SİDAS MEDYA
Akademik Gıda® / Academic Food Journal
ISSN Print: 1304-7582
http://www.academicfoodjournal.com

Akademik Gıda 9(4) (2011) 12-16

Research Paper / Araştırma Makalesi

Biofilm Producing Microorganisms in Dairy Industry and Prevention of Biofilm Formation

Zübeyde Öner, Zeynep Ölmez

Süleyman Demirel University, Engineering and Architecture Faculty, Food Engineering Department, Isparta, Turkey

Received (Geliş Tarihi): 06.07.2011, Accepted (Kabul Tarihi): 06.09.2011
✉ *Corresponding author (Yazışmalardan Sorumlu Yazar): zubeydeoner@.sdu.edu.tr (Z. Öner)*
✆ + 90 246 211 15 96 🖷 +90 246 211 15 38

This paper is a part of master thesis of Zeynep Ölmez. / Bu çalışma Zeynep Ölmez'in yüksek lisans tezinden alınmıştır.

ABSTRACT

In this study, the formation of biofilms in dairy industry was investigated. Biofilm samples were collected from the equipments of various dairy plants in Burdur and Isparta cities in Turkey. A biofilm layer was formed with isolated strains under laboratory conditions, and several disinfection procedures were applied to these biofilms. To remove the biofilms formed on stainless steel plates, six different disinfectants were used at different concentrations and impact times. Following microbial loads were found in samples collected from biofilms; total mesophilic aerob bacteria <10-20x10^8 cfu/mL, coliform <10-75x10^7cfu/mL, *Lactobacillus* spp <10-41.7x10^5 cfu/mL, *Lactococcus* spp <10-36x10^3 cfu/mL, *Staphylacoccus* spp <10-13.6 x10^6 cfu/mL, *Listeria* spp <10-13.6 x10^2 cfu/mL. The incidence of biofilm formations in the samples of small plants were higher than those of larger plants because of ineffective cleaning procedures in small plants. The cleaning agents used in small size plants were ineffective to eliminate biofilms.

Key Words: Biofilm, Attachment, Dairy industry, Disinfectants, Microflora

Süt Sanayisinde Biyofilm Oluşturan Mikroorganizmalar ve Biyofilm Oluşumunun Önlenmesi

ÖZET

Bu çalışmada, süt sanayisinde biyofilm oluşumu üzerinde çalışılmıştır. Bu amaçla Burdur ve Isparta'da süt işletmelerinden biyofilm örnekleri toplanarak, mikroorganizma profili belirlenmiştir. İşletmeden alınan örneklerden izole edilen izolatlarla laboratuar koşullarında biyofilm oluşturulmuş ve oluşan biyofilm üzerine çeşitli ticari dezenfektanlar kullanılmıştır. Laboratuar koşullarında paslanmaz çelik yüzeyde oluşturulan biyofilmin temizlenmesi için uygun olan zaman ve konsantrasyonu tespit etmek için farklı konsantrasyonlarda ve farklı sürelerde işlem uygulanmıştır. Alınan örneklerden izole edilen mikroorganizma grupları şu şekilde bulunmuştur: Toplam mezofilik aerob bakteri grubu <10-20x10^8 kob/mL, koliform <10-75x10^7 kob/mL, *Lactobacillus* spp <10-41.7x10^5 kob/mL, *Lactococcus* spp <10-36x10^3kob/mL, *Staphylococcus* spp <10-13.6x10^6 kob/mL, *Listeria* spp <10-13.6 x10^2 kob/mL değerleri arasında değişim göstermiştir. Küçük işletmelerden alınan numunelerde temizlik işlemlerinin yetersiz olması sonucu biyofilm oluşumu daha sık görülmüştür. Uyguladıkları temizlik ajanları biyofilmi yok etmede yeterli bulunmamıştır.

