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The effectiveness of commercial antimicrobial compounds against saccharolytic microorganisms isolated from a beet sugar production line

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Summary

The antimicrobial activities of five commercial disinfectants containing quaternary ammonium compound-isopropanol (D1), sodium methyl dithiocarbamate (D2), sodium thiocarbamate (D3), sodium dimethyl dithiocarbamate (D4) and formaldehyde (D5) were studied against three main saccharolytic indigenous isolates (*Bacillus cereus*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides*) from a beet sugar extraction line. Preliminary studies suggested that although all the disinfectants were effective against those isolates, the high economic cost in combination with large amounts of the disinfectants D2, D3 and D4 weaken their possibility for industrial use. Therefore, the minimum inhibitory concentration (MIC) of the other two examined disinfectants D1 and D5 was determined and survivor curves were obtained, for a period of 7 days. Bacterial counts against time (h) suggested that D1 was more effective than D5 against the microbial population. In particular, D1 was bacteriolytic above 7 mg/l for *B. cereus* and bactericidal above 80 mg/l for *Lc. mesenteroides* and above 100 mg/l for *L. plantarum*. The disinfectant D5 was bacteriolytic above 25 mg/l for *B. cereus* and bactericidal above 500 mg/l for *Lc. mesenteroides* and *L. plantarum*. Taking into consideration both features, i.e. high concentration and very low cost, the use of D5 (formaldehyde) appeared more suitable to the concerned beet sugar processor.

Introduction

During sugar beet processing, microorganisms mainly originating from the fields are introduced into the production process. Processing conditions such as temperature, pH value, water activity and sugar content favour microbial growth, resulting in sugar losses amounting to 0.1–0.3% in beet weight equivalent (Belami *et al.* 1991). Microbial activity leads to production of secondary metabolites such as slimy extracellular polysaccharides (dextran, levan), which clog pipes and filters, and the production of lactic acid that induces corrosion of steel in the extractor (Sidebotham 1974; Atkins & McCowage 1984; Tallgren *et al.* 1999).

Several soil genera of bacteria (*Bacillus*, *Clostridium*, *Pseudomonas*, *Enterobacter*, *Erwinia*, *Lactobacillus*, *Leuconostoc*, *Serratia*, *Xanthomonas*), have been identified as predominant in the juices produced in beet factories, during the extraction process. Yeasts and moulds are also present (Bugbee *et al.* 1975).

Control of microorganisms is possible by using physical and chemical methods. Franchi & Bocchi (1994) reported that some physical methods are of practical importance (e.g. steaming of tanks or filter presses), while others are not (e.g. u.v. and ultrasound). A large number of antiseptics, disinfectants and germicides are available for the control of microorganisms in food products (Denyer 1995; Russell 1998). The required optimum conditions for the practical application of these chemicals vary among factories depending on climatic and process parameters. In addition the choice of a disinfectant agent should be based on effective results at low concentrations, low cost, environmental and health risks.

The amount of chemical needed for disinfections is usually estimated by the minimal inhibitory concentration (MIC) test. This test determines the minimum concentration of a disinfectant needed to inhibit specific microorganisms under defined conditions and provides useful preliminary information about an antimicrobial compound prior to further testing (Davidson & Parish 1989).

Considering the prolonged practical problems that a sugar-processing plant is facing, the main objective of

the present study was to select the most economic and most efficient of several available commercial disinfectants, using MICs and survivor curves, against three saccharolytic bacterial species i.e. *Bacillus cereus*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* isolated from extracted juices that predominated in a certain beet sugar-processing plant.

