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Biofilm Formation and Biocides Sensitivity of *Pseudomonas marginalis* Isolated from a Maple Sap Collection System

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ABSTRACT

The susceptibility of planktonic and biofilm cells of *Pseudomonas marginalis* toward four commonly used biocides at different temperatures (15 and 30°C) and biofilm growth times (24 and 48 h) was assessed. Using the MBEC biofilm device, biofilm production in maple sap was shown to be highly reproducible for each set of conditions tested. Biofilm formation was influenced by growth temperature and time. A temperature of 15°C and incubation time of 24 h yielded fewer CFU per peg and showed fewer adhered cells and typical biofilm structures, based on scanning electron microscopy observations as compared with other conditions. Minimal biofilm eradication concentration values for *P. marginalis* were significantly greater ($P < 0.001$) than were MBCs for planktonic cells and for every biocide tested, with the exception of minimal biofilm eradication concentration values for peracetic acid at 15°C and 24 h. Sodium hypochlorite and peracetic acid sanitizers were able to eliminate *P. marginalis* biofilms at lower concentrations as compared with hydrogen peroxide– and quaternary ammonium–based sanitizers ($P < 0.001$). According to the results obtained, sodium hypochlorite and peracetic acid sanitizers would be more appropriate for maple sap collection system sanitation.

The prevention of microbial contamination caused by pathogenic and spoilage microorganisms during the manufacturing, processing, and packaging of food products is a major concern for the industry. Therefore, the food industry has adopted cleaning and disinfection practices (cleaning-in-place) to control the microbial contamination of equipment and to improve safety and quality of food items produced as well as process performance. However, the effectiveness of these measures is compromised by adherence of microorganisms and biofilm formation on the surface of equipment. Microorganisms adhered to a surface and contained in a biofilm are more resistant to biocides than when dispersed in a liquid medium (planktonic) *(3, 28).* The recalcitrance of biofilm bacteria toward biocides leads to cycles of regrowth following system disinfection procedures and eventually, bacteria may be transferred to the bulk medium and contaminate food *(7, 23, 34).*

In the maple syrup industry, maple sap is collected during the spring season from tap holes applied to maple trees (*Acer saccharum*) with a tubing network made of plastic material from which maple syrup and other derived products are produced. Because microorganisms found in maple sap are known to be detrimental to the quality of maple syrup, every effort must be made to avoid microbial contamination of maple sap. Microorganisms found in maple sap will be destroyed during the heat evaporation process used to make maple syrup. However, through their metabolic activity on maple sap constituents prior to evaporation, they will have a negative effect on the quality and commercial value of maple sap products *(19, 24, 26).* The bacterial community of maple sap has recently been studied *(20),* and formation of biofilm at the surface of the maple sap collection system has been recently documented *(21).* These studies indicated that *Pseudomonas* species were predominant in maple sap and are largely responsible for the formation of biofilms at the surface of the sap collection system. *Pseudomonas* species are also associated with spoilage of a number of food products *(15).* As it is for the food industry in general, commonly available biocides are used to disinfect the maple sap collection system. Even though disinfection of the maple sap collection system is regularly performed during sap flow season, no real investigation has been made to demonstrate the effectiveness and limitations of this operation and to identify the appropriate chemicals and conditions. Considering the fact that biofilms are present at the surface of the sap collection system, it would be essential to determine efficient procedures of sanitation suitable to eliminate the bacteria associated with biofilm formation and the biofilms themselves.

