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Efficacy of a typical clean-in-place protocol against in vitro membrane biofilms

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ABSTRACT

This study evaluates the effectiveness of a typical clean-in-place (CIP) protocol against in vitro biofilms on whey reverse osmosis (RO) membranes developed under static condition. Bacterial isolates obtained from RO membrane biofilms were used to develop single and multispecies biofilms under laboratory conditions. A typical commercial CIP protocol was tested against the 24-h-old biofilms, and included 6 sequential treatment steps based on alkali, surfactant, acid, enzyme, a second surfactant, and a sanitizer treatment step. Experiments were conducted in 4 replicates and the data were statistically analyzed. The results revealed a variation in the resistance of mixed-species biofilms against the individual steps in the sequential CIP protocol. The overall 6 steps protocol, although resulted in a greater reduction, also resulted in the detection of survivors even after the final sanitizer step, reflect the ineffectiveness of the CIP protocol for complete removal of biofilms. Posttreatment counts of 0.71 log after the sequential CIP of mixed-species biofilm revealed the resistance of biofilm constitutive microbiota. Mixed-species biofilms, constituting different genera including *Bacillus*, *Staphylococcus*, and *Streptococcus*, were observed to be more resistant than most of the single-species biofilms. However, among the single-species biofilms, significantly different resistance pattern was observed for *Bacillus* isolates compared with the other bacterial isolates. All 5 isolates of *Bacillus* were found resistant with survivor counts of more than 1.0 log against the sequential CIP protocol tested. Thus, it can be concluded that the tested CIP protocol had a limited effectiveness to clean membrane biofilms formed on the whey RO membranes.
Key words: biofilms, whey, reverse osmosis, CIP

INTRODUCTION

Pressure-driven membrane filtration technologies are widely used for the processing of whey produced during cheesemaking. Fouling to membrane surfaces, either because of solid deposition or microbial attachment, affects the performance of membranes in terms of flux rate (Daufin et al., 1991; Melo et al., 1992; Ridgway et al., 1999; Chang et al., 2002; Ivnitsky et al., 2007; Susanto and Ulbricht, 2007; Hassan et al., 2010; Anand et al., 2012; Marka and Anand, 2018). Development of biofilm on equipment surfaces can enhance the corrosion and reduce the heat transfer to heat exchangers (Yuan and Pehkonen, 2007). Bacterial cells attach with the surface using flagella and pilli to form the biofilms (van Pelt et al., 1985; van Loosdrecht et al., 1987; Dang and Lovell, 2000). Structure of biofilms is mostly comprised of proteins and polysaccharides. Exopolysaccharides produced by bacterial cells provide survivability against biocides, antimicrobials, and disinfectants (Stewart et al., 2000; Spoering and Lewis, 2001; Donlan and Costerton, 2002). Bacterial irreversible attachment with the surface along with various biological, physical, and chemical growth factors affect the development of biofilm (Gésan-Guiziou et al., 1999; Donlan, 2002). These include substratum properties (roughness and hydrophobicity), type of feed (Marka and Anand, 2018), a conditioning film of macromolecules on the surface, system hydrodynamics, medium characteristics (ionic strength, pH, and the presence of multivalent cations), and cell surface properties (hydrophobicity, expression of flagella and pilli, lipopolysaccharides, and exopolysaccharides; Costerton et al., 1985; Herzberg and Elimelech, 2008; MSU, 2008).

Previous studies illustrated multispecies biofilms including species of *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Escherichia*, *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Streptococcus*, and *Aeromonas* isolated from whey processing reverse osmosis (**RO**) membranes (Biswas et al., 2010; Avadhanula, 2011; Anand and Singh, 2013). Biofilms formed by thermophilic *Streptococcus* species were present with pasteur-

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izers (Flint et al., 1999; Knight et al., 2004), whereas the heat-resistant *Bacillus* species were noticed in milk powder (Flint et al., 2001; Ronimus et al., 2003). Multispecies biofilms developed on different surfaces (Kawarai et al., 2007; Macleod and Stickler, 2007) were reported to be of different nature (Jones et al., 1969; Hassan et al., 2010; Tang, 2011) and more resistant against cleaning agents compared with the biofilms developed using individual bacteria (Ridgway et al., 1983; Kumar and Anand, 1998; Tang et al., 2009).

