Journal of Microbiology, Biotechnology and Food Sciences

Čapla et al. 2012 : 1 (February Special issue) 733-741

REGULAR ARTICLE

SANITATION PROCESS OPTIMALIZATION IN RELATION TO THE MICROBIAL BIOFILM OF *PSEUDOMONAS FLUORESCENS*

Jozef Čapla[1], Peter Zajác[1], Pavol Bajzík[1], Lucia Zeleňáková[1], Jozef Golian[1], Vladimír Vietoris[2]*

Address: [1]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Food Hygiene and Safety, Tr. A. Hlinku 2,949 76 Nitra, Slovak Republic

[2]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Plant Products Storing and Processing, Tr. A. Hlinku 2,949 76 Nitra, Slovak Republic

*****Corresponding author: capla@potravinarstvo.com

ABSTRACT

Biofilms have been of considerable interest in the context of food hygiene. Extracellular polymeric substances play an important role in the attachment and colonization of microorganisms to food-contact surfaces. If the microorganisms from food-contact surfaces are not completely removed, they may lead to biofilm formation and also increase the biotransfer potential. The experimental part was focused on the adhesion of bacterial cells under static conditions and testing the effectiveness of disinfectants on created biofilm. In laboratory conditions we prepared and formed the bacterial biofilms *Pseudomonas fluorescens* in the test surfaces of stainless steel. Over the 72 hours and the next 72 hours were observed numbers of adhesion bacterial cells of *Pseudomonas fluorescens* on solid surfaces of tested materials.

Keywords: biofilm, microbial attachment; *Pseudomonas fluorescens*

INTRODUCTION

In most environments the majority of microorganisms are able to grow as biofilms **(Costerton, 2007)**, where they express a different phenotype from their planktonic counterparts **(Sauer, 2003)**. The main feature of this phenotype is the production of extracellular materials that build an adhesive gel, the matrix, embedding the cells and protecting them from shear forces and harsh conditions, including presence of most antimicrobial agents **(Flemming and Wingender, 2010)**. Food contact surfaces are good substrata for biofilm development. Although strict cleaning and disinfection procedures can generally assure suitable hygienic conditions in the food industry, destroying planktonic cells and biofilms starting to be formed, they may fall short for the elimination of biofilms that are already well developed. These tend to settle on sites that are especially difficult to clean, due to difficult access, surface irregularities or retention of sticky raw materials. Microbial cell transfer from biofilms to foods, particularly after their hygienization, is a hazard for food safety and quality **(Shi and Zhu, 2009; Verran et al., 2008)**. Bacteria of the genus *Pseudomonas fluorescens* are gram negative aerobic rods about the size of cells from 2 to 3 μm. They are usually occured in the wild, in the waste water (in pure water are reproduced) in the intestinal tract of man and animals, which live as saprophytes. A healthy individual has in his digestive tract these microorganisms are present and are not dangerous for him. If a healthy person is given the contaminated environment, are occurring with colonization, but no signs of disease, pseudomonads living saprophytic **(Horáček et al., 2000)**.

The biofilm is resistant to all efforts to eradicate it short of removal of the foreign material. Bacteria may attach to the surface of the foreign material by surface charge attraction, hydrophilic/hydrophobic interactions, and by specific attachment by fimbrae. Growth, colonization, and maturation follow bacterial attachment. A mature biofilm is composed of three layers: a linking film binding the biofilm to the surface; a base film made up of a compact layer of bacteria; and a surface film from which free-floating bacteria can arise and spread **(Silverstein and Donatucci, 2003)**.

Biofilms are communities of microorganisms that live attached to surfaces. Biofilm formation has received much attention in the last decade, as it has become clear that virtually all types of bacteria can form biofilms and that this may be the preferred mode of bacterial existence in nature **(Karatan and Watnick, 2009)**.