Materials and methods

Sample collection – isolation of microorganisms

Process samples were taken aseptically from 15 different selected sample areas of the sugar factory. The samples, in sterile screw cap containers, were cooled in ice and transported to the laboratory. Immediate isolation procedures were carried out on all samples. After appropriate serial dilutions, samples were transferred, as inoculants, in Nutrient Agar (NA, Merck) and de Man, Rogosa and Sharpe Agar (MRSA, LabM) and incubated at 30 °C for 24–48 h. After incubation, all colonies that appeared were selected and purification was carried out by sub-culturing on the same selected media. Stock cultures were stored at −80 °C in 50% v/v glycerol solution.

B. cereus, isolated from NA plates and *L. plantarum* and *Lc. mesenteroides* isolated from MRSA plates, were identified as the predominant saccharolytic species causing sugar losses in the corresponding sugar-processing line.

Identification of microorganisms

Specific tests were performed to categorize the isolates into various groups. Gram staining, spore formation, catalase and oxidase reactions together with the examination of the phenotypic characteristics are some of the tests that were used to identify the organisms.

Furthermore, the isolates were identified using the API 50 CH System (BioMerieux Sa, France). *B. cereus* was grown on NA plates for 18–24 h at 37 °C, whilst *L. plantarum* and *Lc. mesenteroides* were grown on MRSA plates for 18–24 h at 30 °C. Bacterial population was harvested in 2 ml sterile 0.85% (w/v) saline solution to correspond with tube number 2 of the McFarland scale of standard opacities. 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium (for *B. cereus*) or in 10 ml of API 50 CHL medium (for *L. plantarum* and *Lc. mesenteroides*). The strips were inoculated, incubated at 37 °C and 30 °C respectively and read after both 24 and 48 h. The results were scored according to the manufacturers' instructions and the emerging biochemical profile was identified by means of APILAB software V 2.1, 1990.

Chemical agents

Five commercial disinfectants commonly used for the control of biological contamination in the sugar industry were studied. The antimicrobial compounds and the concentrations in the ready-to-use solutions are listed in Table 1. All disinfectants were diluted according to the instructions of the manufacturers. New solutions in sterile distilled water were prepared before each experiment.

Inoculum preparations

Frozen cells of *B. cereus*, *L. plantarum* and *Lc. mesenteroides* in 50% (v/v) glycerol solution, were thawed briefly, vortexed loopfuls inoculated into Nutrient Broth (NB, for *B. cereus*) and MRS Broth (MRSB, for *L. plantarum* and *Lc. mesenteroides*). These were incubated at 37 °C (for *B. cereus*) and at 30 °C (for *L. plantarum* and *Lc. mesenteroides*) on an orbital shaker at 200 rev/min for 18–24 h, to provide the working cultures.

Minimum inhibitory concentration

For MIC determinations, 500 ml of NB or MRSB in 2 l Erlenmeyer flasks were inoculated with the appropriate volume of the working culture, to obtain a starting $OD_{600\ nm}$ of 0.04–0.06 and incubated at 37 °C (for *B. cereus*) and 30 °C (for *L. plantarum* and *Lc. mesenteroides*) as above, until $OD_{600\ nm}$ of 0.1–0.2 and 0.2–0.4, respectively were attained.

At this time different amounts of the various disinfectants were added to individual cultures and incubation was continued. At various times $OD_{600\ nm}$ measurements were made to detect growth.

Survivor curves

A series of cultures were prepared as described above for the bacterial survivor curves. Immediately after inocu-

Table 1. The antimicrobial compounds tested and their concentration in the solutions of the disinfectants agents tested.

