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Optimal Protocol for Enumeration of Attached Bacteria on Glass Slides

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In examining bacterial growth on glass surfaces immersed in sea water, we found serious differences between enumeration methods. Therefore, we compared various methods and found sonication and direct count methods were superior to other methods. Since the direct count method was not suitable for long-term investigation, we chose the sonication method and confirmed that sonication periods 8 times for 30 seconds was optimal for the detachment of bacteria from glass surfaces.

Key words: Attached bacteria, biofilm, direct count, glass surface, sonication

Various surfaces exposed in aquatic systems adsorb dissolved organic materials from surrounding waters, and the conditioned surfaces are invariably colonized by bacterial cells (4). Since ZoBell observed bacteria attached to glass slides exposed to natural soil in 1943 (19), there have been many reports on bacterial attachment to various surfaces (4, 8, 10, 16, 18). Most marine environments contain fewer amounts of organic matter compared to soil environments (7, 15). In such an oligotrophic marine environment the microbes are present as planktonic or sessile forms. Bacterial colonization on abiotic materials such as suspended particles, metal surfaces and concrete or biotic surfaces was thought to be one of the microbial survival strategy (5, 7, 15).

During the process of colonization on particular surfaces, bacteria produce extracellular materials (12). Microalgae and other organisms settle on these surfaces and develope biofilm. Biofilm can be formed on various kinds of surfaces such as metal, glass, plastic, concrete, etc. In some aspect it applies to human beings in remediation processes such as treatment of wastewater, degradation of recalcitrant, and aquaculture. In other aspects biofilm processed on a heat exchanger, pipelines, ship surfaces and other industrial devices could cause serious problems and consume large amounts of time and money in removing it. In addition, biofilm formed on implanted materials has been related to microbial disease (6). In this aspect, control of biofilm formation is an important topic of interest and many studies were performed and are continuing (1, 13, 14, 17). Most of these studies concentrated on the process of biofilm succession or formation of extracellular polymers. However, only a few studies have been performed on the quantitative progress of bacterial attachment and increase in surfaces (8, 11, 13, 14, 17), and some optimal protocol for enumeration of attached bacteria have been developed. Some studies adapted scanning electon microscopy (SEM) (9), while others used direct count for the enumeration of attached bacteria (3).

In this study, 1) we examined the changes in bacterial number on glass slides which were exposed to natural seawater and 2) we compared several methods for counting bacterial numbers on glass slides for the evaluation of the optimal protocol for the enumeration of attached bacteria on glass slides.

Before the tests, glass slides (25 mm in width, 75 mm in height, and 1 mm in thickness) were cleaned with 1 N HCl for 24 h to remove organic materials and washed with filtrated Milli-Q water (designated as filtrated TDW) and then dried at 105°C for 3 h. The treated glass slides were held on an acryl holder. All tests were performed in duplicate except for the direct count.

The changes in bacterial number on glass slides exposed to natural seawater were examined near Dea-Ho bank in Daesan, Chungcheong province, Korea. Pre-cleaned glass slides on acryl holders were placed into seawater and periodically sampled up to 72 h.

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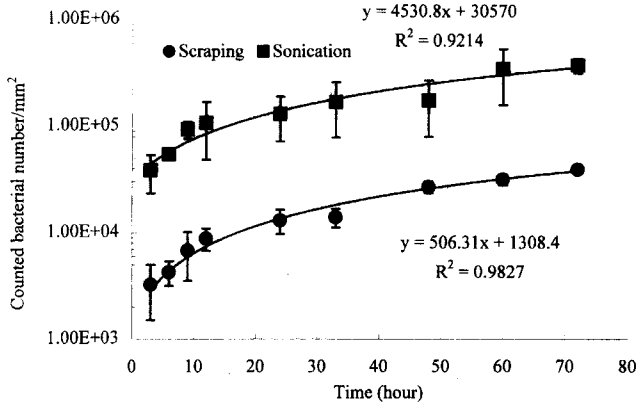

Fig. 1. Number of counted bacteria on glass slides recovered by sonication and scraping methods.