Many methods have already been proposed to evaluate the effectiveness of biocides against biofilm bacteria *(17).* As such, the Calgary biofilm device MBEC (MBEC Biofilm Technologies, Ltd., Calgary, Alberta, Canada) belongs to a category of instruments that are particularly well adapted to easily test many concentrations of biocides simultaneously against planktonic bacteria and reproducible biofilms *(2, 4, 5, 29, 32).* The MBEC device consists of a 96-well microtiter plate equipped with a lid mounted with 96

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TABLE 1. *List of biocides and range of concentrations tested*

Biocides	Range of active ingredient concn tested (mg/liter)	Active ingredients and concn in commercial solution
Sodium hypochlorite (Atomes, St-Laurent, Quebec, Canada)	10–5,120	Sodium hypochlorite, 12%
Bardac 2050 (Lonza, Inc., Allendale, N.J., USA)	10–5,120	Octyl decyl dimethyl ammonium chloride, 25% Dioctyl dimethyl ammonium chloride, 10% Didecyl dimethyl ammonium chloride, 15%
Hydrogen peroxide (Sigma Chemical Co., St. Louis, Mo., USA)	10–5,120	Hydrogen peroxide, 31.3%
Peracetic acid (American Chemicals, Ltd., St-Laurent, Quebec, Canada)	25–11,900	Peracetic acid, 35.5% Acetic acid, 39.5% Hydrogen peroxide, 6.8% Sulfuric acid, 1%

pegs on which equivalent biofilms can be produced. In our study, we used the MBEC device to test commonly used biocides against planktonic- and biofilm-contained *Pseudomonas marginalis* isolated from the maple sap collection system. The aim was to obtain relevant information that could provide guidelines for the maintenance of the maple sap collection system and to improve the overall quality of maple sap products.

MATERIALS AND METHODS

Bacterial culture and growth media. *P. marginalis* PTB2093 was isolated during the 2002 season from the inner surface of the sap collection system tubing located at the Centre ACER experimental sugarbush (St-Norbert, Quebec, Canada) and identified using the Biolog system (Biolog, Inc., Hayward, Calif.). This strain was selected because of its predominance and its particular ability to produce a biofilm on the surface of plastic material (data not shown). Maple sap was used to cultivate *P. marginalis* and to produce biofilm for the experiments. Maple sap was obtained during the early season of 2001 (Centre ACER) and sterilized in an autoclave at 121°C for 20 min. The sap was in the range of an average maple sap, with a total solids composition of 2.3°Brix and a pH of 7.7 *(25). P. marginalis* was recovered from frozen stock cultures by two consecutive transfers of 0.2 ml of culture in 10 ml of maple sap and incubated in static conditions for 18 h at 30°C. The biofilm inoculum was prepared from an 18-h culture in maple sap and standardized by centrifugation at 8,000 rpm (6,797 × *g*) and suspension of the pellet in the same medium for a final concentration of approximately 10^7 CFU/ml, validated by dilution plating on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, Md.) incubated at 30°C for 48 h.

Biofilm formation. Biofilms of *P. marginalis* were produced using the MBEC assay system (MBEC Biofilm Technologies, Ltd.) as previously described, with slight modifications *(5).* A 200-μl volume of *P. marginalis* inoculum prepared in maple sap was added in the wells of the MBEC microtiter plate, and the 96-peg lid was deposited on top of the plate. The plate was then incubated for 24 or 48 h at 15°C (temperature representative of cold sap collection system conditions) or 30°C (temperature representative of warm sap collection system conditions) in a gyratory shaker at 150 rpm for biofilm growth. The biofilm growth was determined by following bacterial counts on the surface of pegs after 24 and 48 h of growth in maple sap at 15 or 30°C. Each peg lid containing the biofilms was rinsed twice by placing the lid on a microtiter plate containing 200 μl of saline for 1 min in each well to remove planktonic cells. After rinsing, the peg lid was transferred to another microtiter plate containing 200 μl of maple sap in each well and sonicated for 5 min (model 5510, Branson Ultrasonics Corp., Danbury, Conn.) to remove biofilms. Viable counts were then performed on removed biofilms on TSA plates as described above. The same procedure was used to control for the numbers of CFU per peg in all biocide susceptibility tests prior to exposure to biocide.

Biocides. Sodium hypochlorite, a commercial mixture of quaternary ammonium compounds (Bardac 2050), hydrogen peroxide, and peracetic acid were selected for this study. Details on the origin and composition of these biocides are shown in Table 1.