Anahtar Kelimeler: Biyofilm, Tutunma, Süt sanayii, Dezenfektan, Mikroflora

INTRODUCTION

Biofilm is commonly used to describe bacteria in the attached state surrounded by an extracellular matrix of polysaccharides. The biofilm state is generally believed to increase the ability of bacteria to survive antibacterial influence, such as heat, dehydration, foam cleaning, UV light, disinfectants, antibiotics, etc. from its surroundings.

The bacterial biofilms create a number of serious problems for industrial fluid processing operations. Mechanical blockages, increased impedance of heat transfer processes and biodeterioration of the components of metallic and polymeric systems result in billions of dollars in losses each year to food industries [1].

Bacterial contamination can adversely affect the quality, functionality and safety of the products of the dairy industry. When contamination of dairy products occurs, evidence suggests that biofilms on the surfaces of milk processing equipment are a major source [2-4].

The attachment of bacteria with subsequent development of biofilms in food processing environments is a potential source of contamination that may lead to food spoilage or transmission of diseases. The surfaces of equipment used for food handling, storage or processing are recognized as major source of microbial contamination. Even with acceptable cleaning in place (CIP) systems, bacteria can remain on equipment surfaces [5, 6].

In this study, the formation of biofilms and their microorganisms in dairy industry were investigated. Biofilm samples were collected from the equipments of seven different dairy plants in Burdur and Isparta cities of Turkey. The samples were analyzed for microorganisms. Six different commercial disinfectants were applied to the surfaces and the performance of them were examined with respect to contact time.

MATERIALS and METHODS

Sample collection

Samples were collected from seven commercial plant. 52 biofilm samples were taken from different sites in the dairy production line and the environment which included the pasteurization inlet, pasteurization outlet, the storage tank, the cheese tank and the feeding unit.

The samples were collected after the cleaning and sanitization treatment and before the milk were taken in for pasteurization using the swab method. The capacity of the commercial plants and application of sanitation are detailed in Table 1.

Table 1. The Production Capacity of the Dairy Plants and the Sanitation Application

Plant Code	Production Capacity (ton/day)	Sanitation
A	30.0	Caustic 0.5 %, nitric acid 1.5% and clor
B	4.0	Caustic
C	8.5	Caustic, nitric acid
D	6.0	Caustic and nitric acid
E	3.5-4.0	Caustic
F	60.0	Caustic and nitric acid
G	350.0	CIP

Isolation of micro-organisms

For the counting process of microorganisms, swabs were swashed in ringer solutions and then serial dilutions were made. Coliform bacteria and *Stapylococcus* spp. were counted Eosin Methylene Blue Agar (EMB), Baird Parker Agar (BPA) 24-48h at 37°C, *Lactobacillus* spp., and *Lactococcus* spp MRS agar and M17 agar 24-48h at 30°C with respectively. *Listeria* spp. were determined in PALCAM agar (with selective supplement) 35-37°C at 48h.

Total mesophilic aerob bacteria were counted on Plate Count Agar (PCA; Merck) and Tryptic Soy Agar (TSA; Merck) incubated during 24-48h at 30°C from equipment from seven dairy plants in Turkey after cleaning and disinfection [7].

Selected colonies from each segment were identified on the basis of colony morphology (color, shape, size) and Gram's reaction. After identification of isolates, mucous colonies (one of the important characteristics of biofilm forming microorganisms) were selected for attachment to stainless steel test [8].

Bactericidal Tests

Test tubes containing 4mL of each disinfectant, which were to be tested, were prepared from disinfectant stock solutions. Skim milk (Dry matter 10%) was mixed with cell culture that the concentrations in the test tube would be 10^7 cfu/mL and cultures incubated at 30°C for 24h. 1mL of this solution was added into the 4mL disinfectants which were prepared in three different concentrations, 100, 200 and 300ppm, respectively. Each culture was suspended in either 10mL of disinfectant solution for eight different contact times (0, 1, 3, 5, 10, 15, 20 and 30 min), and distilled water was used as a control. After 5 min. reaction time 500µL were transferred to 4.5mL D/E Neutralization broth and mixed. Neutralized samples were diluted 10 fold in peptone water. Plates were then vortexed for 3 min, with 5g steril glass beads (3 mm) in 45mL peptone water and then waited in ultrasound water bath for 15 minutes at about 40°C to dislodge attached cells. The suspension was serially diluted and the cells enumerated on Nutrient Agar to measure cfu mL^{-1}[9]. Table 2 lists the disinfectants and concentrations used in the bactericidal suspension tests.

