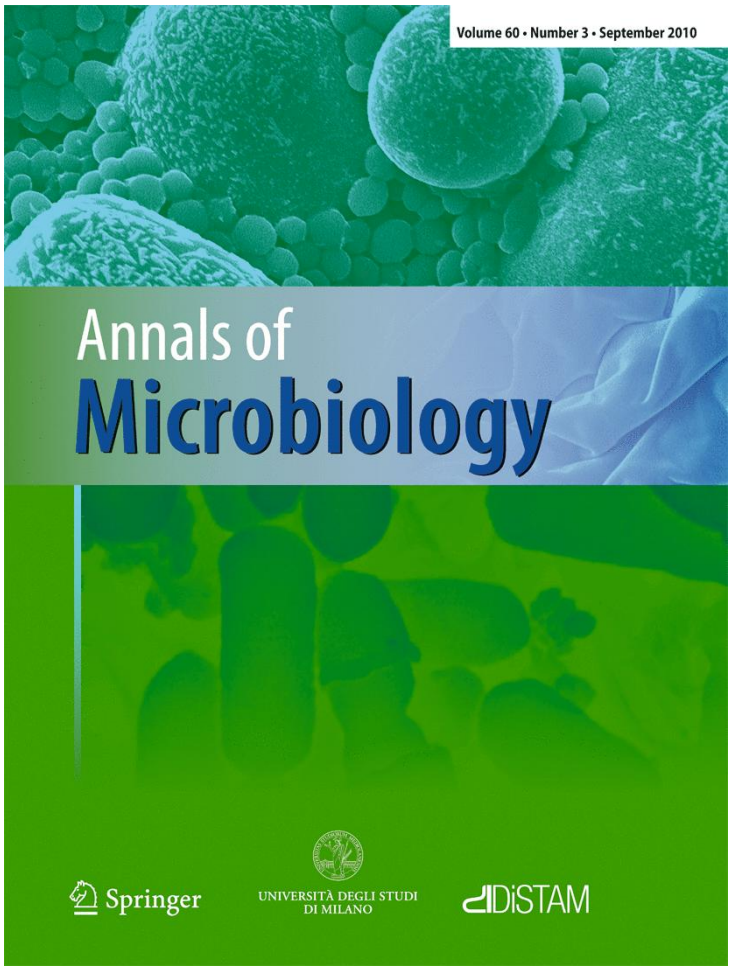
ISSN 1590-4261, Volume 60, Number 3



Ann Microbiol (2010) 60:549–556
DOI 10.1007/s13213-010-0085-5

ORIGINAL ARTICLE

Susceptibility of wine spoilage yeasts and bacteria in the planktonic state and in biofilms to disinfectants

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Received: 5 February 2010 / Accepted: 10 June 2010 / Published online: 4 July 2010

Abstract The aim of this work was to determine the ability of six yeast and two bacterial species associated with wine spoilage to form biofilms in mono- or co-culture using the Calgary Biofilm Device (CBD). Moreover, the efficacy of several disinfectants was evaluated against these spoilage microorganisms, both in the planktonic and the biofilm states. Results showed that *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe* and *Acetobacter aceti* formed biofilms both in wine and in synthetic medium. *Zygosaccharomyces bailii* formed biofilm only in wine and *Pichia guilliermondii* and *Lactobacillus hilgardii* formed biofilms only in synthetic medium. In wine, *D. bruxellensis* presented the same biofilm population when grown in pure culture or in mixed culture with acetic acid bacteria. There was a 3-log increase in biofilm formed by *A. aceti* in mixed culture with *L. hilgardii*. Alkaline chlorine-based disinfectant was the most effective in decontaminating spoilage yeast and bacteria both in planktonic and biofilm tests. Sodium hydroxide-based detergents and peracetic-based disinfectant were also efficient against suspended cells, but at least 10-fold more concentrated solutions were needed to remove the biofilms. Furthermore, the results showed that, except for the neutral detergent VK10, the tested agents were actually effective when used under the conditions recommended by manufacturers. In any case, biofilms showed greater tolerance to biocides when compared to the same microorganisms in the planktonic state. To our knowledge, this is the first study in which the CBD is used to assess the ability of wine spoilage microorganisms to form biofilms and their susceptibilities to disinfectant agents.

