JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 391-398

Optimization of Environmental Conditions for Biofilm Formation of Bacterial Strains *Salmonella aerizonae*, *Micrococcus luteus* and *Aerococcus viridans*

Rejaul Haque Mostafa Chowdhury[1], Nigar Sultana[2], Wahhida Shumi[1]*, Md. Aktar Hossain[3,4*] and Hazandy Abdul Hamid[4,5]

[1]Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh.
[2]Department of Microbiology, University of Science and Technology Chittagong, Chittagong-4000, Bangladesh.
[3]Institute of Forestry and Environmental Sciences, University of Chittagong, Chittagong-4331, Bangladesh.
[4]Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor , Malaysia.
[5]Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.

(Received: 11 November 2014; accepted: 07 January 2015)

Biofilm forming ability is an inherent property of the microorganisms for their multiplication and substantial infection. By forming this protective layer (biofilm) microorganisms gain resistance against antibiotics, antibodies and phagocytosis. An attempt was made to investigate the biofilm forming ability of three bacterial strains *Salmonella aerizonae*, *Micrococcus luteus* and *Aerococcus viridans* isolated from poultry wastes (PW) dental plaques (DP) and hospital effluents (HE) respectively under various environmental conditions. The effects of different environmental factors such as growth media, pH, salt concentration, incubation period, temperature and condition (shaking or stationary) on biofilm forming ability of these isolates were determined for their optimum growth and development. The study reveals that LB was the best media for maximum yield of biofilm after 24 h of incubation for *M. luteus* and 48 h for *S. arizonae* and *A. viridians*. All the strains showed their maximum biofilm formation in LB media at 37°C temperature supplemented with 1% salt concentration at medium pH 6.5 in shaking (*S. arizonae*) or stationary (*M. luteus* and *A. viridians*) condition. The inhibitory effects of penicillin and erythromycin on the biofilm formation of the isolates were also investigated. The results indicate that increasing concentration of antibiotics decreased the biofilm formation of these strains progressively.

Key words: Biofilm, Environment factors, *Salmonella aerizonae, Micrococcus luteus, Aerococcus viridans.*

Biofilm is the aggregates of interactive bacteria attached to solid surface and each other. It may take place on biotic and abiotic surfaces and represents a prevalent mode of microbial life in natural, industrial and hospital settings [1]. The cells of a microorganism growing in a biofilm are physiologically different from those in planktonic states (single-cells that may float or swim in a liquid medium) in the same organism. Although mixed-species biofilms predominate in most environments, like teeth and gut, single-species biofilms exist in a large variety of infections, on surface of medical implants and food industries [2-5]. These single-species biofilms are the focus of the most current researches, which include gram-

* To whom all correspondence should be addressed.
E-mail: wahhida_shumi@hotmail.com ; aktar_forestry@hotmail.com

negative biofilm-forming bacteria like *Pseudomonas aeruginosa* [6], *Escherichia* coli [7] and *Vibrio* cholera [8] and gram-positive biofilm-forming bacteria such as *Staphylococcus epidermidis* [9], *Staphylococcus aureus* [10], *Streptococcus mutans* [11,12], *Bacillus lichienformis*, *Bacillus subtilis* [13,14] and *Bacillus* cereus [15].

In biofilm, bacteria secrete a thick exopolysaccharide (EPS) matrix and exist within this matrix in a dormant or sessile state of low metabolic activity [16]**.** The microcolonies attached to the substratum surrounded by EPS. The biofilm matrix contains EPS, proteins and DNA where EPS constituent with 50% to 90% organic carbon in matrix. Some biofilms have been found to contain water channels that help to distribute nutrients and signalling molecules. EPS protect the microbial communities from the antimicrobial agents such as detergents and antibiotics. The level of potential prevention, by the EPS matrix from antimicrobial agents, is totally dependent on the type, amount and properties (hydrophobicity and charge) of the antimicrobial agent. In some cases antibiotic resistance can even be increased 1000 fold [17]. Furthermore, EPS can provide protection from a wide variety of environmental stress, like UV irradiation, pH shifts, osmotic shock and desiccation. The chemical composition of a surface also impacts on bacterial colonization since it may contain beneûcial or detrimental components[18]. Biofilms are responsible for a significant portion of acute infections. A classic case is that of Legionnaire's disease, an acute respiratory infection resulting from aspiration of clumps of *Legionella* biofilms detached from air and water heating/cooling systems. Many food borne pathogens such as *E. coli* 0157:H7, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella spp.* and *Camphylobacter jejuni* can form either single-species or multi-species biofilms on food surfaces and equipment. Most of the pathogenic bacteria utilize biofilms for their survival and infections. Greater number of chronic and persistent infections in the context of indwelling medical devices is clearly linked to biofilms. Many pathogenic microorganisms grow readily on the materials used for catheters, artificial joints, mechanical heart valves and other devices. In many cases, it is impossible to eradicate the infection using antimicrobial agents, and the implant has to be removed, which is expensive as well as dangerous and traumatic for the patient [1].

