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Research Article

BIOFILM DETECTION AND QUANTIFICATION FOR BIOREMEDIATION BY IRON OXIDISING BACTERIA

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Abstract-The objective of this study was to investigate the initial attachment and biofilm formation of iron-oxidising bacteria, *Leptothrix* sand *Sphaerotilus* spp. Biofilm detection was carried out by 3 methods *viz.* tissue culture plate, tube method and congo red method. All the isolates were positive for biofilm formation. Tissue culture plate method was found to be reliable. Further, the biofilms formed were quantified spectrophotometrically and subjected to different environmental stress conditions. Various factors like temperature, glucose, and NaCl concentration for biofilm formation were studied and determined to be optimal at 30-37ºC, 4-6% and 2-4% respectively. Under different environmental stress conditions biofilm formation decreased with exposure to UV, SDS, lysozyme and trypsin treatments. MATH was performed to determine biofilm hydrophobicity, three of the isolates showed adhesion pattern whereas two of the isolates did not respond.

Keywords- Biofilms, Iron-oxidising bacteria, Tissue culture plate method, Environmental stress condition, MATH

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Introduction

Human health and wellbeing are at stake due to science and technological disproportionate developments not having considerations on environmental issues and their implications. According to a survey conducted by US EPA(1984) showed that heavy metals were the most common contaminants in the 395 remedial action sites in the US causing human hazards. This is a universal phenomenon all around the globe, including our country. It is alarming that, the levels of heavy metals in our environments including air, water and soil are at toxic (lethal) levels, due to industrial and domestic sources endangering our human life and ecosystem.

Heavy metal contamination results from anthropogenic as well as natural calamities. Anthropogenic activities such as mining, smelting operation and agriculture have locally increased levels of heavy metals such as Cd, Co, Cr, Pb, As and Ni in soil up to dangerous levels. Metal contamination has led to different types of medical problems like birth defects, cancer, skin lesions, and growth retardation leading to disabilities, heptorenal and other maladies. Heavy metals are persistent in nature, therefore it get accumulated in soils and plants. Dietary intake of many heavy metals through consumption of plants has long term detrimental effects of human health [19].

Once the metal is out of the earth's crust, it is difficult to put it back into earth despite effort being made. Technologies for reduction should be cost effective and affordable. Most industries use engineered technologies for remediation based on physico-chemical methods. Bioremediation method using plant and microbial systems have been developed for detecting pollution as well as for remediation [14].

Bioremediation is a sustainable strategy that utilizes the metabolic potential of microorganisms and plants to clean-up contaminated environments. It achieves contaminant decomposition or immobilization by exploiting the existing metabolic potential of microorganisms with novel catabolic functions derived from selection or by introduction of genes encoding such functions. Bioremediation is a cost effective eco-friendly means of healing nature with nature [24].

Sheathed iron bacteria of the genus *Leptothrix* are found commonly in aquatic and terrestrial habitats containing aerobic-anaerobic interface zones in which Fe and Mn are cycled between their oxidized (insoluble) and reduced (soluble) forms. These bacteria often are the dominant in such habitats, where their filamentous growth habit and Fe- and Mn- oxidizing ability can cause massive accumulation of ore-forming minerals. Because of the binding of trace metals to the iron and manganese oxides, these bacteria are attractive candidates for use in model expolymer-based metal binding biofilm and metal recovery systems [7].

Heavy metals can be biosorbed by microbes at binding sites present in cellular structure without the involvement of energy. Among the various reactive compounds associated with bacterial cell walls, the extracellular polymeric substances are of particular importance and are well known to have significant effects on acid-base properties and metal adsorption. Studies on the metal binding behaviour of extracellular polymeric substances (EPS) revealed a great ability to complex heavy metals through various mechanisms, which include proton exchange and micro-precipitation of metals. In order to meet the a need for the biofilm formation the present study was focused on optimizing the various factors involved in biofilm formation, detection, environmental conditions and hydrophobicity nature.

