Journal of Environmental Biotechnology
Vol. 21, No. 1, 1–4, 2021

Technical Report

Construction of a Black-Dirt Formation Model Using Microorganisms Obtained from the Toilet Bowl

Michitaka Muramatsu[1], Chie Ito[1], Kazuma Niizeki[2], Mitsuhiro Gomi[2] and Masaaki Morikawa[1]*

[1] *Graduate School of Environmental Science, Hokkaido University, Sapporo 060–0810, Japan*
[2] *Research and Development Center, Kobayashi Pharmaceutical Co., Ltd., Ibaraki, Osaka 567–0057, Japan*
* E-mail: morikawa@ees.hokudai.ac.jp
* TEL/FAX: 011–706–2253

In this study, we constructed a method for preparing microbial black-dirt model on a glass plate under laboratory condition. It clearly demonstrated that indigenous bacterial biofilms function as scaffolds for fungal spore adhesion followed by black-dirt formation by a fungus, *Cladosporium halotolerans*. This black-dirt model should be a useful tool not only for development of the methods for its prevention and removal but also for studying fungi-bacteria complex biofilm formation.

Key words: biofilm, *Pseudomonas azotoformans*, *Methylobacterium goesingense*, *Mycobacterium obuense*, black-dirt, *Cladosporium halotolerans*

(Received: 6 April, 2021/Accepted: 8 May, 2021)

Hygienic environment is important for keeping human in good health. The places where troublesome microorganisms including fungi are likely to colonize in house, are mainly wet area such as a bathroom, a kitchen and a toilet, which often becomes unsanitary area[1,8].

It has been reported that environmental pollution by fungi, especially filamentous fungi, causes not only malodor and defaced appearance but also the onset of asthma, rhinitis, atopy and other allergic diseases[1,2,4,5,6,9]. One of the examples is the black-dirt formed on the toilet bowl. It causes uncomfortable feeling, generation of odors, and increases the risk of tracheal infections. It has been previously reported that the black-dirt visible on the toilet bowl is due to a melanin pigment produced in the spores of two fungi *Exophiara* sp. and *Cladosporium* sp[7]. Microscopic observation of the black-dirt formed on the toilet bowl showed that the fungal hyphae often grow over the bacterial biofilms (Fig. 1). In this paper, we simplified and optimized the experimental method to form fungal black-dirt on a glass plate in order to examine the effect of bacterial biofilms on the fungal spore adhesion followed by black-dirt formation.

We previously reported isolation of a unique biofilm forming *Rhizobium* sp. R8 that have adapted to the conditions on the toilet bowl by acquiring high urease activity instead of lacking nodule formation and nitrogen fixation capabilities[3]. *Pseudomonas azotoformans* 225A, *Methylobacterium goesingense* 124A, and *Mycobacterium obuense* 114B were isolated this time from the surface of a toilet bowl, that are

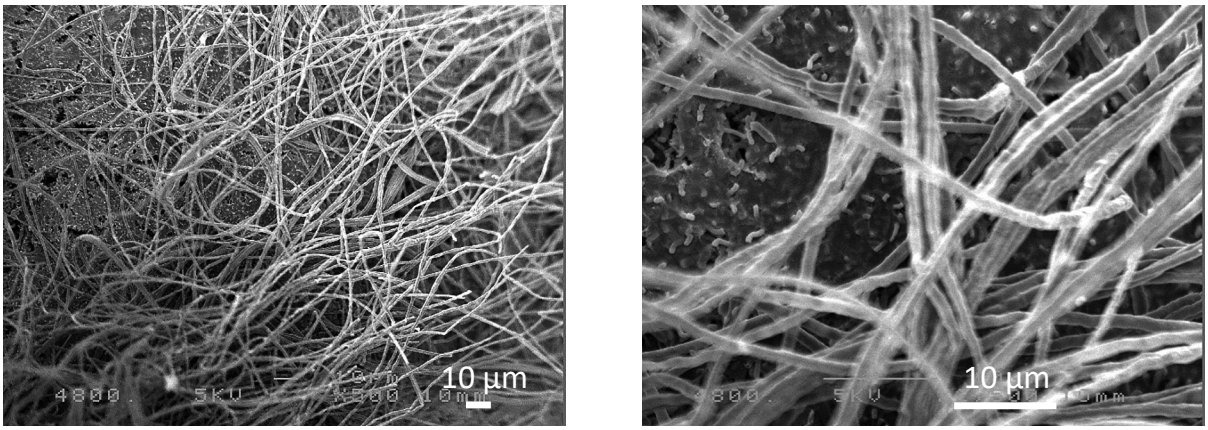

Fig. 1. Observation of black-dirt by scanning electron microscope.

capable of adhering to glass surfaces followed by biofilm formation. In this study, a black-dirt model was tried to construct using two different bacterial biofilms by single species R8 and by mix species of 225A, 124A, and 114B with a fungal strain, *Cladosporium halotolerans* which had been previously isolated from the toilet bowl[7].