Recently, the researcher stated that biofilms may be seen as a source of new bioactive agents. When bacteria are organized in biofilms, they produce effective substances which individual bacteria are unable to produce alone. For the reason, biofilms study is largely dependent upon the innovation of new and powerful techniques to investigate the inside structure and functions of biofilm development. The type of equipment needed to investigate biofilm formation is very much dependent upon the type of investigations (*in situ* or *in vivo*) are required; the experimenter is obviously restricted in his choice since the biofilm itself proliferates in its natural system. Considering above mentioned facts the present research work was undertaken with a view to the following objectives: i) to screen, characterize and identify bacterial strains for biofilm formation from poultry wastes, dental plaques and hospital effluents, ii) to observe the attachment ability of selected bacterial strains, iii) to evaluate the suitable culture condition and environmental factors for the maximum biofilm formation and iv) to observe the inhibitory effects of different commonly used antibiotics on biofilm formation of the selected bacterial strains.

MATERIALS AND METHODS

Sample collection, isolation and identification

Bacterial samples were collected from poultry wastes, dental plaques from teeth and hospital effluents for the present study. Bacterial isolates were isolated through specialized enrichment techniques and purified with repeated plating method, streak plate and pour plate methods on nutrient agar (NA) media for the purposes. Several media, cell morphology, biochemical tests and cultural methods were used for the identification and characterization of the microorganisms.

Screening of biofilm forming ability by test tube assay

Biofilm production procedure was determined by the method proposed by Christensen *et al*. [19] with slight modifications. For the production of biofilm in test tube, fresh single colony was picked and inoculated in 5ml LB broth

medium in test tubes. The tubes were incubated at 37°C for 12h to 16 h for using them as inoculums for the biofilm assay. Fresh inoculum were then inoculated at 1:20 ratio and incubated at 37°C for 24 h. Biofilm assay was performed by the modified method of Stepanovic *et al*. [20] and absorbance of the retained dye was measured by spectrophotometer at 600 nm.

Determination of cultural and environmental conditions for biofilm formation

The strains with positive biofilm formation were selected for mass culture in liquid media. The optimum yield of biofilm is dependent on the optimization of several factors, such as composition of media, incubation period, incubation temperature, incubation condition (Shake or stationary), pH of the medium, medium salt concentration, presence of antibiotics etc. Therefore, biofilm forming ability of the organisms was determined under above mentioned environmental conditions separately or in combined.

Culture media, pH, salt concentration, incubation period, temperature and condition

Two different liquid media (Nutrient broth and Luria-Bertani broth) were used for investigating the biofilm forming ability of three selected bacterial isolates. After sterilization, 5 ml media was transferred into each test tube (6 inches) and inoculum was inoculated at a ratio 1:20. After incubating for 24 h at 37°C biofilm was quantified. The isolates from PW, DP and HE produced significantly higher levels of biofilm in the LB medium than those developed in the nutrient medium. Thus LB medium was selected as a suitable media for further study for the maximum production of biofilm by the selected isolates. To determine the effects of incubation periods on the biofilm formation by the selected isolates, inoculated media were incubated at 37°C for 24h, 48h and 72 h. However, 24 h was found to be the optimum incubation period for the maximum biofilm formation for *M. luteus* and 48 h for *S. arizonae* and *A. viridians*. The effects of medium pH on biofilm formation was determined by incubating the isolates in LB media with pH 4.5, 6.5 and 8.5 at 37°C for optimum incubation periods. The optimum temperature was determined by culturing the isolates in media with optimum pH at 4°C, 27°C, 37°C and 45°C for 24h. To investigate the suitable salt concentration for the biofilm formation, the isolates were grown in the media with 0%, 1%, 5%, 10% and 20% salt keeping other experimental conditions optimum. The effects of aeration on bacterial biofilm formation was assessed by incubating the culture media in stationary or shaking conditions maintaining all other experimental conditions optimum. After growth, the biofilm density was determined by crystal violet (1% solution) absorbance assay since the amount of crystal violet colour absorbed by the cells are correlated with the amount of bacterial cells present in the culture.

