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In Situ Monitoring of Biofilm Formations of *Escherichia coli* and *Pseudomonas putida* by Use of Lux and GFP Reporters

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A plasmid vector containing two reporter genes, *mer-lux* and *lac-GFP*, was transformed to both *Escherichia coli* and *Pseudomonas putida*. Their cellular activities and biofilm characteristics were investigated in flow-cell units by measuring bioluminescent lights and fluorescent levels of GFP. Bioluminescence was effective to monitor temporal cell activities, whereas fluorescent level of GFP was useful to indicate the overall cell activities during biofilm development. The light production rates of *E. coli* and *P. putida* cultures were dependent upon concentrations of $HgCl_2$. Mercury molecules entrapped in *P. putida* biofilms were hardly washed out in comparison with those in *E. coli* biofilms, indicating that *P. putida* biofilms may have higher affinity to mercury molecules than *E. coli* biofilms. It was observed that *P. putida* expressed GFP cDNA in biofilms but not in liquid cultures. This may indicate that the genetic mechanisms of *P. putida* were favorably altered in biofilm conditions to make a foreign gene expression possible.

Key words: biofilm, bioluminescence, GFP, *lux*, mercury

INTRODUCTION

Microorganisms present in aquatic environments tend to attach to exposed surfaces, proliferate, and form gelatinous layers termed biofilms [1]. These biofilms can be either beneficial or detrimental in many practical applications in industry. One beneficial role of biofilms is their use in bioreactors to degrade pollutant substances in wastewater treatment. Biofilms also result in reduction of heat transfer flux in heat exchangers, corrosion inside pipes, and contamination in the food processing industry [2]. The formation of microbial biofilms may cause the release of mercury from fresh amalgam in teeth [3]. The role of microbial biofilms in the process of accumulation of heavy metals has been reported [4, 5]. Many bacterial strains contain genetic determinants of resistance to heavy metals such as Hg^{2+}, Ag^{+}, Cd^{2+}, and Cu^{2+} [6]. These resistance determinants are often found on plasmids and transposons. Mercury resistance operon-luciferase (*mer-lux*) fusion plasmids have been described to evaluate gene expression rates of the *mer* structural gene promoter of transposon *Tn*21 [7]. Binding of inducer Hg(II) to the transcriptional activator *mer*R is known to result in light output. *Mer-lux* fusions provide an unusually sensitive reporter of gene expression [8].

Biofilm formations require a number of complex adhesive interactions. They may be involved with the bacterial surface components, solid surface properties, and dissolved medium components [2]. Initial adhesion to solid surfaces is strongly dependent upon the surface properties of the bacterium so that bacterial strains differ considerably in their attachment processes. Most techniques described in the literature on measuring biofilm formation are destructive because of the requirement of biofilm removal from the system. Recently an *in situ* projection technique for biofilm thickness measurement was reported [9], but this method cannot give information at the molecular level for bacterial cells growing inside biofilms.

Green fluorescent protein (GFP) of *Aequorea victoria* is superior to the *lux* genes in preparing reporter genes because GFP requires no biochemical substrate and is also extremely stable under most environmental conditions. Moreover, the fluorescence of GFP is very bright and bacterial cells can easily be seen under ultraviolet light. Thus, GFP may be useful in monitoring real time cell activity in biofilms without cell disruption. The GFP gene has been cloned and used as a promising reporter gene in both prokaryotes and eukaryotes [10, 11]. This research was aimed at both monitoring biofilm formations and measuring cellular activities *in situ* by construction of a vector containing two reporter genes of *mer-lux* and *lac-GFP*, simultaneously.

