) ethanol was inoculated to give an initial population of approximately 10^4 cells/ml. A volume of 50 ml of this diluted wine was poured into 100 ml cotton plugged Erlenmeyer flasks. After cell inoculation, incubation was performed without shaking at 25 °C. Cells were counted with a counting chamber until approximately 10^6 cells/ml. This wine mixture was then used to inoculate wine with 4% (v/v) ethanol to a concentration of 10^4 cells/ml. This procedure was repeated with wines of increasing ethanol concentration, namely 6, 8, 10, and 12% (v/v). Diluted wine was obtained by mixing with a solution of 5 g/l tartaric acid to give the desired ethanol concentration.

Red wine blends were obtained by mixing several commercial red wines to which acetaldehyde was added in order to combine free sulphur dioxide. Glucose (2 g/l) was added to wines with 12% (v/v) ethanol, pH 3.50 and sterilised by filtration through 0.22 μm pore membranes. 50 ml were poured in 100 ml Erlenmeyer flasks plugged with rubber stoppers and perforated by a hypodermic needle. Wines were inoculated with adapted cells to give an initial population of approximately 10^4 cells/ml and kept at 25 °C without shaking. After 24 h of incubation, the above cultures were utilised to inoculate sterile experimental wines to give initial populations of 500 CFU/ml or 10^5–10^6 CFU/ml. Daily prepared ethanol (Merck) solutions of DMDC (Fluka) were added just after the cell inoculation and thoroughly mixed by hand to the desired concentrations (25, 50, 100, 150, 200 and 300 mg/l).

After appropriate decimal dilution with Ringer solution, viability was measured by spread plating 0.1 ml onto GYP, and grown from 3 to 5 days at 25 °C. At the end of each experiment leading to null viable countings, the remaining wine volume was analysed by membrane filtration (0.22 μm pore size), plated onto GYP, and incubated at 25 °C for 3–5 days.

Results shown are the mean of two independent experiments, and averages did not vary by more than 10%.

Table 1
Origin of analysed strains

Species	Strains ISA[a]	Source[a]
Dekkera bruxellensis	ISA 1791	Red wine
Lachancea thermotolerans	72	Fermenting grape juice, UM 72
Pichia guilliermondii	ISA 2105	Grapes (4-ethylphenol producer)
	ISA 2126	Press roll (4-ethylphenol producer)
	ISA 2131	Red wine (4-ethylphenol producer)
Saccharomyces cerevisiae	ISA 1000	Commercial starter (Fermivin®)
	ISA 1026	Sediments in white wine
Saccharomycodes ludwigii	ISA 1083	Sediments in sweet white wine
Schizosaccharomyces pombe	ISA 1190	CECT 1375
Zygosaccharomyces bailii	ISA 1307	Valve in sparkling wine production line
	ISA 2270	Sour rotten grapes
Zygoascus hellenicus	ISA 2284	Sour rotten grapes
Oenococcus oeni	–	Commercial starter (Viniflora oenos®)
Acetic acid bacterium	–	Catalase positive, gram— rods isolated from red wine

[a]ISA (Instituto Superior de Agronomia, Lisbon, Portugal), CECT (Colección Española de Cultivos Tipo, Madrid, Spain), UM (Minho University, Braga, Portugal).

2.3. Preparation of bacterial inocula

Lactic acid bacteria (Viniflora Oenus®) were prepared according to supplier's instructions by spreading the bacterial starter on the surface of 50 ml wine in 100 ml Erlenmeyer flasks and gently shaken. Red wine blends consisted of 4 g/l malic acid (Merck), 12% (v/v) ethanol, and pH 3.50. Incubation was carried out for 24 h at 28 °C without shaking, after which time experimental wines were inoculated to give an initial bacterial population of approximately 500 or 10^6 CFU/ml.

A loopful of fresh acetic acid bacteria was inoculated in 50 ml of 60 g/l WLN (Difco) in 100 ml Erlenmeyer flasks, and kept at 28 °C for 72 h without shaking. Experimental wines were prepared as described before for yeast experiments and inoculated to give an initial count of about 500 or 10^6 CFU/ml.

