



Effect of enterocin AS-48 singly or in combination with biocides on planktonic and sessile *B. cereus*



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ABSTRACT

Enterocin AS-48 was tested singly or in combination with biocides on a cocktail of six *Bacillus cereus* strains in planktonic state and in biofilms formed on polystyrene microtiter plates. The biocides tested were benzalkonium chloride, cetrimide, hexadecylpyridinium chloride, triclosan, chlorhexidine, polyhexamethylen guanidinium chloride and commercial sanitizers P3 oxonia and P3 topax 66. The numbers of survivors were determined after 60 min incubation with biocides or the biocide-bacteriocin combinations. Addition of enterocin AS-48 (25 mg/l) increased the inactivation of planktonic cells by the quaternary ammonium compounds, bisphenols and biguanines tested in a range of biocide concentrations from 0.25 to 2.5 g/l, and by 4 g/l polyguanine. Increased inactivation of the bacilli was also observed for the combination of enterocin AS-48 with 2.5% P3 oxonia, but not by P3 topax 66. In the sessile state, the bacilli were more resistant to biocides and also to the bacteriocin-biocide combinations. Hexadecylpyridinium chloride was the most active biocide on biofilms in the single treatments. In the combined treatments with 50 mg/l bacteriocin, hexadecylpyridinium (2.5 g/l), polyhexamethylen guanidinium chloride (4 g/l) and P3 oxonia (2.5%) achieved complete inactivation of bacilli populations. P3 topax 66 showed the lowest performance among all treatments tested, either singly or in combination with bacteriocin. A cocktail of endospores was challenged with biocides and enterocin AS-48 for 60 min at temperatures of 22 °C, 40 °C, and 60 °C. Enterocin AS-48 did not significantly ($p > 0.05$) reduce viable counts or increase the lethal effect of biocides. However, treatments with 5 g/l benzalkonium chloride at 60 °C, 2.5 g/l hexadecylpyridinium at 60 °C or P3 oxonia at 0.025% and 60 °C or at 0.25% at 22–60 °C achieved complete inactivation of bacterial endospores, both singly and in combination with bacteriocin. Significant reductions of viable counts (1–2 log cycles) were also obtained for some treatments with cetrimide, triclosan or polyhexamethylen guanidinium chloride, but not for chlorhexidine (up to 5 g/l) or P3 topax 66 (up to 1%). Polystyrene surfaces dosed with enterocin AS-48 (25 or 50 mg/l) remained free of detectable bacilli from 2 to 24 h after being inoculated with a cocktail of endospores, but stainless steel surfaces dosed with 50 mg/l bacteriocin did not prevent bacterial growth from endospores. Results from this study suggest that enterocin AS-48 could be applied as enhancer of biocide activity against planktonic and sessile *B. cereus* cells.

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1. Introduction

Bacillus cereus is a food-poisoning bacterium that may cause emetic and diarrhoeal syndromes, caused by different toxins (Granum & Lund, 1997; Granum, 2007). Due to its ubiquitous

distribution in nature, *B. cereus* occurs frequently in a wide range of food raw materials. It has been isolated from a wide variety of foods like dairy, bakery products, rice and seafoods (Ankolekar & Labbé, 2010; Bailey & von Holy, 1993; Granum, 2007; Rahmati & Labbé, 2008). The persistent contamination of industrial food processing systems by *B. cereus* is due to the formation of endospores, which may survive pasteurization, heating, and gamma-ray irradiation (Nicholson et al., 2000), and to biofilms, which are highly resistant to cleaning and disinfection procedures (Peng, Tsai, & Chou, 2002; Ryu & Beuchat, 2005).

Biofilm formation by *B. cereus* has been described on different substrates such as stainless steel, plastic materials of different

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compositions, borosilicate glass and glass wool (Auger et al., 2009; Elhariry, 2008; Hesham & Elhariry, 2011; Houry, Briandet, Aymerich, & Gohar, 2010; Jan et al., 2011; Lindsay, Brözel, & Von Holy, 2006; Orgaz, Lobete, Puga, & Jose, 2011; Padegar & Singh 2012; Peng, Tsai, & Chou, 2001; Ryu & Beuchat 2005) and also on biotic surfaces such as cabbage and lettuce surfaces (Hesham & Elhariry, 2011). Since biofilm formation enhances bacterial tolerance to environmental factors (Abee, Kovács, Kuipers, & Van der Veen, 2011; Costerton, Stewart, & Greenberg, 1999; Van Houdt & Michiels, 2010), different approaches have been proposed to combat biofilms, based on physico-chemical agents, enzymes, or bacteriophages (de Carvalho, 2012). A few studies have explored the possibility of using bacteriocins or their producer strains against biofilm-forming bacteria (Ammor, Tauveron, Dufour, & Chevallier, 2006; Bower, McGuire, & Daeschel, 1995; Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2012, 2013; Guerrieri et al., 2009; Kumar, Parvathi, George, Krohne, & Karunasagar, 2009; Leriche, Chassaing, & Carpentier, 1999; Minei et al., 2008; Winkelströter et al., 2011; Zhao, Doyle, & Zhao, 2004). Interestingly, bacteriocins could improve the bactericidal effect of biocides on bacterial biofilms (Caballero Gómez et al., 2012, 2013; Lobos, Padilla, & Padilla, 2009). Enterocin AS-48 improved inactivation of *Listeria monocytogenes* and *Staphylococcus aureus* both in planktonic state as well as in biofilms (Caballero Gómez et al., 2012, 2013). Enterocin AS-48 is a cyclic antimicrobial peptide produced by strains of enterococci (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010; Maqueda et al., 2004). Previous studies have shown the effectiveness of enterocin AS-48 against vegetative cells of *B. cereus* in different food systems such as rice, rice pudding, vegetable sauces, or inoculated on the surface of vegetables (Muñoz et al., 2004; Grande et al., 2006, 2007; Cobo Molinos et al., 2008). The purpose of the present study was to investigate the capacity of enterocin AS-48 to potentiate the activity of biocides against planktonic or sessile *B. cereus* cells and its possible effects on endospores from this bacterium.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

