LWT - Food Science and Technology 57 (2014) 782–784

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Research note

Fate of biofilm cells of *Cronobacter sakazakii* under modified atmosphere conditions

Sang-Hyun Park, Dong-Hyun Kang*

Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Food and Bioconvergence, Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, South Korea

ARTICLE INFO

Article history:
Received 5 August 2013
Received in revised form
2 January 2014
Accepted 26 January 2014

Keywords:
Biofilms
Cronobacter sakazakii
N_2 gas
CO_2 gas
Modified atmosphere

ABSTRACT

Survival of biofilm cells of *Cronobacter sakazakii* formed on stainless steel and polyvinyl chloride (PVC) on exposure to different atmosphere conditions was studied. Biofilms were formed on stainless steel and PVC coupons by using three strains of *C. sakazakii*. Six day old biofilms on stainless steel and PVC coupons were stored under N_2 gas, CO_2 gas, and air for up to 20 days. N_2 and CO_2 gases resulted in significant ($p < 0.05$) further reductions of 1.79 and 2.47 log CFU/cm^2 after 20 days of storage, respectively, compared to air storage. N_2 and CO_2 gases led to less reduction of biofilm cells on PVC compared to those on stainless steel. N_2 and CO_2 gases resulted in significant ($p < 0.05$) further reductions of 0.98 and 1.20 log CFU/cm^2 after 20 days of storage, respectively, compared to air storage.

1. Introduction

Cronobacter sakazakii is a group of Gram-negative, motile, and facultative anaerobic bacteria (Healy et al., 2010). *C. sakazakii* is a bacterium considered to be an emerging foodborne pathogen, causing a rare but severe disease in neonates (<28 days) and children under 4 years of age which may present as septicemia, meningitis, or necrotizing enterocolitis (Adekunte et al., 2010; Hartmann et al., 2010; Iversen & Forsythe, 2003). *Cronobacter* spp. can be isolated from a range of foods such as milk, cheese, dried foods, meats, vegetables, rice, bread, tea, herbs, spices, and powdered infant formula (PIF), and PIF is considered the main source of this pathogen (Bowen & Braden, 2006; Healy et al., 2010; Iversen, Lane, & Forsythe, 2004). Also *C. sakazakii* has been isolated from a variety of abiotic surfaces, and spoon and blender used in preparation of PIF are the source of infection (Chenu & Cox, 2009; Iversen & Forsythe, 2003; Kandhai, Reij, Gorris, Guillaume-Gentil, & van Schothorst, 2004). Contaminated PIF preparation equipment, such as spoons, blenders, and brushes have been associated with neonatal infections caused by *C. sakazakii* (Bar-Oz, Preminger, Peleg, Block, & Arad, 2001; Simmons, Gelfand, Haas, Metts, & Ferguson, 1989).

Some strains of *C. sakazakii* have been known to produce biofilms on several materials such as stainless steel, polyvinyl chloride (PVC), latex, silicon, polycarbonate, and glass (Iversen et al., 2004; Kim, Ryu, & Beuchat, 2006; Lehner et al., 2005). Biofilms provide a physical barrier and protect bacterial cells against various environmental stresses such as antibiotics, sanitizers, osmotic stress, heat, and starvation (Borucki, Peppin, White, Loge, & Call, 2003; Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Folsom & Frank, 2006; O'Toole, Kaplan, & Kolter, 2000). Also, biofilms on the abiotic surfaces could be a source of contamination (Furukawa, Kuchma, & O'Toole, 2006; Kim, Bang, Beuchat, & Ryu, 2008).

The survival characteristics of biofilm cells of *C. sakazakii* as affected by temperature, relative humidity (RH), or disinfectants have been studied to develop effective eliminating strategies (Kim et al., 2008; Kim, Ryu, & Beuchat, 2007). However, there have been no studies about the effect of modified atmosphere conditions on the survival of biofilm cells of *C. sakazakii*. Modified atmosphere is an effective food preservation method for various foods and inhibits the growth of microorganisms (Latou, Mexis, Badeka, Kontakos, & Kontominas, 2014; Lu, 2009; Sivertsvik, Jeksrud, & Rosnes, 2002). This study aimed at determining survival characteristics of biofilm cells of *C. sakazakii* as affected by different atmosphere conditions. Survival of cells in biofilms formed on stainless steel and PVC on exposure to air, N_2 gas, and CO_2 gas for up to 20 days was determined.