Wines inoculated with bacterial cultures were incubated at 28 °C without shaking. Viability of bacterial populations

after addition of increasing concentrations of DMDC was assessed as described for yeast experiments.

2.4. Effect of DMDC and potassium metabisulphite (PMB)

In order to evaluate the effect of DMDC plus PMB, we selected four strains of the most resistant yeast species. Yeast strains were adapted to wine and inoculated in red wine blend (12% (v/v) ethanol, pH 3.50, 2 g/l glucose) prepared as described before. After inoculation, 100 mg/l DMDC was added and immediately after PMB (Merck) solution to the desired concentration (25, 50 and 100 mg/l). Titration of free sulphur dioxide by the iodine method showed 15 mg/l free sulphur dioxide before PMB addition. PMB additions resulted in 20, 30 and 51 mg/l of initial free sulphur dioxide, which correspond to 0.4, 0.6 and 1 mg/l of molecular sulphur dioxide, respectively (at pH 3.50, the percentage of the molecular form is 2%, according to Ribéreau-Gayon et al., 2006). Viability measurements were done as described before. When viable cells were not detected after 144 h of incubation, 20 ml of wine were membrane filtered, plated onto GYP and colonies counted after 3 days at 25 °C. Other 20 ml of wine were diluted with 80 ml GYP broth in 200 ml cotton plugged Erlenmeyer flasks, incubated at 25 °C for 72 h to allow the recovery of damaged cells. These cells were evaluated by plating onto GYP.

2.5. Effect of DMDC at the winery

Trials were conducted at the winery on commercial red wines before bottling. Red wine stored in 15.400 l stainless-steel vessels as well as red wine stored in new French oak barrels (225 l) were tested. Samples (100 ml) were taken from the vessels or from the barrels using sterile pipettes and transferred to Schott flasks. In order to measure the effects of DMDC, this product was added at a concentration of 200 mg/l to wine from tanks and 25 mg/l to wine from barrels. Samples with and without added DMDC were incubated, membrane filtered and colonies counted as described before.

3. Results

3.1. Evaluation of DMDC tolerance

The first inactivation trials were conducted in microplates (200 μl) and test tubes (2 ml) but were discarded because full cell inactivation was achieved with 25 mg/l of DMDC for all strains (results not shown). This indicated that low test volumes were not adequate to measure the killing effect of DMDC.

The viability variation of yeast populations in red wine after DMDC addition is shown in Fig. 1 for adapted cells of *D. bruxellensis* ISA 1791 under high inoculation rates and for two different test volumes (50 ml and 1 l). For non-lethal doses of DMDC, the viability decreased during the first 2–3 h and maintained constant afterwards. In 1 l of wine the killing effect was lower demonstrating the importance of test volume standardisation to obtain reproducible results. The lower killing effect was also observed for *Schizosaccharomyces pombe* in 1 l flasks (results not shown). We decided to use 50 ml of wine because of laboratorial constraints when testing a high number of strains.

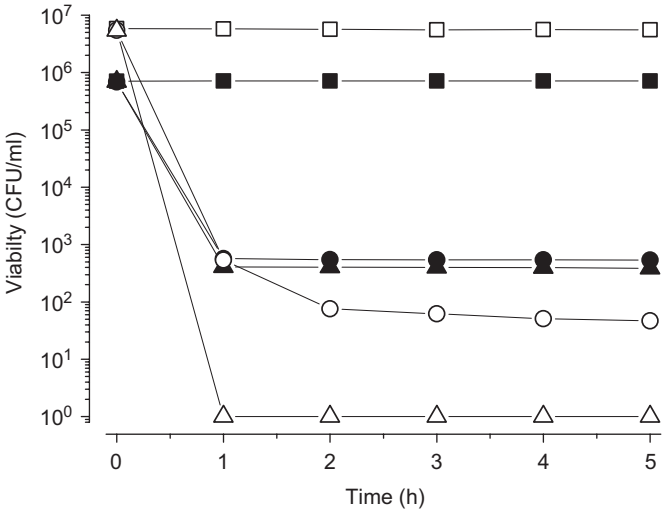

Fig. 1. Effect of DMDC on the viability of *Dekkera bruxellensis* ISA 1791 inoculated in 50 ml (open symbols) and 1 l (filled symbols) of red wine at pH 3.50 and 12% (v/v) ethanol. DMDC concentrations: 0 (■, □), 200 (●, ○) and 300 mg/l (▲, △). The value 10^0 for viability indicates the absence of colonies on the volume of 0.1 ml of wine sample.