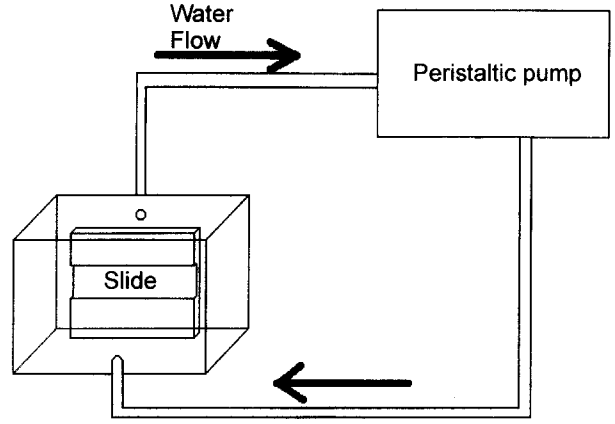

Fig. 2. The flow-system used in this experiment. Ten glass slides were fixed on an acryl holder and placed in the aged sea water flow container.

The withdrawn glass slides were rinsed with filtrated TDW to remove unattached bacteria and further treated with two different procedures. First, the exposed side of glass slide was scraped with a cell scrapper (Corning) and suspended into 10 ml of filter-sterilized aged sea water (designated as filtrated ASW) and fixed with a final concentration 1% of filtrated formalin. Then 1 to 10 ml of this scraped solution was collected on a black polycarbonate filter (25 mm in diameter and pore size of 0.2 μm, Nuclepore) and stained with 5 mg/l of 2,4-diamidino-phenyl-indol (DAPI) solution (2). The filter was observed by fluorescence microscopy at 1,000 × magnification (Axioplan, Carl Zeiss, Germany) and the bacterial number were counted from at least 12 fields.

Second, glass slides were placed into 50 ml conical tube and fixed with 50 ml filtrated formalin solution. These conical tubes were sonicated 5 times for 30 sec (total 150 sec) with a Branson sonic bath (3210R-DTH, 335 watts). The solution in the conical tube was replaced with filtrated ASW after each sonication. The collected solution was used for counting total bacterial number.

The bacterial numbers on glass slides exposed to seawater increased with exposure time (Fig. 1). This suggests that bacterial adsorption increased with time or attached bacterial cells were grown on surfaces of glass slides or both. The rates of attached bacterial numbers were $506 \cdot (mm^2)^{-1} \cdot h^{-1}$ in the scraping method and $4530 \cdot (mm^2)^{-1} \cdot h^{-1}$ in the sonication method.

As shown in Fig. 1. bacterial numbers estimated by the sonication method were approximately 10 times higher than that of scraping method [after 72 h exposure, bacterial numbers detached by scraping was estimated as 3.91×10^4 cells$\cdot(mm^2)^{-1}$, and by the sonication method, it was estimated as 3.74×10^5 cells $\cdot$ $(mm^2)^{-1}$.

Due to its small size, low density, net negative charge and cell surface hydrophobicity, bacterial cells would behave as a living colloid and easily adsorb to some surfaces. In this process a long-range van der Waals force may be the most important factor for strong adhesion of bacterial cells to net negatively charged surfaces (16). For this reason scraping method could not serve in collecting attached bacteria and the bacterial number recovered by the scraping method was one order less than that of the sonication method.

Choi *et al.* (3) reported the number of adhered bacteria on glass and acryl surface that was exposed to seawater for 6 days in Incheon Harbor as $4.0\sim10.7 \times 10^3$ and $15.8\sim46.4 \times 10^3$ cells$\cdot(mm^2)^{-1}$, respectively. 1.2×10^6 cells$\cdot(mm^2)^{-1}$ was counted on glass surface from Woods Hole harbor after 7 days of exposure (9). Bacterial numbers on a metal surface that was exposed to the Arctic river for 3 and 6 days were reported as $1.0\sim4.0 \times 10^3$ and $4.0\sim11.0 \times 10^3$ cells$\cdot(mm^2)^{-1}$, respectively (10). Our results are between the values reported by Choi *et al.* (3) and Dexter *et al.* (9). Yet it is difficult to conduct a direct comparison due to the difference in enumeration methods.