Inhibitory effects of antibiotics against biofilm development

The inhibitory effects of different commonly used antibiotics (Penicillin and Erythromycin) against the biofilm formation by the selected isolates were also studied. For the purpose sterilized media was added with 2µg ml^{-1}, 5µg ml^{-1} and 50µg ml^{-1} antibiotics. The inoculums were then cultured in LM medium with optimum experimental conditions and biofilm formation was assessed.

All data were analyzed using statistical program IBM SPSS (ver 21.0) to explore the possible variation among the treatments. Analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) were performed to determined significant treatment variations at $p \leq 0.05$ probability. Each experiment was repeated at least three times to confirm the reproducibility of the methods.

RESULTS AND DISCUSSION

Bacterial Isolates

Eight bacterial colonies were isolated from three different sources, poultry wastes, teeth and hospital effluents. Off which, three isolates were finally selected for further study based on their source of origin and colony characteristics (Table 1). By comparing the morphological, cultural and biochemical properties with the standard description of "Bergey's Manual of Determinative Bacteriology"[21], the isolates from poultry wastes, teeth and hospital effluents were identified as *Salmonella arizonae*, *Micrococcus luteus* and *Aerococcus viridians* respectively. Colony morphology of the three isolates was somehow different, mostly differed in size, density and in

pigment. As observed, the isolate *S. arizonae* formed relatively larger colonies with translucent density, while *M. luteus* and *A. viridians* formed small colonies with opaque density. For the characterization of the selected isolates as a positive biofilm former, test tube assay was performed (Figure 1). The figure (Figure 1) clearly showed the crystal violet bound ring formation around the air-liquid interface of the tubes. These rings (violet colour) were clearly observed in all positive strains *S. arizonae* (PW), *M. luteus* (DP) and *A. viridians* (HW) with different thickness. After washing the test tube with distilled water, the characteristic of the ring structures formed at the liquid-air interface were interpreted as the numbers of bacterial cells present in the biofilms. The amount of crystal violet colour absorbed by the cells is correlated with the amount of bacterial cells present in the culture.

Effects of growth media and culture conditions on biofilm formation

In the present study, the strains *S. arizonae*, *M. luteus* and *A. viridians* were characterized as positive (V^{e+}) biofilm formers on the basis of the presence of the rings and the level of absorbance by test tube assay. The studies of biofilm formation by the selected strains were with included the determination of the suitable culture and environmental conditions for maximum biofilm formation. Although it is believed that biofilms are formed due to some unique characteristics possessed by some bacterial genera but environmental and physicochemical factors play key roles in the biofilm development. Different environmental parameters such as medium contents, medium pH, salt concentration, air relation, temperature, periods of incubation etc. influence the biofilm formation and thus optimization of these factors are needed for the maximum biofilm formation. In our study two different liquid media - Luria Bertani broth (LB) and Nutrient broth (NB) medium were used for the maximum production of biofilm by the three selected bacterial isolates. The biofilm level of isolates from PW (*S. arizonae*), DP (*M. luteus*) and HE (*A. viridians*) was significantly higher in the

Table 1. Physical, morphological and cultural properties of the three selected isolates

Samples source	Isolates	Form, shape and density	Margin and shape	Pigment
Poultry waste (PW)	*Salmonella arizonae*	Circular and translucent	Entire, smooth	Whitish, non-diffusible
Dental plaque (DP)	*Micrococcus luteus*	Punctiform, circular and opaque	Entire, smooth	Whitish orange, non-diffusible
Hospital effluent (HE)	*Aerococcus viridians*	Punctiform, circular and opaque	Entire, smooth	Lemon yellow, non-diffusible

Table 2. Biofilm formation of the stains measured through crystal violet absorbance (600nm) in different medium pH in LB broth