Cleaning of membrane surfaces for removal of biofilms is performed using circulation of alkali, acids, metal chelating agents, surfactants, and enzymes, using more favorable physical conditions (Thurman, 1985; Rosen, 1989; Tragårdh, 1989; Hong and Elimelech, 1997; Liikanen et al., 2002; Meyer, 2003; Mohammadi et al., 2003; Ang et al., 2006) at certain temperature, time, and flow within closed process equipment without dismantling. A review paper based on typical membrane cleaning techniques, published by Anand et al. (2014), described the importance of each individual clean-in-place (**CIP**) chemicals. Anionic surfactants interact with whey proteins to decrease the surface tension of molecules in contact with each other. Utilization of a combination of enzymes (especially proteases and polysaccharide hydrolyzing enzymes) was considered an effective formulation for removal of biofilm matrix from the membrane surface (Meyer, 2003). Cleaning efficiency can be affected using variety of enzymes by degrading the polymeric foulants (Sutherland, 1995; Fernández García et al., 2013). Effectiveness of cleaning should be evaluated by the number of survivor cells after each step of cleaning and based on the cell residue on the surface (Parkar et al., 2004). Therefore, there is a need to understand the resistance pattern of biofilms based on number of survivor cells against cleaning steps to be used on membrane surfaces.

The present study aimed to evaluate the effectiveness of sequential cleaning steps of a typical CIP protocol against biofilm-embedded cells under static conditions. The resistant biofilm isolates, obtained from 2- to 14-mo-old membrane biofilm consortia using RO membranes (Anand et al., 2012; Anand and Singh, 2013), were used to develop single and mixed-species biofilms, under in vitro conditions, for screening of different chemicals as sequential cleaning steps of an existing CIP protocol. The objective of this study was to understand the resistance pattern of individual isolates within a biofilm consortium, when treated against 6 sequential cleaning steps of a typical CIP protocol.

Table 1. Distribution of constitutive microbiota obtained from the biofilms on the retentate side of used reverse-osmosis whey concentration membranes (2 to 14 mo old)

Membrane consortium	Membrane age (mo)	Biofilm constitutive microbiota
1	2	1.2.1 *Enterococcus* sp.
		1.2.2 *Staphylococcus* sp.
		1.2.3 *Micrococcus* sp.
2	4	2.4.1 *Enterococcus* sp.
		2.4.2 *Klebsiella* sp.
		2.4.3 *Bacillus* sp.
		2.4.4 *Corynebacterium* sp.
3	6	3.6.1 *Enterococcus* sp.
		3.6.2 *Aeromonas* sp.
		3.6.3 *Bacillus* sp.
4	8	4.8.1 *Enterococcus* sp.
		4.8.2 *Staphylococcus* sp.
		4.8.3 *Bacillus* sp.
		4.8.4 *Corynebacterium* sp.
		4.8.5 *Escherichia coli*
		4.8.6 *Pseudomonas* sp.
5	10	5.10.1 *Streptococcus* sp.
		5.10.2 *Staphylococcus* sp.
		5.10.3 *Bacillus* sp.
6	12	6.12.1 *Escherichia coli*
		6.12.2 *Klebsiella* sp.
		6.12.3 *Bacillus* sp.
7	14	7.14.1 *Enterococcus* sp.
		7.14.2 *Staphylococcus* sp.
		7.14.3 *Escherichia coli*
		7.14.4 *Klebsiella* sp.