* Corresponding author. Tel.: +82 2 880 4927; fax: +82 2 883 4928.
E-mail address: kang7820@snu.ac.kr (D.-H. Kang).

0023-6438/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2014.01.038

2. Materials and methods

2.1. Bacterial cultures and cell suspension

Three strains of *C. sakazakii* (ATCC 12868, ATCC 29004, and FSM 30) were obtained from the School of Food Science bacterial culture collection, Washington State University (Pullman, WA, USA). These strains were cultured in 10 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 °C for 24 h, harvested by centrifugation at 4000 × *g* for 20 min at 4 °C, and washed three times with buffered peptone water (BPW; Difco). The final pellets were resuspended in sterile phosphate-buffered saline (PBS; pH 7.4), corresponding to approximately 10^7–10^8 CFU/ml.

2.2. Biofilm formation

The method used for biofilm formation was similar to that described by Kim et al. (2007). Stainless steel (thickness, 0.01 cm), type 304 with number 4 finished, and PVC (thickness, 0.02 cm) were purchased from Kahee metal (Incheon, Korea) and cut into coupons (5 cm × 2 cm). Each prepared coupon was immersed in a sterile 50-ml conical centrifuge tube (SPL Lifesciences, Pocheon, Korea) containing 30 ml of cell suspension of *C. sakazakii* in PBS (ca. 10^7–10^8 CFU/ml). Conical centrifuge tubes were incubated at 4 °C for 24 h to facilitate attachment of cells. After incubation, coupons were removed from conical centrifuge tubes with a sterile forceps, and washed in 500 ml of sterile distilled water for 10 s (22 ± 2 °C). Washed coupons were transferred to 50 ml conical centrifuge tubes containing 30 ml of TSB, and incubated at 25 °C for 6 days.

2.3. Survival of biofilm cells of C. sakazakii as affected by different atmosphere conditions

Nylon–polyethylene vacuum bags (16 cm × 25 cm) with a film thickness of 0.07 mm manufactured by Wonchang vinyl packaging (Seoul, South Korea) were used in this study. Each stainless steel and PVC coupon was transferred to nylon-polyethylene vacuum bags separately, and packed under different atmosphere conditions (N_2 gas 100%, CO_2 gas 100%) with a vacuum packaging machine (Airzero, Ansan, Korea). N_2 and CO_2 gas were purchased from KumKang gas (Bucheon-si, South Korea). Also, stainless steel and PVC coupons were air (control) packaged. The samples were stored at 25 °C (RH, 20 ± 2%) for 20 days and analyzed before packaging (0 day) and on 5th, 10th, 15th and 20th day of storage. Eight samples for each packaging condition were prepared and two samples were randomly taken at each storage time (5, 10, 15, and 20 days). Gas contents in the packages were measured immediately after packaging and before microbial analysis at each sampling day using a gas analyzer (OXYBABY, WITT-Gasetechnik, Germany) to confirm the leakage of gas. At each sampling day, biofilm cells were removed from stainless steel and PVC coupons by swabbing with a sterile cotton swab moistened with BPW (Ibusquiza, Herrera, Vázquez-Sánchez, & Cabo, 2012; Kim & Wei, 2007). The swab was transferred to a tube containing 10 ml of BPW and vortex-mixed for 2 min to suspend the cells. Cell suspension was ten-fold serially diluted in BPW, and 0.1 ml of undiluted cell suspension or diluents was spread-plated onto HiCrome Ent. sakazakii Agar (HiMedia, Bombay, India). The plates were incubated at 37 °C for 24 h, and colonies were counted.

2.4. Statistical analysis

All experiments were repeated three times. Data were analyzed by ANOVA using Statistical Analysis System (SAS Institute, Cary, NC, USA) and separation of means by Duncan's multiple range test at a probability level of $p < 0.05$.