R8 and 114B were cultured in TY medium and R2A medium for 72 h at 30°C, respectively. 124A and 225A were cultured in R2A medium overnight at 30°C. TY contained per liter 5 g of Difco Bacto tryptone, 3 g of Difco Bacto yeast extract, and 0.83 g of $CaCl_2$-$2H_2O$ (pH 7.0). R2A contains per liter 0.5 g each of Difco Bacto proteose peptone No.3, Difco Bacto yeast extract, casamino acid, glucose, soluble starch, and 1.3 g each of K_2HPO_4, sodium pyruvate, and 0.05 g of $MgSO_4$-$7H_2O$ (pH 7.0). A fungus *C. halotolerans* was previously grown on Difco PD agar plate at 22°C for several days and the spores were recovered using cell scraper (AS ONE Corp.) followed by filtratration by polypropyrene cell strainer (40 mm pore size, Corning Inc.) to remove hypha.

The optimized protocol for easy formation of a black-dirt model is as follows (Fig. 2):

(1) Bacterial biofilms were formed on the surface of 1 mm thick cover glass (MICRO COVER GLASS 18×18 No.5, CS00500, Matsunami glass Ind., Ltd.). Planktonic cells in the above mentioned culture were quantified by measuring optical density at 600 nm (OD_{600}) and diluted to OD_{600} value 1.0. Each bacterial strain was inoculated at 1% (v/v) to 2 mL R2A medium in 12 well plates (Costar® 12-well Clear TC-treated Multiple Well Plates, 3513, Sigma-Aldrich). Sterilized cover glass was stood up in each well and incubated at 30°C for 3 d to form biofilms on the surfaces.

(2) The biofilms can be visualized by 1% (w/v) crystal violet staining for 15 min (Fig. 3 left). The amount of biofilms was measured by OD_{595} after solubilizing crystal violet in 1 mL of 33% acetic acid for 15 min (Fig. 2). It was shown that the amount of biofilms formed was more in single species R8 than 114B, 124A, and 225A mixed-species.

(3) After formation of the biofilms, "seesaw shaking" was applied to allow selective adhesion of fungal spores onto the cover glasses with different preparations. The cover glasses with and without bacterial biofilms were fixed in lines near the wider edges of a rectangle petri dish (AW2000, Eiken Chemical Co. Ltd.) using ethylene oxide gas (EOG) sterilized 4 mm thick double-sided adhesive tape (3M 7140-12-AAD, 3M Japan Ltd.). Fungal spore in 40 mL sterilized water (300 CFU/μL) was added to the dish.

