

BIOFILMS

REMOVAL OF *PSEUDOMONAS PUTIDA* BIOFILM AND ASSOCIATED EXTRACELLULAR POLYMERIC SUBSTANCES FROM STAINLESS STEEL USING ALKALI CLEANING

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Alkali (NaOH)-based compounds are commonly used in the food industry to clean food contact surfaces. However, little information is available on the ability of alkali and alkali-based cleaning compounds to remove polymeric substances (EPS) produced by biofilm bacteria. The objectives of this study were to determine the temperature and NaOH concentration necessary to remove biofilm EPS from stainless steel under turbulent flow conditions (clean-in-place simulation), and to determine the ability of a commercial alkali cleaner to remove biofilm EPS from stainless steel when applied under static conditions without heat. Biofilms were produced by growing *Pseudomonas putida* on stainless steel for 72 h at 25°C in a 1:10 dilution of trypticase soy broth. The biofilms were treated using NaOH at concentrations from 1.28 to 6.0% and temperatures ranging from 66 to 70°C. Other biofilms were treated with commercial alkali cleaner at 25 or 4°C for 1 to 30 min. Removal of EPS was determined by direct microscopic observation of samples stained with fluorescent-labeled peanut agglutinin (PNA) lectin. Treatment with 1.2% alkali at 66°C for 3 min was insufficient to remove biofilm EPS. A minimum of 2.5% NaOH at 66 °C and 2.0% NaOH at 68°C for three min were effective at EPS removal. Commercial alkali cleaner removed over 99% of biofilm EPS within 1 min at 4 and 25°C under static conditions. Selection of appropriate cleaning agent formulation and use at recommended concentrations and temperatures is critical for removal of biofilm EPS from stainless steel.

LETHALITY OF CHLORINE, CHLORINE DIOXIDE, AND A COMMERCIAL PRODUCE SANITIZER TO *BACILLUS CEREUS* AND *PSEUDOMONAS* IN A LIQUID DETERGENT, ON STAINLESS STEEL, AND IN BIOFILM

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The majority of cells in *Bacillus* biofilms are in a vegetative form. However, during the course of biofilm formation and aging, sporulation may occur. If *Bacillus cereus* produces spores within a biofilm matrix on a food-contact surface, those spores may be exceptionally resistant to environmental stresses. Formation of spores by *B. cereus* in biofilms on food-contact surfaces as a result of exposure to stresses and their subsequent resistance to sanitizers commonly used in food preparation and processing environments warrant further research attention. *Pseudomonas* species have been isolated from a wide range of environmental sources, including ready-to-eat foods. *Pseudomonas* in biofilms on food-contact surfaces can cross-contaminate foods and biofilms may provide a protective matrix for pathogens. Little is known about the effectiveness of sanitizer treatments in eliminating pathogens and spoilage microorganisms that may remain in food or detergent residues on food-contact surfaces in foodservice, hospital, and home kitchen settings.

We undertook a study to determine the sensitivities of vegetative cells and spores of *B. cereus* and cells of *Pseudomonas* suspended in a light-duty, liquid, dishwashing detergent (LLDD) to treatment with chlorine and chlorine dioxide. LLDD was used as a medium in which to suspend cells, a situation that may exist if a detergent were to be left as a residue after cleaning surfaces in food preparation areas. The efficacy of chlorine, chlorine dioxide, a commercial produce sanitizer (Fit™), and combinations of these sanitizers in killing *B. cereus* and *Pseudomonas* spot-inoculated on stainless steel was also determined. Suspensions of vegetative cells and spores in water and horse serum, the latter carrier representing a high-organic matrix, were dried on the surface of stainless steel, which simulated contamination of a biologically inert surface in a food processing or preparation area. A third objective was to determine the

efficacy of chlorine, chlorine dioxide, and Fit in killing vegetative cells and spores of *B. cereus* in biofilm formed on the surface of stainless steel of the type used in food processing and preparation areas.