MATERIALS AND METHODS

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

Source of Bacterial Isolates

During a previous study in our laboratory, 2- to 14-mo-old RO whey concentration membranes were obtained from a commercial cheese plant (Anand et al., 2012) for isolating biofilm-embedded bacteria, using standard methods (Wehr and Frank, 2004), that were divided into 7 consortia (Table 1). For identification, the isolates were outsourced for biotyping at Veterinary Science Department (South Dakota State University). Ten different genera with a total number of 26 different isolates were finally selected (Table 1) and were referred as biofilm isolates in the present investigation. The isolates were coded as 3 digits to specify the consortium, age of membrane, and the isolate, respectively. The biofilm isolates as listed in our previous work (Anand and Singh, 2013) were stored in cryovials at −80°C in a deep freezer (Nuaire), activated in 10 mL of Brain Heart Infusion broth (Difco Laboratories Inc.), and used for biofilm development.

Table 2. The existing clean-in-place (CIP) protocol being used by a reverse-osmosis whey concentration plant

Step number	CIP steps in sequence	Temperature (°C)	Target pH range[1]	Time (min)
1	Alkali rinse	50	11.0–11.4	12
2	Surfactant 1	50	11.0–11.4	30
3	Acid	50	1.9–2.3	30
4	Enzyme	50	10.5–11.0	45
5	Surfactant 2	50	11.0–11.4	10
6	Sanitizer	21.1	3.0 –4.0	1

[1]Target pH range of CIP chemicals used by the dairy plant.

Preparation of Cell Suspensions

The biofilm isolates were activated by 3 transfers in Brain Heart Infusion broth, followed by incubating at 37°C. The overnight grown cultures were pelleted by centrifugation (2,900 × g) for 15 min. The cell pellet was washed twice with sterile PBS (pH 7.0) and finally suspended in 1 to 2 mL of sterile PBS. The required amount of the bacterial suspension was added to 10 mL of sterile phosphate buffer to obtain the optical density of the final solution, around 0.3 at a wavelength of 600 nm. This bacterial suspension was serially diluted using 9.0 mL of sterile PBS and plated on the plate count agar (Difco Laboratories Inc.) to enumerate the viable counts expressed as $\log_{10}$ colony-forming units per milliliter.

Development of Biofilms Under In Vitro Static Conditions

An unused spiral wound RO membrane was cut into small pieces (3 cm × 3 cm) using an electric cutter (powered hand saw, Black and Decker) under aseptic conditions, and sterile cheese whey was used as a medium to grow single- and mixed-species biofilms (Anand and Singh, 2013) under static conditions. In case of mixed-species biofilms, the isolates of a particular consortium (Table 1) were mixed in equal ratio and added to the whey medium to get a final level of about 7.0 log cfu/mL. The biofilms were developed in a manner similar to single-species biofilms by incubating at 37°C for 24 h. The objective of the in vitro biofilm was to develop this in 24 h of time and perform CIP to simulate the industrial whey processing. All the isolates obtained from the old RO membrane were able to grow at an optimum temperature of 37°C. This temperature of 37°C was selected for the experiment to have adequate biofilm development within 24 h.

Effectiveness of Complete CIP Cycles in Cleaning Biofilms Under Static Conditions

The 6 steps of the CIP protocol followed in this study were similar to the one detailed in our previous study (Anand and Singh, 2013). In brief, the 6 steps included alkali rinse, surfactant 1, acid, enzyme, surfactant 2, and sanitizer treatments (Table 2). The sequential cleaning process for single- and mixed-species biofilms included all 6 steps of a typical CIP cycle (Table 2), as previously described (Anand and Singh, 2013). All chemicals were obtained from the commercial dairy plant and tested in accordance with the cleaning process being used in the day-to-day operations of the dairy plant. The enzyme used for the experiment was basically protease-based enzyme obtained from the commercial whey processing facility to be used for cleaning of RO membranes. The routine membrane cleaning process was conducted at a temperature of 50°C, whereas the sanitizer treatment was performed at 21.1°C. The concentrated chemical solutions obtained from the dairy plant were diluted with distilled water to maintain the pH as per the recommended CIP protocol (Table 2).