Keywords Biofilms · Disinfectants · Spoilage yeasts · Acetic acid bacteria · Lactic acid bacteria

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Introduction

Microbial spoilage of wine can occur at multiple stages of the winemaking process. Spoilage microorganisms mainly include lactic acid and acetic acid bacteria and yeasts of the genera *Dekkera/Brettanomyces, Candida, Hanseniaspora, Pichia, Metschnikowia, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces* (Enrique et al. 2007). The common spoilage effects are film formation in stored wines, cloudiness or haziness, sediments and gas production in bottled wines, and off-odours and off-tastes. *S. cerevisiae*, the dominant yeast during fermentation, is regarded as a spoilage organism when associated with re-fermentation of bottled wines (du Toit and Pretorius 2000; Loureiro and Malfeito-Ferreira 2003).

The maintenance of high hygienic standards during the various steps of wine production is essential to prevent the contamination of wine. The selection of detergents and disinfectants in the food industry is dependent upon several factors, such as the efficacy in removing a wide range of microorganisms, the safety issues of handling, the rinsability of the agent, its corrosiveness for the common surfaces and its effect on sensory values of the products manufactured (Salo

and Wirtanen 2005). The key to effective cleaning and disinfection of food plants is the understanding of the type and nature of the soiling agent (e.g. sugar, fat, protein, mineral salts) and of the microbial growth to be removed from the surfaces (Czechowski and Banner 1992).

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton 2002). Studies have shown that biofilms may have greater than a 100-fold increase in tolerance to biocides and antibiotics when compared to the same microorganisms in a planktonic (free-floating) state (Ceri et al. 1999). Once formed, biofilms are very difficult to remove and control, and can represent a significant source of contamination in the food and beverage industries (Kumar and Anand 1998).

Over the last decades, a broad range of model systems have been described for the in vitro study of biofilm formation and development (Peeters et al. 2008). The Calgary Biofilm Device (CBD) is a system that allows, within a single experiment, the production of 96 biofilms and the evaluatation of the susceptibility to several biocide agents or concentrations (Ceri et al. 1999). To date, we are not aware of studies using this system with microbial species related with wine.

The aim of this work was to test the ability of major wine spoilage microorganims to form biofilms in mono- and co-culture using the CBD and to assess the efficacy of various types of commonly used disinfectants against these spoilage microorganisms both in the planktonic state and in biofilms.

Materials and methods

Microbial strains and culture mediums

Six yeast and two bacterial strains obtained from culture collections or isolated in our laboratory were used in the present study (Table 1). Yeast strains were maintained on GYP slants [20 g/L glucose (Copam, Loures, Portugal), 5 g/L yeast extract (Oxoid, Hampshire, UK), 10 g/L peptone (Biokar Diagnostics, Beauvais, France) and 20 g/L agar, pH 6.0]. *Dekkera bruxellensis* was kept in GYP plus 5 g/L of calcium carbonate (Merck, Darmstadt, Germany). Lactic acid bacteria were maintained on MRS slants (Biokar). Acetic acid bacteria were maintained on GY slants (50 g/L glucose, 10 g/L yeast extract, and 13 g/L agar, pH 4.5, at 4°C).

Experimental red wine was obtained by a blend of commercial red wines without residual sugar (ethanol 12%, volatile acidity 0.29 g/L, total sulphur dioxide 27 mg/L, free sulphur dioxide 6 mg/L, pH 3.54). The wine ethanol content was then adjusted to 10% (v/v) with a solution of 5 g/L of tartaric acid (Merck), sterilized by filtration through cellulose acetate membranes (0.22 μm pore size, 47 mm diameter; Millipore) and used as growing medium.

Disinfectants and cleaning agents

Six commercial disinfectants and cleaning agents commonly used in the food industry were tested against free-floating cells and biofilms. The antimicrobial compounds and the in-use concentrations as suggested by the manufacturers are listed in Table 2. The agents were diluted to various concentrations within the in-use range.