From the result of the above study, we need to evaluate the optimal protocol for detachment of bacteria from glass slides. Several methods based on bacterial dislodgment methods from sediment particles were compared.

Pre-cleaned glass slides were exposed to 2 μm filtered ASW contained in the flow system (Fig. 2). Flow rate of sea water was adjusted to 40 l/h with a peristaltic pump (Gelman Science Posi-flo II). During the experiment temperature was maintained at 15 to 25°C. After 24 h, the glass slides were collected and rinsed with filtrated TDW and then treated as four different methods as follows. I) Sonication, and II)

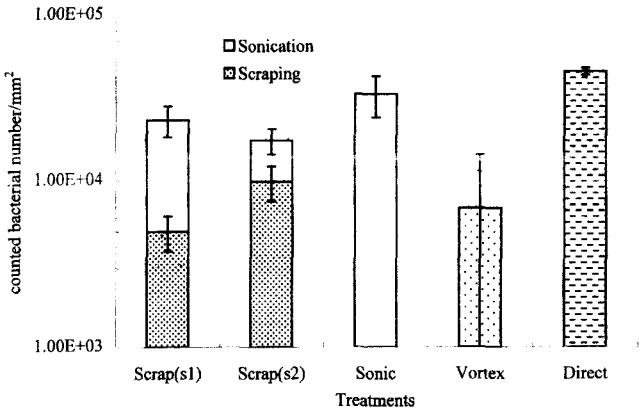

Fig. 3. Comparison of bacteria numbers recovered by various methods. Scrap(s1); scraped one side of glass slide and then sonicated glass slide, Scrap(s2); scraped both sides of glass slide and then sonicated glass slide, Sonic; sonicated the glass slide, Vortex; vortexed the glass slide, Direct; direct count of the glass slide after staining with DAPI.

scraping methods were the same as mentioned above. In case of the scraping method, the shielded side was also scraped and the scraped glass slide was sonicated as above. III) The vortexing method was tested as follows. Collected glass slide was put into a 50 ml conical tube containing 50 ml of 1% formalin solution and vortexed for 1 min. IV) Direct count method on glass slides was examined. DAPI solution was dropped on the surface of the collected glass slide and washed with filtrated TDW. The treated glass slide was observed under fluorescence microscopy.

The bacterial numbers estimated by sonication and direct count methods [3.35×10^4 and 4.6×10^4 cells·$(mm^2)^{-1}$, respectively] were approximately one order higher than those of the scraping and vortexing methods [4.97×10^3 and 6.9×10^3 cells·$(mm^2)^{-1}$, respectively]. The bacterial numbers were increased approximately two times when both sides were scraped [9.90×10^3 cells·$(mm^2)^{-1}$]. When the scraped side was further treated by the sonication method, 1.83×10^4 cells·$(mm^2)^{-1}$ were counted when one side was scraped and 7.51×10^3 cells·$(mm^2)^{-1}$ when both sides were scraped. It seemed that the attached bacteria adhered so strongly, only a small portion of it could be detached by the scraping method (Fig. 3 and 4). The accumulated bacterial numbers by scraping followed by sonication were 2.32×10^4 when one side was scraped and 1.74×10^4 cells·$(mm^2)^{-1}$ when both sides were scraped. Large amounts of bacteria may be lost during the scraping procedure.

From this result we confirmed that the direct count method and sonication method are useful for enumeration of attached bacteria on a glass surface. Comparing these two methods, the direct count method was less labor-consuming and counted bacterial numbers were higher than those of the sonication method. But this result was obtained from the initial stage of film formation, as the glass slides were exposed to seawater for only one day. Microbes may aggregate and the biofilm thickness could increase with time if the adhered surfaces were exposed to seawater for a long period. In this case the direct count could no longer be a valid method. Thus, we chose the sonication method as the optimal treatment and to optimize the number and frequency of sonication, recovered glass slides were rinsed and sonicated 3, 5, 6, 7 and 8 times for 30 sec and 15 sec, respectively.