Biofilms are characterized by the environmental conditions and surfaces that favor their formation, the gene products that are required for their formation, the genes that are

activated and required to maintain the biofilm, the architecture of the biofilm, and the types of extracellular products that are concentrated in the biofilm matrix. There are as many different types of biofilms as there are bacteria, and even one bacterium may make several different types of biofilms under different environmental conditions **(Beech et al., 2006; Brady et al., 2008; Bryers, 2008; Pavithra and Doble, 2008)**.

Bacterial biofilm is naturally resistant to many antimicrobials. For the control of biofilms were developed alternative methods such as bacteriophages. Phage-φIBB PF7A is highly effective in removing biofilm *Pseudomonas fluorescens* in a short period of time. Terms of biofilm formation and application during phage infection are critical factors for the effectiveness of sanitation process. The integration of phages into biofilm matrix and its capture on the surface may be useful in phage treatment, considered either individually or as a supplement to chemical biocides in industrial environments where *Pseudomonas fluorescens* causes degradation **(Sulakvelidze et al., 2004)**. The aim of this study was verify the effectiveness of the dosage form and exposure time tested disinfectants on the viability of selected bacterial biofilm *Pseudomonas fluorescens*.

MATERIAL AND METHODS

For a biofilm preparation and testing its sensitivity to the selected disinfectant and sanitation procedure was used in our work the following microorganism: *Pseudomonas fluorescens* - CCM 7141 (the Czech collection of microorganisms).

The isolates of microorganism were stored in micro-petri dishes on a medium GSP (GSP agar, cat. n. 1.10230.0500, Merck KGaA, Germany, a selective agar for pseudomonads and aeromonads by Kielwein) at the temperature below 4 °C.

For the experiments we selected the following materials: The plate made of stainless steel - STN 17 240, 17 241 W Nr. 1.4301 AISI 304 (plate with dimensions approximately 30 x 20 mm).

We also selected four disinfectants with different active ingredients that are most used in the food industry with regard to the test material and safety (Tab 1).

Table 1 Recommended parameters for the application of disinfectants tested by the manufacturer

Preparation	Active substance	Concentration %	Exposure time min.	Temperature °C
Type A	generated peracetic acid	1	15	20 - 25
Type B	chloramin T (natrium-tozylchloramid)	2	15	20
Type C	hydrogen peroxide, didecyldimetylamonium chlorid, alkyldimetylbenzylamonium chlorid	1	15	20 - 25
Type D	quaternary ammonium compounds	0,5	15	20

To prepare the starting microorganism suspension was applied pure culture model of bacterium *Pseudomonas fluorescens* CCM 7141. The prepared culture was cultivated 24 hours

on a shaker with the frequency 130 min^{-1} at room temperature from 22 to 25 °C. The prepared bacterial suspension was diluted with sterile stock paste so that their value OD_{615} is equal to 0.32 which corresponds to 10^8 $CFU.cm^{-3}$.

Biofilm formation on surfaces of solid materials

The plates of tested surfaces of stainless steel were placed in the Petri dish and glass shower trays in which they were embedded in a standardized suspension so that it was submerged surfaces. The surfaces were then in Petri dishes and a glass beaker incubated at 25 ° C for 3, 6, 24 and 72 hours with occasional stirring. The surfaces were then removed from solution and washed with sterile saline phosphate buffer (PBS pH 7.4) to remove uncaptured cells. Subsequently, the surfaces after 72 hours of culture shock rinsed with sterile water, bathed and prepared a standardized suspension cultured again for 72 hours.

Application of disinfectants on the test surfaces

We have prepared solutions of disinfectant on the volume of 1000 ml. The surfaces were washed with PBS to remove free bacterial cells. Plate of surfaces were immersed in solution dosage forms of disinfectants the exposure time recommended by the manufacturer.

Consequently was made the calculation of viable cells. The effectiveness of each disinfectant was calculated from the difference between the two observations (before and after using disinfectant for 1, 5, 10 and 15 minutes).