Significant reductions ($P \leq 0.05$) in populations of vegetative cells of *B. cereus* ($1.92 \log_{10}$ CFU/ml) but not spores, and a significant reduction in the number of *Pseudomonas* ($2.71 \log_{10}$ CFU/ml) occurred within 16 - 18 h at 21°C. Surviving *Pseudomonas* cells were more sensitive than *B. cereus* cells or spores to treatment with chlorine and chlorine dioxide. At 50 µg/ml, chlorine dioxide killed a significantly higher number of *Pseudomonas* ($3.82 \log_{10}$ CFU/ml) compared to a reduction of $1.34 \log_{10}$ CFU/ml caused by treatment with 50 µg/ml chlorine. Treatment of spot-inoculated stainless steel with chlorine was more effective than chlorine dioxide in killing cells and spores of *B. cereus* enmeshed in an organic matrix. Treatment with a 0.5% solution of a commercial produce sanitizer (Fit™) was ineffective in killing *B. cereus* on stainless steel. The lethality of chlorine dioxide, but not chlorine, was greatly enhanced by combining with Fit, regardless of the presence of organic material in the inoculum carrier. Treatment of *Pseudomonas* spot-inoculated on stainless steel with 0.5% Fit or 100 or 200 µg/ml chlorine dioxide or chlorine, alone or in combination with Fit, significantly reduced populations. Treatment of *B. cereus* biofilm that had formed on the surface of stainless steel coupons with chlorine dioxide or chlorine at a concentration of 200 µg/ml caused reductions in total populations (vegetative cells plus spores) of $\geq 4.42 \log_{10}$ CFU/coupon; reductions in the number of spores were $\geq 3.80 \log_{10}$ CFU/coupon. Fit (0.5%) was ineffective in killing *B. cereus* in biofilm but treatment with mixtures of Fit and chlorine dioxide caused greater reductions than treatment with chlorine dioxide alone. In contrast, when chlorine was combined with Fit, the lethality of chlorine was completely lost. These observations provide insights to developing more effective strategies for cleaning and sanitizing contact surfaces in food preparation and processing environments.

BIOFILM FORMATION AND SPORULATION BY *BACILLUS CEREUS* ON A STAINLESS STEEL SURFACE, AND SUBSEQUENT RESISTANCE OF VEGETATIVE CELLS AND SPORES TO CHLORINE, CHLORINE DIOXIDE, AND A PEROXYACETIC ACID-BASED SANITIZER

(J.-H. Ryu and L. R. Beuchat)

One of the most distinct features of *Bacillus cereus* is its ability to produce heat resistant spores. As a result of sporulation, resistance to wet heat, dry heat, radiation, desiccation, extreme pH, chemicals, enzymes, and high pressure is greatly enhanced. This resistance enables the bacterium to survive commercial food pasteurization and cooking at ambient pressure. Sublethal heat treatment of foods containing *B. cereus* spores can select for the pathogen among other microorganisms that might be present. It is known that *B. cereus* can form biofilms on food contact surfaces. These biofilms may originate from vegetative cells or from spores that become attached to surfaces. It has been reported that spores of *Bacillus* spp. can attach more readily than vegetative cells on stainless steel surfaces because of their hydrophobic properties. The majority of cells in *B. cereus* biofilms are in a vegetative form. However, during the course of biofilm formation and aging, sporulation may occur. The rate and extent of spore production by *B. cereus* in biofilm would be anticipated to be affected by environmental conditions. If *B. cereus* produces spores with a biofilm matrix on food contact surfaces, those spores may have greater resistance to environmental stresses, including sanitizers. We did a study to determine the effects of temperature on biofilm formation by *B. cereus* on stainless steel coupons, to investigate the influence of nutrient availability, temperature, and relative humidity on sporulation of *B. cereus* in the biofilm, and to evaluate the resistance of vegetative cells and spores in biofilms to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer.

Total counts in biofilms formed on coupons immersed in tryptic soy broth (TSB) at 12 and 22°C consisted of 99.94% of vegetative cells and 0.06% of spores. Coupons on which biofilms had formed were immersed in TSB or exposed to air with 100, 97, 93, or 85% relative humidity. Biofilms on coupons immersed in TSB at 12°C for an additional 6 days or 22°C for an additional 4 days contained 0.30 and 0.02% of spores, respectively, whereas biofilms exposed to air with 100 or 97% relative humidity at 22°C

for 4 days contained 10 and 2.5% of spores, respectively. Sporulation did not occur in biofilms exposed to 93 or 85% relative humidity at 22°C. Treatment of biofilm on coupons that had been immersed in TSB at 22°C with chlorine (50 µg/ml), chlorine dioxide (50 µg/ml), and a peroxyacetic acid-based sanitizer (Tsunami 200[®]) (40 µg/ml) for 5 min reduced total cell counts (vegetative cells plus spores) by 4.7, 3.0, and 3.8 log₁₀ CFU/coupon, respectively; total cell counts in biofilms exposed to air with 100% relative humidity were reduced by 1.5, 2.4, and 1.1 log₁₀ CFU/coupon, respectively, reflecting the presence of lower numbers of vegetative cells. Spores that survived treatment with chlorine dioxide had reduced resistance to heat. It is concluded that exposure of biofilm formed by *B. cereus* exposed to air at high relative humidity (≥ 97%) promotes the production of spores. Spores and, to a lesser extent, vegetative cells embedded in biofilm are protected against inactivation by sanitizers. Results provide new insights to developing strategies to achieve more effective sanitation programs to minimize risks associated with *B. cereus* in biofilms formed on food contact surfaces and in foods.