Table 2. Disinfectants used in the bactericidal suspension tests

Main active components	Concentration (ppm)
Hypochlorite	100, 200, 300
Mixture of sodium hydroxide and sodium hypochlorite	100, 200, 300
Sodium hypochlorite	100, 200, 300
Sodium formaldehyde bisulphate	100, 200, 300
Aldehyde-based	100, 200, 300
Heterocyclic compounds	100, 200, 300

Attachment to stainless steel

Sterile steel coupons (75x22x1mm) were placed vertically in 50mL falcon tubes with 45mL TSB. The medium was then inoculated with overnight culture, the final cell concentration being 10^5-10^6 cfu mL^{-1}. The tubes were incubated in an incubation shaker at 100 rev min^{-1} at 30°C. The steel coupons were transferred to a new 50mL falcon tubes containing 45mL of peptone water. Attached cells were detached from the coupon by sonication in an ultra sound bath at 40°C for 15min. The cfu mL^{-1} was then measured by serial dilution in peptone water, spreading on Nutrient Agar at 30°C for 6 days. The experiments were performed three times on different days and with all solutions freshly prepared [9].

Statistical Analysis

Bactericidal activities of disinfectant and efficient of between the disinfectants were found with the Kruskal Wallis. Moreover, duration of application of disinfectant, concentration and changes in the differences between samples were analyzed with statistical applications. Determining the differences between groups was obtained using the Duncan's multiple-comparison test ($P<0.05$). Statistical analysis was performed using SPSS (version 10.0, SPSS Inc., Chicago, IL).

RESULTS

Isolation of micro-organisms

Samples were collected in four small size (<10 ton/day), two medium-size (30-60 ton/day), and one large-scale enterprises (350 ton/day). 52 biofilm samples were selected in different sites of these dairy plants. Isolated microorganisms were found as follows. Total mesophilic aerob bacteria <10-2,0x10^9 cfu/mL, coliform bacteria <10-7,5x10^8 cfu/mL, *Lactobacillus* spp. <10-4,1x10^6 cfu/mL, *Lactoccocus* spp <10-36x10^4 cfu/mL, *Staphylococcus* spp. <10-1,3x10^7 cfu/mL, *Listeria* spp. <10-1,7x10^3 cfu/mL (Figure 1). There were significant differences between the number of microorganisms of small plants and bigger plants ($p<0.05$).

