SHORT COMMUNICATION

Biofilm removal technique using sands as a research tool for accessing microbial attachment on surface

Nathanon Trachoo

Abstract

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Biofilms have profound impacts on improved survival of the constituent microorganisms in nature. Biofilms were believed to protect constituent microorganisms from sanitizer treatment, provide a more suitable habitat for microorganisms, and become a site for genetic material exchanges between microorganisms. As we realize more about the significance of biofilm, methods used for biofilm study should be consistently developed and evaluated. To determine microbial attachment on surfaces, usually biofilms are grown on substratum surfaces and removed by vortexing with glass beads or scraping. However, scraping is not as effective as vortexing with glass beads. Another approach is direct-agar overlaying which cannot be used with high density biofilm. In this experiment, we compared effectiveness of glass beads (298±28 μm in diameter) and sands (width: 221±55 μm and length: 329±118 μm) in removing biofilm of *Pseudomonas aeruginosa* by vortexing method. The results suggested that acid-washed sands, which are significantly less inexpensive than glass beads, were as effective as ($P \geq 0.05$) analytical grade glass beads in *Pseudomonas aeruginosa* biofilm removal without inhibiting growth of the organism.

Key words : biofilm, biofilm removal method, sands, microbial attachment

Ph.D.(Food Science and Technology), Department of Food Technology and Nutrition, Faculty of Technology, Mahasarakham University, Mahasarakham 44000, Thailand
E-mail: nathanon.t@msu.ac.th
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บทคัดย่อ

ณฐนนท์ ตราชู

การใช้ทรายในการลอกแผ่นชีวะเพื่อศึกษาการเกาะติดของจุลินทรีย์บนพื้นผิว

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แผ่นชีวะสามารถเพิ่มอัตราการรอดชีวิตของจุลินทรีย์ที่อาศัยอยู่ภายในแผ่นชีวะได้ และสามารถปกป้องจุลินทรีย์จากสารฆ่าเชื้อ ทั้งยังมีสภาพที่เหมาะสมต่อการมีชีวิตของจุลินทรีย์บางชนิด เนื่องจากแผ่นชีวะมีความสำคัญอย่างมากต่อความสะอาดของพื้นผิว โดยเฉพาะพื้นผิวที่สัมผัสอาหาร วิธีที่ใช้ในการศึกษาการเกาะติดของแผ่นชีวะบนพื้นผิวต่าง ๆ จึงมีความสำคัญอย่างมาก วิธีการดั้งเดิมคือการขูดแผ่นชีวะและการเขย่าด้วยเม็ดแก้วขนาดเล็ก (glass beads) อย่างไรก็ตามวิธีการขูดนั้นไม่สามารถลอกเอาแผ่นชีวะออกมาจากพื้นผิวได้ทั้งหมด ส่วนวิธีการเทอาหารแข็งลงไปบนพื้นผิวโดยตรงเพื่อเพาะเชื้อนั้น อาจจะไม่เหมาะสมกับตัวอย่างที่มีแผ่นชีวะเกาะอยู่อย่างหนาแน่น แม้ว่าเม็ดแก้วทางการค้าจะมีประสิทธิภาพสามารถลอกแผ่นชีวะได้ดีในการศึกษาการเกาะติดของจุลินทรีย์ แต่มีราคาสูงมาก งานวิจัยนี้ทดสอบประสิทธิภาพการใช้ทราย (กว้าง: 221±55 μm และยาว: 329±118 μm) ในการลอกแผ่นชีวะ เปรียบเทียบกับเม็ดแก้วทางการค้า (เส้นผ่านศูนย์กลาง 298±28 μm) โดยศึกษากับแผ่นชีวะของ *Pseudomonas aeruginosa* ที่เกาะติดบนผิวเหล็กสแตนเลส จากผลการทดลองพบว่าทรายซึ่งมีราคาถูกว่าเม็ดแก้วทางการค้ามีความสามารถในการลอกแผ่นชีวะของ *P. aeruginosa* ที่เกาะอยู่บนผิวเหล็กสแตนเลสได้ดีเสมอกัน จึงแสดงให้เห็นได้ว่าทรายมีศักยภาพในการนำมาใช้เพื่อศึกษาการเกาะติดของแผ่นชีวะของแบคทีเรีย

ภาควิชาเทคโนโลยีการอาหารและโภชนศาสตร์ คณะเทคโนโลยี มหาวิทยาลัยมหาสารคาม อำเภอเมือง จังหวัดมหาสารคาม 44000