Biocide susceptibility testing. Biofilms used for biocide susceptibility testing were prepared in maple sap as described above after incubation periods of 24 and 48 h. After incubation, the peg lid was removed and rinsed by deposition on a microtiter plate containing 225 μl of saline per well for 1 min. Each test biocide was serially twofold diluted with sterile purified water and placed in one lane of another 96-well microtiter plate. After rinsing, the peg lid containing biofilms was secured over a 96-well plate containing the biocides to be tested and challenged for 30 min at the same temperature (15 or 30°C), as was used for the generation of the biofilms. Saline was used to replace biocide solutions for control biofilms. Following the challenge incubation, the peg lid was rinsed twice with 225 μl of saline for 1 min to remove any residual biocide on the pegs. The peg lid was transferred to another 96-well plate containing 200 μl of maple sap per well, sonicated, and plated on TSA as described above to determine viable counts. To establish the susceptibility of biofilms to biocides, the minimal biofilm eradication concentration (MBEC) was determined and corresponded to the minimal concentration of biocide to which no bacterial growth was detected. The susceptibility of planktonic populations of *P. marginalis* was determined by the MBC of biocide. For the MBC testing, identical serial twofold dilutions of biocides used to challenge biofilms were prepared in a 96-well plate to which inoculum of *P. marginalis* was added to a final concentration of approximately 10^7 CFU/ml. After the 30-min challenge at 15 or 30°C, dilution plating was performed as described above to detect any residual population of viable bacteria for each biocide dilution.

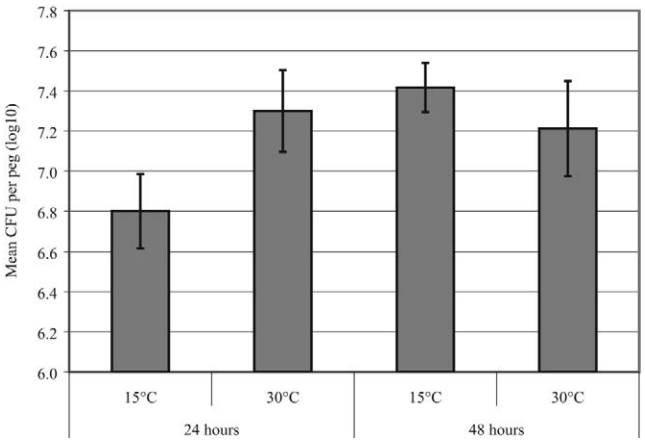

FIGURE 1. *Biofilm formation of* P. marginalis *PTB2093 on the pegs of the MBEC system in maple sap after 24 and 48 h at 15 and 30°C. Error bars are standard deviations of five independent determinations.*

Scanning electron microscopy. Pegs were broken from the lid of the MBEC system after biofilm growth of *P. marginalis* in maple sap as described above. After fixation for 2 to 3 h in 100 mM cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde, the pegs were rinsed (six to eight times) in 100 mM cacodylate buffer, and then sequentially dehydrated in graded series of ethanol (30, 50, 80 and 100%) for 15 min each. Pegs were then critical point dried (SPI Supplies, West Chester, Pa.) and were immediately sputter coated with gold (Kurt J. Lesker Company, Clairton, Pa.). Visualization of pegs was performed by using a Hitachi S-3000N scanning electron microscope (Hitachi, Mountain View, Calif.) at 5.0 kV.