Materials and Methods

Biofilm Detection Methods:

A) Tube method (TM)

This is a qualitative method for biofilm detection. A loopful of test organisms inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes. Incubate the tubes at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with distilled water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible thick film lined the wall and the bottom of the tube [12].

B) Congo Red Agar method (CRA)

CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and congo red indicator 8 g/L. Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other

medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose. CRA plates with test organisms were inoculated and incubated at 37ºC for 24 h aerobically. Black colonies indicate biofilm production [11]

C) Tissue culture plate method

Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37ºC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates (Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37ºC for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm [5].

Biofilm Formation

Spore formation

Sporulation was induced by adding 10mg $MnSO_4.H_2O$ per litre of nutrient broth. When at least 95% spores were presented, the cultures were harvested and subsequently washed by repeated centrifugation (4000Xg for 20 mins) and re-suspended in sterile distilled water at 4ºC. Spore suspension was treated with 50% ethanol for 1 hr to kill any remained vegetative cells. After 3 times of washing with PO_4-buffer, the spore pellet was re-suspended in P-buffer (7-10ml) and then stored at 4ºC [12].

Quantification of Biofilm Formation by Vegetative Cells on Polystrene

The commonly used microtitre-plates method for determining bacterial adhesion to plastic surface was applied. Briefly, the wells of sterile 96 well polystyrene microtitre-plates were filed with 230µl of tryptone soy broth (TSB). A quantity of 20µl of each culture was added into every single well. The negative control wells contained TSB only. The plates were incubated aerobically for 24 hrs at tested temperature (30, 37, 45 and 55ºC). The content of the microtitre-plates was poured off and the wells were washed 3 times with 300µl of PO_4-buffer. The remaining attached bacteria were fixed with 250µl of methanol per well. After 15 mins microtitre- plates were emptied and air dried. The microtitre-plates were stained with 250µl per well of 1% crystal violet used for gram staining for 5 mins. The excess of stain was rinsed off by placing the microtitre- plates under running tap water. After the microtitre plates were air dried, the dye bound to the adherent cells was extracted with 250µl of 33% (V/V) glacial acetic acid per well. The absorbance of each well was measured at 570nm using UV-visible spectrophotometer. Based on the absorbance (A570nm) produced by bacterial films, strains were classified into 4 categories according to the classification of Christensen and others which modified by Stepanovic and others [12].

Briefly, the cut off absorbance (Ac) was the mean absorbance of the negative control.

Strains were classified as follows:

A= Ac= no biofilm producer (0);

Ac< A= (2 X Ac) = weak biofilm producer (+);

(2X Ac) < A= (4X Ac)= moderate biofilm producer (++);

(4X Ac)< A= strong biofilm producer (+++).

All tests were carried out in triplicate and the results were averaged.

Influence of Food- Related Stress Conditions on the Production of Biofilm

The concentration of glucose and NaCl used are within the range relevant for the food industry. In addition to glucose (0.25%) and NaCl (0.5%) contents of TSB medium, different concentration (2, 4, 6, 8%) of glucose and (0, 2, 4, 6) of NaCl were added. Biofilm formation under these food-related stress conditions was examined at different incubation temperatures respectively 30, 37, 45 and 55˚ C [12].

Influence of Environmental Stress Conditions on the Production of Biofilm

The ability of vegetative cells to form biofilm was studied after heat treatment (100ºC for 10 min) or after exposure to UV light (254nm) for 10 mins in sterilized plates opened in a laminar flow. Biofilm formation was also studied under dynamic conditions by incubating the microtitre- plate in at 150 rpm. A diluted TSB medium was used as mimic food industry conditions. This medium was prepared by mixing TSB with sterilized distilled water (1: 20, V/V). In these experiments, biofilm formation was studied at the optimum growth temperature for each tested bacterium [12].