Biofilm has become an interesting topic in many research areas in recent years including food safety. Biofilm formation can compromise the cleanliness of food contact surfaces and environmental surfaces by spreading detached organisms to food and other areas of processing plants. These detached organisms are not similar to normal microorganisms suspended in an aquatic environment because they can be more resistant to environmental and chemical stresses including some food preservation methods (de-Beer *et al*., 1994, Mosteller and Bishop, 1993; Nickle *et al*., 1985, Ronner and Wong, 1993). There are a few methods to quantitatively determine biofilm organism attached on surfaces. Low number of attached cells can be determined by overlay technique. Overlaying a dense biofilm can result in overgrowth on the surface. A teflon scraper was used to remove attached cells from flat and solid surfaces such as stainless steel and plastic coupons (Frank and Koffi, 1990; Marion-Ferey *et al*., 2003). Although biofilm removal by scraping attached cells is simple and requires an inexpensive device, it may not be suitable on samples with irregular shape and rough surfaces. Sonication was used to improve the removal efficacy of scraping techniques (Busscher *et al*., 2000). A more rapid method was used and evaluated by Trachoo and Frank (2002). Vortexing a *Pseudomonas* biofilm grown on plastic in 0.1% peptone water with 5 g of glass beads for 90 s removed 99% of biofilm population when percent coverage of vortexed and non-vortexed samples was microscopically determined by using a digital camera and computer software. Acid-washed glass beads are costly. We propose here to use acid-washed sands to remove biofilms. If we could substitute glass beads with sands, research cost would be substantially lowered. The objective of this research was thus to compare the effectiveness of biofilm removal agent using sands and glass beads.

Materials and Methods

Culture and Biofilm formation. Freeze-dried culture of *Pseudomonas aeruginosa* (357 K.

Komagata) was obtained from Thailand's Microbiological Resources Center (Bangkok MIRCEN). The culture was recovered in tryptic soy broth (TSB, HiMedia Laboratories, Mumbai) at 37ºC for 18 h. Before each use, the stock culture of *P. aeruginosa* was activated by a series of three transfers on tryptic soy agar (TSA, HiMedia) at 37ºC for 18 h. Sterile stainless steel type 304 finish 4b was cut into 1- by 5- by 0.1-cm coupons. The coupons were incubated in 10^8 CFU/ml of *P. aeruginosa* in diluted TSB (1:1) at 25ºC for 3 days. Each coupon was removed and rinsed for 10 s with 45-ml sterile water in a sterile 50-ml beaker and placed into fresh diluted TSB every 24 h.

Preparation of removal agents. Sands were sorted by using a 40-mesh sieve. The yield was 90.58%. The sands were thoroughly washed with mild detergent and water. The sands were acid-washed with 2% HCl for 20 min. Distilled water was used to wash away acid from the sands until pH 7 can be obtained. Sands were then dried in a hot-air oven at 50ºC and kept in a cool place before use. Glass beads were washed in a similar manner to sands. Five gram of washed sands or glass beads was autoclaved in a plastic bottle with 10-ml of peptone water.

Size measurement. Fifty sand crystals and glass beads were randomly selected for size measurement under a light microscope (Olympus BX40F, Olympus, Tokyo, Japan) using a stage micrometer at a magnification of 40 times. The microscope was connected to a CCD color video camera (SSC-C350P, Sony Cooperation, Tokyo, Japan). Photographs were digitally captured using a video capture device, Dazzle Digital Video Creator (Dazzle Multimedia, Singapore).

Effectiveness of biofilm removal method using glass beads and sands. After the three-day incubation, each coupon was rinsed with sterile water to remove unattached cells and placed into a sterile plastic bottle containing 10-ml of peptone water and 5 g of sands or glass beads (Sigma, St. Louis) (425- to 600-µm diameter). Biofilms were removed from the stainless steel surfaces by vortexing (vortex model G-560E, Scientific Industries, NY) for 30, 60, and 90 s at the speed of 8. Controls were not vortexed. Then 0.1 millimeter of this cell suspension from each treatment was spread plated on plate count agar (HiMedia). The plates were incubated at 25ºC for 24 h.

Microscopic analysis. After the removal of biofilm, coupons were stained with acridine orange (0.01% in acetate buffer, pH 4.0) for 1 min (Hood and Zottola, 1997). Samples were then washed with distilled water and air-dried. Stained samples were observed under an epifluorescence microscope (Olympus AX 70, Japan) at magnification of 1000 times with an excitation wavelength of 595 nm and emission wavelength of 615 nm. Photomicrographs were obtained on 35-mm color films (Kodak-400).

Effect of removal agents on *P. aeruginosa* growth. To study the effect of removal agents on the viability and growth of biofilm, 10^8 CFU/ml *P. aeruginosa* was grown in 50% TSB with 5 g of sands or glass beads (no stainless steel coupons were used). Control treatment was the culture in diluted TSB without sands and glass beads. Growth of *P. aeruginosa* was determined by plate count on plate count agar.