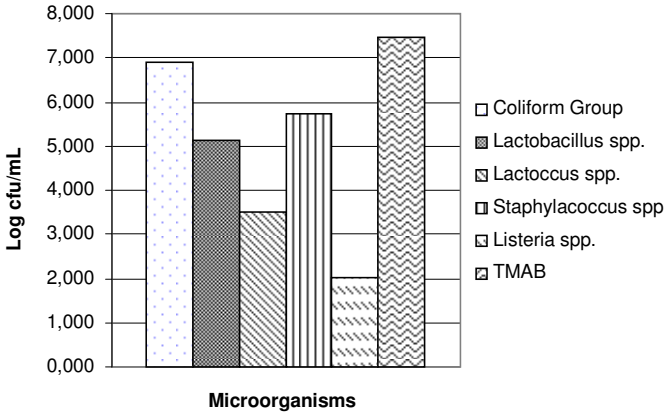

Figure 1. Average microorganism profile of commercial plants

Control of biofilm formation is difficult. A number of authors have reported that biofilm bacteria are more resistant to antimicrobial agents than suspended bacteria of the same species [10, 11]. Biofilm can be removed and/or destroyed by chemical and physical treatments. To eliminate the biofilm that has been formed on the stainless steel plates at the laboratory conditions, six different disinfectants were used. Certain variations to the method of for removal of microorganisms on the biofilm formations were also applied. These were varying the application time, concentration and the base of disinfectant. 4th disinfectant which was composed of the solution of sodium formaldehyde bisulphate compounds was chosen as the most affective disinfectant to control biofilm sanitation. The most effective contact time of disinfectants were seen between 15-30 minutes ($p<0.05$). The use of disinfectants at appropriate concentrations has been found to be very important to eliminate microorganisms in the biofilm matrix. At this project, the effective concentration was found as 300 ppm ($p<0.05$). Furthermore, it was observed that microbial removal increased with duration of cleaning time.

Attachment to steel coupons

Isolates were tested for attachment to steel coupons. Seven isolates showed attachment more than 10^6 cfu mL^{-1}. The degree of attachment to stainless steel is shown in Figure 2. *Bacillus* spp. F9 strain showed the lowest attachment to the steel surface while *Bacillus* spp. F5 the highest attachment after 48h. The remaining isolates showed lower levels of attachment.

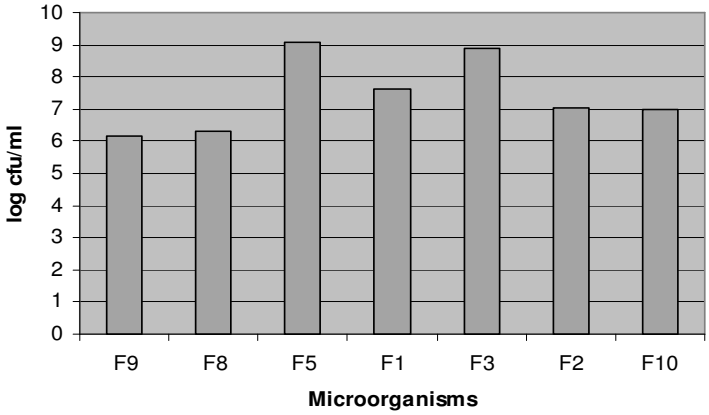

Figure 2. Microorganism's (*Bacillus* spp. isolates) attachment to stainless steel

DISCUSSION

Adsorption of microorganisms by the contact surfaces of dairy products in processing plants made from materials such as stainless steel and plastic is a major public health and economic concern. It is clear that cells, which are attached to equipment, increase resistance to sanitizers and in turn become sources of cross contamination. Distribution of isolates of the commercial plant was given Table 3. Sharma and Anand [8] isolated 105 isolates from dairy plant, and they found Gram-positive microflora and Gram negative microflora included *Lactobacillus* spp, *Streptococcus* spp. and *Staphylococcus* spp., *Shigella* spp., *Escherichia coli* and *Enterobacter aerogenes* respectively. Frank and Koffi [12] showed that the attachment of *Listeria monocytogenes* enhanced the resistance of cells against sanitizers.

Table 3. Distribution of isolates of the commercial dairy plants.

Plants	Coliform bacteria (cfu/mL)	*Lactobacillus* spp. (cfu/mL)	*Lactoccocus* spp. (cfu/mL)	*Staphylococcus* spp. (cfu/mL)	*Listeria* spp. (cfu/mL)	Total Mesophilic Aerob (cfu/mL)
A	<10-10,3x10^5	<10-41,7x10^5	<10-3,6x10^4	<10-41,6x10^3	<10-9,9x10^2	5,2x10^2-65x10^5
B	<10-55x10^4	<10-5x10^2	<10-7x10^2	<10-18,3 x10^5	<10-13,6x10	2,7x10^2-2,8x10^8
C	<10-7.5x10^8	<10-3,5x10^2	<10-6.6x10^2	<10-1,6x10^3	<10-1,7x10^3-	<10-2x10^9
D	<10-1,6x10^3	<10x3,3x10^2	<10-2.83x10^2	<10-1,3x10^7	<10-7x10^1	3,5x10^2-2,3x10^7
E	<10-2,3x10^2	6,6x10-1,6x10^3	3,3x10-1,17x10^2	<10	<10	1,7x10^2-4,1x10^3
F	<10-2,7x10^2	1x10^2-3,2x10^6	<10	<10-3,6x10^3	<10	1,1x10^3-4,1x10^6
G	<10	<10	<10	<10	<10	<10