Influence of Destroying and Removing Surface Layers on Biofilm Formation

Surface layers of the vegetative cells were removed or destroyed. Briefly, stock of either cells or spores were centrifuged (1000g for 10 mins) and then resuspended to the original volume in one of the following solutions: 2% Sodium dodecyl sulphate (SDS, Aldrich) at 100ºC for 10 mins or 1% trypsin (Sigma) at 37ºC for 1 hr. A control preparation was made in distilled water. After treatment, the cells or spores were centrifuged (1000g for 10 mins), re-suspended to the original volume in PO_4- buffer and stored at 4ºC. After aforementioned treatments, biofilm formation by vegetative cells and attachment of spores to substratum were carried out at the optimum growth temperature of each tested bacterium [12].

Determination of Hydrophobicity

Percentage of hydrophobicity was determined using the Microorganism Adhesion To Hydrocarbon (MATH) test. The prepared spore suspension was centrifuged at 3000g for 10 mins and the pellet was re-suspended in sterile distilled water to an A600nm of 1.2-1.6. Samples of each suspension (3ml) were added to 3ml hexadecane, mixed on a vortex mixer for 1min, incubated at 30ºC for 10 min and then vigorously remixed again on a vortex mixer at ambient temperature for 2 min. The absorbance of the aqueous phase was measured at 600nm after standing at ambient temperature for 20 min. The percentage of hydrophobicity (% h) was determined from the absorbance of the initial bacterial cell, or spore suspension (Ai) and the absorbance of the aqueous phase after mixing with hexadecane (Af) using the following equation:

$$\% h = [(Ai- Af) X 100] / Ai$$

Result

Biofilm detection and quantification

Biofilm detection for all the isolates was carried out by tissue culture plate, tube and congo red method. All the isolates were positive for biofilm formation. In the present study, tissue culture plate method showed best result than tube and congo red method. Hence, all the five isolates were chosen for biofilm quantification test.

Quantification of biofilms formed by the isolates was done by staining 96 well microtitre plate with crystal violet. Deeply and faintly stained well were observed. Further absorbance was read at 660nm using spectrophotometer. According to the calculation, IB-29 isolate was a strong biofilm producer, whereas, IB-06 and IB-16 was moderate and IB-07 and IB-22 was week biofilm producer [Table-1].

Table-1 *Biofilm film detection and quantification*

Isolates	Od at 590nm Average	Result
IB06	1.6	Moderate
IB07	1.1	Weak
IB16	1.98	Moderate
IB22	0.33	Weak
IB29	1.97	Strong

Effect of incubation temperature on biofilm formation

At the optimum growth, temperature of 30ºC IB-29 showed maximum ability to

form biofilm. This isolate was still a strong biofilm producer above their optimum growth temperature (37ºC). IB-06 and IB-16 showed moderate biofilm producing ability at optimum temperature and continued to be a moderate biofilm producer at 37ºC. Isolates IB-07 and IB-22 was a week producer both at 30ºC and at 37 º C. At 45ºC, IB-06, IB-22 and IB-29 became weak biofilm producers, whereas at 550C, there was no biofilm activity [Table-2].

Table-2 *Effect of incubation temperature on biofilm formation*

Isolates	Incubation temperature in ^{0}C			
	30	37	45	55
IB-06	++	++	+	-
IB-07	+	+	-	-
IB-16	++	++	+	-
IB-22	+	+	-	-
IB-29	+++	+++	+	-

The combined effect of temperature, glucose and NaCl on biofilm formation

The ability of all tested isolate to form biofilm on polystyrene surface was enhanced by increasing glucose concentration in TSB upto 8% [Fig-1a and 1b]. Isolate IB-06 and IB-29 became strong biofilm producers at 30ºC and 37ºC, when the glucose concentration ranged between 4 to 6%. On the other hand, slight enhancement in biofilm formation by all the strains was obtained by increasing NaCl concentration upto 2 to 4% [Fig-2a and 2b]. Biofilm production was even seen at zero NaCl.