MATERIALS AND METHODS

Plasmid Construction

All DNA manipulation, including plasmid isolation, molecular cloning, and transformation, were performed by the standard methods described previously [12]. The *mer-lux* fusion in pRB28 carries the regulatory gene *mer*R, the *mer* operon, a portion (87bp) of *mer*T, and *lux*CDABE derived from *Vibrio fischeri* [13]. pGFP cDNA vector was purchased from Clontech Laboratories (Palo Alto, CA) and digested with restriction enzymes *Sap*I and *Stu*I to isolate a DNA fragment of GFP

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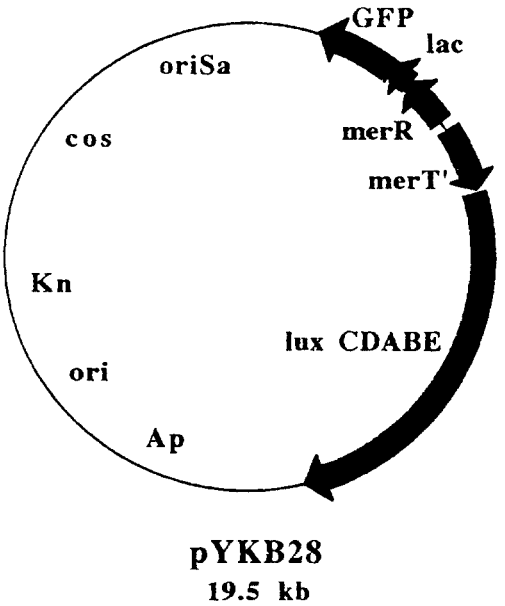

Fig. 1. Construction of a pYKB28 vector: GFP cDNA fragment under *lac* promoter was inserted in *EcoR*I site of pRB28 vector that was described previously (Selifonova *et al.*, 1993).
Abbreviations: *oriSa*, origin of replication from plasmid pSa; *ori*, origin of replication from pBR322; cos, packaging site of phage lambda; *Kn*, kanamycin resistance; *Ap*, ampicillin resistance.

cDNA under the *lac* promoter. Staggered ends of this fragment were made blunt-ends by Klenow and then linked with *EcoR*I polylinkers by T4 DNA ligase. This GFP fragment was integrated into the *EcoR*I restriction site of the pBluescript IIKS+ vector (Stratagene, La Jolla, CA) and then transformed to *E. coli* DH5α strain. *E. coli* colonies harboring GFP cDNA fragment were directly screened from agar plates by observing green fluorescence under UV light. The *EcoR*I fragment separated from the pBluescript vector was then integrated into the pRB28 vector, which had a *mer-lux* fusion gene (Fig. 1). The resulting plasmid vector, pYKB28, was screened with green fluorescence under UV light and confirmed with digestions of *EcoR*I and *BamH*I. The pYKB28 vector was then transformed into both *E. coli* DH5 and *P. putida* strains using an electroporator (BTX ECM600, BTX Inc., San Diego, CA). Electroporation conditions were followed as described in the protocol of BTX operating manuals.

Biofilm Experiments

E. coli and *P. putida* harboring pYKB28 were incubated overnight at 37°C and 28°C, respectively, in a low nutrient medium containing per liter: tryptone 1g, yeast extract 0.5g, NaCl 10g, and ampicillin 50 mg. An aliquot of the overnight culture (10 ml) was inoculated into flow-cell units using a sterile syringe and incubated for 1 hour at 25°C to attach cells to the glass surface of flow-cell units as shown in Fig. 2. Sizes of top and bottom flow-cell units were 73(w)×178(l)×19(h) mm with flow channels at 25(w)×130(l)×1(h) mm. Diameters of inlet and outlet ports were 3.2 mm and 9.5 mm respectively. Flow-cell units were sealed with silicon gaskets and sterilized with ethylene oxide gas. Cells attached to the glass in flow-cell units were growing with a continuous supply of low nutrient medium