	Medium pH		
	4.5	6.5	8.5
S. arizonae	0.43±0.1	0.82±0.21*	0.51±0.08
M. luteus	0.01±0.0	0.25±0.12*	0.17±0.04
A. viridians	0.005±0.0	0.32±0.14*	0.13±0.02

Note: *Maximum biofilm development

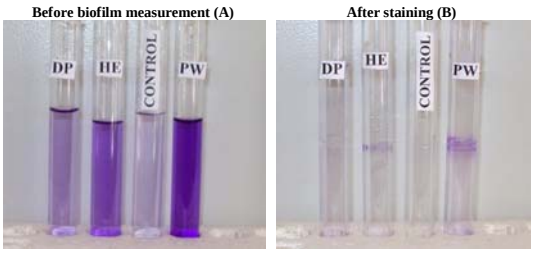

Fig. 1. Biofilm ring inside the tubes after 48 h of incubation (A) followed by staining with crystal violet (B). Figure showed that ring formed in all tubes i.e, DP (+), HE (+), Negative control (-) and PW (+) where PW, DP and HE represents the isolate *S. arizonae, M. luteus and A. viridians* respectively. After addition of 95% ethyl alcohol, crystal violet was released from the cells and the violet colours absorbance was measured at 600nm

LB medium (Figure 3) compared to those developed in the nutrient medium. Therefore LB medium was used for the subsequent study for the maximum production of biofilm by the selected isolates.

Optimum environmental conditions for biofilm formation

Figure 4 showed the effects of incubation periods on biofilm formation of the selected isolates. The optimum incubation period for maximum biofilm formation were found at 24 h for *M. luteus* and 48 h for *S. arizonae* and *A. viridians*. Medium pH 6.5 was found to be optimum for the formation of biofilm for all the isolates (Table 2). At the same pH value *S. arizonae* produced the highest level of biofilm (absorbance 0.82) among the isolates. The suitable temperature for the maximum biofilm formation by the isolates *S. arizonae*, *M. luteus* and *A. viridians* was found 37°C (Table 3). The isolates were found to develop biofilm at wide range

Table 3. Different incubation temperature affects biofilm produced by the strains measured by the absorbance (600nm) of the **c**rystal violet colour

	Isolates Biofilm absorbance (600nm) Incubation Temperature			
	4°C	27°C	37°C	45°C
S. arizonae	0.05±0.0	0.45±0.07	0.79±0.2*	0.31±0.1
M. luteus	0.00±0.0	0.12±0.01	0.29±0.1*	0.04±0.0
A. viridians	0.005±0.0	0.13±0.01	0.80±0.19*	0.075±0.0

Note: Medium pH 6.5, *maximum biofilm development

Table 4. Effects of salt concentrations in culture media on biofilm production of the isolates measured by the absorbance (600nm) of the crystal violet colour

Isolates	Biofilm absorbance (600nm) Salt concentrations				
	0%	1%	5%	10%	20%
S. arizonae	0.05±0.01	0.79±0.17*	0.13±0.01	0.07±0.0	0.00±0.0
M. luteus	0.00±0.0	0.27±0.11*	0.01±0.0	0.00±0.0	0.00±0.0
A. viridians	0.02±0.0	0.74±0.22*	0.19±0.02	0.21±0.01	0.015±0.0

Note: LB medium pH 6.5, Incubation temperature 37°C, *maximum biofilm development

Table 5. Inhibitory effects of various concentrations of antibiotics on biofilm development by the isolates measured by the absorbance (600nm) of crystal violate colour. The result showed the mean value of triplicate result

Isolates	Antibiotics	Biofilm absorbance (600nm) Antibiotic concentration 10µg	25µg	50µg	Positive control
S. arizonae	Penicillin	0.65±0.14*	0.635±0.11	0.605±0.09	0.96±0.03
	Erythromycin	0.73±0.12*	0.54±0.22	0.52±0.17	
M. luteus	Penicillin	0.43±0.08*	0.34±0.03	0.14±0.01	0.445±0.01
	Erythromycin	0.18±0.02*	0.135±0.01	0.11±0.00	
A. viridians	Penicillin	0.83±0.21*	0.70±0.04	0.455±0.05	0.905±0.12
	Erythromycin	0.81±0.16*	0.78±0.02	0.76±0.15	