Until 7 times of sonication for 30 sec, the cumulative bacterial numbers increased in proportion to the number of sonication times and equilibrated afterwards. In case of sonication for 15 sec, cumulated bacterial number did not reach equilibrium until 8 times of sonication and more than 8 times of sonication was needed for maximal recovery of attached bacteria. Dif-

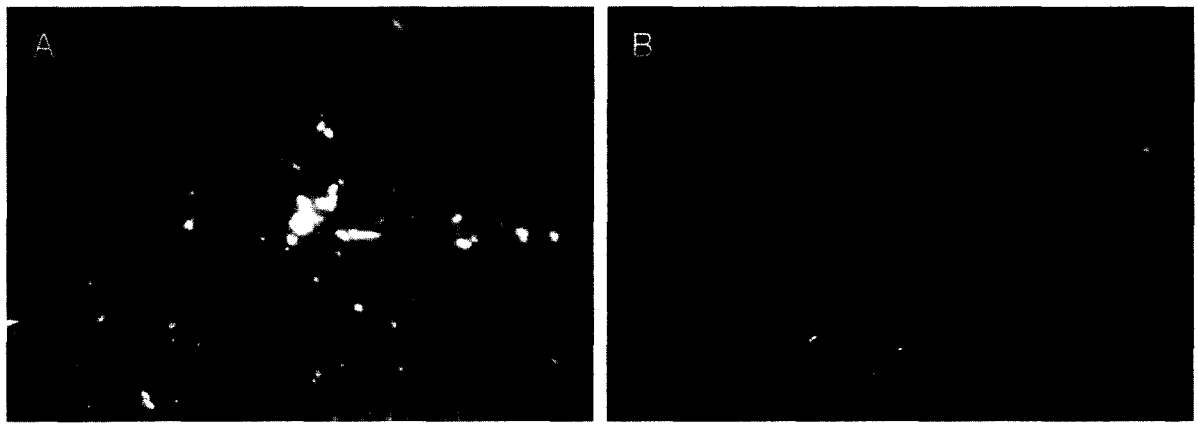

Fig. 4. Photographs of the biofilm on glass slides. A; the glass slide was submerged 24 hours into aged sea water flow container as in Fig. 2 and then stained with DAPI. B; the image of glass slide after the surface was scraped and stained with DAPI.

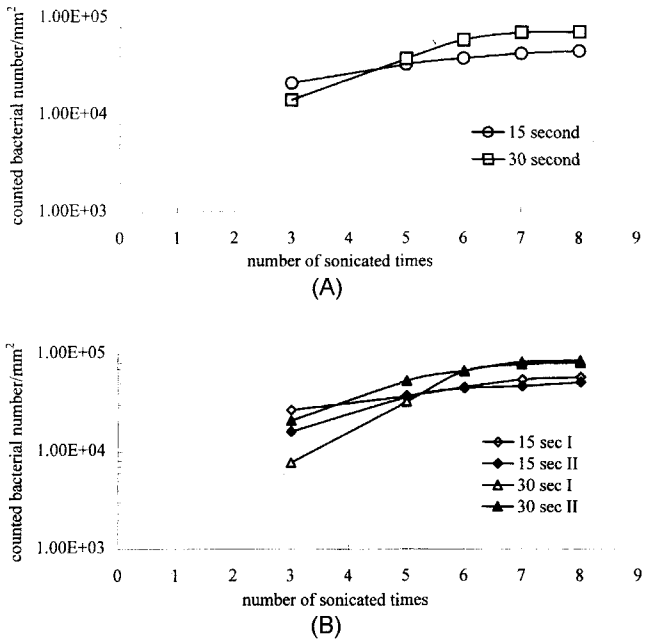

Fig. 5. Cumulative bacterial numbers after treatment of various frequency of sonication. A; average of the counted bacterial number of duplicated treatment. B; counted bacterial number of each treatment. 30 sec; sonicated the glass slide for 30 seconds. 15 sec; sonicated the glass slide for 15 sec.

ferent results were not shown between two duplicate glass slides (Fig. 5A and B). Compared to the 8 times sonication for 30 sec, detachment efficiency was 52%, 81% and 98% at 5, 6 and 7 times, respectively.

Finally we confirmed that 8 times for 30 sec of sonication (total 240 sec) was the optimal procedure for the detachment of bacteria from glass surfaces.

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