Test with planktonic cells

The minimum inhibitory concentrations (MIC) and the minimum lethal concentrations (MLC) of the disinfectants and cleaning agents were determined using a microdilution method. The range of tested concentrations are presented in Table 3. These concentrations were established on the basis of preliminary tests carried out in order to allow to intercept the MIC and MLC of each product against all micro-organisms studied. Twofold dilutions of each agent were performed, in nutrient broth in 96-well microtitre plates (Nunc, Copenhagen, Denmark) in volumes of 100 μL per well. The inocula were prepared from fresh broth cultures in GYP, GY or MRS, according to the microorganism, to a concentration of 1×10^6 CFU/mL. The concentration of each inoculum was confirmed by plate counts in GYP, GY

Table 1 Origin of the strains used

Strain[a]	Species	Source
ISA 1000	*Saccharomyces cerevisiae*	Commercial starter (Fermivin®)[b]
ISA 1083	*Saccharomycodes ludwigii*	Sediments in sweet white wine
ISA 1190	*Schizosaccharomyces pombe*	CECT[c] 1375
ISA 1791	*Dekkera bruxellensis*	Red wine
ISA 2131	*Pichia guilliermondii*	Red wine (4-ethylphenol producer)
ISA 2270	*Zygosaccharomyces bailii*	Sour rotten grapes
ISA 3962	*Lactobacillus hilgardii*	CECT 4786
ISA 4201	*Acetobacter aceti*	Red wine

[a] ISA (Instituto Superior de Agronomia, Lisbon, Portugal)
[b] Fermivin® (DSM, Delft, Netherlands)
[c] CECT (Colección Española de Cultivos Tipo, Valencia, España)

Table 2 Cleaning agents and disinfectants tested

Product	Manufacturer	Description	Active ingredient	In-use recommended concentration (%)
VK10 Shureclean	JohnsonDiversey	Neutral foaming liquid detergent	Sodium alkylbenzenesulphonate	0.5–5
VV3 Spraygrap	JohnsonDiversey	Alkaline detergent (powder)	Sodium hydroxide	1–10
VV9 Omegrap	JohnsonDiversey	Alkaline liquid detergent	Sodium hydroxide	0.75–20
TOPAX 99	Henkel-Ecolab	Mild alkaline to neutral disinfectant	Alkylamine acetate	0.5–3
TOPAX 66	Henkel-Ecolab	Alkaline disinfectant	Sodium hydroxide, sodium hypochlorite	2–5
VT5 Divosan activ	JohnsonDiversey	Oxidising disinfectant	Peracetic acid	0.09–3.6

or MRS. After inoculation of 100 μL per well, the microtitre plates were incubated aerobically for 48 h at 28°C (for bacteria) or 25°C (for yeast). The MIC was defined as the lowest concentration of the disinfectant agent that prevents visible growth, by production of turbidity or pellet, after an incubation period of 48 h.

The procedure for MLC evaluation was similar to MIC evaluation, except that the inocula as well as the disinfectants and cleaning agents dilutions were prepared in sterile water. In order to prepare the inocula, fresh broth cultures were collected and washed with sterile water by centrifugation for 5 min at 4,000*g* (Eppendorf centrifuge 5810R, Hamburg, Germany). Thirty minutes after inoculation in the microtitre plates, the total volume was removed from each well and transferred to tubes containing 20 mL of GYP, GY or MRS broth, according to the microorganism. The tubes were then aerobically incubated for 48 h at 28°C (for bacteria) or 25°C (for yeast). The MLC was defined as the lowest concentration of the agent that prevents visible growth, by production of turbidity or pellet, after a 1/100 dilution of the agent.

Biofilm test

Biofilm assays were performed in monoculture in GYP (for yeasts), GY (for acetic acid bacteria), MRS (for lactic acid bacteria) broth or red wine (for all microorganisms). Mixed-culture biofilms in a proportion of 50/50 of *D. bruxellensis*/*A. aceti* and *A. aceti*/*L. hilgardii* were also assayed in red wine.