3. Results and discussion

Fig. 1 shows the survival of biofilm cells of *C. sakazakii* on stainless steel stored under different atmosphere conditions. *C. sakazakii* formed biofilms on stainless steel coupons with a cell density of 7.67 log CFU/cm^2. There were significant ($p < 0.05$) differences on levels of biofilm cells of *C. sakazakii* among samples stored under different atmosphere conditions. N_2 gas resulted in significant ($p < 0.05$) further reductions of 0.98, 1.34, 1.96, and 1.79 log CFU/cm^2 after 5, 10, 15, and 20 days of storage, respectively, compared to air storage. CO_2 gas exhibited significant ($p < 0.05$) further reductions of 1.31, 1.45, 2.04, and 2.47 log CFU/cm^2 after 5, 10, 15, and 20 days of storage, respectively, compared to air storage. N_2 and CO_2 gases led to less reduction of biofilm cells on PVC compared to those on stainless steel (Fig. 2). The initial biofilm cells of *C. sakazakii* on PVC were 7.09 log CFU/cm^2. N_2 and CO_2 gases resulted in significant ($p < 0.05$) further reductions of 0.98 and 1.20 log CFU/cm^2 after 20 days of storage, respectively, compared to air storage.

Kim et al. (2008) reported the numbers of biofilm cells of *C. sakazakii* on stainless steel coupon decreased significantly within 5 days at RH 23%, remained constant until 35 days of storage. Similarly, significant reduction of air packaged biofilm cells of *C. sakazakii* was observed within 5 days at RH 20%, in this study. CO_2 gas is widely used due to its antimicrobial effect. It could affect the cell membrane function, enzymatic reactions, intracellular pH, or physiochemical properties of proteins of bacteria (Davies, 1995; Farber, 1991). N_2 gas is commonly used as filler gas to prevent collapse of packaging (Mullan & McDowell, 2003). Although it could inhibit the growth of aerobic microorganisms, little is known about the antimicrobial effect of N_2 gas (Cutter, 2002). In this study, N_2 gas along with CO_2 gas significantly reduced biofilm cells of *C. sakazakii* compared to air storage. The treatment of radish seeds with ClO_2 (100 μL/mL) followed by storage under modified atmosphere (10% O_2, 10% CO_2 and 80% N_2; 5% O_2, 10% CO_2 and 85% N_2; or 10% O_2, 0% CO_2 and 90% N_2) for 1 day significantly reduced the number of *Cronobacter* spp. compared to air storage (Kim, Ryu, &

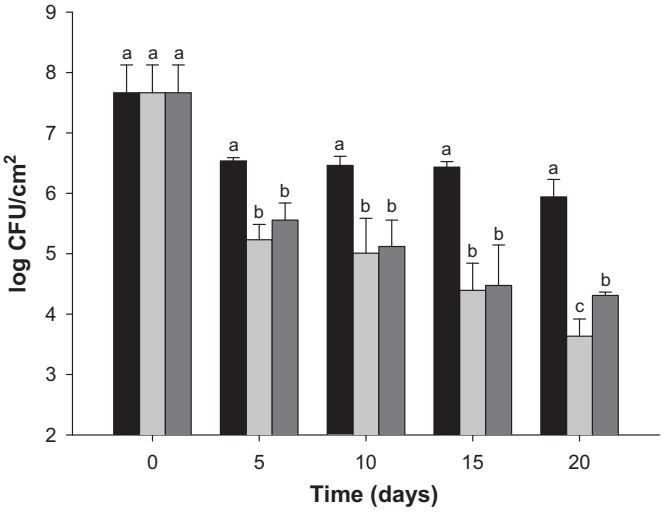

Fig. 1. Survival of biofilm cells of *C. sakazakii* on stainless steel coupons stored under different atmosphere conditions for up to 20 days. ■, air; ■, N_2 gas; ■, CO_2 gas. Means with the same letter within each treatment level are not significantly different ($p < 0.05$).