Data analysis. A 3×2 factorial design in a randomized completed block design with four replications was used for data obtained from the biofilm removal study. The same frozen stock cultures and equipment were used in all replicates. The main effects were vortexing time (30, 60, and 90 s) and removal agent (sands and glass beads). Data was analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA and GLM. Significant differences between means were determined using Least Significant Difference (LSD) test. Significance was determined by least square means at $P = 0.05$.

Results and Discussion

Seawater sands were purchased from an aquarium store (20 baht for 1000 g). The sands were then treated as described in materials and methods. There was about 10% loss during the preparation process. The treated sands cost 0.022 baht/g while the imported commercial glass beads

costed 30 baht/g (1 US dollar = 43.50 Thai baht, as of March 30, 2003). Sand crystals were irregular-shaped and their sizes were varied while glass beads were more uniform in size (Table 1 and Figure 1).

The three-day old biofilms were subjected to vortexing with 5 g of sands or glass beads for 30, 60 or 90 s. Data from plate counts showed that vortexing times of 30, 60 and 90 s removed the same (P>0.05) amount of *P. aeruginosa* biofilm from stainless steel coupons. Trachoo and Frank (2002) determined the biofilm attachment of *Pseudomonas* sp. and isolates from chicken farms by vortexing samples (biofilms on plastic coupons) with 5 g of glass beads from 90 s. They could removed 99% of *Pseudomonas* sp. biofilm. Although their work was done on plastic surface, according to the result in this study a shorter vortexing time may be used to potentially give similar efficacy. A series of rinsing and shaking biofilm samples in sterile saline with a flask shaker for 10 min was used to remove 99% attached *P. aeruginosa* (Wood *et al*., 1996). Although the biofilm samples were in a shaker for 10 min, the high removal rate of 99% is unlikely. This may be because the authors used different calculation methods. Biofilm removal protocol can also be specific for each type of sample. For example, biofilm with little area coverage may require longer vortexing time. Area coverage, type of organism,

Table 1. Sizes of sands and glass beads

	Measurement[a]	
	Width±SD (μm)	**Length±SD (μm)**
Sands	**221±55**	**329±118**
	Diameter ± SD (μm)	
Glass beads	298±28	

[a]**Determined by using a light microscope equipped with a stage micrometer. Data are average values of fifty measurements.**

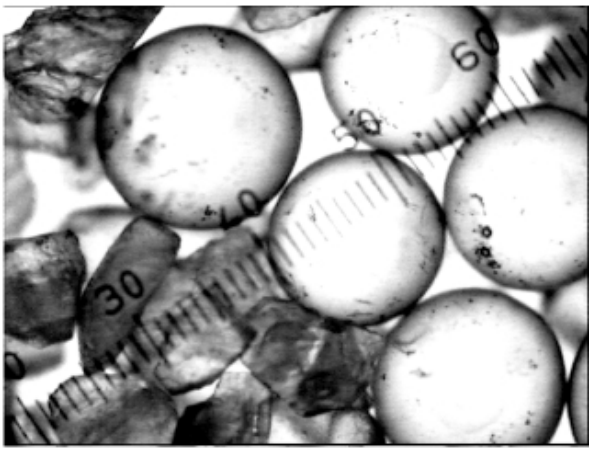

Figure 1. Photomicrographs of sands and glass beads (round-shaped). Each scale represents 25 μm.

Table 2. Efficacy of biofilm removal method by vortexing with acid-washed sands or glass beads

Vortexing time (s)	Removal agent[a]	
	Sands	Glass beads
	Log CFU/cm^2 ± SD[b]	
30	5.9±0.3	6.4±0.4
60	6.0±0.4	6.3±0.2
90	6.4±0.5	6.1±0.4
Average[ns]	6.1	6.3

[a] **Three-day-old *P. aeruginosa* biofilms on stainless steel coupons were removed by vortexing with 5 g of sands or glass beads. The cell suspension was then spread plated on plate count agar to determine the microbial attachment.**
[b] **Data are the means of four replications.**
[ns] **Values across vortexing times are not significantly different (*P*>0.05).**

biofilm age, and biofilm removal agent affect the efficacy of biofilm removal. In this study, the effect of biofilm removal agents (sands and glass beads) was insignificant (P>0.05). Sands removed 6.1 log CFU/cm^2 of *P. aeruginosa* biofilm while glass beads removed 6.3 log CFU/cm^2 (Table 2). This indicates that sands can be used to remove biofilm of *P. aeruginosa* on stainless steel as effectively as glass beads. Sand vortexing is expected to be able to remove biofilms from surfaces of irregular shapes where scraping method cannot efficiently be employed. Busscher *et al*. (2000) studied biofilm formation on artificial voice prostheses which had dents and curved surfaces. Scraping off followed by sonication was used to access microbial attachment. In an experiment to quantify the population size of biofilm on leaves, endive and parsley leaves were subjected to washing, rinsing, sonication and grinding (Morris *et al*., 1998). It is believed that the biofilms on these leaves could be effectively removed by vortexing with sands as described in this study since it simultaneously simulates the actions of washing, rinsing and brushing. However, if the leaves were to be preserved, sonication may be the method of choice for removal biofilms that attach on this kind of soft and thin surfaces.

