Removal of *Pseudomonas putida* Biofilm and Associated Extracellular Polymeric Substances from Stainless Steel by Alkali Cleaning

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**ABSTRACT**

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 extracellular 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 alkaline 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 of 1.28 to 6.0% and temperatures ranging from 66 to 70°C. Other biofilms were treated with commercial alkaline 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 lectin. Treatment with 1.2% NaOH 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 3 min were both effective for EPS removal. Commercial alkaline 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.

Biofilms are formed by living microorganisms growing on a surface. Biofilm microorganisms produce extracellular polymeric substances (EPS) that act as a biological glue to firmly attach the cells to the surface (4). EPS protects these cells from environmental stresses such as desiccation and chemical inactivation, entraps nutrients, and restricts diffusion of some antimicrobial molecules (15, 18). When biofilm cells detach, portions of the EPS remain attached to the underlying surface. Removal of this residual EPS should be a goal in food industry cleaning because this EPS residue increases the subsequent ability of other bacteria to attach to the surface (17).

Periodic cleaning controls biofilm accumulation by detaching and inactivating microorganisms and removing food residues that promote microbial growth (6). Chemical sanitizers effectively inactivate biofilm cells after the biofilm has been disrupted by cleaning (11). Previous research demonstrated that clean-in-place processes for stainless steel that utilize hot alkali effectively remove food residues and prepare the surface for effective chemical sanitation (7). However, various studies have provided evidence that biofilms and associated EPS may not be effectively removed by normal cleaning procedures (1, 2, 9, 12, 14, 16, 20). Cleaning processes that are effective for removal of food residues are not necessarily effective in removal of biofilm because the chemical properties of EPS differ from those of fats and proteins normally targeted by the cleaning process. Alkaline cleaners, which act by hydrolyzing and dissolving organic soil, should be able to remove biofilm EPS, but required exposure conditions for effective removal may differ from those determined for protein and lipid soils. Alkali concentration, temperatures, and exposure times published in the Model Pasteurized Milk Ordinance (19) are based on removal of milk fat and protein and have not been evaluated for effectiveness on removal of EPS.

One objective of this study was to determine the ability of NaOH to remove biofilm EPS from stainless steel when applied in hot turbulent solution as might be done for circulation applications. A second objective was to determine the ability of a commercial alkaline cleaner designed for application in the processing environment as a foam or thin film to remove biofilm EPS from stainless steel. To accomplish these objectives, we developed a method for locating residual EPS produced by *Pseudomonas putida* on stainless steel by microscopic observation of fluorescent-labeled lectin bound to the EPS.

**MATERIALS AND METHODS**

Stainless steel coupons. Stainless steel coupons (2 by 5 cm) were cut from type 304 stainless steel with a no. 4B finish. The coupons were cleaned with acetone to remove grease associated with the steel manufacturing process and then washed by sonication (Ultrasonic bath model 550 HT, VWR, Atlanta, Ga.) in 1.28% NaOH solution for 1 h at 80°C, rinsed in deionized water, sonicated in 15% phosphoric acid solution for 20 min at 80°C,
and again rinsed in deionized water. Cleaned coupons were submerged in deionized water and autoclaved before use.

**Attachment and biofilm formation.** *P. putida* recently isolated from a food processing plant environment was used for this study. The isolate was identified using fatty acid methyl ester profiles (13). The culture was stored frozen at −80°C on cryogenic beads (Microbank, Pro-Lab, Inc., Weston, Fla.). Stock cultures were activated by transferring them into 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) and incubating at 32°C for 24 h. Prior to each use, cultures were transferred twice into fresh TSB and incubated under similar conditions. This culture was used to inoculate (0.1%) 500 ml of a 1:10 TSB dilution, and the mixture was incubated at 32°C for 24 h. Each stainless steel coupon was submerged in 25 ml of the inoculated broth in a test tube (25 by 150 mm). The submerged coupons were incubated at 25°C for 4 h to allow the bacteria to attach. Uninoculated controls were incubated in sterile medium. After the 4-h attachment period, coupons were rinsed in sterile potassium phosphate buffer (0.01 M, pH 7.0) to remove unattached cells, submerged again in 25 ml of a 1:10 TSB dilution, incubated at 25°C for 48 h, rinsed in phosphate buffer, transferred to 25 ml of fresh broth, and incubated for an additional 24 h to complete biofilm formation.