Disinfectant agent	Antimicrobial compound	Concentration of the compound in the working solution (% w/v)
Disinfectant 1 (D1)	Quaternary ammonium-isopropanol (QAC)	3.5–1.5
Disinfectant 2 (D2)	Sodium methyl dithiocarbamate	5
Disinfectant 3 (D3)	Sodium thiocarbamate	2.5
Disinfectant 4 (D4)	Sodium dimethyl didithiocarbamate	2.5
Disinfectant 5 (D5)	Formaldehyde	~3.7

lation, addition of the selected disinfectant into the appropriate concentration occurred and the culture was incubated as before. When the concentration of the disinfectant was doubled, the concentration of the inoculum was also doubled in order to maintain a constant proportion between the two parameters. Samples (1 ml) were removed from the flask at 10 min intervals for the first 30 min of incubation, at 30 min intervals for the following 3 h and once or twice per day for the next 7 days of the incubation period. Bacterial biomass was estimated by culture absorbance at 600 nm and total cell counts by using a Neubauer chamber. Each sample was diluted immediately in 9 ml of sterile Ringer's solution to reduce residual chemical agent effects and to produce serial dilutions necessary for total plate counts of microorganisms. Further serial dilutions were elaborated and plated onto NA for *B. cereus* or MRSA for *L. plantarum* and *Lc. mesenteroides* plates in triplicate. Plates were dried upright for half an hour, then inverted and incubated for 24–48 h at 37 °C (for *B. cereus*) and at 30 °C (for *L. plantarum* and *Lc. mesenteroides*). Survivor curves were obtained by expressing the log colony forming units ml^{-1} against time (h).

Statistical analysis

Statistical analyses were accomplished using the MINITAB statistical package (Minitab Statistical Software, State College. Pa.). Minimum significant differences (MSD) were calculated from analysis of variance using the Tukey–Kramer method (Petersen 1985; Fry 1989). All points on graphs are the means of three replicate samples each counted in triplicate for c.f.u.

Results

Estimation of MIC

MICs are shown in Table 2 and are the concentrations that totally inhibited all growth of the tested microorganisms.

The results indicated that *L. plantarum* and *Lc. mesenteroides* showed great resistance to disinfectants D2 containing sodium methyl dithiocarbamate, D3 containing sodium thiocarbamate and D4 containing sodium dimethyl dithiocarbamate. Due to logistics we did not proceed to determine further MIC values for the above disinfectants, since their high economic cost made them inexpedient.

The data demonstrated that the membrane disinfectant D1 was bacteriolytic for *B. cereus* above 7 mg/l and had a killing effect of 8 log units within the first 2 h, as it was estimated by c.f.u. ml^{-1} (Figure 1a). Also 50 mg/l of D1 had a bacteriostatic activity for *L. plantarum* (the growth stopped for 2 days) but it was bactericidal above 100 mg/l after 3 h (Figure 1b). Regarding *Lc. mesenteroides,* D1 had approximately the same behaviour. It had a bacteriostatic activity up to 40 mg/l but it was bactericidal above 80 mg/l after 24 h (Figure 1c).

Table 2. MICs of disinfectants.

Disinfectant	*B. cereus* (mg/l)	*L. plantarum* (mg/l)	*Lc. mesenteroides* (mg/l)
D1	7	100	50
D2	20	>1000	>1000
D3	12.5	>1000	>1000
D4	20	> 2500	>2500
D5	25	500	500