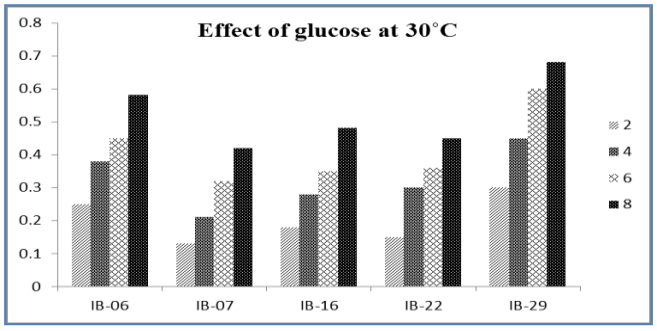

Fig-1a Effect of Glucose at 30˚C

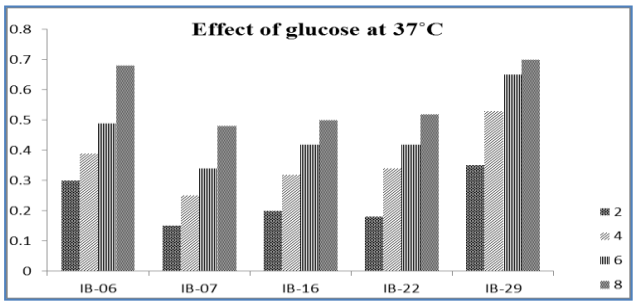

Fig-1b Effect of Glucose at 37˚C

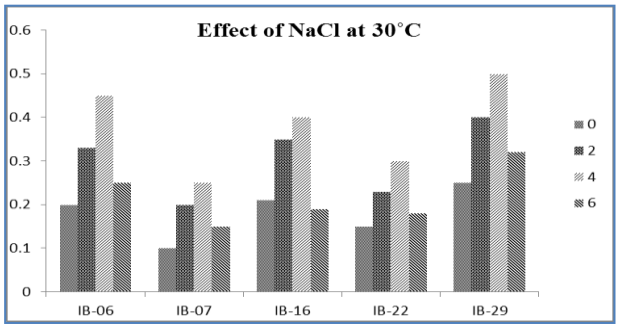

Fig-2a Effect of NaCl at 30˚C

Effect of environmental stress on biofilm formation

The effect of environmental stress on the ability of isolates to form biofilm on polystyrene surface was studied at the optimum growth temperature of each tested isolates [Table-3].

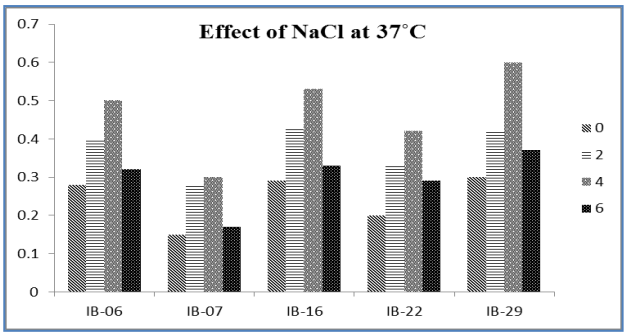

Fig-2b Effect of NaCl at 37˚C

Under conditions of nutrient starvation (diluted TSB 1:20), the ability to produce biofilm on plastic surface was significantly enhanced by all the tested isolates. In contrast, significant reduction was recorded by all the isolates after heating (100ºC for 10 min) the cell suspension and after exposure to UV (254nm for 10min). Under dynamic conditions (150 rpm) isolates IB-06, IB-07, IB-16, IB-22 lost their abilities to produce biofilm and interestingly, isolate IB-29 a strong biofilm producer became weak.

Table-3 *Effect of environmental stress on biofilm formation*

Isolates	Incubation temperature	Control	Nutrient starvation	Heating	UV	Dynamic Conditions
IB-06	30 and 37^{0}C	++	-	+	+	-
IB-07		+	+	-	-	-
IB-16		++	++	+	+	-
IB-22		+	+	-	-	-
IB-29		+++	+++	++	++	+

Role of outer layers of vegetative cells in adhesion to polystyrene

The ability of the isolate to form biofilm on polystyrene surface was drastically decreased after treatment with 1% lysozyme, except IB-29, which became a weak biofilm producer [Table-4]. All the five isolates became poor biofilm producer after treatment with SDS, whereas, isolate IB-29 became a weak biofilm producer in trypsin.