Note: Conditions used here were the optimum for the production of biofilm. *maximum biofilm development

of temperature from as low as 4°C to as high as 45°C but the quantity was very low (Table 3). Significantly higher level of absorbance was noticed at 37°C compared to the other temperature for the isolates. The highest level of biofilm formation was also observed in 1% salt concentration in LB medium. Low concentration (0%) or higher concentrations (>1%) of salt inhibited the biofilm development of the isolates. The biofilm production level by *S. arizonae*, *M. luteus* and *A. viridians* were 0.79, 0.27 and 0.74 respectively in 1% salt concentrations in LB medium (Table 4). The results were correlated with the biofilm development by the *Salmonella* in neutral pH and at 37°C temperature [20,22]. The *S. arizonae* isolate showed the maximum biofilm formation in shaking condition whereas the *M. luteus* and *A. viridians* in stationery condition (Figure 5). Since the isolate *S. arizonae* is aerobic bacteria, it formed higher level of biofilm in well aerated (shaking) conditions. On the other hand, *M. luteus* and *A. viridians* are microaerophilic and

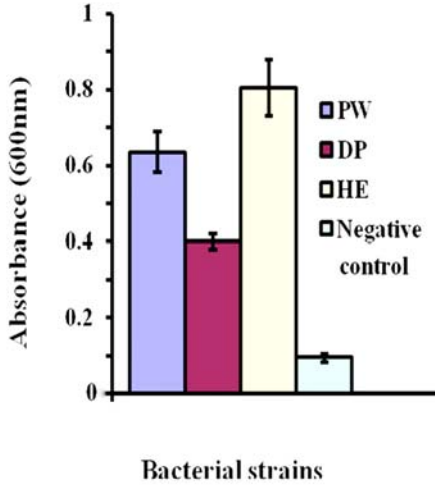

Fig. 2. Biofilm formation of the *S. arizonae* (PW), *M. luteus* (DP) and *A. viridians* (HE) and negative control after 24 h of incubation. The crystal violet colour absorbance was measured at 600nm

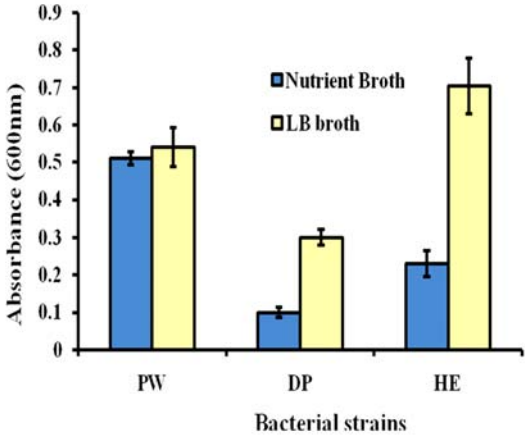

Fig. 3. Biofilm formed in nutrient broth and LB medium. Biofilm assay was measured by crystal violet absorbance at 600nm 24h after incubation. PW, DP and HE represented *S. arizonae, M. luteus and A. viridians* respectively

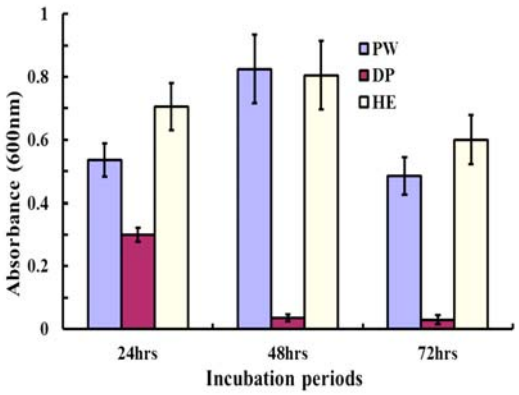

Fig. 4. Effects of various incubation periods (24 h, 48 h and 72 h) on biofilm formation followed by staining the absorbance of crystal violet colour at 600nm. PW, DP and HE representes the *S. arizonae, M. luteus and A. viridians* respectively

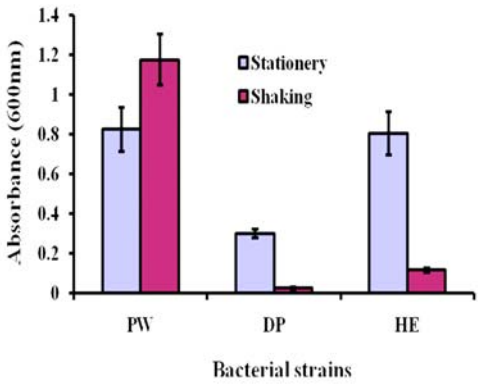

Fig. 5. Biofilm formation measured by the crystal violet absorbance (600nm) after incubation with stationery and shaking condition. PW, DP and HE represented *S. arizonae, M. luteus and A. viridians* respectively

facultative anaerobic bacteria produced maximum amount of biofilm in static condition where less O_2 was supplied.