**Hot alkali cleaning.** Hot alkali cleaning was accomplished by submersion of coupons in hot NaOH under turbulent flow using the procedure and apparatus described by Frank and Chmielewski (9). The cleaning apparatus consisted of a Buchi 461 controlled temperature water bath (Fisher Scientific, Norcross, Ga.) with a spindle model 1750 mixer (VWR) and an impeller (6.35 cm in diameter). A stainless steel frame designed to hold six stainless-steel coupons had a radius of 11.75 cm. Six coupons were placed 9.5 cm apart in holders on the stainless steel frame. Coupons were exposed to turbulent flow (Reynolds number 14680) in NaOH solution for 3 min at a specified temperature. Treated coupons were immediately rinsed in sterile water, and residual NaOH was neutralized with a final rinse in sterile potassium phosphate buffer (0.01 M, pH 7.2). After treatment, coupons were allowed to air dry before analysis.

**Static low-temperature cleaning.** Static cleaning was accomplished by the procedure of Frank et al. (10), which involved submersion of coupons in commercial alkaline cleaner (TFC Green II, Ecolab, Inc., St. Paul, Minn.) for 1, 5, or 10 min at either room temperature (23 ± 2°C) or at 4 ± 1°C. This cleaner consisted of 10% NaOH, 5% tallow bis (hydroxyethyl) amine oxide, 3% diethyleneglycol methyl ether, 3% propylene glycol monomethyl ether, and 3% dipropylene glycol methyl ester. Neutral cleaner (TFC Pink, Ecolab), consisting of 9% surfactant mixture, 5% tribasic potassium phosphate, 4% trimethyl tallowalkyl quaternary ammonium chloride, 3% diethylene glycol methyl ether, 3% propylene glycol methyl ether, and 1% isopropyl alcohol, was tested only at room temperature. Cleaning agents were used at a concentration of 34 ml/liter as recommended by the manufacturer for removal of light soil. The chemicals were removed by rinsing as previously described. For 4°C cleaning, the cleaning and rinsing solutions and test surfaces were tempered at 4°C before treatment.

**Biofilm staining.** Hoescht 33258 (0.05 mg/ml, Sigma Chemical Co., St. Louis, Mo.) was used to stain biofilm cells. Biofilms were flooded with the stain, incubated in the dark for 30 min at room temperature, rinsed with deionized water, and allowed to air dry. Lectins from the following sources were evaluated for their ability to label EPS: *Erythrina christagalli* (coral tree), *Sophora japonica* (from Japanese pagoda tree), wheat germ agglutinin, and peanut agglutinin (PNA) from *Arachis hypogaea*. All lectins were Alexa Fluor conjugates (Molecular Probes, Inc., Eugene, Oreg.). Lectin solutions of 100 and 150 µg/ml were prepared by dissolving the protein in an aqueous buffer at neutral pH containing 0.05 mM CaCl$_2$ and 0.05 mM MgCl$_2$. Flooded surfaces were incubated in the dark for 45 min, rinsed with deionized water, and allowed to air dry. Positive and negative control surfaces and treated surfaces were stained.

The coupons stained with Hoescht 33258 were viewed under an epifluorescence microscope using a filter with an excitation wavelength of 330 to 380 nm, a dichroic mirror of 400 nm, and an emission wavelength of 435 to 485 nm (Nikon Eclipses E600, Nikon, Tokyo, Japan). The lectin-stained coupons were viewed using a filter with an excitation wavelength of 450 to 490 nm, a dichroic mirror of 500 nm, and an emission wavelength of 515 nm. Coupons were viewed by using a ×10 water immersion lens. Images were captured by using a Magnafire camera (Optronics, Goleta, Calif.). Ten randomly selected fields per coupon were captured and processed using Image Pro Plus Imaging software (Media Cybernetics, Silver Spring, Md.). Processing included image thresholding to improve contrast and reduce noise, with black pixels representing the stainless steel background and white pixels representing the stained biofilm. The black-and-white images were analyzed for percentage of area covered by white pixels using UTHSCSA Image Tool version 2.0 (Health Science Center, University of Texas, San Antonio, Tex.).