Treated biofilms were swabbed, and viable cells were enumerated by serial dilution and plating method. Sterile neutralized phosphate buffer was used as a diluent, and plating was performed using plate count agar (Thermo Fisher Scientific). The plates were incubated at 37°C for 24 h, and the results were expressed as colony-forming units per square centimeter of the membrane samples (Laird et al., 2004). The final counts were presented as $\log_{10}$ values in the ensuing data. The posttreatment counts were enumerated and compared with their respective pretreatment counts. All the experiments were conducted in duplicate and repeated 2 times, resulting in a total of 4 replicates for each test.

Statistical Analysis

Data were analyzed by ANOVA using the general linear-models procedure of the SAS statistical analysis software package (Version 8, 1999, SAS Institute Inc.), and means were compared using the Tukey test. Differences in all experiments were considered significant at $P < 0.05$. The experiment was performed in duplicates and repeated 2 times with a total number of 4 replicates for each of the experiment.

Table 3. Posttreatment counts of membrane biofilm ($\log_{10}$ cfu/cm^2; presented as mean ± SD) in embedded state (24-h-old mixed-species biofilms[1]) by sequential application of clean-in-place (CIP) chemicals[2] under static conditions

Chemical	Consortium 1	2	3	4	5	6	7
Pretreatment count	5.24 ± 0.12	5.17 ± 0.18	5.30 ± 0.11	5.28 ± 0.06	5.19 ± 0.16	5.13 ± 0.07	5.26 ± 0.19
CIP step (sequential)	Posttreatment count ($\log_{10}$ cfu/cm^2) after sequential treatment step						
Step 1, alkali	3.13 ± 0.01^{a,E}	3.24 ± 0.13^{a,E}	3.27 ± 0.02^{a,D}	3.25 ± 0.02^{a,E}	3.28 ± 0.05^{a,D}	3.18 ± 0.04^{a,D}	3.17 ± 0.04^{a,D}
Step 2, surfactant 1	2.64 ± 0.00^{a,D}	2.69 ± 0.02^{a,D}	2.61 ± 0.03^{a,C}	2.57 ± 0.08^{a,D}	2.74 ± 0.07^{a,C}	2.67 ± 0.01^{a,C}	2.47 ± 0.11^{a,C}
Step 3, acid	0.59 ± 0.03^{a,C}	0.83 ± 0.06^{b,C}	0.85 ± 0.18^{b,B}	0.78 ± 0.03^{b,C}	0.98 ± 0.02^{c,B}	0.88 ± 0.10^{bc,B}	0.85 ± 0.00^{b,B}
Step 4, enzyme	0.36 ± 0.11^{a,B}	0.67 ± 0.03^{b,C}	0.68 ± 0.15^{ab,AB}	0.58 ± 0.04^{ab,B}	0.91 ± 0.04^{c,B}	0.83 ± 0.21^{bc,B}	0.78 ± 0.04^{b,B}
Step 5, surfactant 2	0.28 ± 0.11^{a,B}	0.45 ± 0.05^{ab,B}	0.43 ± 0.14^{ab,A}	0.47 ± 0.04^{ab,AB}	0.83 ± 0.00^{c,AB}	0.67 ± 0.11^{b,AB}	0.53 ± 0.06^{b,B}
Step 6, sanitizer	0.05 ± 0.00^{a,A}	0.20 ± 0.00^{b,A}	0.32 ± 0.17^{b,A}	0.28 ± 0.11^{b,A}	0.71 ± 0.02^{c,A}	0.52 ± 0.11^{bc,A}	0.44 ± 0.12^{b,A}

[a–c]Means within the same row not sharing a common superscript are significantly different ($P < 0.05$).

[A–E]Means within the same column for individual consortia not sharing a common superscript are significantly different ($P < 0.05$).

[1]Mixed consortium biofilms were developed using respective isolates of 2- to 14-mo consortia at an interval of 2 mo.

[2]Existing CIP protocol was followed.

RESULTS AND DISCUSSION

Effect of Sequential Cleaning Steps Against 24-Hour-Old Mixed-Species Biofilms Under Static Conditions

To simulate the industrial cleaning protocol, the CIP steps were evaluated in a sequential manner against the 24-h-old biofilms developed using the 7 individual consortia (Table 1), with an initial count of 5.13 to 5.30 log. After each treatment step, the posttreatment counts were calculated and statistically analyzed (Table 3).