In order to develop biofilms on the pegs of the Calgary Biofilm Device (MBEC™ P & G assay; Innovotech, Calgary, Canada), 150 μL of a cell suspension of 10^7 CFU/mL (in GYP, GY, MRS or wine) was pipetted into each well. The CBD assembly was placed on a Titertek Model DSG orbital microplate shaker (Flow Laboratories, Meckenheim, Germany) at speed 8 at room temperature for 180 min for the initial adhesion phase and then incubated on a orbital shaker (Agitorb 200IC; Aralab, Lisbon, Portugal) at 200*g*, for 48 h at 28°C (for bacteria) or 25°C (for yeast) in a humidified environment.

The CBD lid containing the biofilms on the pegs was then rinsed in microtitre plates with 200 μL Ringer's solution in each well for 90 s in order to remove the loosely adhering planktonic cells. The CBD lid was then transferred to fresh medium in a new 96-well microtiter plate and it was incubated for another 48 h in the same conditions. After incubation, the pegs were rinsed as previously described and the culture medium was discarded. For each tested strain, two pegs were broken off from the lid of the CBD using sterile pliers and immersed in 200 μL of Ringer's solution in a new 96-well plate. The microtitre plate containing the cut pegs was transferred onto the tray of the sonicator (P-Selecta, Barcelona, Spain) where it was sonicated for 10 min. The vibrations created by the sonicator disrupted the biofilms from the surface of the pegs into the saline solution. The biofilm population was enumerated by plating decimal dilutions of the disrupted biofilm on GYP, GY or MRS agar according to the microorganism. The enumeration of the co-culture biofilm of *D. bruxellensis* and *A. aceti* was performed by plating on GYP agar supplemented with 100 mg/L chloramphenicol (Sigma, Steinheim, Germany) (selective for *D. bruxellensis*) and on GY agar supplemented with 200 mg/L Delvocid® (DSM, Delft, Netherlands) (100 mg/L natamycin) selective for *A. aceti*. The co-culture biofilm of *A. aceti* and *L. hilgardii* was enumerated on GY supplemented with 3,000 U/L penicillin (Sigma) selective for *A. aceti* and MRS supplemented with 0,5 g/L L-cysteine (Sigma) selective for *L. hilgardii*.

The lid of the CBD with the remaining pegs was then immersed for 15 min in a microtiter plate containing a twofold gradient of the disinfectant agents or detergent to be tested (Table 3) in a final volume of 200 μL. After contact time, the pegs were rinsed twice as previously described. The lid of the CBD was then transferred into the "recovery" plate filled with 200 μL of recovery medium, for instance GYP, GY or MRS, in each well. The recovery plate was sonicated as previously described. The lid of the CBD was discarded and replaced with the original lid of the microtiter plate. The recovery plate was incubated for 48 h at 28°C (for bacteria) or 25°C (for yeast).

Table 3 Maximum and minimum concentration of cleaning agents and disinfectants tested expressed in %

	VK10			VV3			VV9			TOPAX 99			TOPAX 66			VT5		
	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC
Yeast	5–0.08	5–0.08	20-0.31	1.25–0.02	1.25–0.02	5-0.08	5–0.08	5–0.08	5-0.08	1.25-0.02	1.25-0.02	5-0.08	1.25–0.02	1.25–0.02	1.25-0.02	1.25-0.02	1.25-0.02	1.25-0.02
Bacteria	5-0.08	5-0.08	nd	1.25-0.02	1.25-0.02	5-0.08	0.63-0.01	0.63-0.01	nd	1.25-0.02	1.25-0.02	nd	0.63-0.01	0.63-0.01	1.25-0.02	0.63-0.01	0.63-0.01	1.25-0.02

MIC Minimum inhibitory concentrations, *MLC* minimum lethal concentrations, *MBEC* minimal biofilm eradication concentration, *nd* not determined

On the recovery plate, wells that corresponded to the pegs where the biofilm had not been exposed to disinfectant/detergent agents were used as positive growth control while the wells that corresponded to cut-off pegs or non inoculated wells on the CBD were used as negative growth control.