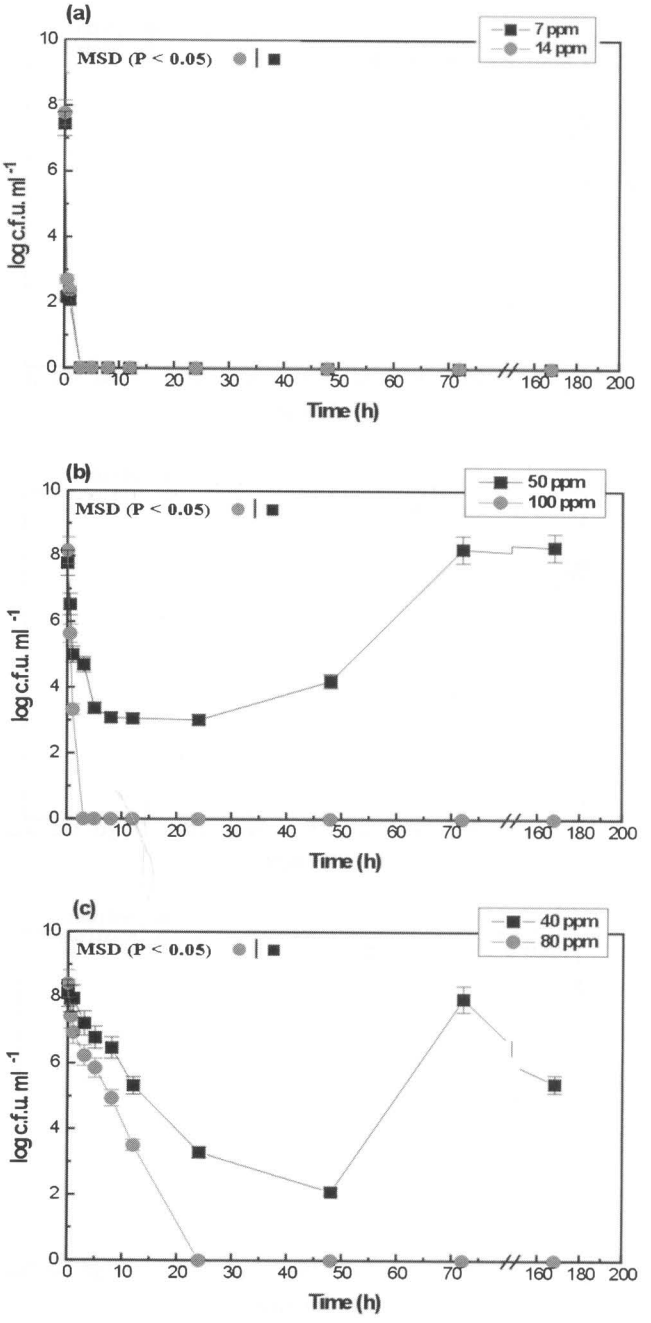

Figure 1. The antimicrobial activity of D1 against (a) *B. cereus*, (b) *L. plantarum* and (c) *Lc. mesenteroides.* Bacterial population was expressed as log c.f.u. ml^{-1} in all cases. The examined concentration of D1 varied between 7 and 100 mg/l.

In the case of D5, it has for many years been the practice to add formaldehyde in rather large doses, assuming that a high concentration is needed to kill the bacteria in the trouble spot. D5 had a bacteriolytic activity for *B. cereus* above 25 mg/l (Figure 2a) and a killing effect within the first 4 h. The same disinfectant had a bactericidal activity above 500 mg/l for both *L. plantarum* and *Lc. mesenteroides* after 48 h (Figures 2b and c).

In all the cases cell viability was recorded for a period of 1 week.