Relatively high number of microorganisms was found in A, B, C and D plants. Only three plants did not contain *Listeria* spp. It was observed that the samples taken from smaller plants contained more biofilm formations than those taken from larger plants. This is believed to be resulting from difficulties encountered in cleaning the smaller plants.

The applied cleaning agents were not appropriate to eliminate biofilm. Hypochlorite, mixture of sodium hydroxide and sodium hypochlorite, sodium formaldehyde bisulphate, aldehyde-based heterocyclic compounds that have a bactericidal activity, act at the cytoplasmic membrane in Gram positive and Gram negative bacteria. In this study sodium formaldehyde bisulphate had a greater bactericidal effect on microorganisms. But this disinfectant produces poisonous gases with acid solutions. Therefore, it is more commonly used in cooling units. Alkaline solutions facilitate protein denaturation, fat saponification and have a bactericidal activity. Concentrations of disinfectants were used three different ratios and the best result was obtained at 300ppm. Chlorine, iodophors and quaternary ammonium products have been shown ineffective at removing biofilms. However, peroxide and peroxide containing sanitizers have been found effective in removal of biofilms [13]. This study showed the difficulty in obtaining a disinfectant that was effective both on spoilage and pathogenic bacteria.

The effectiveness of cleaning in removing gram-positive bacteria known to form biofilms in dairy plants, such as Bacillus species, has only recently received attention [3, 14]. *Bacillus* spp. are spore forming bacterium groups commonly contaminating raw milk and considered a major microbiological problem in the dairy industry [15]. Isolated *Bacillus* spp. indicated different attachment from each other. *Bacillus* spp. F5 showed the highest attachment among them. Heat stable spores of *B. cereus* in milk are a source of contamination for milk

derived products, such as milk powder, infant food formulas [16, 17] and many food commodities [18]. It is known that *B. cereus* spores occur in low numbers (10^2-10^3 per liter) in farm collected milk ([19, 20]. Thus, the farms are not the sole source of *B. cereus* in dairy milk. Additional contamination of milk occurs after the arrival to the dairy plant. A modern dairy plant is not an easy environment for *B. cereus* to colonize. The incoming milk is stored at cold temperature, heat treated, and the equipment is washed with hot, highly alkaline (pH > 13) and acid (pH <1) liquids. It has been shown that certain genotypes of *B. cereus* found in dairy silo tanks [21, 22].

CONCLUSIONS

Microbial control in dairy plants has the main aims of reduction/eradication of microbes and their activity. However in this study was shown microbial activity was present after thorough cleaning and fogging disinfection. Samples taken from seven different plants showed that there were pathogenic micro-organisms such as *Coliform* bacteria, *S.aureus, Listeria* spp. on contact surfaces of stainless steel food-processing. There were quite a few variations observed in the constitutive microflora of the six plants. It was possible to select a concentration of sanitizer to effectively reduce the biofilms. Biofilms in dairy processing is an important sources pathogenic and spoilage microflora which can lead to spoilage of finished product and spread of diseases. The discovery of new biofilm control strategies, following the specifications needed to be used in food industry and based on the use of biological-based solutions with high antimicrobial activity.

REFERENCES

[1] Mittelman, M.W., 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. *J.Dairy Sci*. 81: 2760-2764.