The behaviour pattern of the other yeasts strains tested during 5 h of incubation was similar to that displayed in Fig. 1 (results not shown). When DMDC doses induced total viability loss after 5 h, death was checked after 72, 144 and 216 h of incubation. This procedure enabled the determination of minimum inhibitory concentrations (MIC) necessary to achieve absence of viable counts. In Table 2 are shown the MIC values as a function of initial inoculation rate. For all strains the inactivation effect was dependent on the inoculum size, being the lowest MIC's obtained for inocula of 500 CFU/ml. At high inoculation rates, *S. pombe* was the most resistant species, showing viability with 300 mg/l of DMDC (results not shown). Under low inoculation rates, *S. pombe*, *D. bruxellensis*, *Saccharomyces cerevisiae* and *Pichia guilliermondii* were found to be the most resistant yeast strains, maintaining viability with 50 mg/l of DMDC but not 100 mg/l. The most sensitive strains were *Zygosaccharomyces bailii*, *Zygoascus hellenicus* and *Lachancea thermotolerans*, with MIC of 25 mg/l for inocula of 500 CFU/ml.

The viability of lactic acid and acetic acid bacteria was not affected during the extent of the experiments for all tested concentrations of DMDC (0–300 mg/l) and therefore the MIC value was considered to be higher than 300 mg/l.

3.2. Combined effect of DMDC and PMB

The simultaneous effect of DMDC and PMB was determined using the four most resistant species. The viability results during incubation time are shown in Table 3. The addition of 100 mg/l of DMDC alone did not provoke viability loss during 144 h of incubation, as expected from the results presented in Table 2. However, the addition of PMB induced different responses according to the strain. After 144 h of incubation, no viable cells were detected for *D. bruxellensis* and *S. cerevisiae* under 25 mg/l PMB and for *P. guilliermondii* under 50 mg/l PMB. The most resistant species was *S. pombe*, tolerating 100 mg/l DMDC and 100 mg/l PMB.

The absence of cell viability in the 0.1 ml of wine does not exclude the presence of viable cells in higher wine volumes. Therefore we analysed 20 ml wine by plating and another 20 ml by incubation in resuscitation GYP broth. No viable cells were detected for *D. bruxellensis* and *P. guilliermondii* after membrane plating or inoculation in resuscitation broth. On the other hand, although *S. cerevisiae* did not show viability after membrane plating (non-culturable state), viable cells were recovered after broth culture. This indicates the presence of the so-called viable but non-culturable (VBNC) populations. Thus, for this latter species, the addition of 100 mg/l DMDC and 100 mg/l of PMB was not fully inhibitory.

Table 2
Minimum inhibitory concentrations (MIC) of DMDC against wine associated microorganisms inoculated in red wine with 12% (v/v) and pH 3.50, at two different rates

Strains	DMDC (mg/l)	
	500 CFU/ml	10^6 CFU/ml
S. pombe ISA 1190	100	>300
D. bruxellensis ISA 1791	100	300
S. cerevisiae ISA 1000	100	300
S. cerevisiae ISA 1026	100	300
P. guilliermondii ISA 2105	100	300
P. guilliermondii ISA 2126	100	300
P. guilliermondii ISA 2131	100	200
Z. bailii ISA 1307	25	200
Z. hellenicus ISA 2284	25	200
S. ludwigii ISA 1083	25	200
Z. bailii ISA 2270	25	150
L. thermotolerans 72	25	100
Oenococcus oeni	>300	>300
Acetic acid bacterium	>300	>300