In the case of biofilms developed using consortium 1, after the alkali treatment (first step of CIP), the initial counts reduced to 3.13 log, whereas, after next step of treatment with surfactant 1, the posttreatment counts were observed to be 2.64 log. After the next sequential treatment with acid (step 3), the posttreatment counts for the consortium 1 were only 0.59 log. With the remaining steps after the acid treatment, the cells were further reduced. The posttreatment counts were 0.36 and 0.28 log after enzyme treatment and cleaning with surfactant 2, respectively. The final counts after the sanitizer treatment steps were observed to be 0.05 log. This revealed the survival of biofilm-embedded bacterial cells, even after the completion of all 6 steps of the tested CIP protocol. Survivability of cells after the CIP is likely due to variations in resistance of the constitutive bacterial species of each consortium. Some of those bacterial cells could be resistant to 1 or more chemical compounds used at each CIP step.

The sequential CIP was also evaluated against other consortia as well, in a similar manner. For each of the consortium, the survivability of cells was observed after all 6 sequential steps of the tested CIP protocol. The higher posttreatment counts were observed in older biofilms, with 0.71 log of survivors for the consortium 5 (obtained from the 10-mo-old membrane). One of the possible reasons for resistance of mixed-species biofilms may be due to the stable biofilm matrix formed by mixed bacterial cells (Jones et al., 1969). Our previous studies also revealed the greater resistance of some of the isolates in older biofilms against CIP chemicals (Anand and Singh, 2013).

Presence of some viable cells, even after all the steps of CIP protocol in a sequential manner, indicated the limited effectiveness of the tested cleaning process. Another observation of significance is that the steps after the acid treatment (step 3), did not show substantial effectiveness against biofilms, indicating that these steps were not as efficient as they were anticipated to be. Survivability of microbes against different cleaning agents may possibly be due to conversion of microbial irreversible fouling to the 3-dimensional biofilm matrix on the surface (Stoodley et al., 2002). Also, the bacterial adherence is reported to be influenced by the microbial strain and by the hydrophobicity of different surfaces (Araújo et al., 2009). Cell attachment with the surface was reported to be influenced by proteins present in the cell wall of lactococci (Habimana et al., 2011). Based on the previous studies the application of enzymes in any typical cleaning process would hydrolyze the exopolysaccharide matrix, in which the microorganisms are embedded (Whittaker et al., 1984; Böckelmann et al., 2003). However, chlorine-based sanitizers were reported to be not so effective against the embedded cells (Frank and Chmielewski, 1997). The results from this part of the study thus established that the sequential steps-based evaluation is a more accurate measure to evaluate the effectiveness of any CIP process against in vitro membrane biofilms.

Table 4. Posttreatment counts of membrane biofilm (log_{10} cfu/cm^2; presented as mean ± SD) in embedded state (24-h-old individual biofilms of representative resistant isolate[1]) by sequential application of clean-in-place (CIP) chemicals[2] under static conditions