B. cereus strains B47, B70, CRG5, ERG1 and LWL1 (all of them from food sources) and *B. cereus* CECT 148T (type strain, Spanish Type Culture Collection CECT) were cultivated on trypticase soy broth (TSB, Scharlab, Spain) or trypticase soy agar (TSA, Scharlab) and stored at 4 °C for routine use or as stocks in TSB supplemented with 30% glycerol at –80 °C. For preparation of inocula, bacilli were grown in TSB for 18 h at 37 °C. One ml cultures from each strain were mixed in a 50 ml sterile plastic tube to prepare the cocktail of strains.

2.2. Antimicrobials

Enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Bacteriocin concentrates were filtered through 0.22 µm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Bacteriocin concentrates were diluted 20–50-fold in biocide solutions in order to achieve the desired final bacteriocin concentrations of 25 or 50 µg/ml.

The commercial sanitizers P3 oxonia (25–35% hydrogen peroxide, 0.83–2.5 N acetic acid, and 0.26–0.66 N peracetic acid) and P3 topax 66 (2–5% sodium hydroxide, 2–5% sodium

hypochlorite, and 2–5% alkylamine) were from ECOLAB (Barcelona, Spain). Poly-(hexamethylen guanidinium) hydrochloride (PHMG) solution (containing 7.8% of PHMG, by weight) was a kind gift of Oy Soft Protector Ltd (Espoo, Finland). These commercial solutions are widely used for sanitation in the food industry. Benzalkonium chloride (BC), cetrimide (CT), hexadecylpyridinium chloride (HDP), triclosan (TC) and chlorhexidine (CH) were from Sigma–Aldrich (Madrid, Spain). Benzalkonium chloride commercial solution contained 50% (wt/v) of the active compound. Triclosan was dissolved (10% wt/v) in 96% ethanol. The remaining biocides were dissolved aseptically in sterile distilled water at final concentrations of 5% (CH and HDP) or 10% (CT), and stored at 4 °C for a maximum of 7 days.

2.3. Biocide treatment of planktonic cells

A cocktail of the six *B. cereus* strains prepared from overnight cultures as described in Section 2.1 was inoculated in duplicate Eppendorf test tubes containing 1 ml TSB to achieve a final cell density of ca. 5.5×10^5 CFU/ml. TSB broth was previously warmed at 30 °C and supplemented or not with biocides and/or enterocin AS-48 (25 mg/l). After inoculation, TSB broths were incubated at 30 °C for 60 min. Then, 0.5 ml of triple-strength D/E neutralizing broth (Difco, Barcelona) was added, followed by vortexing and centrifugation ($12,000 \times g$, 10 min). Supernatants were discarded, and the sediments were resuspended in 1.5 ml sterile saline solution by vortexing, followed by centrifugation as above. The resulting cell pellets were resuspended in 1 ml sterile saline solution, serially diluted in the same diluent and plated on TSA for viable cell counting. Three replicate experiments of the duplicate samples were performed.

2.4. Biocide treatment of sessile cells

The cocktail of *Bacillus* strains obtained from overnight cultures was inoculated (0.1% vol/vol) in duplicate onto diluted TSB broth (5.0 g/l) to achieve a cell density of ca. 5.5×10^5 CFU/ml. Inoculated broths were distributed (200 µl per well) on U-shaped 96-well polystyrene microtiter plates (Beckton Dickinson Labware, Franklin Lakes, NJ). Microtiter plates were incubated at 30 °C for 24 h. Then, the cultured broths were discarded and the biofilms formed on the microtiter plates were washed with 200 µl of sterile saline solution to remove loosely associated bacterial cells. Solutions containing biocides, enterocin AS-48 or biocide/bacteriocin combinations were added to the wells and the plates were further incubated at 30 °C for 60 min. After treatments, the biocidal solutions were removed and the wells were washed with 200 µl of D/E Neutralizing broth (Difco, Barcelona) followed by 200 µl of phosphate buffered saline (PBS) (Merck, Darmstadt, Germany). Biofilms were resuspended in 200 µl PBS by sonication for 1 min in a sonicator bath (Mod 3510, Branson; Danbury, CT, USA) followed by pipetting vigorously for 30 s (Caballero Gómez et al., 2012). Removal of biofilm cells was confirmed by the crystal violet staining method described by Djordjevic, Wiedmann, & McLandsborough (2002). For each treatment, samples from two wells were pooled together and vortexed, followed by serial dilution in sterile saline solution and surface plating in triplicate on TSA. Viable cell counts obtained after 24 h incubation at 37 °C were used to calculate the average numbers of viable cells per well. To determine the number of spores in biofilm, suspensions (400 µl) were heated at 80 °C for 10 min, serially diluted in sterile saline solution and plated on TSA for viable counts as described above. Three replicate experiments (with two wells per replicate) were performed.

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