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BIO-BARRIER FORMATION BY BACTERIUM/FUNGUS INJECTION INTO SOILS

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ABSTRACT: If microorganisms are injected into porous medium such as soils along with appropriate substrate and nutrients, soil pore size and shape are changed from the initial condition as a result of biofilm formation, which make hydraulic conductivity reduced. In this research, hydraulic conductivity reduction was measured after specific bacterium or fungus was inoculated into soil pore. Hydraulic conductivity was decreased to 10 % ~ 1 % and maintained constant while substrate was provided. Under the adverse conditions such as no substrate, chemical solution permeation, and freeze-thaw cycles, hydraulic conductivity was increased 30~50%. Hydraulic conductivity decrease of fungus-soil mixture was faster than that of bacterium-soil mixture. Fungus-soil mixture, however, was more sensitive to the adverse conditions.

KEYWORDS: Hydraulic conductivity; microorganism; biofilm; EPS.

INTRODUCTION

Microorganism growth in soil and the consequent reduction in permeability is often associated with groundwater recharge, wastewater land application, enhanced oil recovery schemes, and more recently, *in situ* bioremediation of organic contaminants in subsurface environment. If an appropriate microorganism is injected into soil along with substrate, the immobilized cells grow, reproduce, and produce extracellular polymers (EPS) which frequently extend from the cell forming a tangled matrix of fibers which provide structure to the assemblage termed a biofilm. A biofilm consists of cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin. EPS also acts as shield to prevent a damage of biofilm (Characklis and Marshall, 1990). This microorganism and soil mixture is called as bio-barrier (Cunningham, 1993; Rijnaarts *et al.*, 1997).

Several researches indicated that microorganisms in soil have a potential on remediation of subsurface pollution. Shaw *et al.* (1985) and Stoodley *et al.* (1994) measured the permeant flow velocity and found that deposition of biofilm on soil pore decrease the velocity significantly. Brough *et al.* (1997) observed the decrease of chemical oxygen demand when filling sand in the column. In their experiments, permeability and COD were decreased about 60.6 % and 90 % respectively and proved that biofilm can be applied to install bio-barrier and its capability of biodegradation. Precedent researches were carried out using homogeneous porous medium such as glass beads and thus involve difficulties on applying to the field condition. In order to install bio-barrier in the field, sustainability of bio-barrier should be evaluated under adverse conditions. In this research, microorganisms were inoculated into residual soil and cultured. Possibility of using as bio-barrier was evaluated. In addition, sustainability of bio-barrier under the adverse condition was evaluated.

MATERIALS AND METHODS

Soils used in this research were sand and residual soil. Sand was used for observing permeability change and biofilm formed after inoculation. Diameter of sand was in the range of 250 μm ~ 420 μm.

Physical characteristics of sand were; uniformity coefficient = 1.58; curvature coefficient = 1.03; D_{50} = 0.47mm. Residual soil was used for observing permeability change and testing physicochemical characteristics of the biofilm. Residual soil was taken a sanitary landfill construction site. Physical characteristics of the residual soil were; class SP by unified classification system; D_{50} = 0.63 mm; liquid limit = 30 %; plastic limit = 24.3 %; plastic index = 5.8 %; specific gravity = 2.67; and optimum water content = 15.8 %.

The bacterium and fungus used in this research were *Azotobacter chroococcum* and *Aureobasidium pullulans*, respectively. *A. chroococcum* is a mesophilic and nitrogen fix bacteria, and its optimum pH range for growth is between 5.5 and 8.5. (Stainer *et al.*, 1986; Atlas and Bartha, 1992). All experiments are carried out under the temperature controlled condition at 25±2 °C. Substrate for *A. chroococcum* was consisted of KH_2PO_4, 200 mg/L; K_2HPO_4, 800 mg/L; $MgSO_4$, 200 mg/L; $CaSO_4$, 100 mg/L; trace amount of $FeSO_4$ and Na_2MoO_4; yeast extract 500 mg/L; and mannitol, 20 g/L.

Reagents for substrate were dissolved in the distilled water and adjusted to optimum pH 7.6 with 1N NaOH or 1N HCl. In order to remove any microorganism in the substrate solution, it is sterilized in the autoclave at 121℃, 1.5 kg/cm^2 for 15 minutes. Number of bacterium was measured using cell count method (Atlas and Bartha, 1992) before inoculation. *Aureobasidium pullulans* is an imperfect fungi and produce exopolysaccaride pullulan during growth period. *A. pullulans* was cultured in the media composed of diced potatoes 200 g/L, glucose 20 g/L, agar 15 g/L, and potato dextrose agar 39 g/L at 25 °C for 2 days. Cell count of fungi was measured indirectly by weighing. Inoculum was diluted and centrifuged at 10000 rpm for 30 minutes. Isolated cells are dried in the vaccum oven at 80 °C for 24 hours and weighed.