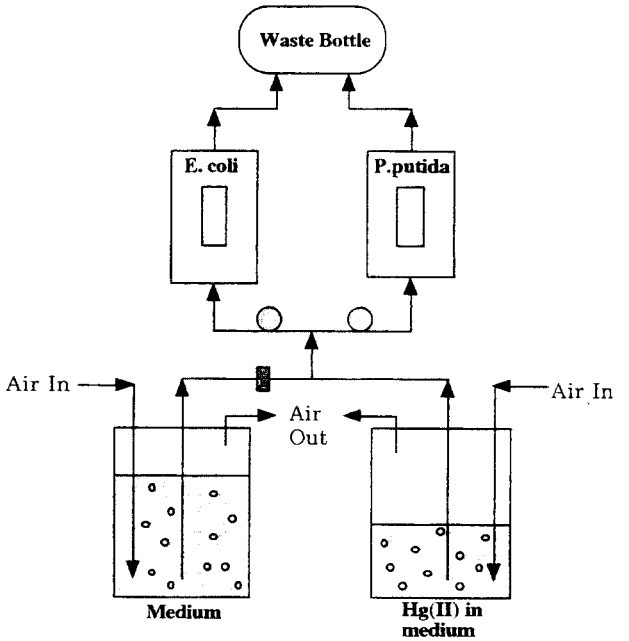

Fig. 2. Schematic diagram of flow-cell units. Low nutrient medium containing 1 μM of $HgCl_2$ was supplied to each flow-cell unit by use of a two-channel peristaltic pump (circles) for the induction of bioluminescence.

containing per liter: tryptone 0.1g, yeast extract 0.05g, NaCl 10g, and ampicillin 50 mg. The low nutrient medium was sterilized through a 0.2 μm filter unit. Sterile air was provided into the medium vessel to supply oxygen. The flow rate of the low nutrient medium was adjusted to 2.5 ml/min by a peristaltic pump. Induction of bioluminescence was performed with 1 μM $HgCl_2$.

Bioluminescent and Fluorescent Assays

Light outputs from liquid cell cultures were measured using a luminometer (Turner TD-20e, Sunnyvale, CA) after loading mercury chloride solutions. Bioluminescence emitted from biofilms was measured using an Oriel light-pipe-photomultiplier tube-ammeter assembly (Oriel Corp., Stratford, CT). A fluorescence spectrophotometer (Perkin Elmer LS50, Norwalk, CT) was used to scan emission wavelengths from liquid cell cultures with excitation of 395nm. Fluorescence from biofilms was measured with a Spex Fluorolog F212 fluorometer (Spex Industries Inc., Edison, NJ) with double grating spectrophotometers for both excitation and emission light. Light wavelengths of excitation and emission were 395 nm and 509 nm for GFP, respectively. Data were collected in a dark room after electronic probes vertically touched to the top glass of the flow-cell units.

RESULTS AND DISCUSSION

Since pYKB28 vector did not contain *mer*T and *mer*A genes, of which products are responsible to mercury transportation and mercury reduction respectively, Hg^{+2} molecules that would be uptaked to cells by passive diffusion could not be reduced to Hg^0, which is volatile to air. *Lux* gene expression was tested by light induction performed with various concentrations of $HgCl_2$. Each cell was cultured to mid-log phase (OD_{600nm} $=1.0\pm0.2$) in LB media before loading $HgCl_2$ solutions.

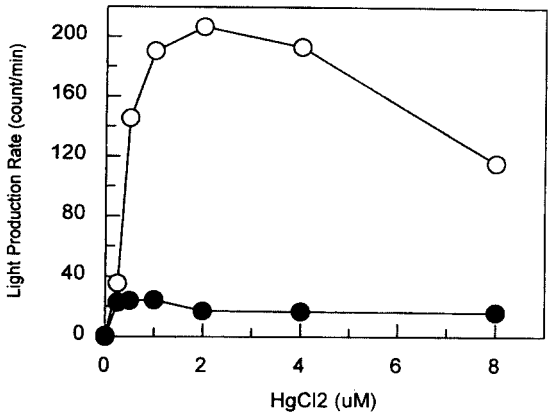

Fig. 3. *Mer-lux* fusion gene expression in *E. coli* and *P. putida* liquid cultures. Various concentrations of $HgCl_2$ were added to mid-log phase cells grown in LB media. Initial light production rates were calculated from the slope of light outputs during the first 30 min incubations at 25°C. -○- *E. coli*, -●- *P. putida*

Initial light production rates were determined from the slope of light outputs for the first 30 min incubation at 25°C. Fig. 3 showed that initial light production rates from *E. coli* culture were much higher than those from *P. putida* culture. In *E. coli* culture, the light production rates began to decrease in higher concentrations than 2 μM $HgCl_2$, indicating that the rapid uptake of Hg(II) inhibited other cellular functions due to the toxicity of mercury molecules. In contrast, initial light production rates of *P. putida* culture did not increase linearly along with higher concentrations of $HgCl_2$. The reason is not known but it may be caused by the different genetic and/or physiological systems of *P. putida*.