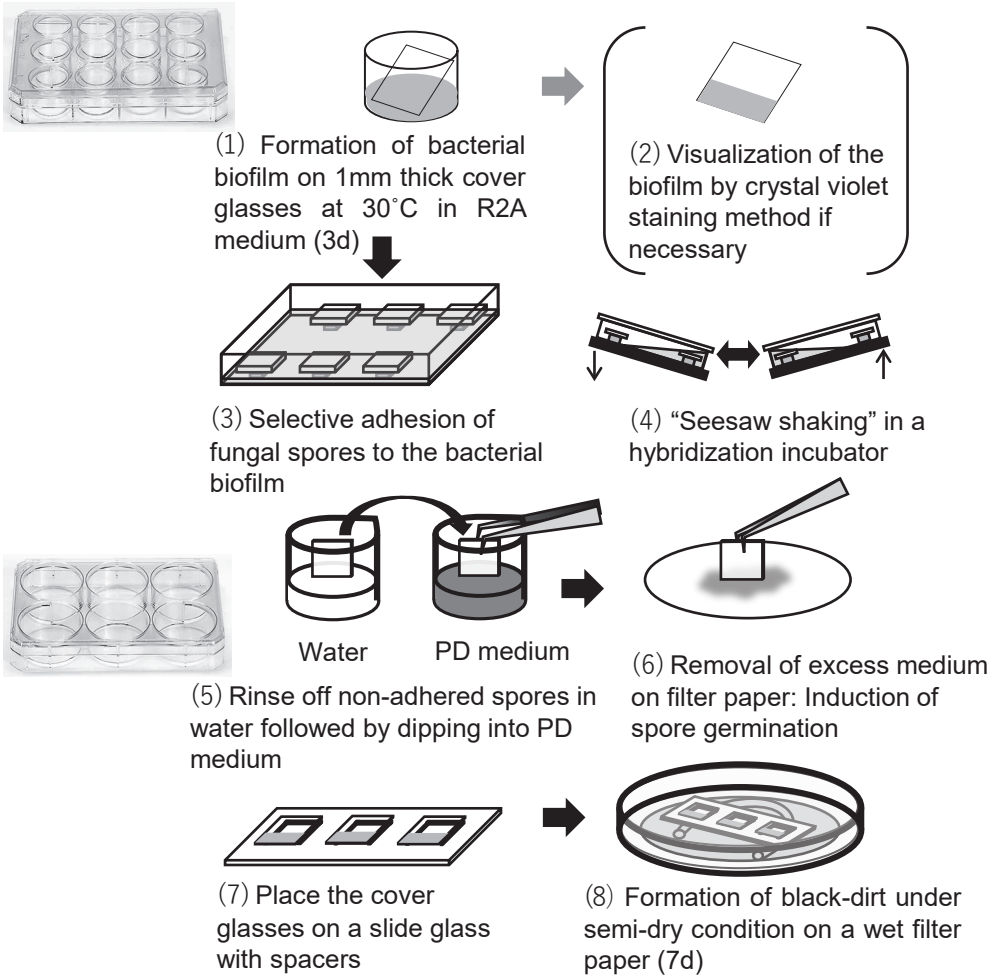

Fig. 2. Method for preparing black-dirt model in 10 d.

(4) The dishes were put in a hybridization incubator (HB-100, TAITEC Corp.) followed by "seesaw shaking" at the minimum speed of three reciprocations per min for 2 h at room temperature. This enabled to mimic the condition in part of a toilet such as intermittent water flushing on the bowl surfaces.

(5) After the 2 h seesaw shaking, each cover glass was carefully peeled off from the adhesive tape with tweezers and gently rinsed with sterilized water to remove non-adhered spores. The cover glass was then immersed in PD medium containing nutrients needed for germination and growth of the fungus.

(6) We found that removal of excess medium by touching the edge of the cover glass to the filter paper was useful to shorten the time for black-dirt formation. Direct drop of PD medium onto the cover glass was not appropriate for the rapid fungal growth.

(7) The cover glass was laid on a slide glass (SLIDE GLASS No. S1111, Matsunami glass Ind., Ltd.) by keeping distance using a staple (1224FA-H, MAX Co., Ltd.). This set of glasses was placed in a Petri dish containing a wet filter paper at the bottom.

(8) The Petri dishes were incubated at 22.5°C for 3 to 10 d until the black-dirt was observed. It was also important to keep the filter paper wet during cultivation. For example, 2 mL of sterilized water was added on the first day and another 1 mL was added after 5 or 6 d to keep the semi-dry condition of the cover glass in the petri dish.

Different formation of bacterial biofilms and fungal

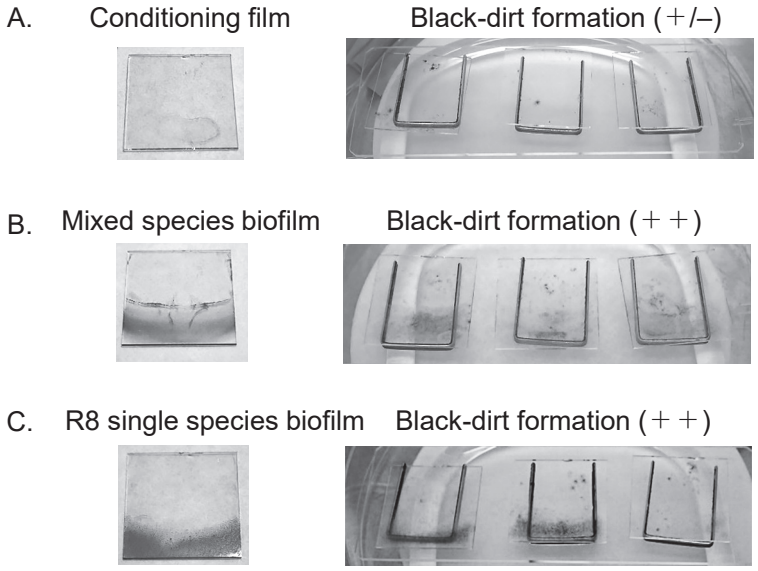

Fig. 3. Bacterial biofilm formation (3d) and black-dirt formation (7d).
Pictures on the left are cover glasses stained with crystal violet. Dark area indicates the bacterial biofilms. Pictures on the right are a cover glasses with black-dirt formation, dark area, after 7 days. "Conditioning film" is a deposit of chemicals contained in the surface water, which helps adhesion of bacterial cells followed by biofilm formation[10].