RESULTS AND DISCUSSION

Testing of the sensitivity of Pseudomonas fluorescens biofilms on the surface made of stainless steel

Table 2 The average number of viable bacteria *Pseudomonas fluorescens* biofilms cultivated 72 h on the surface made of stainless steel after application of disinfectants during the exposure time of 1, 5, 10 and 15 minutes.

Method of treating with the disinfectant	**The average number of microorganisms (CFU.cm^{-2}) after the exposure time**									
	0 min.		**1 min.**		**5 min.**		**10 min.**		**15 min.**	
	x ± sd	v (%)	x ± sd	v (%)	x ± sd	v (%)	x ± sd	v (%)	x ± sd	v (%)
type A 1 %	$7{,}5.10^{4}$ ±1202,61	16	$1{,}2.10^{2}$ ±11,44	65	$5{,}0.10^{1}$ ±4,54	72	**0**	-	**0**	-
type B 2 %	$7{,}5.10^{4}$ ±1202,61	16	$1{,}8.10^{2}$ ±9,09	51	$8{,}0.10^{1}$ ±5,25	65	$3{,}0.10^{1}$ ±5,25	17	**$1{,}2.10^{1}$** ±6,94	57
type C 1 %	$7{,}5.10^{4}$ ±1202,61	16	$6{,}7.10^{1}$ ±2,63	39	$6{,}5.10^{1}$ ±2,63	41	**0**	-	**0**	-
type D 0,5 %	$7{,}5.10^{4}$ ±1202,61	16	$1{,}5.10^{2}$ ±38,66	26	$7{,}7.10^{1}$ ±4,54	58	$2{,}1.10^{1}$ ±6,94	32	**0**	-

Legend: x - arithmetic mean, SD - standard deviation, v - the coefficient of variation (%)