GFP expression was examined by scanning emission wavelengths when 0.1 ml of *E. coli* overnight culture in 2 ml of phosphate buffer saline (pH 7) was excited with 395 nm. As shown in Fig. 4, there were peaks observed around 509 nm due to the GFP expression, which were not appeared in a control *E. coli* culture that did not contain a pYKB28 vector (data not shown). The GFP expression level was not shown to be dependent upon IPTG concentration, possibly because of the absence of *lac*I gene in the pYKB28 vector. Thus it was not necessary to include IPTG in a low nutrient medium to induce the expression of GFP during the following biofilm experiments. In *P. putida* grown on LB agar plates, the GFP expression yielded much less light of fluorescence than that of *E. coli*. Surprisingly, the GFP expression was not observed in liquid cultures of *P. putida* even with a microscope (data not shown).

E. coli and *P. putida* harboring the pYKB28 vector were grown in low nutrient media overnight and then transferred to flow cell units. Since exopolymers are responsible for the attachment of bacterial cells to a surface and they are different from each microorganism, the attachment phenomena and growth of biofilms would be strongly dependent upon the type of microbial species. In this biofilm experiment, two different microbial strains of *E. coli* and *P. putida* were attached to the glass of flow cell units and monitored by measuring both bioluminescence and green fluorescence. Bioluminescent induction was continued for 120 min providing 1 μM $HgCl_2$ dissolved in a low nu-

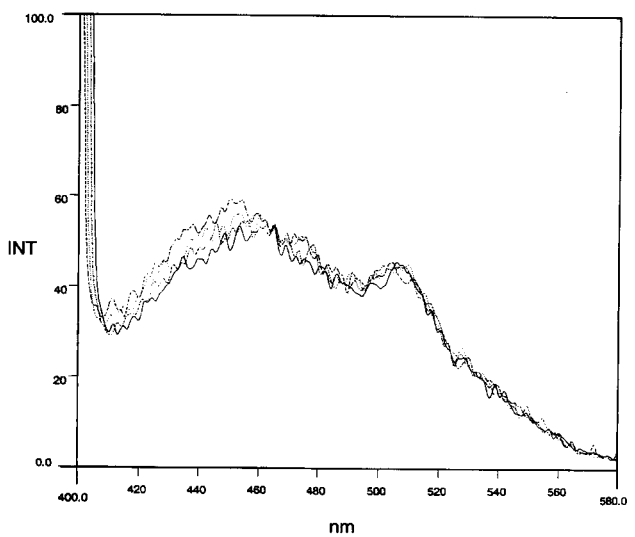

Fig. 4. GFP expression in *E. coli* liquid culture. The excitation was performed with 395 nm. *E. coli* was incubated at 37°C overnight in the presence of IPTG, of which concentrations were 0 mM, 1 mM, 2 mM, 4 mM from left to right at 400 nm position of wavelength. A peak of light emission appeared at 509 nm due to the expression of GFP.