**Effectiveness of cleaning for removing EPS.** The effectiveness of different NaOH-temperature combinations for EPS removal was evaluated by staining the cleaned stainless steel coupons with the PNA Alexa Fluor 488 conjugate. Treated surfaces were subjected to the following cleaning conditions: 1.28% (0.32 mM), 2.0% (0.5 mM), 2.5% (0.63 mM), 4.0% (1 mM), and 6.0% (1.5 mM) NaOH at 66°C; 1.5% (0.38 mM) and 2.0% (0.5 mM) NaOH at 70°C. Each NaOH-temperature combination was tested in three trials, and each trial included duplicate coupons. The percentage of the area covered by EPS was calculated for each trial by averaging the area covered for two stainless steel coupons. The percentage of the area covered for the negative controls (cleaned but containing no biofilm) was subtracted from the percentage of the area covered for the corresponding treated samples.

**Data analysis.** Data were analyzed using SPSS version 10.1 (SPSS, Inc., Chicago, Ill.) to calculate the analysis of variance. Significant differences (*P* = 0.05) between means for biofilm coverage after cleaning treatments was determined using the least significant difference test. We also determined whether there were significant differences in biofilm coverage on the control samples before cleaning treatments commenced.

**RESULTS**

**Biofilm staining.** A cleaning treatment using 1.28% NaOH at 66°C for 3 min as recommended in the Model Pasteurized Milk Ordinance (19) was selected for initial study. Various lectins were tested for their ability to label EPS produced by the *P. putida* biofilm. Lectin PNA conjugated with Alexa Fluor 488 produced the brightest images of biofilm EPS before and after NaOH treatment and was therefore selected for use in subsequent cleaning experiments (data not shown).

Images of biofilms stained with lectin PNA and Hoescht 33658 were obtained from coupons cleaned at 66°C with various concentrations of NaOH, and the per-
TABLE 1. Biofilm coverage on stainless steel before and after cleaning with NaOH at 66°C for 3 min as determined by staining with Hoescht 33258 (cellular coverage) and PNA lectin (peanut agglutinin) for polysaccharide coverage

<table>
<thead>
<tr>
<th>NaOH (%)</th>
<th>Trial</th>
<th>Cellular coverage (% area)</th>
<th>Polysaccharide coverage (% area)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before cleaning&lt;sup&gt;a&lt;/sup&gt;</td>
<td>After cleaning</td>
</tr>
<tr>
<td>1.28</td>
<td>1</td>
<td>32.9 AB</td>
<td>0.05 c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35.3 AB</td>
<td>0.04 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30.7 AB</td>
<td>0.14 c</td>
</tr>
<tr>
<td>2.0</td>
<td>1</td>
<td>33.9 AB</td>
<td>0.26 c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>38.9 B</td>
<td>0.04 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25.7 A</td>
<td>0.05 c</td>
</tr>
<tr>
<td>2.5</td>
<td>1</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
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<tr>
<td></td>
<td>2</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4.0</td>
<td>1</td>
<td>31.6 AB</td>
<td>0.05 c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.1 A</td>
<td>0.15 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25.5 A</td>
<td>0.22 c</td>
</tr>
<tr>
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<td>26.9 A</td>
<td>0.05 c</td>
</tr>
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<td></td>
<td>2</td>
<td>24.6 A</td>
<td>0.05 c</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24.1 A</td>
<td>0.03 c</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data in columns with no common letters differ significantly at P < 0.05.
<sup>b</sup> ND, no data were obtained.