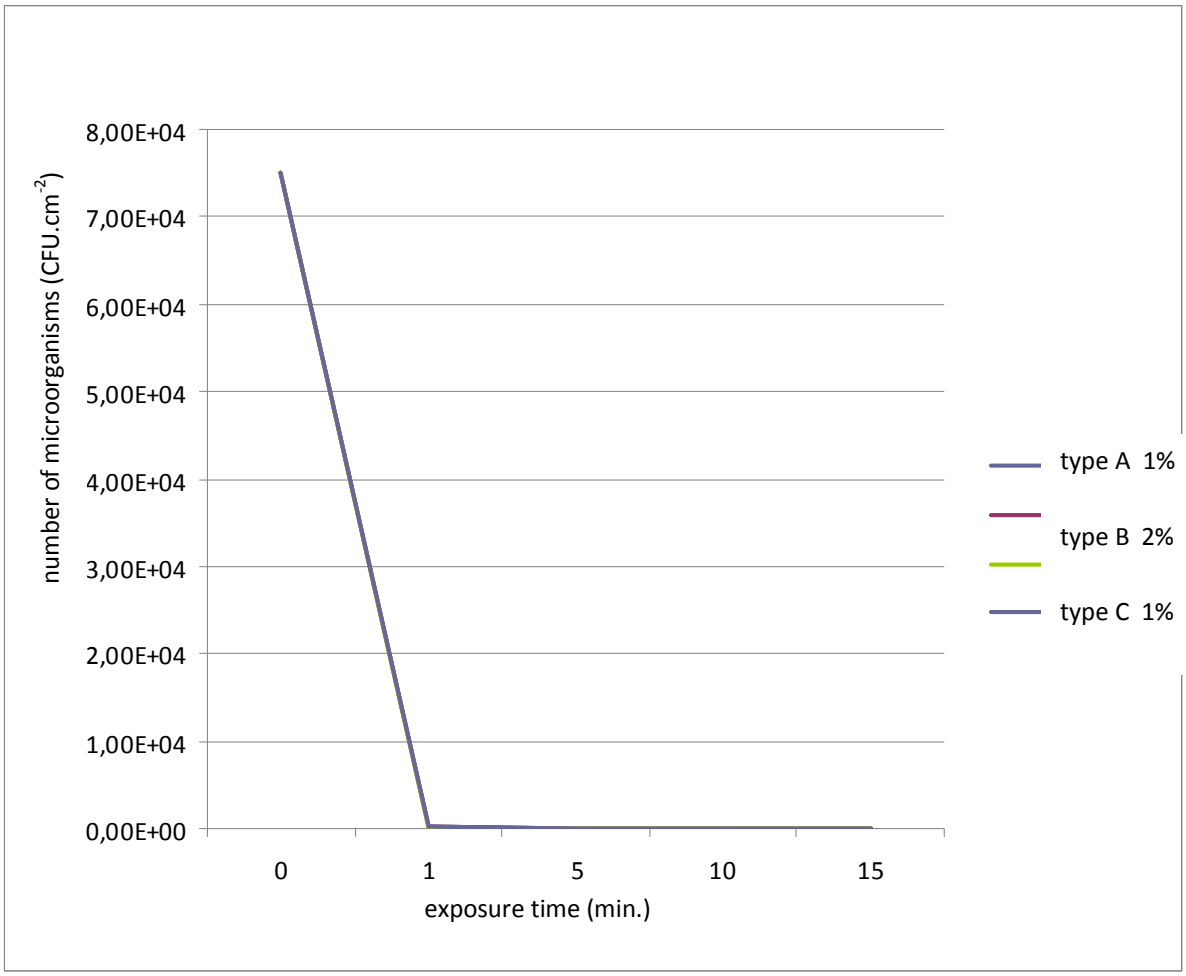

Figure 1 Effect of the exposure time and the action of four disinfectants on the viability of biofilm formed from bacteria *Pseudomonas fluorescens* prepared on surfaces made of stainless steel (shown by the initial values of microorganisms at the beginning of exposure)

Table 3 Efficiency of the chosen disinfectants depending on the time of exposure

Disinfectants	1 % disinfectants type A with generated peracetic acid				2 % disinfectants type B with chloramin T				1 % disinfectants type C with hydrogen peroxide, didecyldimetylamonium chlorid, alkyldimetylbenzylamonium chlorid				0,5 % disinfectants type D with quaternary ammonium compounds			
Exposure time in minutes	**1**	**5**	**10**	**15**	**1**	**5**	**10**	**15**	**1**	**5**	**10**	**15**	**1**	**5**	**10**	**15**
stainless steel	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	+

Zottola (1994) also tried to treat the attached bacteria *Pseudomonas fraga*, *Salmonella montevideo*, and *Bacillus cereus* on solid surfaces with different materials used in food with the disinfectant sodium hypochlorite at a concentration of application recommended by the manufacturer and found that rinsing with water and then rinse the plant sanitation has not been sufficiently effective to remove attached organisms. However, the author found that the micro-organisms after treatment preparations were viable. We can say that in our case with an effective disinfectant chlorine component is unable to penetrate the biofilm formed on the surfaces made of stainless steel, it is not penetrating or wetting properties and the formation of biofilms react with other compounds that may not be bactericidal. Efficacy of disinfectants becomes meaningless when it reaches the microorganisms in the biofilm.

Similar results with the test disinfectant an active ingredient with active oxygen as we **Koreňová et al. (2008)**, who tested G-biofilm forming *Pseudomonas aeruginosa* and contaminants that were decayed plant with active oxygen, in the case of biofilm formed at 37 °C and 20 °C. Test results in both cases reduced the number of bacterial cells adhered to the zero-CFU.cm^{-2}.

Microbiological risk is even more serious because the bacteria in biofilms have increased resistance to with the disinfectant compared with their counterparts in the state plankton **(O'Toole a Mah, 2001)**.

CONCLUSION

The sensitivity of biofilm was tested by immersing the surfaces in solutions of disinfectants with peracetic acid, chlorine, hydrogen peroxide and quaternary ammonium compounds as active ingredients in concentrations recommended by the manufacturer and the same exposure time of 15 minutes. As the most effective product has been evaluated disinfectant 1 % of active ingredient peracetic acid, which was very effective not only at the recommended concentration and exposure time, but also have less time at work as the manufacturer. Only 2 % of the disinfectant chlorine with an active ingredient for 15-minute exposure time, the number of bacterial cells could not be 100 % effective devitalize test surfaces of stainless steel.