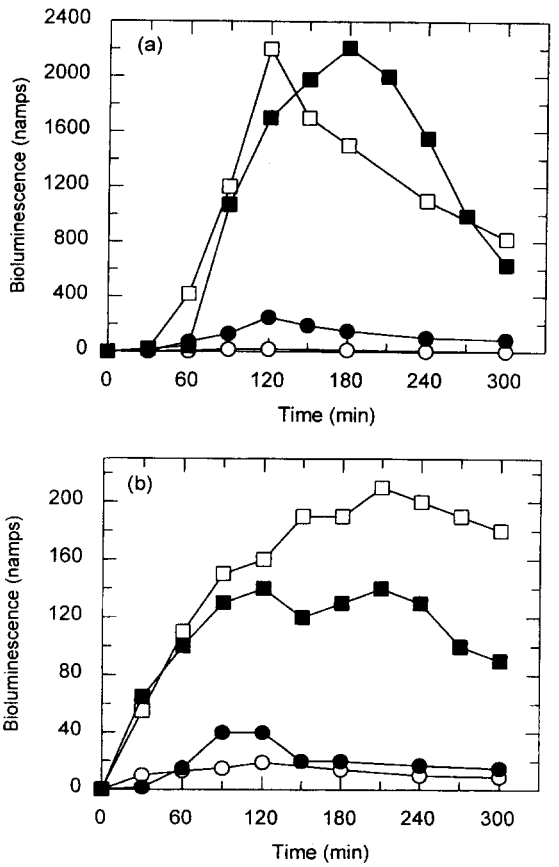

Fig. 5. Bioluminescence emitted from biofilms of (a) *E. coli* and (b) *P. putida*. Light induction by $HgCl_2$ (1 μM) was continued for the first 120 min and then fresh media were supplied until next experiments to remove residual mercury molecules inside biofilms. Light unit was indicated as nanoamperes. Data were obtained from the average of two measurements.
(a) -○- Day 1, -●- Day 3, -□- Day 5, -■- Day 7,
(b) -○- Day 1, -●- Day 3, -□- Day 5, -■- Day 7

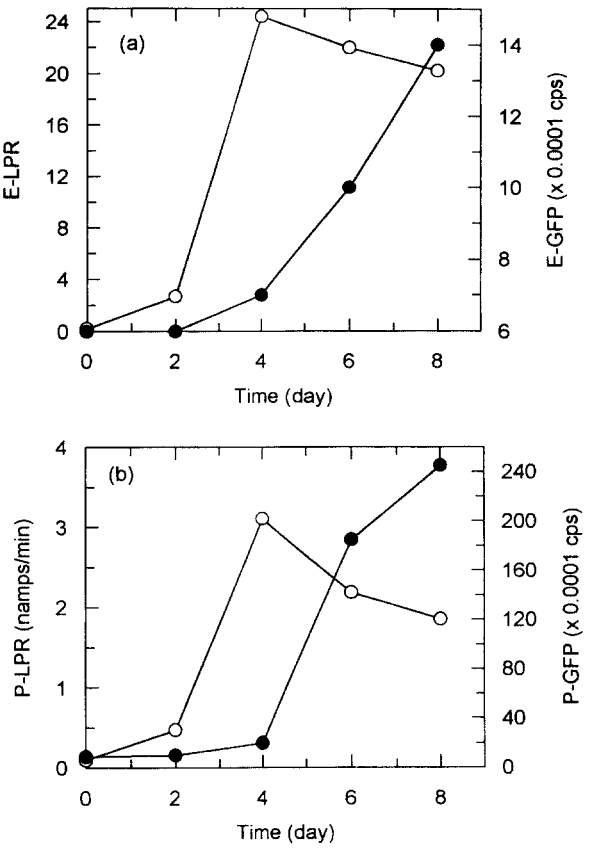

Fig. 6. Light production rates (LPR) and fluorescent levels (GFP) emitted from biofilms of (a) *E. coli* and (b) *P. putida*. CPS represents light counts per second. Data were obtained from the average of two measurements.
(a) -○- E-LPR, -●- E-GFP, (b) -○- P-LPR, -●- P-GFP

trient medium, and then fresh medium was supplied to wash out the residual mercuries from biofilms. As shown in Fig. 5, it was possible to detect bioluminescent light from *P. putida* biofilms in no more than 24 hours, while the light from *E. coli* biofilms was detected at a later time. This phenomena could be explained by the differences in attachment characteristics of the two strains since *Pseudomonas* strains were reported to be good surface colonizers [14, 15]. On the other hand, the ambient room temperature (25°C), which was used to make biofilm growth, might contribute to a more rapid biofilm development of *P. putida*. Biofilms of the two strains had been fully developed in 5 days. The maximum light output of *P. putida* biofilms was about 10 fold less than that of *E. coli*, agreeing with the results obtained from liquid cultures. It has been reported that the diffusivity of lower molecular weight compounds was not limited in biofilms [16]. Thus it is unlikely that the transport of mercury in the *P. putida* biofilm was inhibited by the biofilm structure. During a 4-hour washing time after supplying mercury molecules for 120 mins, the light outputs from *E. coli* biofilms declined progressively but not those from *P. putida* biofilms, indicating that exopolymers of *P. putida* biofilms may have higher binding affinity to mercury molecules than those of *E. coli* biofilms.