Data analysis. MBCs and MBECs were obtained from five independent replicates of planktonic or biofilm growth and biocide challenge. Values were normalized by $\log_2$ transformation before being compared by analysis of variance and the Tukey multiple comparison test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Biofilm production on the MBEC device. In this work, we have tested the susceptibility of *P. marginalis* isolated from the surface of a maple sap collection system to four different biocides commonly used in the food industry. First, the MBEC system was selected to rigorously control biofilm production and to reproduce, as much as possible, identical conditions between biocide susceptibility tests. The results for biofilm formation at different growth temperatures and times are presented in Figure 1. These results representing the data from separate experiments show that the final biofilm concentration of *P. marginalis* averaged between 6.8 and 7.4 log CFU per peg among the conditions tested. According to the standard deviations presented, biofilm growth was highly reproducible for each set of conditions tested (temperature and time), and equivalent biofilms were produced in terms of biofilm cell counts (log CFU per peg). Ceri et al. *(5)* have also demonstrated that the MBEC system produced equivalent biofilms of *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* grown in tryptic soy broth for 24 h. The time and temperature influenced biofilm growth, as those produced after 24 h at 15°C had significantly lower ($P < 0.05$) cell counts (log CFU per peg) as compared with other growth conditions under which cell counts did not significantly differ (Fig. 1). Temperature is known to be an important factor for biofilm formation. Chavant et al. *(6)* showed that biofilm formation was affected by a low temperature in combination with the nature of the surface for the psychotropic bacterium *Listeria monocytogenes.* According to these authors, a low temperature will affect bacterial growth as well as physicochemical properties of the cells (hydrophobicity), leading to lower bacterial adhesion.

In addition to biofilm cell counts, scanning electron micrographs of the biofilm formed by *P. marginalis* on the MBEC system were obtained (Fig. 2). Scanning electron microscopy observations show adherent *P. marginalis* and the high-density biofilm produced on the surface of pegs. These results demonstrate that it is possible to reproduce under laboratory conditions biofilm formation by a bacterial strain isolated and grown in natural maple sap. From our observations of the biofilm production, it was of note that

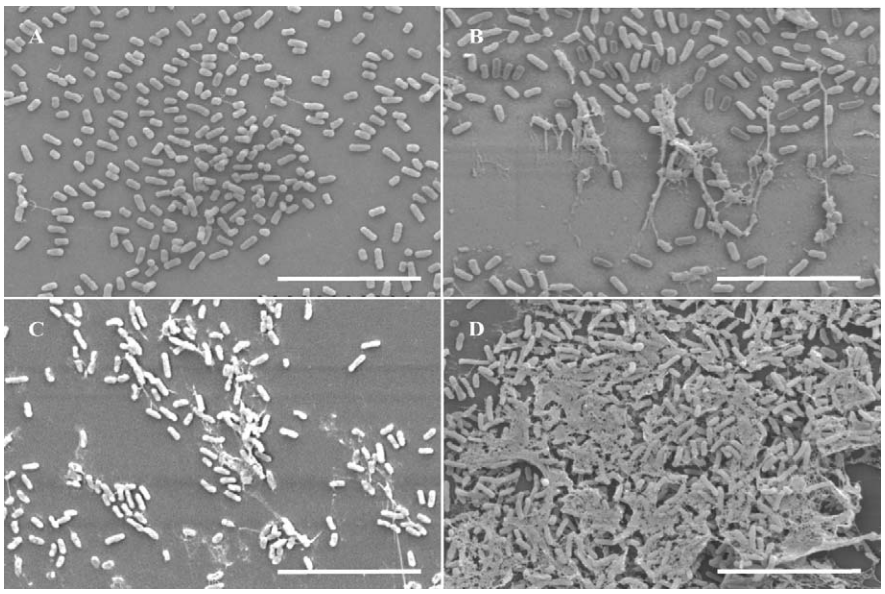

FIGURE 2. *Scanning electron micrographs of biofilms formed by* P. marginalis *PTB2093 on the pegs of the MBEC system in maple sap after incubation for 24 h at 15°C (A), 48 h at 15°C (B), 24 h at 30°C (C), and 48 h at 30°C (D). Bars correspond to 10 μM.*

TABLE 2. *Biocide susceptibility (30-min challenge) of* P. marginalis *PTB2093 as a planktonic population (MBC) and a biofilm population (MBEC) evaluated by the MBEC system with 24 and 48 h of incubation at 15 and 30°C in maple sap as growth medium*