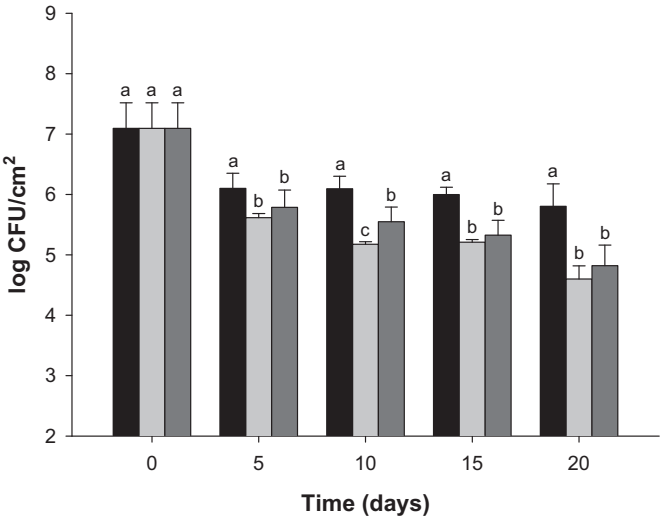

Fig. 2. Survival curves of biofilm cells of *C. sakazakii* on PVC coupons stored under different atmosphere conditions for up to 20 days. ■, air; ■, N_2 gas; ■, CO_2 gas. Means with the same letter within each treatment level are not significantly different ($p < 0.05$).

Kim, 2013). Further study is needed to determine the mode of action of CO_2 and N_2 gases on survivor of biofilm cells of *C. sakazakii*.

Biofilm cells of *C. sakazakii* showed greater resistance against N_2 and CO_2 gases on PVC coupons than stainless steel coupons. Characteristics of the substratum may affect the formation of the matrix material and the structure of the biofilms (Bremer, Monk, & Butler, 2002; Krysinski, Brown, & Marchisello, 1992). Bremer et al. (2002) reported that biofilm cells of *Listeria monocytogenes* on PVC/polyester surfaces were more resistant to chlorine treatment than those on stainless steel surfaces. The resistance of biofilm cells of *L. monocytogenes* on stainless steel was lower than that on polyester or polyester/polyurethane (Krysinski et al., 1992).

In conclusion, reductions of biofilm cells of *C. sakazakii* were greater under N_2 and CO_2 gases conditions rather than air condition. These results provide insights to predicting fate of biofilm cells of *C. sakazakii* on abiotic surfaces under modified atmosphere conditions. In this study, cocktails of *C. sakazakii* strains were used. Further studies are required to compare survival patterns of *C. sakazakii* strains with different ability in biofilm formation.

Acknowledgments

This research was supported by the Public welfare & Safety research program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012M3A2A1051679). This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A1A2003838).

References

Adekunte, A., Valdramidis, V. P., Tiwari, B. K., Slone, N., Cullen, P. J., Donnell, C. P. O., et al. (2010). Resistance of *Cronobacter sakazakii* in reconstituted powdered infant formula during ultrasound at controlled temperatures: a quantitative approach on microbial responses. *International Journal of Food Microbiology, 142*, 53–59.

Bar-Oz, B., Preminger, A., Peleg, O., Block, C., & Arad, I. (2001). *Enterobacter sakazakii* infection in the newborn. *Acta Paediatrica, 90*, 356–358.

Borucki, M. K., Peppin, J. D., White, G., Loge, F., & Call, D. R. (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology, 69*, 7336–7342.

Bowen, A. B., & Braden, C. R. (2006). Invasive *Enterobacter sakazakii* disease in infants. *Emerging Infectious Diseases, 12*, 1185–1189.

Bremer, P. J., Monk, I., & Butler, R. (2002). Inactivation of *Listeria monocytogenes*/*Flavobacterium* spp. biofilms using chlorine: impact of substrate, pH, time and concentration. *Letters in Applied Microbiology, 35*, 321–325.

Chenu, J. W., & Cox, J. M. (2009). *Cronobacter* ("*Enterobacter sakazakii*"): current status and future prospects. *Letters in Applied Microbiology, 49*, 153–159.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Review of Microbiology, 49*, 711–745.

Cutter, C. N. (2002). Microbial control by packaging: a review. *Critical Reviews in Food Science and Nutrition, 42*, 151–161.

Davies, A. R. (1995). Advances in modified-atmosphere packaging. In G. W. Gould (Ed.), *New methods of food preservation* (pp. 304–320). New York: Blackie Academic & Professional.

Farber, J. M. (1991). Microbiological aspects of modified atmosphere packaging technology–a review. *Journal of Food Protection, 54*, 58–70.

Folsom, J. P., & Frank, J. F. (2006). Chlorine resistance of *Listeria monocytogenes* biofilms and relationship to subtype, cell density, and planktonic cell chlorine resistance. *Journal of Food Protection, 69*, 1292–1296.