[2] Koutzayiotis, D., 1992. Bacterial biofilms in milk pipelines. *S. Afr. J. Dairy Sci*. 64: 19-22.

[3] Flint, S.H., Bremer, P.J., Brooks, J.D., 1997. Biofilms in dairy manufacturing plant-description, current concerns and methods of control. *Biofouling* 11: 81-97.

[4] Bremer, P., Suzanne Fillery, J., James McQuillan, A., 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *Int. J. Food Microbiol*. 106: 254-262.

[5] Austin, J.W., Berferon, G., 1995. Development of bacterial biofilms in dairy processing lines. *J.Dairy Res*. 62: 509-519.

[6] Amy, C., Wong, L., 1998. Biofilms in food processing environments. *J. Dairy Sci*. 81: 2765-2770.

[7] Halkman, A.K., 2005. Gıda Mikrobiyolojisi Uygulamaları. Başak Matbaacılık ve Tanıtım Hizmetleri Ltd. Şti. Ankara. 358p.

[8] Sharma, M., Anand, S.K., 2002. Characterization of constitutive microflora of biofilms in dairy processing lines. *Food Microbiol*. 19: 627-636.

[9] Bore, E., Langsrud, S., 2005. Characterization of microorganisms isolated from dairy industry after cleaning and fogging disinfection with alkyl amine and peracetic acid. *J. of Appl. Microbiol*. 98: 96-105.

[10] Brown, M.R., Allison, D.G., Gilbert, P., 1988. Resistance of bacterial biofilms to antibiotics: a growth rate related effect. *J. Antimicrob. Chemotherapy* 22: 777-783.

[11] Brading, M.G., Jass, J., Lappin-Scott, H., 2003. Dynamics of Bacterial Biofilm Formation. Microbial Biofilms. Edited by H.M.Lappin-Scott and J.W.Costerton Cambridge University pres.301p.

[12] Frank, J.F., Koff, R.A., 1990. Surface adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *J. Food Protect.* 53: 550-554.

[13] Deibel, V., 2005. Biofilms. *Internet Journal of Food Safety* 1: 6-7.

[14] Parkar, S.G., Flint, S.H., Brooks, J.D., 2003. Physiology of biofilms of thermophilic bacilli-potential consequences for cleaning. *J. Ind. Microbiol. Biotech*. 30: 553-560.

[15] Andersson, A., Rönner, U., Granum, P., 1995. What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *Int. J. Food Microbiol*. 28: 145-155.

[16] Becker, H., Schaller, G., Wiese, W., Terplan, G., 1994. *Bacillus cereus* in infant foods and dried milk products. *Int. J. Food Microbiol*. 23: 1-15.

[17] Shaheen, R., Svensson, B., Andersson, M.A., Christiansson, A., Salkinoja-Salonen, M., 2010. Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiol.* 27: 347-355.

[18] Wijnands, L.M., Dufrenne, J.B., Rombouts, F.M., Veld, P.H., Van Leusden, F.M., 2006. Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the Nether. *J. Food Protec*. 69: 2587-2594.

[19] Banyko, J., Vyletelova, M., 2009. Determining the source of *Bacillus cereus* and *Bacillus licheniformis* isolated from raw milk, pasteurized milk and yoghurt. *Letters in Appl. Microbiol*. 48: 318-323.

[20] Bartoszewicz, M., Hansen, B.M., Swiecicka, I., 2008. The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. *Food Microbiol.* 25: 588-596.

[21] Svensson, B., Ekelund, K., Ogura, H., Christiansson, A., 2004. Characterization of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. *Int. Dairy J*. 14: 17-27.

[22] Svensson, B., Monthan, A., Shaheen, R., Andersson, A., Salkinoja-Salonen, M., Christiansson, A., 2006. Occurrence of emetic toxin producing *Bacillus cereus* in the dairy production chain. *Int. Dairy J*. 16: 740-749.