			MBEC (mg/liter)			
	MBC (mg/liter)[b]		24 h		48 h	
Biocides[a]	15°C	30°C	15°C	30°C	15°C	30°C
Sodium hypochlorite	10 C[c]	10 C	30 B	92 BC	34 B	135 B
Bardac 2050	52 B	34 B	88 B	200 B	226 B	320 B
Hydrogen peroxide	218 A	125 A	3,875 A	5,113 A	4,299 A	4,096 A
Peracetic acid	25 BC	25 BC	25 B	30 C	63 B	121 B

[a] Concentrations of biocides used ranged from 10 to 5,120 mg/liter for sodium hypochlorite, Bardac 2050, and hydrogen peroxide, and from 25 to 11,900 mg/liter for peracetic acid.
[b] MBCs were significantly lower ($P < 0.001$) than MBECs for all biocides, except for the MBEC of peracetic acid after 24 h.
[c] Values in a column not followed by the same letter are significantly different, $P < 0.05$.

the biofilm did not develop uniformly on the surface of the peg, but as dispersed aggregates. Larger numbers of adherent cells were found in areas of the peg closer to the interface with liquid as compared with the tip of the peg. The extent of biofilm formation according to bacterial cell density and thickness of the structure appeared to be enhanced after 48 h of biofilm growth (Fig. 2B and 2D) and especially at 30°C (Fig. 2D). After 24 h of growth, however, cells adhered to the surface of the peg, but biofilm formation and extracellular polymeric material production were not as evident (Fig. 2A and 2C). Under prolonged incubation time (48 h), *P. marginalis* developed a dense biofilm composed of bacterial cells surrounded by extracellular polymeric material. However, this typical biofilm structure was only observed at much dispersed locations of the peg surfaces. This may explain why it had no influence on cell counts, and that no difference in cell counts (Fig. 1) was found between biofilms formed after 24 h at 30°C, and 48 h at 15 and 30°C.

Susceptibility of *P. marginalis* to biocides. In this study, the susceptibility of *P. marginalis* to biocides was investigated according to the mode of growth (planktonic or biofilm), time of biofilm growth, and type and concentration of biocides. Overall, the mode of growth has a significant influence on the susceptibility of *P. marginalis* to biocides (Table 2). MBCs (concentration obtained on planktonic cells) were significantly lower ($P < 0.001$) than MBECs (concentration obtained on biofilm cells) for most of the biocides and conditions tested. However, MBEC results obtained from biofilm (24 h) exposed to peracetic acid were similar to MBCs for both temperatures (15 and 30°C).

Pseudomonas spp. are well recognized as food spoilage microorganisms *(15, 16, 27).* This bacterial genus was found the most abundant in maple sap *(20)* and closely related to the formation of biofilms in maple sap collection system tubing *(21).* Biofilm formation is associated with the persistence of microbial contamination and spoilage of maple sap. The efficacy of biocides against *Pseudomonas* biofilm associated with maple sap production is therefore necessary to identify appropriate products that would maintain collection system sanitation. The extent of the bacterial inactivation by biocides is generally governed by five principal factors: the concentration of biocide, nature of bacterial cells and density, time of contact, temperature of medium, pH, and presence of foreign matter *(18, 30, 31).* In addition, biofilms may exhibit high levels of resistance to biocidal treatments *(12, 13).* Recalcitrance of *P. marginalis* biofilm grown in maple sap against four commonly used biocides (sodium hypochlorite, quaternary ammonium compounds, hydrogen peroxide, and peracetic acid) was also demonstrated in our study (Table 2). The increase in susceptibility according to the difference between MBCs and MBECs ranged from none (peracetic acid at 24 h) to nearly 50 times (hydrogen peroxide). This resistance in part may be explained by the production of extracellular polymeric material (Fig. 2A and 2C), which may react with chemical biocides and act as a diffusion barrier, or by the physiological status of the biofilm cells *(14).* While the effectiveness of all biocides was affected to a certain extent by the presence of *P. marginalis* biofilm, hydrogen peroxide was the one most affected (Table 2). Time of biofilm growth also had an overall effect on biocide susceptibility of *P. marginalis* (Table 2). Biofilms grown for 24 h were more susceptible to biocides than were biofilms grown for 48 h ($P < 0.001$, Table 2). This result is in accordance with the development of biofilm (Fig. 1) and scanning electron microscopy observations (Fig. 2). Previous studies have also suggested that the resistance of microorganisms to sanitizers is affected by the age of the biofilm *(11, 22).* Temperature, however, did not show any significant effect on biocide susceptibility of *P. marginalis.* Biofilms obtained and challenged at 15°C tended to be more susceptible than did those obtained and challenged at 30°C, but the difference was not statistically significant ($P = 0.261$).