Acknowledgments: The project was supported by KEGA 237-011SPU-4/2010.

REFERENCES

BEECH, I. B. - SUNNER, J. A. - ARCIOLA, C. R. – CRISTIANI, P. 2006. Microbiallyinfluenced corrosion: damage to prostheses, delight for bacteria. In *J. Artif. Organs* vol. 29, 2006, p. 443–452.

BRADY, R. A. - LEID, J. G. - CALHOUN, J. H. - COSTERTON, J. W. - SHIRTLIFF, M. E. 2008. Osteomyelitis and the role of biofilms in chronic infection. FEMS In *Immunol. Med. Microbiol.*, vol. 52, 2008, p. 13–22.

BRYERS, J. D. 2008. Medical biofilms. In *Biotechnol. Bioeng*. vol. 100, 2008, p. 1–18.

COSTERTON, J. W. 2007. Toward a unified biofilm theory. In *The Biofilm Primer*, 1st ed., Heidelberg, C.E., Ed.; Springer: Berlin, Germany, 2007, vol. 1, p. 169–180.

FLEMMING, H. C. - WINGENDER, J. 2010. The biofilm matrix. In *Nat. Rev. Microbiol.*, vol. 8, 2010, p. 623–632.

HORÁČEK, J. et al. 2000. *Základy lékařské mikrobiologie*. Nakladatelství Karolinum, p. 309.

KARATAN, E. - WATNICK, P. 2009. Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms In *Microbiol.*, *Mol. Biol. Rev.* vol. 73, no. 2, 2009, p. 310-347.

KOREŇOVÁ, J. – LOPAŠOVSKÁ, J. – KUCHTA, T. 2008. Comparison of three microtitre plate-based methods for quantification of biofilm formation ability of bacteria contaminating

food technologies. In *Journal of Food and Nutrition Research*, vol. 47, 2008, s. 100-104.

O´TOOLE, G. A. – MAH, T. F. 2001. Mechanisms of biofilm resistance to antimicrobial agents. In Trends Microbiol., vol. 9, 2001, p. 34-39.

PAVITHRA, D. – DOBLE, M. 2008. Biofilm formation, bacterial adhesion and host response on polymeric implants-issues and prevention. In Biomed. Mater. vol. 3 no. 034003, 2008. doi:10.1088/1748-6041/3/3/034003.

SAUER, K. 2003. Minireview: The genomics and proteomics of biofilm formation. In Genome Biol., vol. 4, 2003, p. 1-4.

SILVERSTEIN, A. - DONATUCCI, C. F. 2003. Bacterial Biofilms and implantable prosthetic devices. In International Journal of Impotence Research*. vol. 15, 2003, p. S150-S154.*

SULAKVELIDZE, A. – MORRIS, J. G. Jr. – ALAVIDZE, Z. – PASTERNACK, G. R. – BROWN, T. C. 2004. Method and device for sanitation using bacteriophages, In *Journal of Clinical Microbiology*, vol. 42, no. 1, 2004, p. 276-285.

SHI, X. - ZHU, X. 2009. Biofilm formation and food safety in food industries. In *Trends Food Sci. Technol.*, vol. 20, 2009, p. 407-413.

VERRAN, J. - AIREY, P. - PACKER, A. - WHITEHEAD, K.A. 2008. Microbial retention on open food contact surfaces and implications for food contamination. In *Adv. Appl. Microbiol.*, vol. 64, 2008, p. 223–246.

ZOTTOLA, E. A. 1994. Scientific status, summary, Microbial attachment and biofilm formation, a new problem for the food industry. In *Food Technology*, vol. 48 no. 7, 1994, p. 107-117.