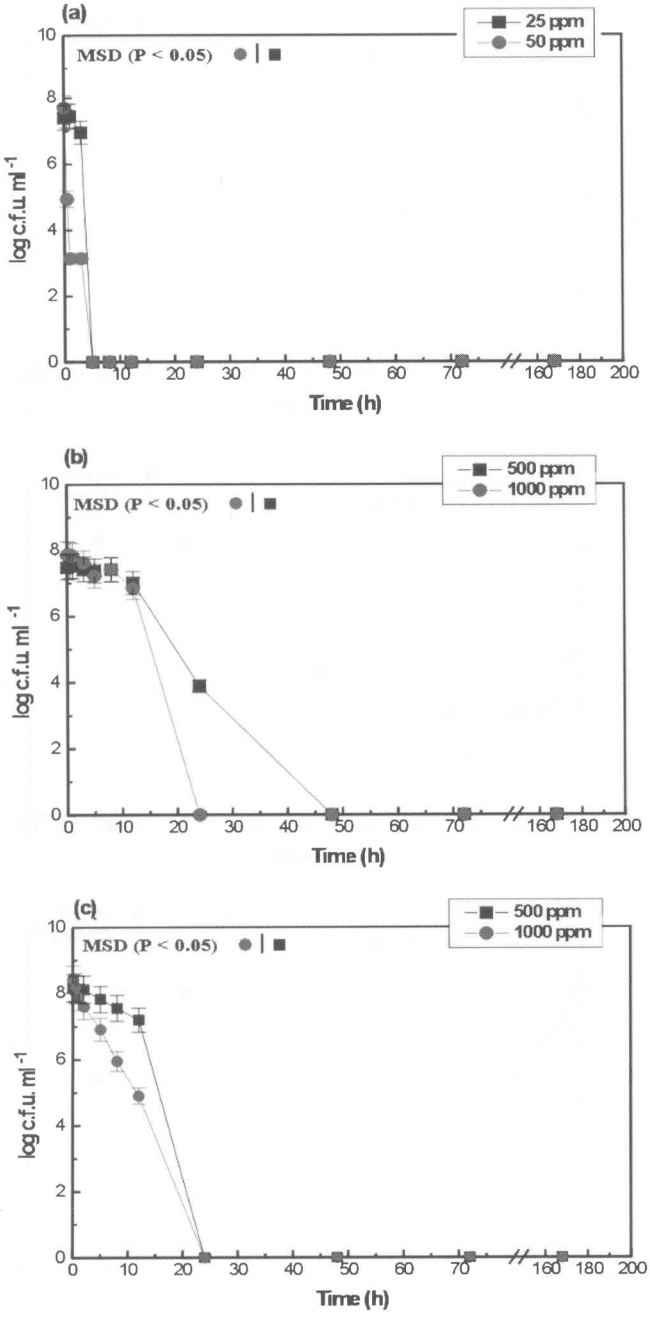

Figure 2. The antimicrobial activity of D5 against (a) *B. cereus*, (b) *L. plantarum* and (c) *Lc. mesenteroides*. Bacterial population was expressed as log c.f.u. ml^{-1} in all cases. The examined concentration of D5 varied between 25 and 1000 mg/l.

Relationship between disinfectant concentration and microbial biomass

The fact that 50 mg/l of D1 was effective on *L. plantarum* (or 40 mg/l for *Lc. mesenteroides*) for 2 days, led us to conduct a series of experiments, aimed at observing the influence of increment biomass of the saccharolytic bacteria upon the disinfectants concentrations required. The results indicate that it may not be necessary to double the concentration of disinfectant D1 when the biomass is doubled. Thus, with reference to *L. plantarum* (Figure 3a), when the initial log c.f.u. ml^{-1} was 7.78 (representing 6.05×10^7 cells), the concentration of D1 had to be above 50 mg/l in order to be effective for more than 2 days. On the other hand, the double concentration of D1 (100 mg/l) was effective on the double initial biomass (log c.f.u. $ml^{-1} = 8.16$ representing 1.5×10^8 cells) for a period for more than a week. The results were similar for *Lc. mesenteroides* (Figure 3b). Further investigation on the influence of biomass to the concentration of D1 is under consideration.

These results were not observed with the disinfectant D5. Thus, in survivor curves, disinfectant D5 was