Table-4 *Role of outer layers of vegetative cells in adhesion to polystyrene*

Isolates	Control	SDS	Trypsin	Lysozyme
IB-06	++	-	-	-
IB-07	+	-	-	-
IB-16	++	-	-	-
IB-22	+	-	-	-
IB-29	+++	-	+	+

Determination of hydrophobicity

Among the tested isolates, IB-29 showed maximum percentage hydrophobicity for hexadecane closely followed by a moderate biofilm producer, IB-07. IB-16 showed a very least adhesive pattern with adhesion value 4. Based on direct adhesion values of the isolates to plastic surface, two isolates namely IB-06 and IB-22 did not respond to any adhesion pattern [Fig-3].

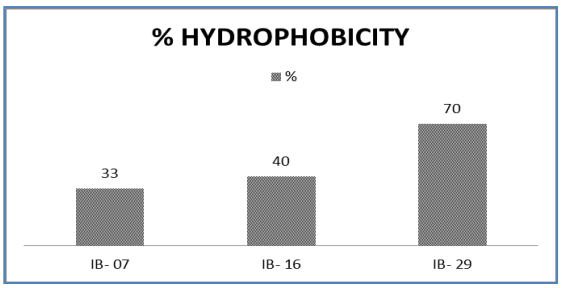

Fig-3 Determination of hydrophobicity on adherence to hexadecane

Discussion

Biofilm detection for all the isolates was carried out by tube and congo red method. All the isolates were positive for biofilm formation. Hope and Bott [13] have reported the biofilm formation in *Leptothrix sps*. Likewise Lee *et al.,* [10] also mentioned the biofilm formation of *Leptothrix* isolated from aqueous environment. Emerson *et al.,* [6] reported the ability of biofilm formation by *Gallionella* from the marine environment.

In the present study quantification of biofilms formed by the isolates were done by staining 96 well microtitre plates with crystal violet. In another study, PCR was used for the quantitative analysis of biofilm [8]. 4% of total cells were shown to form strong biofilm which was in much disagreement with the present study where it was only 0.2%. This may be due to different environmental conditions.

In the present study, the 96 well polystyrene microtitre plates were filled with cultures and were incubated at different temperatures i.e. 30, 37, 45 and 55ºC. At the optimum growth temperature (30ºC), IB-29 showed the maximum ability to form biofilm. IB-06 and IB-16 showed moderate biofilm producing ability at optimum temperature and continued to be a moderate biofilm producer at 37˚C. Isolates IB-07 and IB-22 was a week producer both at 30ºC and at 37˚C. At 45˚C, IB-06, IB-22 and IB-29 became weak biofilm producers, whereas at 55ºC, there was no biofilm activity. The results obtained in a previous study demonstrated that the optimum temperature for biofilm formation by *Bacillus spp* was 30ºC. This could be explained by the strong relationship between the bacterial attachment conditions and the metabolic activity. In a study by [22], showed biofilm formation has been limited not only by elevated temperature but also by individual microbial ability to produce EPS. When native microorganisms from the subsurface at Yucca mountains were tested, temperatures from 60-70˚C at 100% RH inhibited biofilm formation, resulting in few culturable counts above the level of detection. Also the authors in this study observed that the boundary conditions too required 100% RH and 30˚C for even minimal biofilm production. Other factors such as metal type, nutrient availability and toxicity may have played a role in biofilm development as well. High temperatures predicted slow or eliminated biofilm formation.

The food related stress in the present study was analysed using different concentrations of glucose and NaCl. Isolate IB-06 and IB-29 became strong biofilm producers at 30ºC and 37ºC. Slight enhancement in biofilm formation by all the strains was obtained by increasing NaCl concentration upto 2 to 4%. Biofilm production was even seen at zero NaCl. Similar studies, including the combined effect of temperature and some additives were not previously undertaken for iron bacteria. At a temperature higher than the optimum growth temperature the ability of the species to form biofilm may be due to heat protective effect of glucose. Rode, [20] also demonstrated the increase biofilm formation by *S. aureus* at 46˚C comparing with those obtained at optimum temperature (37˚C). Pertaining to the NaCl concentration studies, Hesham [12] reported a decrease in biofilm formation with increase in NaCl concentration, which was very contrast to the present study. This may be due to different osmotic stress caused by NaCl on polystyrene.

