J. Dairy Sci. 96:1–10
http://dx.doi.org/10.3168/jds.2013-7012

Resistance of the constitutive microflora of biofilms formed on whey reverse-osmosis membranes to individual cleaning steps of a typical clean-in-place protocol

Sanjeev Anand[1] and Diwakar Singh[2]
Midwest Dairy Foods Research Center, Dairy Science Department, South Dakota State University, Brookings 57006

ABSTRACT

This experiment evaluates the effectiveness of individual steps of a clean-in-place protocol against the biofilm constitutive microflora isolated from the biofilms developed on whey reverse-osmosis membranes, aged 2 to 14 mo, under industrial processing conditions. The isolates used for the in vitro resistance studies included species of *Bacillus*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Aeromonas*, *Corynebacterium*, *Pseudomonas*, *Klebsiella*, and *Escherichia*. The 6 cleaning steps (alkali, surfactant, acid, enzyme, a second surfactant, and sanitizer treatment) revealed resistance of isolates in both planktonic and biofilm-embedded cell states. The most effective step was the acid treatment, which resulted in 4.54 to 7.90 and 2.09 to 5.02 log reductions of the planktonic and biofilm-embedded cells, respectively. Although the sanitizer step causing a reduction of 4.91 to 8.33 log in the case of planktonic cells, it was less effective against the biofilm-embedded cells, resulting in a reduction of 0.59 to 1.64 log. *Bacillus* spp. showed the highest resistance in both planktonic, as well as embedded cell states.

Key words: biofilm, reverse osmosis, membrane, cleaning

INTRODUCTION

Previous studies in our laboratory demonstrated the presence of bacterial biofilms on whey reverse-osmosis (**RO**) membranes obtained at 2-mo intervals during a total of 14 mo of whey-processing operations. These multispecies biofilms had, on average, about 5.0 log counts that constituted diverse bacterial species, including *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Escherichia*, *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Micrococcus*, *Streptococcus*, and *Aeromonas* (Biswas et al., 2010; Hassan et al., 2010; Anand et al., 2012). These biofilms developed on RO membranes despite regular clean-in-place (**CIP**) protocols followed by the whey processing plant, carried out under dynamic flow conditions [around 300–400 psi (2,068.43–2,757.90 kPa) and 80–100 GPM (302.83–378.54 L/min)]. A typical membrane-cleaning process includes the application of alkaline solution, acids, metal chelating agents, surfactants, and enzymes (Tragardh, 1989; Mohammadi et al., 2002). Chelating agents bind metal ions from the complex organic molecules, with increased effectiveness of cleaning (Hong and Elimelech, 1997), whereas surfactants remove the foulants by solubilizing macromolecules by forming micelles around them (Rosen, 1987). Addition of enzymes provided enhanced cleaning efficiency by breaking proteinaceous materials and polymeric foulants (Sutherland, 1995). Resistance of biofilm-embedded bacteria to cleaning processes has also been previously reported to be different from that of their planktonic counterparts (Sternberg et al., 1999; Loo et al., 2000; Stewart and Costerton, 2001; Donlan and Costerton, 2002; Keevil, 2002; Sauer et al., 2002; Chmielewski and Frank, 2003; Shi and Zhu, 2009). These enhanced resistances of biofilm-entrapped cells are due to their different transcriptional programs (Asad and Opal, 2008) and their complex distribution (Stoodley et al., 2002). To inactivate these biofilm-embedded cells, it is essential for the sanitizer to penetrate first the surrounding polysaccharide material. Bacterial cells attached to biofilms were reported to be about 1,000 times more resistant to antimicrobial stress than free-flowing bacteria of the same species. Studies have also indicated that selection of high persisters within biofilm matrices may also be responsible for the recalcitrance to antimicrobials (Lewis, 2010). This resistance capacity was shown to be dependent on the type of organism and the antimicrobial system (Lewis, 2001; Mah and O'Toole, 2001; Stewart, 2002). The high tolerance of mature biofilms to chlorine-based sanitizers and antimicrobials was reported to be due to their lower penetration into the biofilm matrix (Gilbert et al.,

Received May 8, 2013.
Accepted June 9, 2013.
[1]Corresponding author: Sanjeev.anand@sdstate.edu
[2]Current affiliation: Jerome Cheese Company, a division of Davisco Foods International Inc., Jerome, ID 83338.

1990; Marshall, 1992; Stewart, 2002). The other related factors are the biofilm maturation stage (Drenkard, 2003), lower growth rate, and the associated changes in the cell physiology (Stewart, 2002; Shah et al., 2006). Chlorine transport was shown to improve due to weakening of the biomass at the periphery of cell clusters by increased fluid shearing (Davison et al., 2010). Corbin et al. (2011) concluded that the in vitro time for activity against biofilm was much longer than diffusive penetration time, using an in vitro oral biofilm model. In addition, microbial activity was enhanced with biofilm formation, facilitating a protective shield against environmental stresses such as desiccation, starvation, or the presence of heavy metals.

The presence of multispecies biofilms on whey RO membranes observed in our previous study (Anand et al., 2012) led us to hypothesize the enhanced resistance of constitutive microflora to typical CIP protocols as the membranes age. The present study evaluates the effectiveness of individual cleaning steps of CIP protocols against both planktonic and biofilm-embedded cells. Although no set standards exist for logarithmic reductions, attempts were made to simulate the biofilms developed under industrial conditions by achieving embedded cell levels up to 5.0 log using membrane biofilm isolates. These resistant isolates were obtained from membrane biofilm consortia of 2- to 14-mo-old used membrane cartridges. This study also helps identify the most effective cleaning steps of a typical CIP protocol.

MATERIALS AND METHODS

Source of Bacterial Isolates

Used whey RO membranes were aseptically collected from a commercial dairy plant at intervals of 2, 4, 6, 8, 10, 12, and 14 mo (Anand et al., 2012). Standard microbiological methods were used to isolate the constitutive microflora of biofilms (Wehr and Frank, 2004). For further identification to genus level, the isolates were biotyped using microbiological culturing techniques and biochemical identification protocols in the Veterinary Science Department, South Dakota State University (Brookings). A total of 26 isolates, belonging to 10 genera, were finally selected (Table 1) and termed biofilm isolates in this experiment. The isolates were coded as 3 digits to represent consortium, age of membrane cartridge, and isolate number, respectively.

Maintenance of the Isolates

The bacterial isolates, as listed in Table 1, were stored in Cryovials (CRYO/B; Copan Diagnostics Inc., Murrieta, CA) at −80°C (−112°F) in a deep freezer

Table 1. Biofilm constitutive microflora on the retentate side of whey reverse-osmosis (RO) membranes

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