Chemical	*Bacillus* sp. (5.10.3)	*Enterococcus* sp. (4.8.1)	*Staphylococcus* sp. (5.10.2)	*Klebsiella* sp. (6.12.2)	*Escherichia coli* (6.12.1)	*Corynebacterium* sp. (4.8.4)
Pretreatment count	3.39 ± 0.21	5.42 ± 0.37	5.53 ± 0.21	5.44 ± 0.26	5.13 ± 0.28	5.18 ± 0.18
CIP step (sequential)	Posttreatment count (log_{10} cfu/cm^2) after sequential treatment step					
Step 1, alkali	2.20 ± 0.09^{a,B}	3.00 ± 0.09^{c,D}	3.09 ± 0.11^{c,F}	3.04 ± 0.07^{c,D}	2.56 ± 0.22^{b,D}	2.81 ± 0.01^{b,E}
Step 2, surfactant 1	2.02 ± 0.08^{b,B}	2.15 ± 0.20^{b,C}	2.64 ± 0.06^{c,E}	2.51 ± 0.17^{bc,C}	1.60 ± 0.06^{a,D}	2.22 ± 0.06^{b,D}
Step 3, acid	1.35 ± 0.04^{c,A}	0.89 ± 0.06^{b,B}	0.84 ± 0.04^{b,D}	0.89 ± 0.10^{b,B}	0.62 ± 0.04^{a,B}	0.82 ± 0.21^{ab,C}
Step 4, enzyme	1.26 ± 0.01^{c,A}	0.39 ± 0.16^{ab,A}	0.63 ± 0.04^{b,C}	0.67 ± 0.03^{b,B}	0.24 ± 0.00^{a,A}	0.51 ± 0.25^{ab,BC}
Step 5, surfactant 2	1.20 ± 0.03^{b,A}	—[3]	0.36 ± 0.11^{a,B}	0.28 ± 0.11^{a,A}	—	0.31 ± 0.15^{a,B}
Step 6, sanitizer	1.13 ± 0.03^{b,A}	—	0.05 ± 0.00^{a,A}	0.10 ± 0.02^{a,A}	—	0.05 ± 0.00^{a,A}

[a–c]Means within the same row not sharing a common superscript are significantly different ($P < 0.05$).

[A–F]Means within the same column for individual microorganisms not sharing a common superscript are significantly different ($P < 0.05$).

[1]Different genera were isolated from 2- to 14-mo consortia at an interval of 2 mo.

[2]Existing CIP protocol was followed.

[3]Dashes indicate that all detectable culturable cells were inactivated.

Effect of Sequential Cleaning Steps Against 24-Hour-Old Single-Species Biofilms Under Static Conditions

Further studies were conducted to observe the effect of the sequential CIP protocol against the biofilms of individual isolates under static conditions. This also helped to confirm the most resistant organism among the entire constitutive microbiota in the 7 consortia tested. To evaluate the efficacy of the sequential CIP protocol, isolates occurring most frequently in different consortia, such as *Bacillus*, *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Escherichia coli*, and *Corynebacterium*, were selected to form individual in vitro biofilms on membranes. The pretreatment counts and posttreatment cumulative reductions for different microbiota are presented in Table 4.

Among the individual biofilm microbiota, *Bacillus* species were found to be most resistant of all. The following narrative discusses this unique aspect of the greater survival of *Bacillus* species within the constitutive microbiota of different consortia. The pretreatment counts for *Bacillus* was 3.39 log, which reduced to 2.2 log against the first step of cleaning. The next cleaning step of surfactant 1 resulted into a cumulative posttreatment count of 2.02 log, whereas, after acid treatment, the cumulative posttreatment count was 1.35 log for *Bacillus* sp. The next 3 steps of enzyme, surfactant, and sanitizer resulted in cumulative posttreatment counts of 1.26, 1.20, and 1.13 log, respectively. After all the 6 steps of CIP protocol, we found that more than 1 log of resistant cells of *Bacillus* remained viable in the biofilm matrix.

In comparison, a complete inactivation was observed for *Enterococcus* sp. and *E. coli* after fifth step of cleaning. The pretreatment counts for *Enterococcus* sp. was 5.42 log, which reduced to 3.0 log against the first step of cleaning. The next cleaning step of surfactant 1 resulted into a cumulative posttreatment count of 2.15 log, whereas, after acid and enzyme treatment, the cumulative posttreatment counts were 0.89 and 0.39 log. We found no survivor cells after the next CIP step of surfactant for *Enterococcus* sp. The pretreatment counts for *E. coli* were 5.13 log, which reduced to 2.56 log against the first step of cleaning. The next cleaning step of surfactant 1 resulted into a cumulative posttreatment count of 1.6 log, whereas, after acid and enzyme treatment, the cumulative posttreatment counts were 0.62 and 0.24 log. We found no survivor cells after the next CIP step of surfactant for *E. coli* isolate.