Under conditions of nutrient starvation (diluted TSB 1:20), the ability to produce biofilm on plastic surface was significantly enhanced by all the tested isolates. In contrast, a significant reduction was recorded by all the isolates after heating (100ºC for 10 min) the cell suspension and after exposure to UV (254 nm/10min). Under dynamic conditions (150rpm) isolates IB-06, IB-07, IB-16, IB-22 lost their abilities to produce biofilm, whereas, isolate IB-29 a strong biofilm producer became weak. Nutrient availability has a major influence on biofilm structure and composition of microbial community. The enhancement of biofilm formation at different nutrient condition is supported by an earlier research carried out by Van Horn [25] and Chemier [3]. Also a study done by Faisal [9] showed an increase in biofilm formation under different nutrient condition. According to Hesham [12], biofilm formation of many Bacilli initiated by starvation and is assumed to be advantageous for bacteria in oligotrophic environment. Morikawa [16] showed that in spore forming bacteria, nutrient starvation induces some genes encoding starvation-activated transcription factors, EPS biosynthesis and surfactin production. These alterations led to alter bacterial surface characteristics such as hydrophobicity, charge and irreversible attachment, which are essential factors in biofilm formation. In contrary, Ngwai [17] demonstrated reduction in biofilm formation due to nutrient starvation in gram-negative bacteria.

Changing the ability of the isolates from moderate biofilm producer to weak biofilm producer after heat or UV treatment indicates that both treatments significantly affected the cell viability but did not affect the attachment of cells to polystyrene surface. The dynamic conditions (150 rpm) had negative effect not only on attachment of isolates to polystyrene surface but also on biofilm development, were four of the tested strains were no biofilm producer. In accordance with Chmielewski and Frank [4] the steady-state conditions play an important role in initial adhesion of bacteria to surfaces. They mentioned that adhesion to a substratum can be active or passive depending on cell motility.

Among the tested isolates, IB-29 showed maximum percentage hydrophobicity for hexadecane closely followed by a moderate biofilm producer, IB-07. IB-16 showed a very least adhesive pattern with adhesion value 4. Based on direct adhesion values of the isolates to the plastic surface, two isolates namely IB-06 and IB-22 did not respond to any adhesion pattern in the present study. According to Avery and Tobin [1] the surface treatment seems to have an influence on the removal of percentage of Lead and Iron. The best results obtained with granular activated carbon (GAC) treatment with HNO_3 seem to be connected with the higher presence of acidic groups on the surface of this kind of GAC. This acidic group contributes to a better adhesion of biofilm and consequently to a higher removal. The better adhesion of the biofilm to the GAC-HNO_3 is confirmed by the higher production of polysaccharides and total polymers [18].

The ability of the isolate to form biofilm on polystyrene surfaces was drastically decreased after treatment with 1% lysozyme, except IB-29, which became a weak biofuel producer. All the 5 isolates became no biofilm producer after treatment with SDS, whereas, isolate IB-29 became a weak biofilm producer in trypsin. According to Severin [21] after lysozyme treatment, the *Bacilli sp* were less adherent because they lack N-acetyl group. Hesham [12] showed that all bacilli spores had fewer adherences to lysozyme, which was similar to the present observation. According to Cazemier et al., [2] treatment with trypsin or SDS appear to be more effective as a reducer of spore hydrophobicity compared with lysozyme treatment. They found that SDS treatment was effective for obtaining coat-defective spores. Therefore, the appreciable changes in number of attached spores may be referred to changes in spore hydrophobicity after treatment with SDS/ trypsin. Kolari [15] suggested that *D. geothermalis* were not detectably removed from polystyrene surfaces by 1hour washing with 0.2% SDS/ NaOH. *D. geothermalis* formed more biofilm on surfaces of tissue culture treated polystyrene than on regular polystyrene, which differ much with the present observation.

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