Furukawa, S., Kuchma, S. L., & O'Toole, G. A. (2006). Keeping their options open: acute versus persistent infections. *Journal of Bacteriology, 188*, 1211–1217.

Hartmann, I., Carranza, P., Lehner, A., Stephan, R., Eberl, L., & Riedel, K. (2010). Genes involved in *Cronobacter sakazakii* biofilm formation. *Applied and Environmental Microbiology, 76*, 2251–2261.

Healy, B., Cooney, S., O'Brien, S., Iversen, C., Whyte, P., Nally, J., et al. (2010). *Cronobacter* (*Enterobacter sakazakii*): an opportunistic foodborne pathogen. *Foodborne Pathogens and Disease, 7*, 339–350.

Ibusquiza, P. S., Herrera, J. J. R., Vázquez-Sánchez, D., & Cabo, M. L. (2012). Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *Listeria monocytogenes* and *Pseudomonas putida*. *Food Control, 25*, 202–210.

Iversen, C., & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology, 14*, 443–454.

Iversen, C., Lane, M., & Forsythe, S. J. (2004). The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. *Letters in Applied Microbiology, 38*, 378–382.

Kandhai, M. C., Reij, M. W., Gorris, L. G., Guillaume-Gentil, O., & van Schothorst, M. (2004). Occurrence of *Enterobacter sakazakii* in food production environments and households. *Lancet, 363*, 39–40.

Kim, H., Bang, J. H., Beuchat, L. R., & Ryu, J. H. (2008). Fate of *Enterobacter sakazakii* attached to or in biofilms on stainless steel upon exposure to various temperatures or relative humidities. *Journal of Food Protection, 71*, 940–945.

Kim, H., Ryu, J. H., & Beuchat, L. R. (2006). Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. *Applied and Environmental Microbiology, 72*, 5846–5856.

Kim, H., Ryu, J. H., & Beuchat, L. R. (2007). Effectiveness of disinfectants in killing *Enterobacter sakazakii* in suspension, dried on the surface of stainless steel, and in biofilm. *Applied and Environmental Microbiology, 73*, 1256–1265.

Kim, E. G., Ryu, J. H., & Kim, H. (2013). Effect of chlorine dioxide treatment and storage in a modified atmosphere on the inactivation of *Cronobacter* spp. on radish seeds. *Journal of Food Safety, 33*, 172–178.

Kim, S. H., & Wei, C. I. (2007). Biofilm Formation by multidrug-resistant *Salmonella* enterica serotype Typhimurium phage type DT104 and other pathogens. *Journal of Food Protection, 70*, 22–29.

Krysinski, E. P., Brown, L. J., & Marchisello, T. J. (1992). Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *Journal of Food Protection, 55*, 246–251.

Latou, E., Mexis, S. F., Badeka, A. V., Kontakos, S., & Kontominas, M. G. (2014). Combined effect of chitosan and modified atmosphere packaging for shelf life extension of chicken breast fillets. *LWT–Food Science and Technology, 55*, 263–268.

Lehner, A., Riedel, K., Eberl, L., Breeuwer, P., Diep, B., & Stephan, R. (2005). Biofilm formation, extracellular polysaccharide production, and cell-to-cell signaling in various *Enterobacter sakazakii* strains: aspects promoting environmental persistence. *Journal of Food Protection, 68*, 2287–2294.

Lu, S. (2009). Effects of bactericides and modified atmosphere packaging on shelf-life of Chinese shrimp (*Fenneropenaeus chinensis*). *LWT–Food Science and Technology, 42*, 286–291.

Mullan, M., & McDowell, D. (2003). Modified atmosphere packaging. In R. Coles, D. McDowell, & M. J. Kirwan (Eds.), *Food packaging technology* (pp. 304–311). Florida, USA: Blackwell Publishing. CRC Press.

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa. *Journal of Bacteriology, 182*, 425–431.

Simmons, B. P., Gelfand, M. S., Haas, M., Metts, L., & Ferguson, J. (1989). *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. *Infection Control and Hospital Epidemiology, 10*, 398–401.

Sivertsvik, M., Jeksrud, W. K., & Rosnes, J. T. (2002). A review of modified atmosphere packaging of fish and fishery products-significance of microbial growth, activities and safety. *International Journal of Food Science & Technology, 37*, 107–127.