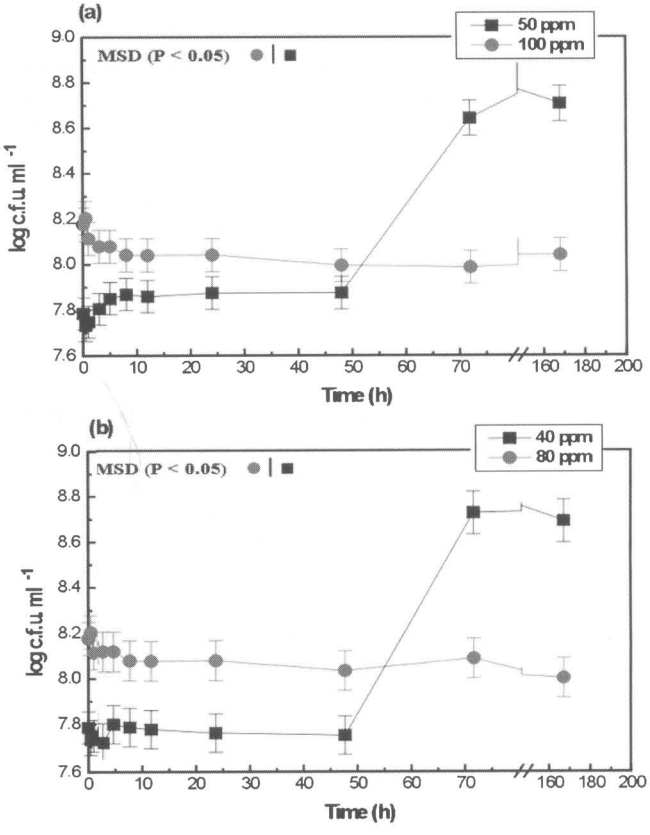

Figure 3. (a) The antimicrobial activity of D1 against *L. plantarum*. Initial bacterial population was expressed as 7.78 and 8.17 log c.f.u. ml^{-1} and the D1 concentration was expressed as 50 and 100 mg/l respectively. (b) The antimicrobial activity of D1 against *Lc. mesenteroides*. Initial bacterial population was expressed as 7.88 and 8.28 log c.f.u. ml^{-1} and the D1 concentration was expressed as 40 and 80 mg/l respectively.

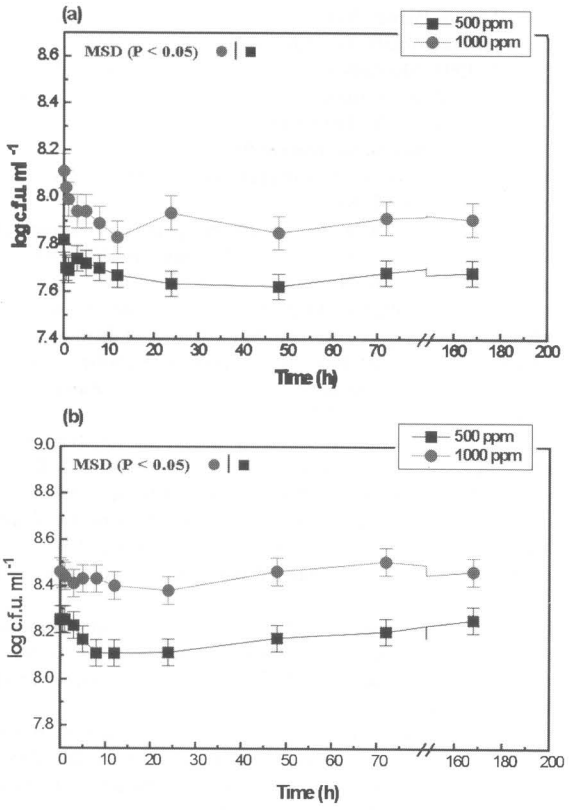

Figure 4. (a) The antimicrobial activity of D5 against *L. plantarum*. Initial bacterial population was expressed as 7.82 and 8.11 log c.f.u. ml^{-1} and the D5 concentration was expressed as 500 and 1000 mg/l respectively. (b) The antimicrobial activity of D5 against *Lc. mesenteroides*. Initial bacterial population was expressed as 8.25 and 8.46 log c.f.u. ml^{-1} and the D5 concentration was expressed as 500 and 1000 mg/l respectively.

bactericidal when the concentration was above 500 mg/l, which was the estimated MIC, for both *L. plantarum* and *Lc. mesenteroides* (Figure 4a and b).