Substantial cumulative reduction patterns were also observed for *Staphylococcus*, *Klebsiella*, and *Corynebacterium* species after applying all the cleaning steps, including sanitizer treatment; however, none of them were completely inactivated. The pretreatment counts for *Staphylococcus* sp. was 5.53 log, which reduced to 3.09 log against the first step of cleaning. The next cleaning step of surfactant 1 resulted into a cumulative posttreatment count of 2.64 log, whereas, after acid treatment, the cumulative posttreatment counts were 0.84 log for *Staphylococcus* sp. The next 3 steps of enzyme, surfactant, and sanitizer resulted in cumulative posttreatment counts of 0.63, 0.36, and 0.05 log, respectively. Low number of survivor cells were found after all the 6 steps of sequential CIP with *Klebsiella* and *Corynebacterium* species, similar to *Staphylococcus* sp.

Based on the comparison of posttreatment cumulative counts, *Bacillus* was observed to be the most resistant isolate, which remained viable with 1.13 log counts, even after the application of sanitizer treatment. Previous experiments related to the individual steps of cleaning also provided evidence with regard to

Table 5. Posttreatment counts of membrane biofilm isolates ($\log_{10}$ cfu/cm^2; presented as mean ± SD) in embedded state (24-h-old *Bacillus* isolate biofilms[1]) by sequential application of clean-in-place (CIP) chemicals[2] under static conditions

Chemical	First isolate (2.4.3)	Second isolate (3.6.3)	Third isolate (4.8.3)	Fourth isolate (5.10.3)	Fifth isolate (6.12.3)
Pretreatment count	3.76 ± 0.18	4.07 ± 0.21	3.88 ± 0.28	3.39 ± 0.21	3.91 ± 0.15
CIP step (sequential)	Posttreatment count ($\log_{10}$ cfu/cm^2) after sequential treatment step				
Step 1, alkali	$2.20 \pm 0.06^{a,D}$	$2.68 \pm 0.06^{b,D}$	$2.33 \pm 0.23^{a,D}$	$2.20 \pm 0.09^{a,D}$	$2.36 \pm 0.04^{a,D}$
Step 2, surfactant 1	$1.98 \pm 0.06^{a,C}$	$2.38 \pm 0.03^{b,C}$	$2.04 \pm 0.13^{a,C}$	$2.02 \pm 0.08^{a,C}$	$2.19 \pm 0.01^{a,C}$
Step 3, acid	$1.44 \pm 0.09^{a,B}$	$1.56 \pm 0.04^{a,B}$	$1.39 \pm 0.13^{a,B}$	$1.35 \pm 0.04^{a,B}$	$1.55 \pm 0.06^{a,B}$
Step 4, enzyme	$1.32 \pm 0.09^{a,AB}$	$1.42 \pm 0.04^{a,B}$	$1.22 \pm 0.11^{a,AB}$	$1.26 \pm 0.01^{a,B}$	$1.41 \pm 0.01^{a,B}$
Step 5, surfactant 2	$1.20 \pm 0.11^{a,AB}$	$1.34 \pm 0.02^{a,AB}$	$1.15 \pm 0.10^{a,AB}$	$1.20 \pm 0.03^{a,AB}$	$1.29 \pm 0.00^{a,A}$
Step 6, sanitizer	$1.11 \pm 0.04^{a,A}$	$1.25 \pm 0.01^{a,A}$	$1.05 \pm 0.04^{a,A}$	$1.13 \pm 0.03^{a,A}$	$1.21 \pm 0.05^{a,A}$

[a,b]Means within the same row not sharing a common superscript are significantly different ($P < 0.05$).

[A–D]Means within the same column for individual isolates not sharing a common superscript are significantly different ($P < 0.05$).

[1]Five *Bacillus* isolates were obtained from 4- to 12-mo consortia at an interval of 2 mo.