Impacts of antibiotics on bacterial grow and biofilm formation

Biofilms are the protective covering of the organisms against the antibiotics compared to the planktonic cells but not completely resistant [23]. To control or protect the infection or biofilm formation, around 100 to 1,000 times concentrated antibiotics is required[17]. In the present research work we observed the biofilm formation of *S. arizonae*, *M. luteus* and *A. viridians* in the presence of common antibiotics such as penicillin and erythromycin. Three different concentrations (10µg ml^{-1}, 25µg ml^{-1} and 50µg ml^{-1}) of antibiotics were used and the absorbance shown in Table 5. The results indicate that all biofilms formed by the three selected isolates were resistant against these two antibiotics. Otherwise (if sensitive), it could not be possible to grow and form the biofilms by the organisms. Since the isolates were resistant against the antibiotics, they formed biofilm but with less quantity compared to the control indicates that the presence of antibiotics have inhibitory effects on the biofilm formation of the isolates. With the increasing concentration of antibiotics the biofilm development was decreased gradually. Our results were correlated with the previous studies reported by Biedlingmaier *et al*.[4] and Coenye [24].

CONCLUSIONS

The formation of biofilm by bacterial isolates is thought to be an important part of some infections in human body and at least partly responsible for the survival of some bacteria in natural environments which doesn't support their normal growth [25]. The discovery of biofilm has earned a lot of attentions of the microbiologists throughout the world for its advantageous and detrimental impacts on human beings both in health and economy [26]. Although it is believed that biofilms are formed due to some unique characteristics possessed by some bacterial genera, environmental and physicochemical factors play key roles in the development biofilms. Disturbance or maintenance of the conditions which are required for the maximum production of biofilm can decrease or increase the ability of biofilm formation respectively. In our experimental result showed that cells of bacterial strains attached on the solid support and formed biofilm in control condition (without antibiotic), but their attachments in presence of various concentrations of antibiotics and adverse environmental conditions. Similar trend of results was observed in all three stains in the study (Table 5). The study unveiled the effects of different factors and environmental parameters on biofilm formation of the three selected isolates. The results also showed the resistance of the isolates against antibiotics some extents which explore the infection and antibiotics sensitivity. Attempt was also made to discover the relationship between the disease causing microorganisms and the used antibiotic for their treatment or control would be the basis for getting information about the drug discovery and delivery system for a particular disease.

REFERENCES

1. Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol*., 2004; **2**:95-108.
2. Adal, K.A. and Farr, B.M. Central venous catheter-related infections. *Nutri. Rev.*, 1996;**12:**208-213.
3. Archibald, L.K. and Gaynes, R.P. Hospital acquired infection in the United States: the importance of interhospital comparisons. *Nosocom. Infect.*, 1997;**11:**245-255.
4. Biedlingmaier, J.F., Samaranayake, R. and Whelan, P. Resistance to biofilm formation on otologic implant materials. *Otolaryngol. Head Neck Surg.*, 1998;**118:**444-51.
5. Johansen, T.B., Agdestein, A., Olsen, I., Nilsen, S.F., Holstad, G. and Djonne, B. Biofilm formation by *Mycobacterium avium* isolates originating from humans, swine and birds. *Bio. Medi. Cent. Microbiol.*, 2009;**9:**159. doi:10.1186/1471-2180-9-159.
6. Flemming, H.C. Biofilms and environmental protection. *Water Sci. Technol*., 1993;**27:**1-10.
7. Lim, J., Lee, K.S., Kim, H., Nam, S.W., Oh, Y.J., Yun, H-S., Jo, W., Oh, S-J., Kim, S-H. and Park, S. Nanoscale characterization of *Escherichia coli* biofilm formed under laminar flow using atomic force microscopy (AFM) and scanning electron microscopy (SEM). *Bull. Korean Chem. Soc.*, 2008;**29**(11):2114-2118.
8. Yildiz, F.H. and Schoolnik, G.K. *Vibrio cholerae*

O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci*., 1999;**96:**4028-4033.