Bacterial luciferase was unstable in microbial cells so that its bioluminescence was known to be easily extinguished. Thus, another reporter, GFP, was used to compensate the bioluminescent results. Fluorescent levels of GFP were measured once a day just before supplying $HgCl_2$ to the flow cell units in an attempt to avoid possible light interactions with bioluminescence. Fig. 6 showed that the initial light production rates from *E. coli* biofilms sharply increased after 2 days, indicating that cells were most active after this time period. In contrast, fluorescent levels began to increase rapidly after 4 days while the bioluminescent production rates decreased. GFP expressed in biofilms was so stable that its fluorescent quantity was accumulated along with cells growing inside biofilms. Thus GFP level was able to be used as an indicator to figure out the overall cell numbers and activities attached on the glass surface without disassembling the biofilm apparatus. It is noteworthy that GFP was expressed in *P. putida* biofilm conditions although it was not in *P. putida* liquid cultures.

More researches should be done to find out optimal conditions for lux/GFP gene expression systems and/or different plasmid copy numbers in *E. coli* and *P. putida* biofilms. However, the use of lux/GFP reporters in biofilm studies may be of use in other research area, such as characterization of heavy metals binding to biofilms and antimicrobial agents against biofilm microorganisms [17].

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REFERENCES

[1] Blenkinsopp, S. A. and J. W. Costerton (1991) Understanding bacterial biofilms. *Trends. Biotech.* 9: 138-143.
[2] Melo, L. F., T. R. Bott, M. Fletcjer, and B. Capdeville (1992) Biofilms-science and technology. NATO ASI series E, vol. 223, Kluwer Academic Publishers, London.
[3] Lyttle, H. A. and G. H. Bowden (1993) The level of mercury in human dental plaque and interaction in vitro between biofilms of *Streptococcus* mutants and dental amalgam. *J. Dent. Res.* 72: 1320-1324.
[4] Brown, M. and J. N. Lester (1979) Metal removal in activated sludge: the role of bacterial extracellular polymers. *Wat. Res.* 13: 817-837.
[5] Hintelmann, H., R. Ebinghaus, and R. D. Wilken (1993) Accumulation of mercury (II) and methylmercury by microbial biofilms. *Wat. Res.* 27: 237-242.
[6] Silver, S. and T. K. Misra (1988) Plasmid-mediated heavy metal resistances. *Ann. Rev. Microbiol.* 42: 717-743.
[7] Condee C. W. and A. O. Summers (1992) A *merlux* transcriptional fusion for real-time examination of *in vivo* gene expression kinetics and promoter response to altered superhelicity. *J. Bacteriol.* 174: 8094-8101.
[8] Virta, M., J. Lampinen, and Karp, M. (1995) A luminescence-based mercury biosensor. *Anal. Chem.*

67: 667-669.
[9] Freitas dos Santos, L. M. and A. G. Livingston (1995) Membrane-attached biofilms for VOC wastewater treatment 1: Novel *in situ* biofilm thickness measurement technique. *Biotechnol. Bioeng.* 47: 82-89.
[10] Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher (1994) Green fluorescent protein as a marker for gene expression. *Science.* 203: 802-805.
[11] Crameri, A., E. A. Whitehorn, E. Tate, and W. P. C. Stemmer (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* 14: 315-319.
[12] Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (1994) Current protocols in molecular biology, John Wiley & Sons, Inc., New York.
[13] Selifonova, O., R. Burlage, and T. Barkay (1993) Bioluminescence sensors for detection of bioavailable Hg(II) in the environment. *Appl. Environ. Microbiol.* 59: 3083-3090.
[14] Stewart, P. S., A. K. Camper, S. D. Handran, C.-T. Huang, and M. Warnecke (1977) Spatial distribution and coexistence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in biofilms. *Microb. Ecol.* 33: 2-10.
[15] Lawrence, J. R., P. J. Delaquis, D. R. Korber, and D. E. Caldwell (1987) Behaviour of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microb. Ecol.* 14: 1-14.
[16] DeBeer, D., P. Stoodley, and Z. Lewandowski (1997) Measurements of local diffusion coefficients in biofilms by microinjection and confocal microscopy. *Biotechnol. Bioeng.* 53: 151-158.
[17] Srinivasan, R., P. S. Stewart, T. Griebe, C. I. Chen, and X. Xu (1995) Biofilm parameters influencing biocide efficacy. *Biotechnol. Bioeng.* 46: 553-560.