Discussion

In a sugar beet production line, certain microbes can form many different types of heteropolysaccharides, causing sugar losses and filtration problems that are not systematically avoided by the use of commercially available dextranase (Tallgren *et al.* 1999). Moreover, the slime deposit of dextran protects the microorganisms so that they can tolerate even higher temperatures. In consequence, although most of the mesophiles are killed in the diffuser, a significant number of them survive (mostly slime bacteria) in an inactive state and pass through with the diffusion juice. Therefore, in order to avoid any further foodborne spoilage, which may be caused by the revival of these microorganisms, the addition of disinfectant is considered essential. *B. cereus*, *L. plantarum* and *Lc. mesenteroides* were the isolates causing sugar losses in the particular sugar installation studied.

Since duration of treatment is one of the important factors affecting the antimicrobial activity of disinfectants, it is desirable, in the food industry, to use persistent disinfectants. However, not only the contact time but also the concentration, the composition and possible combination with other agents, affect the antimicrobial activity (Grönholm *et al.* 1999). According to our study, both of the disinfectants, D1 and D5, provided satisfactory activity against the tested organisms for 1 week. A sufficient inhibition of culture growth and cell activity was achieved with a five times higher concentration of formaldehyde (D5), than the successful quaternary ammonium compound (D1). This is consistent with the classification of antibacterial compounds employed by Russell (1998). Quaternary ammonium compounds (D1), which cause disorganization of the cytoplasmic membrane, belong to group A and are bactericidal (or at lower concentrations, bacteriostatic). Formaldehyde (D5), which interacts with cell proteins, DNA and RNA, belongs to group B and is bactericidal as well, although in most cases much higher concentrations are needed to achieve the target effect. According to the results of this investigation, the antimicrobial activity of D1 was independent of the bacterial biomass since it was not necessary to double the concentration of D1 with simultaneous doubling of biomass. In contrast, the antimicrobial activity of D5 was expressed above the threshold concentration of 500 mg/l and the two parameters (disinfectant concentration vs. microbial biomass) were proportional.

It is known that Gram-positive bacteria are generally more susceptible to disinfectant action than are Gram-negative bacteria, due to their lack of the outer cell layer, which restricts entry of many types of chemicals agents including antibacterials (Nikaido 1994; Paulsen *et al.* 1997). Under the specific conditions of cultivation, the microorganisms used for testing disinfectant efficacy belong to Gram-positive non-sporulating bacteria. They possess cell walls that composed essentially of peptidoglycan and techoic acid (Russell 1995). Neither of these appears to act as an effective barrier to the entry of disinfectants. Nevertheless, there are significant differences in response to the above disinfectants, which may be ascribed to the manner in which the uptake of an antimicrobial agent into a cell is influenced. Since high-molecular-weight substrates can readily traverse the cell wall of *Bacillus* spp. this may explain the sensitivity of these organisms to many antibacterial agents including QACs and formaldehyde (Russell 1995). Gilbert & Brown (1995) demonstrated that growth rate and any growth-limiting nutrient affect the physiological state of the *Bacillus* cells and hence the sensitivity to disinfectants will be altered.

To date, there is little or no experimental evidence about specific resistant mechanism in the tested bacterial strains, except for some lactobacilli that might be protected by exopolysaccharides (Sundheim *et al.* 1998). The formation of extracellular polymeric substances by *L. plantarum* and *Lc. mesenteroides* may play a protective role, either as a physical barrier, to disinfectant penetration or as a loose layer interacting

with (or absorbing) the active molecule. It is necessary to break up or to destabilize the sticky consistency of these slime substances in order to make the microorganisms accessible to disinfectants.

The results presented in this work clearly show that the choice of disinfectant along with the optimum concentration and the time of action are very important parameters aiming to the inhibition of microbial activity. Practical studies are recommended on the use of selected disinfectants in a sugar-processing line. In addition, our results provided much-needed information for the use of QACs and formaldehyde, inferring that formaldehyde seemed less costly for the particular sugar beet processor.

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