The minimal biofilm eradication concentration (MBEC) was defined as the lowest concentration of the disinfectant agent required to eradicate the biofilm of a selected isolate after a recovery period of 48 h.

Interpretation of the results

Each experiment was performed in three replicates for each strain and agent at each concentration.

When interpreting MIC, MLC or MBEC data, the result was considered positive when at least two out of three repetitions presented visible growth. If only in one of the three repetitions was detected visible growth, the result was regarded as negative.

Results

Determination of biofilm formation

Dekkera bruxellensis, *S. cerevisiae*, *S. ludwigii*, *S. pombe* and *A. aceti* formed biofilms in monoculture, both in wine and in synthetic medium, while *Z. bailii* formed a biofilm only in wine. *Pichia guilliermondii* and *L. hilgardii* formed biofilms only in synthetic medium (Fig. 1).

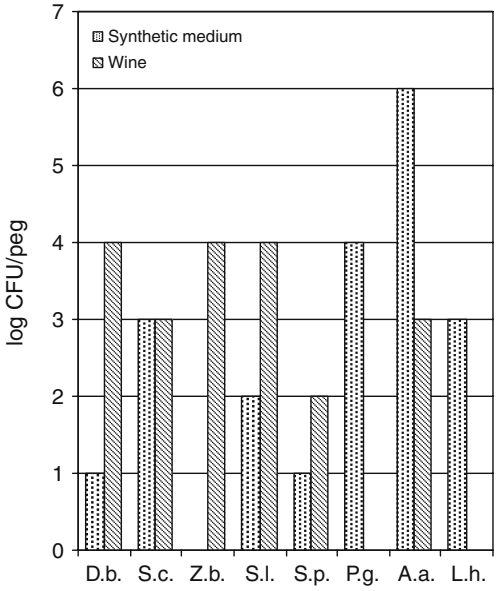

Fig. 1 Biofilm formation in monoculture, in synthetic medium and wine. *D.b. D. bruxellensis*, *S.c. S. cerevisiae*, *Z.b. Z. bailii*, *S.l. S. ludwigii*, *S.p. S. pombe*, *P.g. P. guilliermondii*, *A.a. A. aceti*, *L.h. L. hilgardii*

The mixed culture of *D. bruxellensis* with the acetic acid bacteria did not show any effect on the amount of the formed biofilm. Instead, there was a 3-log increase in biofilm formed by *A. aceti* in mixed culture with *L. hilgardii* (Fig. 2).

Determination of MIC, MLC and MBEC

The MIC, MLC and MBEC for all disinfectants and cleaning agents are reported in Table 4. The neutral foaming liquid detergent *VK10* was not effective in removing biofilms. In fact, the MBEC of this agent against biofilms of *D. bruxellensis*, *S. cerevisiae* and *Z. bailii* was twice the maximum in-use recommended concentration. Moreover, it is interesting to note that, for this detergent, the MIC was higher than the MLC, except in the case of *L. hilgardii*, *P. guilliermondii* and *A. aceti*. The value of the highest MIC, recorded against *D. bruxellensis* and *P. guilliermondii* was equal to the maximum recommended in-use concentration (5%). Furthermore, the strain *P. guilliermondii* ISA 2131 showed the highest value of MLC (5%) for this agent.

The alkaline powder detergent VV3 was effective in removing biofilms of *D. bruxellensis*, *S. cerevisiae*, *Z. bailii* and *A. aceti*, with the highest value of MBEC of 5% for the biofilm of *D. bruxellensis*, yet equal to half the maximum in-use concentration recommended. For this agent, the MIC was equal or lower than the MLC against the yeast strains. Instead, against bacteria, the MLC was lower than the MIC.