3.3. Effect of DMDC on indigenous microbial populations

The effect of DMDC was tested under conditions adapted from winery practice. Table 4 shows the lethal effect of the maximum legal amount of DMDC (200 mg/l) on the indigenous microbial populations of red wine prepared to be bottled. In all samples, yeast counts were reduced to acceptable levels, mostly zero in 100 ml of wine. On the contrary, bacterial counts were only reduced to zero

Table 3
Effect of 100 mg/l DMDC and potassium metabissulphite (PMB) on the viability (CFU/ml) of several yeast strains inoculated in red wine with 12% (v/v) ethanol and pH 3.50

Strains	Time (h)	PMB (mg/l)			
		0	25	50	100
D. bruxellensis ISA 1791	5	$9.7(\pm0.2)\times10^5$	$6.5(\pm0.2)\times10^1$	$1.8(\pm0.1)\times10^1$	<10
	72	$9.6(\pm0.1)\times10^5$	<10	<10	<10
	144	$9.5(\pm0.1)\times10^5$	<10	<10	<10
	216[a]	Nd[b]	0	0	0
	216[c]	Nd	Negative	Negative	Negative
P. guilliermondii ISA 2126	5	$2.6(\pm0.1)\times10^5$	$2.0(\pm0.1)\times10^2$	$6.1(\pm0.1)\times10^1$	<10
	72	$2.5(\pm0.1)\times10^5$	$1.2(\pm0.1)\times10^5$	<10	<10
	144	$2.6(\pm0.2)\times10^5$	$1.6(\pm0.1)\times10^5$	<10	<10
	216[a]	Nd	Nd	0	0
	216[c]	Nd	Nd	Negative	Negative
S. cerevisiae ISA 1000	5	$9.6(\pm0.2)\times10^5$	$5.9(\pm0.1)\times10^2$	$5.6(\pm0.2)\times10^2$	$1.8(\pm0.2)\times10^2$
	72	$9.4(\pm0.1)\times10^5$	$4.0(\pm0.1)\times10^2$	$2.2(\pm0.1)\times10^2$	<10
	144	$9.5(\pm0.1)\times10^5$	<10	<10	<10
	216[a]	Nd	0	0	0
	216[c]	Nd	Positive	Positive	Positive
S. pombe ISA 1190	5	$6.7(\pm0.2)\times10^5$	$2.0(\pm0.1)\times10^2$	$2.6(\pm0.2)\times10^3$	$2.7(\pm0.1)\times10^3$
	72	$6.8(\pm0.1)\times10^5$	$2.0(\pm0.2)\times10^2$	$3.1(\pm0.2)\times10^2$	$1.8(\pm0.1)\times10^2$
	144	$6.8(\pm0.2)\times10^5$	$6.3(\pm0.2)\times10^3$	$4.0(\pm0.1)\times10^3$	$9.8(\pm0.1)\times10^2$

[a]Number of CFU after membrane filtration of 20 ml of wine sample.
[b]Not determined.
[c]Negative or positive growth in 80 ml GYP broth added of 20 ml of wine sample.

Table 4
Range of microbial counts (CFU/100 ml) before and after the addition of DMDC to finished red wine[a] prior to bottling

Number of samples	DMDC (mg/l)					
	0			200		
	Yeasts	Bacteria	Moulds	Yeasts	Bacteria	Moulds
18	1–308	0	0	0	0	0
4	0–4	9-TNTC[b]	1	0	0	0
14	0–534	17-TNTC	0-TNTC	0–3	2-TNTC	0-TNTC

[a]Wine analysis range: free sulphite 26–58 mg/l; pH 3.42–3.63; ethanol 12.7–14.6% (v/v).
[b]Too numerous to count.

in four out of 18 contaminated samples. In 14 samples, bacterial populations were not affected by 200 mg/l DMDC. These results indicate that DMDC is not effective against bacteria.

To mimic cumulative additions of DMDC during storage in the winery, a separate trial was done in red wine matured in oak barrels. In five different samples initial yeast counts ranged from 16 to 146 CFU/100 ml. Utilisation of 25 mg/l of DMDC decreased counts to zero in four samples and to 1 CFU/ml in one sample, showing its effectiveness to keep wines with very low levels of yeast contamination.