All test programs carried out in this research are shown in Table 1. In Test 1, nutrient solution inoculated with *A. chroococcum* permeated sand to form biofilm. SEM (Scanning Electron Microscopy) was utilized to observe the biofilm. Constant head test was carried out to measure the permeability of sand and a rigid wall mold with 10.0 cm of diameter and 12.4 cm of height was used.

Cell concentration of the bacterium was 3×10^7 cell/ml and the specific weight of sand was 1.6 kg/cm^3. In Test 2, permeability of residual soil was measured as a function of time after *A. chroococcum* was inoculated. Compaction energy on the residual soil was 5.64 kg/cm^2. Variable head test was carried out according to ASTM D5856-95. In Test 3, permeability change when chemical solutions permeated through biofilm formed. In general, pH of leachate from landfill is in the range of 4.0～8.5 (Sharma and Lewis, 1994). With considering the optimum pH of *A. chroococcum* is between 5 and 8, acidic solution which adjusted to pH 3 with HCl and alkaline solution which adjusted to pH 11 with NaOH were used in this research. In Test 4, probability of impair of biofilm by freeze and thaw was tested.

Four compaction mold of equally compacted residual soil were subjected to test. One mold is for measuring permeability change and other three molds are for measuring porosity at each freeze-thaw cycle. Initial porosity of all four mold were assumed identical because of same water content and compaction energy. Soil specimen was frozen completely at –4°C for 24 hours (Moo Young and Zimmie, 1996), and thawed at 5 °C for 24 hours. Tab water was permeated to measure permeability. Test 5～8 are similar to Test 1～4 respectively except inoculating *A. pullulans* instead of bacterium.

Table 1. Test program and concentration of bacteria used for the test

Test	Microorganism	Cell Conc.*1,*2	Soil	Test Method	Permeant
1	Bacterium (*Azotobacter chroococcum*)	3×10^7	Sand	CH*4	nutrient solution → tap water
2		$5\times10^6\sim3\times10^7$	SP*3	VH*5	nutrient solution → tap water
3		2×10^6		VH	nutrient solution → chemical solution
4		8×10^6		VH	nutrient solution → three freeze-thaw cycle
5	Fungus (*Aureobasidium pullulans*)	5.45	Sand	CH	nutrient solution → tap water
6		2.60	SP	VH	nutrient solution → tap water
7		2.60		VH	nutrient solution → chemical solution
8		2.60		VH	nutrient solution → three freeze-thaw cycle

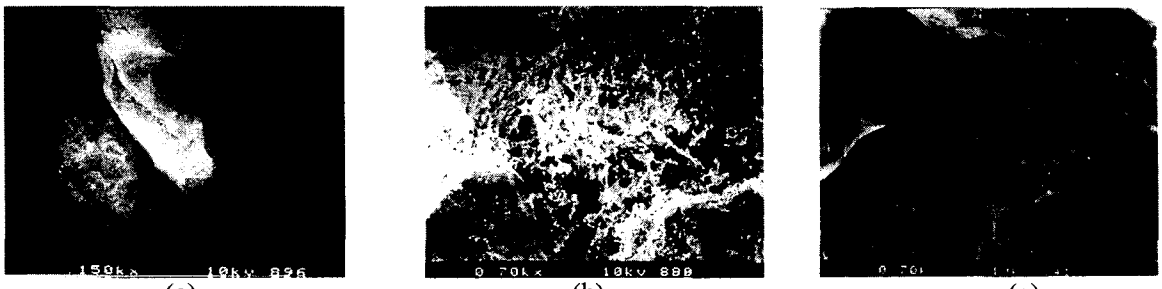
*1 Bacteria concentration (cell/ml) by plate count method
*2 Fungus concentration (g/l): dry cell weight
*3 Poorly graded sand by Unified Classification System
*4 CH : Constant Head Permeability Test
*5 VH : Variable Head Permeability Test

DISCUSSION

Sand was mixed with *A. chroococcum* along with substrate and cultured for 10 days in the shaking incubator. Initial permeability of sand was 4.3×10^{-2} cm/s, which was decreased to 2.6×10^{-3} cm/s after 10 days. To understand the clogging phenomena of biofilm, SEM was utilized to observe the soil surface with bacterium inoculation. Figure 2 (a) is the 700 times enlarged image of the sand surface before bacterium inoculation. Figure 2 (b) is the SEM images of soil aggregates taken from the head of the soil specimen after substrate permeation, enlarged 700 times.

From the Figure 2 (b), it can be observed that biofilm clog the soil pore between sand particles. Taylor *et al.* (1990) proposed a set of model to describe permeability reduction. The model assumes that microorganism cells adsorbed on soil surface increase the soil diameter, thus make soil less permeable. However, that bacterium forms several layers of mesh forms of biofilm between soil particles as well as soil surface as shown in Figure 2 (b). The mesh layers effectively clog the soil pores result in permeability reduction. The model proposed by Taylor *et al.* (1990) thus needed to be modified to consider this observation.