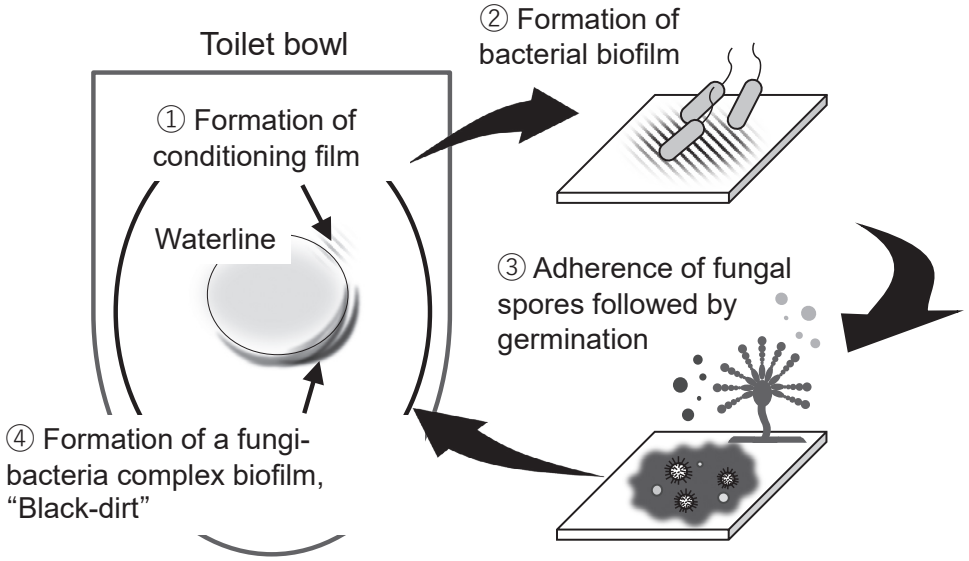

Fig. 4. Processes of black-dirt, a fungi-bacteria complex biofilm, formation.

black-dirt was shown in Fig. 3. It was evident that black-dirt was scarcely formed on the cover glass with R2A medium conditioning film only (Fig. 3A). On the other hand, cover glasses with mixed-species biofilm and single R8 biofilm showed significant black-dirt formation (Fig. 3B, 3C). Moreover, the area of biofilm formation visualized by crystal violet staining coincided with that of black-dirt formation. This observation indicated that fungal spores were dominantly adhered to the bacterial biofilms followed by germination. The biofilm of indigenous bacteria from the toilet bowl was shown for the first time to function as scaffolds of the fungal spore adhesion in the initial stage of black-dirt formation processes (Fig. 4). In addition, we developed a method for black-dirt formation in a shorter period compared to the natural black-dirt formation that usually takes 3–4 weeks.

References

1) Ara, K., M. Aihara, M. Ojima, Y. Toshima, C. Yabune, H. Tokuda, S. Kawai, N. Ueda, T. Tanaka, K. Akiyama, and K. Takatori. 2004. Survey of fungal contamination in ordinary houses in Japan. Allergology International. 53: 369–377.
2) Fouquier, J., T. Schwartz, and S.T. Kelley. 2016. Rapid assemblage of diverse environmental fungal communities on public restroom floors. Indoor Air. 26: 869–879.
3) Fukano, T., M. Gomi, Y. Osaki, and M. Morikawa. 2015. Isolation and characterization of an early colonizing *Rhizobium* sp. R8 from a household toilet bowl. 2015. Biosci. Biotechnol. Biochem. 79: 1207–1215.
4) 福冨友馬. 2016. 真菌とアレルギー疾患. Japanese Society of Allergology. 65: 113–117.
5) 井原　望，濱田信夫. 2010. 天然系抗菌・防カビ剤の利用の現状と将来. 生活衛生. 54: 304–311.
6) Jacob, B., B. Ritz, U. Gehring, A. Koch, W. Bischof, H.E. Wichmann, and J. Heinrich. Indoor exposure to molds and allergic sensitization. 2002. Environ. Health Perspectives. 110: 647–653.
7) Mori, M., Y. Nagata, K. Niizeki, M. Gomi, and Y. Sakagami. 2014. Characterization of microorganisms isolated from the black dirt of toilet bowls and componential analysis of the black dirt. Biocontrol Sci. 19: 173–179.
8) Ojima, M., Y. Toshima, E. Koya, K. Ara, H. Tokuda, S. Kawai, F. Kasuga, and N. Ueda. 2002. Hygiene measures considering actual distributions of microorganisms in Japanese households. J. Appl. Microbiol. 93: 800–809.
9) Sharpe, R., C.R. Thornton, and N.J. Osborne. 2014. Modifiable factors governing indoor fungal diversity and risk of asthma. Clinical and Experimental Allergy. 44: 631–641.
10) Lorite, G.S., C.M. Rodrigues, A.A. de Souza, C. Kranz, B. Mizaikoff, and M.A. Cotta. 2011. The role of conditioning film formation and surface chemical changes on Xylella fastidiosa adhesion and biofilm evolution. J. Colloid. Interface Science. 359: 289–295.