[2]Existing CIP protocol was followed.

the resistance of *Bacillus* in planktonic- and biofilm-embedded cell states (Anand and Singh, 2013). The resistance of *Bacillus* against CIP chemicals revealed that the isolates of this genus were the most resistant among the entire constitutive microbiota. Previous researchers have also identified the presence of spore-forming thermoresistant species, *Bacillus* sp., on whey processing surfaces (Schreiber, 2001). The biofilms produced by spore formers are difficult to clean and cause cross contamination to the dairy products. The prolonged existence of heat-resistant spore-forming *Bacillus* sp. was reported to affect the whey ultrafiltration membranes (Chamberland et al., 2017). Biofilms developed by *Pseudomonas* sp. were noticed to be resistant against different chemicals (Pagedar and Singh, 2015). In another biofilm-related study, *Bacillus subtilis* was established as a predominant strain within a used RO membrane biofilm consortium (Verma et al., 2021), and an antibacterial substance was also identified as one of the factors responsible for the predominance of the *B. subtilis* isolate (Verma and Anand, 2020).

Effect of Sequential Cleaning Against 24-Hour-Old Biofilms of Bacillus Isolates Under Static Conditions

As the *Bacillus* species emerged as the most resistant genus among the entire constitutive microbiota of 7 consortia tested, further studies were carried out to select the most resistant isolate among all the 5 isolates of *Bacillus*. Such a screening for the most resistant isolate was considered to be useful as a test organism for any future modifications in the cleaning process. Keeping this in mind, the present study was conducted using 5 *Bacillus* isolates obtained from different consortia. Individual biofilms were developed using these isolates and treated against the sequential CIP protocol steps, as explained above. The pretreatment counts and posttreatment cumulative reductions are presented in Table 5.

The results indicated that all the 5 isolates of *Bacillus* had similar resistance pattern against the existing CIP protocol. After treatment with alkali (step 1), the first isolate of consortium 2 remained viable with a count of 2.20 log, whereas, after second step of surfactant 1, the cumulative posttreatment count was 1.98 log out of 3.76 log pretreatment counts. After acid treatment, the survivor count was 1.44 log. Enzyme, surfactant 2, and sanitizer treatments were not effective and resulted in posttreatment counts of 1.32, 1.20, and 1.11 log, respectively. Even after sanitizer treatment (step 6), we found some survivors for all the *Bacillus* isolates. The cumulative posttreatment count for *Bacillus* isolate (5.10.3) obtained from a 10-mo-old membrane consortium was 1.13 log out of 3.39 log. Some of the previous studies also concluded *Bacillus cereus* as important postpasteurization contaminants in the dairy industry, because of their ability to form spores (Flint et al., 1997; Svensson et al., 2004; Lindsay et al., 2006). Moreover, the attachment of spores to food contact surfaces is greater than vegetative cells, due to hydrophobicity and hair-like structures on cell surface (Ronner et al., 1990; Husmark and Ronner, 1992; Kumar and Anand, 1998). Spores were also found to be resistant to cleaning regimen (Lindsay et al., 2006).

CONCLUSIONS

The present study concluded that multispecies biofilms developed for all the 7 consortia and treated against the sequential CIP protocol revealed the presence of survivors, even after all the steps of cleaning, and hence the ineffectiveness of the tested CIP protocol. The results also concluded a similar pattern for all the membrane consortia, with older consortia demonstrat-

ing greater resistance. Based on the results for sequential CIP protocol applied against single-species biofilms under static conditions, the complete inactivation of *Enterococcus* and *Escherichia coli* was noticed, whereas maximum resistance was observed for *Bacillus* isolates. Lower resistance was observed for the biofilms of *Staphylococcus*, *Klebsiella*, and *Corynebacterium*. This study supported our previous findings with individual cleaning steps that *Bacillus* was the most resistant isolate among entire constitutive microbiota. Studies further illustrated a similar pattern of resistance for all 5 isolates of *Bacillus*. Resistance of 24-h-old biofilms against CIP chemicals under static conditions revealed the irreversible nature of biofilm formed by isolates. Further studies are being conducted under a dynamic condition using a Centers for Disease Control biofilm reactor. This study illustrated the requirement to modify the existing cleaning with a focus to break down the biofilm matrix using proper enzymes.

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