The alkaline liquid detergent VV9 was effective in removing biofilms of *D. bruxellensis*, *S. cerevisiae* and *Z. bailii*, with a highest value of MBEC of 5%, four times lower than the maximum recommended concentration. For this agent, the MIC was equal to the MLC against all

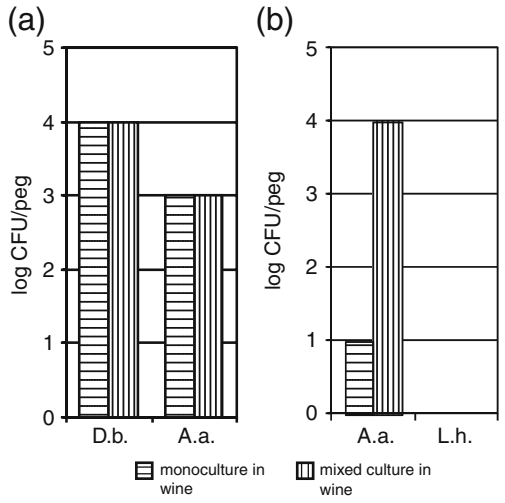

Fig. 2 Biofilm formation in mixed culture, in wine. **a** *D. bruxellensis* ISA 1791 + *A. aceti* ISA 4201; **b** *L. hilgardii* ISA 3962 + *A. aceti* ISA 4201

Table 4 Minimum inhibitory concentrations (*MIC*), minimum lethal concentrations (*MLC*) and minimal biofilm eradication concentration (*MBEC*) expressed in %

Species [a]	VK10			VV3			VV9			TOPAX 99			TOPAX 66			VT5		
	(0.5–5%)[b]			(1–10%)			(0.75–20%)			(0.5–3%)			(2–5%)			(0.09–3.6%)		
	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC	MIC	MLC	MBEC
D.b.	5.00	0.31	10.00	0.04	0.08	5.00	0.16	0.16	5.00	0.04	0.04	0.16	0.08	0.08	0.08	0.08	0.08	1.25
S.c.	0.63	0.31	10.00	0.16	0.31	2.50	0.16	0.16	5.00	0.16	0.16	0.16	0.08	0.08	0.31	0.08	0.08	1.25
Z.b.	0.63	0.31	10.00	0.08	0.08	2.50	0.16	0.16	5.00	0.04	0.08	0.16	0.08	0.16	0.63	0.16	0.31	1.25
S.l.	1.25	0.31	nd [c]	0.16	0.16	nd	0.16	0.16	nd	0.08	0.08	nd	0.04	0.04	0.63	0.08	0.08	0.31
S.p.	1.25	0.63	nd	0.04	0.04	nd	0.16	0.16	nd	0.04	0.04	nd	0.04	0.04	0.04	0.08	0.08	0.63
P.g.	5.00	5.00	nd	0.31	0.31	nd	0.16	0.31	nd	0.08	0.04	nd	0.04	0.04	nd	0.16	0.08	1.25
A.a.	0.16	0.31	nd	0.63	0.04	1.25	0.02	0.02	nd	0.04	0.04	nd	0.04	0.02	0.04	0.02	0.16	0.63
L.h.	0.16	0.16	nd	0.08	0.04	nd	0.02	0.02	nd	0.04	0.04	nd	0.04	0.02	nd	0.02	0.02	0.63

[a] *D.b. D. bruxellensis, S.c. S. cerevisiae, Z.b. Z. bailii, S.l. S. ludwigii, S.p. S. pombe, P.g. P. guilliermondii, A.a. A. aceti, L.h. L. hilgardii*

[b] Recommended in-use concentrations

[c] Not determined

organisms except *P. guilliermondii* for which the MLC was twice the MIC. Furthermore, the MIC and MLC of the product VV9 against yeasts were twofold higher than against bacteria.

The alkaline disinfectant Topax 66 was indeed effective in removing biofilms with MBEC largely below the maximum concentration of use recommended by the manufacturer. For this agent, the MIC was equal to the MLC with respect to all except the yeast *Z. bailii* for which the MLC was twice the MIC. Instead, against bacteria, the MIC of the product Topax 66 were twofold higher than the MLC.

The oxidant disinfectant VT5 was effective in removing the biofilm of all tested organisms, with maximum values of MBEC (1.25%) against *D. bruxellensis*, *S. cerevisiae*, *Z. bailii* and *P. guilliermondii* and minimum (0.3125%) against *S. ludwigii*. For this agent, the MIC was equal to the MLC with respect to *D. bruxellensis*, *S. cerevisiae*, *S. ludwigii*, *S. pombe* and *L. hilgardii*. Instead, with respect to *Z. bailii* and *A. aceti*, the MLC of the product VT5 were greater than the MIC, while for *P. guilliermondii* the MIC was equal to about twice the MLC.