When biocides were compared for their ability to eliminate *P. marginalis,* hydrogen peroxide was shown to be significantly less efficient ($P < 0.001$) among all the conditions tested, with MBCs and MBECs ranging from 125 to 5,113 mg/liter (Table 2). The protective role of catalase has already been related to the resistance of *P. aeruginosa* biofilm to hydrogen peroxide *(9, 33).* Peracetic acid and sodium hypochlorite, however, were generally more effective than were other biocides, with relatively low MBCs and MBECs ranging from 10 to 135 mg/liter. Quaternary

ammonium compounds (Bardac 2050) had intermediate MBCs and MBECs of 34 to 320 mg/liter.

Sodium hypochlorite is the most widely used biocide for sanitation of maple sap collection systems. A concentration of 50 to 100 ppm available chlorine is usually recommended for sanitizing food equipment and utensils *(8).* For sanitation of maple sap collection systems, a concentration of 600 ppm is generally recommended *(1).* This treatment is normally applied at the end of the sap flow season by injecting a 600-ppm hypochlorite solution with a pressure pump in the sap collection lines, followed by through rinsing with water. According to the MBECs obtained in this study for sodium hypochlorite against *P. marginalis* biofilms, the 600-ppm concentration used in the field would be high enough to sanitize the sap collection system. However, the use of a higher-than-necessary concentration of biocides increases the risk for biocide residues to be concentrated by the maple syrup process (reverse osmosis concentration and evaporation), especially for sanitation treatments applied during the season. Furthermore, high concentrations of biocides increase the potential for personal safety and environmental hazards, especially when manipulated in the field. To minimize these risks, a concentration of sodium hypochlorite lower than 600 ppm could be used. Moreover, because peracetic acid (which is chlorine free) was found in this study to be as effective as sodium hypochlorite to eliminate *P. marginalis* biofilms, a relatively low concentration (150 to 200 mg/liter) of this biocide could replace sodium hypochlorite and minimize potential environmental hazards. According to Fatemi and Frank *(10),* peracid sanitizers are more effective than chlorine for inactivating biofilm of *Pseudomonas* spp. and *Listeria monocytogenes* in the presence of organic challenge. This observation is supported by our findings that peracetic acid is highly effective against *Pseudomonas* biofilms produced in maple sap.

Field conditions are difficult to reproduce, and for this reason, a model system was used in this study to produce as much as possible equivalent biofilms for each experiment and to compare biocide products. However, conditions used in the model system to produce biofilms only partially represent the conditions observed in the field. For this reason, some discrepancy may arise with highly variable field conditions (system age, sap flow duration, temperature, shearing forces, etc.). Nevertheless, this study indicates that higher concentrations of biocides were necessary to eliminate biofilms of *P. marginalis.* According to the results obtained using the MBEC system, sodium hypochlorite and peracetic acid sanitizers would be more appropriate to maintain sap collection system sanitation. Because sanitizer residues and environmental hazards are important issues for the production of maple syrup, lower concentrations of sodium hypochlorite than currently recommended or the use of peracetic acid should be considered. This, however, would have to be validated with field experiments in which mixed species biofilms are able to grow in maple sap collection systems.

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