...percentage of area stained by each fluorochrome was calculated. Biofilm coverage before cleaning was variable (Table 1), but there was no correlation between residual coverage and coverage before cleaning. Data from each trial are reported separately because there was a significant difference between trials for the PNA lectin–stained surfaces treated with 2% NaOH (Table 1). Examples of images obtained in these trials are presented in Figure 1. The cleaning treatments removed more Hoescht-binding material (cells) than lectin-binding material (EPS). The Hoescht-binding material was effectively removed (defined by percentage of area covered on the treated coupons not significantly different from the percentage of area covered on the biofilm-free control, P = 0.05) using 1.28% NaOH, but at least 2.5% NaOH was required for removal of lectin-binding substances (Table 1). Because the Hoescht stain is specific for nucleic acids, these results indicate that NaOH at 66°C is more effective at detaching or lysing P. putida cells (thus removing nucleic acids) than it is at removing the associated biofilm EPS. Thus, a surface can appear microbiologically clean but still retain microbial EPS. Because lectin staining was a more sensitive measure of residual biofilm than was...
TABLE 2. Removal of polysaccharides associated with Pseudomonas putida biofilm by treatment with NaOH at 68°C for 3 min

<table>
<thead>
<tr>
<th>NaOH (%)</th>
<th>Trial</th>
<th>Before cleaning (%)</th>
<th>After cleaning (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>1</td>
<td>21.8 A</td>
<td>0.1 A</td>
</tr>
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<td></td>
<td>2</td>
<td>23.1 A</td>
<td>2.5 B</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32.4 B</td>
<td>0.9 A</td>
</tr>
<tr>
<td>2.0</td>
<td>1</td>
<td>25.2 A</td>
<td>0.4 A</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25.8 A</td>
<td>0.2 A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23.4 A</td>
<td>0.8 A</td>
</tr>
</tbody>
</table>

* Determined by staining with PNA lectin. Data in columns with no common letters differ significantly at P < 0.05.

Biofilm removal at 68 and 70°C. Biofilm-containing coupons were treated at 68 and 70°C using 1.5 and 2.0% NaOH. Results from each of three trials are presented in Tables 2 and 3. When coupons were treated at 68°C, one trial using 1.5% NaOH indicated a higher EPS residual than did all the other trials. This high coverage area by residual EPS was not associated with high initial biofilm coverage; coupons in the trial in which the initial coverage was highest (trial 3, 1.5% NaOH) were cleaned as effectively as coupons in the trials starting with less biofilm coverage. Data in Table 2 indicate that treatment with 1.5% NaOH at 68°C for 3 min was marginal in its ability to remove EPS and that increasing the concentration of NaOH to 2.0% was effective for EPS removal.

When treatment temperature was increased to 70°C, results were similar to those obtained at 68°C. However, in the trial with the greatest residual EPS, coverage was only at 1.0%. Although this area percentage was not significantly different from that for the biofilm-free control (which had no detectable EPS coverage), this result indicates marginal cleaning treatment. Data at all temperatures indicate that use of 1.5% NaOH is marginal for removing biofilm EPS even at higher temperatures, whereas use of 2.0% NaOH provided effective removal at temperatures of 68°C and higher, and 2.5% NaOH was effective at 66°C and higher.

EPS removal using commercial cleaners. The commercial alkaline cleaner effectively removed EPS at both 4 and 23°C after 1 min of exposure (Table 4). The neutral cleaner did not effectively remove EPS at 23°C after 30 min of exposure and was therefore not tested at 4°C (Table 4).