The mild alkaline disinfectant Topax 99 was effective in removing biofilms of *D. bruxellensis*, *S. cerevisiae* and *Z. bailii*, with a highest value of MBEC of 0.16%, largely below the maximum recommended concentration. For this agent, the MIC was equal to the MLC against all micro-organisms, except *P. guilliermondii*, for which the MIC was equal to twice the MLC, and *Z. bailii*, for which the MLC was twice the MIC.

Discussion

In the food and beverages industries, the formation of biofilms represents a significant source of contamination that, once established, can be very persistent and difficult to remove and control (Kumar and Anand 1998). These complex microbial communities have been studied for over 20 years, but their true nature is not yet fully understood (Parsek and Greenberg 2005).

The CBD™, developed by Ceri et al. (1999, 2001), was designed and successfully used to monitor the formation of biofilms and the sensitivity to antibiotics of bacterial species. Recently, it has also been utilized for the study of biofilm formation by different species of the genus *Candida* (Parahitiyawa et al. 2006). To our knowledge, this is the first study in which the CBD is used to test the ability of yeast and bacteria contaminating the wine to form biofilms.

Storgårds et al. (1997) showed that contaminant yeasts isolated from a brewery, *Dekkera anomala*, *Candida krusei* var. *krusei*, *Rhodotorula mucilaginosa*, *Pichia anomala*, *Pichia membranifaciens* and *S. cerevisiae* formed biofilms on stainless steel surfaces in semi-static conditions, especially when sugar was added to the media. A recent study has demonstrated the ability of *Brettanomyces* isolates tested to adhere to a plastic surface under conditions of low sugar concentration, and about half the strains tested formed biofilms with a high sugar concentration (Joseph et al. 2007). Our results have shown that the yeast strains belonging to the species *D. bruxellensis*, *S. cerevisiae*, *S. ludwigii*, *S. pombe* and the bacterium *A. aceti* were able to form biofilms, both in wine (sugar concentration <0.2 g/L) and in synthetic media (sugar concentration ≥20 g/L), while *Z. bailii* formed biofilm only in wine, and *P. guilliermondii* and *L. hilgardii* formed biofilms only in synthetic media.

Other authors have reported that strains of *S. cerevisiae* grown in co-culture with different species of lactic acid bacteria formed mixed biofilms, and that the biofilm biomass formed was greater than the monoculture (Kawarai et al. 2007). They have also demonstrated that the yeast-biofilm-forming factor was produced by the lactic acid bacteria and was still effective, even after the removal of bacterial cells from the media. Likewise, in our study, we could observe a similar interaction between a strain of *L. hilgardii* and a strain of *A. aceti*. In fact, there was an increase in the amount of the biofilm formed (in wine) by the acetic acid bacteria in the presence of the lactic acid bacteria, although the latter was unable to form biofilm. In contrast, the co-cultivation of a strain of *D. bruxellensis* with *A. aceti* did not cause any change on biofilm biomass, compared with monocultures.

The aim of disinfection is to reduce the population of viable microbes after cleaning and to prevent microbial growth on surfaces before restarting the production. However, it is acknowledged that disinfectants agents cannot penetrate the matrix of the biofilm remaining on surfaces after inefficient cleaning procedures, and therefore they are not able to destroy all viable cells in biofilms (Bloomfield 1988; Brackett 1992; Carpentier and Cerf 1993). In addition, factors such as the presence of interfering organic substances (e.g., fats, sugars and proteins), pH, temperature, concentration and contact time determines the efficiency of disinfectants (Czechowski and Banner 1992; Mosteller and Bishop 1993; Cerf et al. 2010). Relying on MIC measurement to evaluate or compare disinfection activity can be misleading, as there is no universal relation between MIC and MLC (Cerf et al. 2010). For this reason, in our study, other than MIC, we also have considered the MLC and MBEC to establish the effectiveness of the in-use concentration of disinfectants.