4. Discussion

The first results on the effectiveness of DMDC against wine yeasts used *S. cerevisiae* at low inoculation rates (less than 100 CFU/ml) and showed that less than 60 mg/l of DMDC were effective in avoiding visible growth in wine (Ough, 1975; Ough et al., 1978). Further work showed that variable initial populations (17–10^4 CFU/ml) of several strains belonging to *S. cerevisiae*, *S. pombe*, *Brettanomyces* spp., *D. bruxellensis* and *Rhodotorula rubra* were killed by 50–100 mg/l of DMDC after 48 h, in 50 ml wine with 10% (v/v) ethanol and 20 g/l sugar (Daudt and Ough, 1980). Porter and Ough (1982) showed that *S. cerevisiae* was effectively killed by 100 mg/l DMDC within 10 min in 10% (v/v) wine with 20 g/l sugar. The use of 25 mg/l free sulphur dioxide and 50 mg/l of DMDC were enough to control *S. cerevisiae* (inocula of 330–460 CFU/ml) and avoid malolactic fermentation by *Oenococcus oeni* starters (Ough et al., 1988). Doses of 100 mg/l DMDC fully killed initial unadapted populations of 580–758 CFU/ml *S. cerevisiae* var *bayanus* or prevented visible fermentations in wine with 11% (v/v) and 12 g/l sugar (Threlfall and Morris, 2002). These authors also showed that when 10 mg/l sulphur dioxide was added, 50 mg/l DMDC was enough to ensure wine stability. More recent publications have addressed grape juice treatments rather than wine (Delfini et al., 2002; Divol et al., 2005; Terrell et al., 1993). Thus, our results provided an update and a clarification of several aspects related with the killing effect of DMDC in wines, following its recent approval in the EU.

Using several contamination yeast species adapted to grow in wine with 12% (v/v) ethanol and pH 3.50 at 25 °C, we showed that the maximum legal dose (200 mg/l) of DMDC is an effective preservative when contamination loads are lower than 500 CFU/ml. The inactivation effect of 200 mg/l DMDC was also observed against indigenous yeast populations in commercial wine previous to bottling. For high microbial loads (10^6 CFU/ml) the effectiveness of DMDC depended on PMB addition. *S. pombe* and *S. cerevisiae* were particularly resistant, surviving under 100 mg/l DMDC and 1 mg/l molecular sulphur dioxide. This latter species seems survive due to the induction of a VBNC state, as has also been observed by Divol et al. (2005) in fermentations of botrytised juices.

Due to its rapid hydrolysis, the effect of DMDC is instantaneous and product suppliers advise the use of a dosing apparatus to ensure adequate homogenisation (Anonymous, 2006b). Our results showing that DMDC was much more effective in small test volumes (up to 2 ml) rather than larger ones (50 ml and 1 l) may be explained by different volume dependent homogenisation. Perhaps the products of DMDC hydrolysis (Stafford and Ough, 1976) or the products of its reaction with alcohols and ammonia (Peterson and Ough, 1979) have a prolonged inhibitory effect. However, in winery practice, the prolonged preservation effect is mainly obtained by the presence of molecular sulphur dioxide. DMDC could therefore be used in regular additions during storage to decrease sulphur dioxide utilisation. Although this is common practice in USA, this procedure is not actually permitted by EU regulations.

Several reports showed that lactic acid bacteria in wines (Ough et al., 1988) or in juices (Delfini et al., 2002; Winniczuck and Parish, 1997) were more resistant to DMDC than yeasts. In this work we clearly establish that, in wines, DMDC under the authorised concentration limits is not an effective preservative against lactic acid and acetic acid bacteria. This lack of efficiency was observed for pure cultures as well as for indigenous populations before bottling. In conclusion, winemakers should regard DMDC as another hurdle for the prevention of yeast spoilage, being advisable to companies with the technological ability to maintain low microbial contamination levels in bottled wines.

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