**DISCUSSION**

Biofilms can form on processing equipment at protected sites, such as crevices created by joints with gaskets (1). Formation of biofilms at these sites indicates lack of adequate exposure to the cleaning solution at a sufficiently high temperature. It is useful to know whether postcleaning biofilm residues are the result of lack of contact with the cleaning solution or inadequate solution temperature so that appropriate remedial actions can be implemented. Lack of contact with cleaning solution could also indicate lack of contact with sanitizing solution, which creates more problems. Results of this study indicate that solution temperature is critical for NaOH to be effective at dissolving biofilm EPS but that the commercial alkaline cleaning compound, formulated as an NaOH-based solvent gel, was effective at EPS removal even at low temperatures. The quaternary ammonia surfactant-based neutral cleaner was not as effective at EPS removal as was the alkaline cleaner, most likely because the static conditions used in this experiment provided inadequate force to allow the surfactant to react with the EPS. These static application conditions, however, are consistent with low-pressure foam applications used in environmental cleaning. The neutral cleaner and alkaline cleaners were not tested in the high-temperature turbulent system because they were formulated for environmental cleaning using foam or thin-film application.

Exposure to hot 1.28% NaOH for 3 min at 66°C was employed in this study because it is one of several equivalent cleaning treatments recommended in the Food and Drug Administration Model Pasteurized Milk Ordinance (19). Alkaline cleaners typically employed by the food industry contain chlorine and chelator additives, which increase the ability of these cleaners to dissolve biofilm EPS at the temperatures employed in this study (8). Wirtanen et al. (20) observed that chelation with EDTA enhanced biofilm removal by alkaline cleaners. Therefore, current commercially available cleaning solutions used in circulation contact with sanitizing solution, which creates more problems. Results of this study indicate that solution temperature is critical for NaOH to be effective at dissolving biofilm EPS but that the commercial alkaline cleaning compound, formulated as an NaOH-based solvent gel, was effective at EPS removal even at low temperatures. The quaternary ammonia surfactant-based neutral cleaner was not as effective at EPS removal as was the alkaline cleaner, most likely because the static conditions used in this experiment provided inadequate force to allow the surfactant to react with the EPS. These static application conditions, however, are consistent with low-pressure foam applications used in environmental cleaning. The neutral cleaner and alkaline cleaners were not tested in the high-temperature turbulent system because they were formulated for environmental cleaning using foam or thin-film application.

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cleaning can be expected to exceed EPS removal ability of the time-concentration-temperature alkaline conditions employed in this study.

In this study, we demonstrated that biofilm-associated EPS can be considered a type of soil distinct from bacterial cells, and we have defined conditions necessary to remove EPS from a surface. Our observation that biofilm cells are more readily removed from the surface than is EPS differs from the conclusion of Wirtanen et al. (20). However, Wirtanen et al. used nonspecific staining of EPS rather than specific fluorescent labeling.

The ability of low-pressure application of NaOH to effectively remove EPS, as observed in this study, applies only to lightly soiled surfaces containing biofilm of limited development. Surfaces on which extensive biofilms have developed may require mechanical scrubbing or application of high-pressure sprays for removal. Gibson et al. (12) found that commercial cleaning agents were by themselves ineffective at removing biofilms of *Pseudomonas* and *Staphylococcus*. Bredholt et al. (2) also observed that a low-pressure cleaning system using alkaline foam cleaner in combination with a peracetic acid sanitizer did not remove all bacteria from a test surface.

In various studies directed toward controlling biofilms in food processing facilities, researchers have emphasized use of chemical sanitizers to inactivate biofilm bacteria (3, 8). A chemical sanitizer approach to biofilm control is often a response to ineffective cleaning; release of biofilm cells from the surface renders them susceptible to commonly used chemicals (11). Even though some chemical sanitizers are more effective than others at killing intact biofilm microflora, overreliance on a sanitizer approach to biofilm control in the food industry ignores the potential for EPS-food residue accumulation at unclean sites, which over time could make chemical sanitation measures less effective. Dunsmore (5) observed that when a cleaning procedure does not remove all accumulated soils, then repeated cleaning-processing cycles result in soil accumulation and eventually in loss of product quality and safety. The benefit in treating biofilm control primarily as a cleaning problem should be better long-term control over microbial contamination of the product, because clean surfaces are easier and less costly to sanitize. Residual biofilm soil acts as a microbial attachment site that allows rapid reestablishment of the biofilm (17).

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REFERENCES