In order to verify that this protocol will not affect the viability and growth of biofilm organism, *P. aeruginosa* was incubated with 5 g of sands or glass beads at 25ºC for 10 h. Control without sands and glass beads was included. *P. aeruginosa* increased in number with incubation time. Sands and glass beads did not (P>0.05) affect the growth of *P. aeruginosa* as compared to the control over a period of 10 h.

Photomicrographs of control (Figure 2A), glass bead-cleaned (Figure 2B) and sand cleaned (Figure 2C) coupons show the attachment of *P. aeruginosa* on stainless steel coupons. *P. aerugi-*

Table 3. Effect of sands and glass beads on the growth of *P. aeruginosa* in 50% TSB at 25ºC

Time (h)	Control[a]	Sands	Glass beads
		Log CFU/ml ± SD[b]	
0	8.0±0.2	7.9±0.5	7.8±0.5
5	8.3±0.1	8.4±0.2	8.3±0.1
10	9.3±0.3	9.3±0.2	9.1±0.1
Average[ns]	8.5±0.1	8.5±0.5	8.4±0.0

[a] **Control without sands and glass beads.**
[b] ***P. aeruginosa* (10^8 CFU/ml) was incubated with 5 g of sands or glass beads in 50% TSB. Log CFU/ml was determined by plate count on PCA. Data are the means of three replications.**
[ns] **Average values across time are not significantly different (*P*>0.05).**

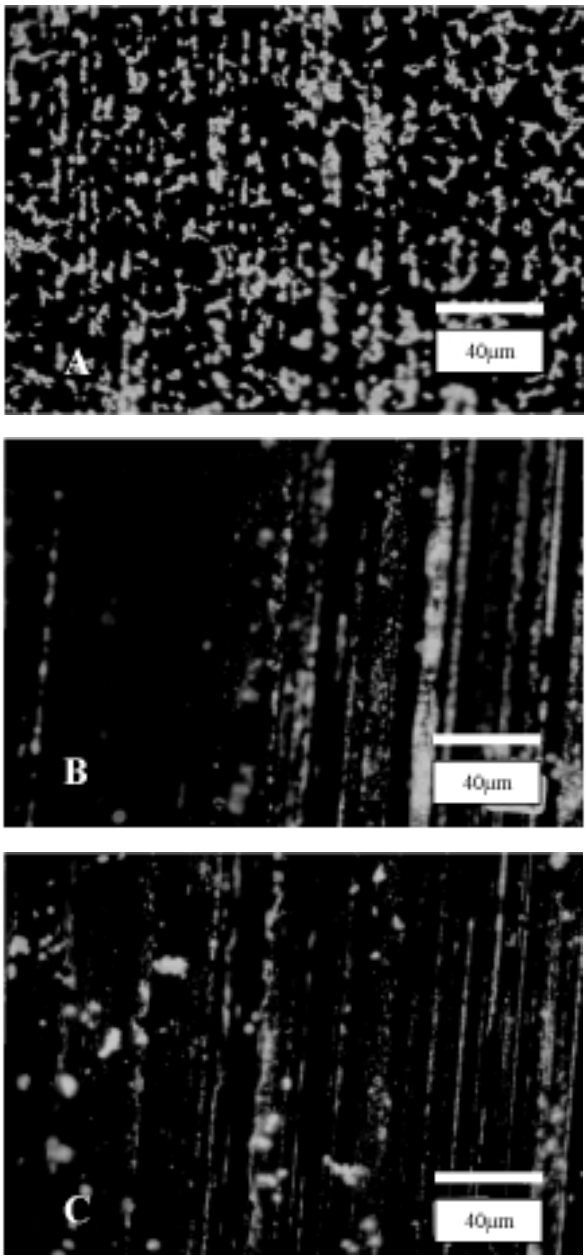

Figure 2. Photomicrographs of *P. aeruginosa* biofilm (A) and biofilms that have been removed by vortexing with glass beads (B) and sands (C) for 60 s. These biofilms were grown on stainless steel type 304 finish 4b at 25ºC for 3 days and stained with acridine orange.

nosa cells attached on scratches that may have occurred during the finishing process of the stain-less steel. It has been illustrated in other report (Trachoo and Frank, 2002) that inactivation of attached cells may be achieved but a complete elimination of biofilm cells is very difficult.

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