To date, there are few published studies that deal with the efficacy of disinfectants against foodborne yeasts and bacteria, both in suspensions and on surfaces, and the removal of biofilms (Wirtanen and Salo 2003). The effectiveness of disinfectants and antimicrobial agents is

usually determined in tests with cells in suspension, which do not faithfully represent the growth conditions on surfaces, where the agents are required to inactivate microbes (Frank and Koffi 1990; Wirtanen 1995). It is clear that tests with cells adhered to surfaces are more difficult to perform (Bloomfield et al. 1994). However, the "model" biofilms have many of the characteristics of "wild" biofilms: the microbial cells are attached to test surfaces, they produce slime, and they show a greater resistance to disinfectant agents. In this regard, the Calgary Biofilm Device is a relatively realistic, simple and reliable miniature device, which allow the growing of 96 equivalent biofilms simultaneously and thus enable rapid screening of anti-biofilm activity of disinfectant agents (Ceri et al. 1999, 2001).

McGrath et al. (1991) showed that a hypochlorite-based disinfectant, used in the recommended concentration, was able to kill *S. cerevisiae* strains in suspension tests with contact time of 15–20 min. In addition, the ascospores of *Saccharomyces* and *Pichia* were more resistant to disinfectants than vegetative cells (McGrath et al. 1991). In another study, hypochlorite, peracetic acid, phosphoric acid and anionic compounds, have been shown to be effective against yeast strains isolated from orange juice (Winniczuk and Parrish 1997). Chlorine dioxide has proven to be effective against yeast and mold contaminants of fruit juice storage tanks (Han et al. 1999). Wirtanen and Salo (2003) showed that the alcohol-based disinfectants were more effective against yeast cells both in suspension and in a biofilm test. Moreover, these authors argue that the persulphate-based and sulphamic acid-based agents were the only ones not suitable in the disinfectant treatment of yeast cells.

In our study, free cell suspension tests and biofilm tests have shown that the sodium hydroxide/sodium hypochlorite-based disinfectant (Topax 66) was the most effective, since the higher values of MIC and MLC were 92% lower than the minimum concentration recommended by the manufacturer. We found that the alkylamine-based disinfectant (Topax 99) was also effective on vegetative cells. Moreover, it was the most effective in biofilm removal tests, with a maximum value of MBEC 95% lower than the maximum recommended concentration. Similar to the study conducted by Wirtanen and Salo (2003), in cell suspension tests, the minimum recommended ready-in-use concentration of peracetic acid was unsuccessful against *Z. bailii*, *P. guilliermondii* and *A. aceti*, while all strains were effectively decontaminated with a fourfold higher concentration. The alkylbezenesulphonate-based detergent (VK10), was the least effective in all decontamination of free cells suspension and in biofilm removal. It is possible that this was the agent most affected by the interference of organic matter (Cerf et al. 2010). This could also explain the fact that, for this agent, most of the MICs (tested on synthetic medium) were higher than the MLCs (tested on water). In any case, for all agents and strains, we confirmed the evidence that cells in biofilms are more resistant than free-floating cells and, therefore, require higher concentrations of disinfectant for an effective elimination of these microorganisms (Mattila-Sandholm and Wirtanen 1992; Cerf et al. 2010).

Conclusions

In conclusion, it was shown that the CBD is a simple, low cost, useful miniature device for the parallel study of biofilms formed by spoilage microorganisms. It was confirmed that biofilms have greater tolerance to biocides when compared to the same microorganisms in the planktonic state. In both types of tests, with free-floating cells and with biofilms, the bacteria showed higher sensitivity to cleaning agents and disinfectants than yeasts.

According to both suspension and biofilm tests, alkaline chlorine-based disinfectant and peracetic-based disinfectant was the most effective in decontaminating spoilage yeast and bacteria. Sodium hydroxide-based detergents were efficient against suspended cells although at least 10-fold more concentrated solutions were needed to remove most biofilms.

Furthermore, our results showed that, except for the neutral detergent VK10, all tested agents were actually effective when used under the concentrations recommended by manufacturers.

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