9. Heilmann, C. and Gotz, F.F. Further characterization of *Staphylococcus epidermidis* transpose mutants deficient in primary attachment or intercellular adhesion. *Zentralbl. Bakteriol.*, 1998;**287:**69-83.
10. Zhang, Y., Lei, Y., Khammanivong, A. and Herzberg, M.C. Identification of a novel two-component system in *Streptococcus gordonii* V288 involved in biofilm formation. *Infect. Immun.*, 2004;**72**(6):3489-3494.
11. Shumi, W., Lim, J., Nam, S.W., Kim, S.H., Cho, K.S., Yoon, J. and Park, S. Fluorescence imaging of the spatial distribution of ferric ions over biofilms formed by Streptococcus mutans under microfluidic conditions. *Biochip J.*, 2009;**3**(2):119-124.
12. Shumi, W., Lim, J., Nam, S.W., Lee, K., Kim, S.H., Kim, M-H., Cho, K-S. and Park, S. Environmental factors that affect *Streptococcus mutans* biofilm formation in a microfluidic device mimicking teeth. *BioChip J.*, 2010;**4**(4):257-263.
13. Morikawa, M. Beneficial biofilm formation by industrial bacteria *Bacillus subtilis* and related species. *J. Biosci. Bioeng*., 2006;**101**(1):1-8.
14. Murray, E.J., Strauch, M.A. and Stanley-Wall, N.R. SigmaX is involved in controlling *Bacillus subtilis* biofilm architecture through the AbrB homologue Abh. *J. Bacteriol.*, 2009;**191**(22):6822-6832.
15. Hsueh, Y.H., Somers, E.B., Lereclus, D. and Wong, A.C.L. Biofilm formation by *Bacillus cereus* is influenced by *plcR*, a pleiotropic regulator. *Appl. Environ. Microbiol.*, 2006;**72**(7):5089-5092.
16. Ledeboer, N.A. and Jones, B.D. Exopolysaccharide sugars contribute to biofilm formation by *Salmonella enterica* serovar typhimurium on HEp-2 cells and chicken intestinal epithelium. *J. Bacteriol.* 2005;**187**(9):3214-3226.
17. Stewart, P.S. and Costerton, J.W. Antibiotic resistance of bacteria in biofilms. *J. Bacteriol.*, 2001;**358**(9276):135-8.
18. Roberts, A.P., Pratten, J. Wilson, M. and Mullany, P. Transfer of a conjugative transposon, Tn5397 in a mixed oral bioûlm. *FEMS Microbiol. Lett.*, 1999;**177:**63–66.
19. Christensen, G.D., Simpson, W.A., Bisno, A.L. and Beachey, E.H. Adherence of slime–producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun.*, 1982;**37:**318–26.
20. Stepanovic, C.S., Cirkoric, M.L. and Svabic-Vlahovic, M. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella spp*. *Food Microbiol.*, 2003;**20:**329-343.
21. Buchanan, R.E. and Gibbons, N.E. (eds.). Bergey's manual of determinative Bacteriology. 8th Edition, The Williams and Wilkins, Baltimore (1974).
22. Decho, A.W. Microbial bioûlms in intertidal systems: an overview. *Cont. Shelf Res*., 2000;**20**: 1257–1273.
23. Zhang, W., Sturm, B.S., Knapp, C.W. and Graham, D.W. Accumulation of tetracycline resistance genes in aquatic biofilms due to periodic waste loadings from swine lagoons. *Environ. Sci. Technol.*, 2009;**43**(20):7643-7650.
24. Coenye, T. Response of sessile cells to stress. from changes in gene expression to phenotypic adaptation. *FEMS Immunol. Med. Microbiol*., 2010;**59**(3):239-52.
25. O'Toole, G.A., Kaplan, H.B. and Kolter, R. Biofilm formation as microbial development. *Ann. Rev. Microbiol*., 2000;**54:**49-79.
26. Zahller, J. and Stewart, P.S. Transmission Electron Microscopic Study of Antibiotic Action on *Klebsiella pneumoniae* Biofilm. *Antimicrob. Agents Chemother.*, 2002;**46**(8):2679–2683.