Figure 2 (c) is the SEM image enlarged 700 times of the soil surface with fungus inoculation. With comparing Figure 2 (b), cell size of fungus is much larger and biofilm thickness is much thicker. It can be also observed that soil pore is completely plugged with fungus cells.



(a) (b) (c)

Figure 1. Comparison of SEM images of pore sand before and after biofilm formation. (a) sand aggregates before injection of microorganism (×150); (b) after biofilm formation by bacterium (×700); (c) after biofilm formation by fungus (×700).

Figure 2 is the permeability change of residual soil when bacterium or fungus was inoculated. Nutrient solution was permeated for 15 or 20 days followed by the permeation of tab water. Initial permeability of

the residual soil inoculated with *A. chroococcum* is 1.1×10^{-4} cm/s ~1.05×10^{-4} cm/s, which decreased to 9.1×10^{-7} cm/s ~ 7.8×10^{-7} cm/s after 15 days and 20 days of nutrient solution permeated, respectively. This value is approximately 1/130 compare to the initial permeability. When tab water was permeated, permeability was increased to 2.1×10^{-6} cm/s ~ 1.7×10^{-6} cm/s which is the 1/50 of the initial permeability.

Specimen inoculated with *A. pullulans* showed permeability decrease to 1/10 ~ 1/100 of the initial value. By the comparison of culturing time required for permeability reduction to reach the least permeability, soil specimen inoculated with *A. pullulans* showed much less time required compared to that of *A. chroococcum*. When nutrient supply was discontinued and resumed, bacterium showed better recovery rate of biofilm than fungus. From the Figure 2, it can be expected that if bacterium or fungus is inoculated into the soil of permeability in the range of 1×10^{-5} cm/s ~ 1×10^{-6} cm/s, resultant permeability of soil after culture would be less than 1×10^{-7} cm/s.

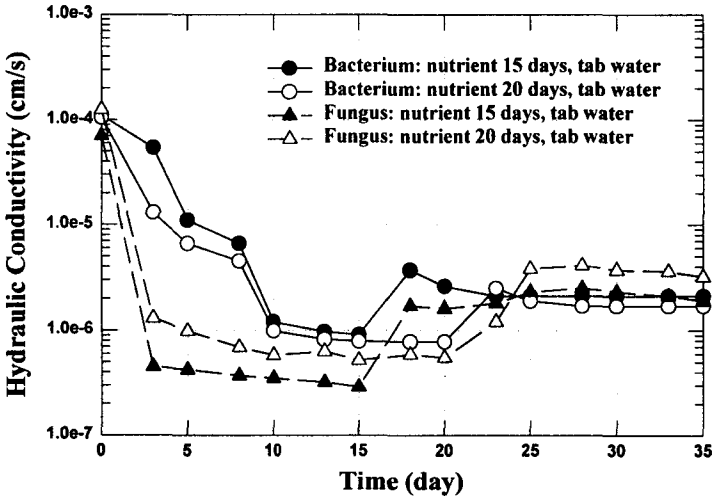


Figure 2. Hydraulic conductivity change of specimens permeated with nutrient solution and tap water

Figure 3 is the hydraulic conductivity change of specimens permeated with nutrient solution followed by chemical solution. Resistance to chemical substances of biofilm formed on soil surface was evaluated. Acidic permeant (0.1N HCl) or basic permeant (0.1N NaOH) is permeated. With the soil specimen inoculated with *A. chroococcum*, no drastic permeability increase was observed when acidic liquid was permeated while basic liquid permeation increase permeability significantly. When acidic or basic liquid was permeated the soil inoculate with *A. pullulans*, permeability was increase approximately 30 % for both cases. This implies that fungus is much sensitive to chemical substances than bacterium.

Figure 4 is the hydraulic conductivity changes with freeze-thaw cycles. Durability of biofilm to temperature change was evaluated by repeated freeze-thaw cycles with residual soils inoculated with bacterium or fungus. Permeability of residual soil mixed with *A. chroococcum* was 1.7×10^{-5}cm/s initially, which increased to 7.3×10^{-5} cm/s by three freeze-thaw cycles.

Porosity was also increased from 0.58 to 0.59 possibly due to impaired biofilm. If substrate was provided to the soil, however, initial permeability and porosity were almost recovered. Residual soil mixed with *A. pullulans* showed the permeability increase by freeze-thaw cycles from 5.7×10^{-6} cm/s to 2×10^{-6} cm/s. Porosity of the specimen was also increased from 0.53 to 0.57. This means that bio-barrier formed by fungus can be impaired by the adverse environment, such as low temperature and chemical permeant.

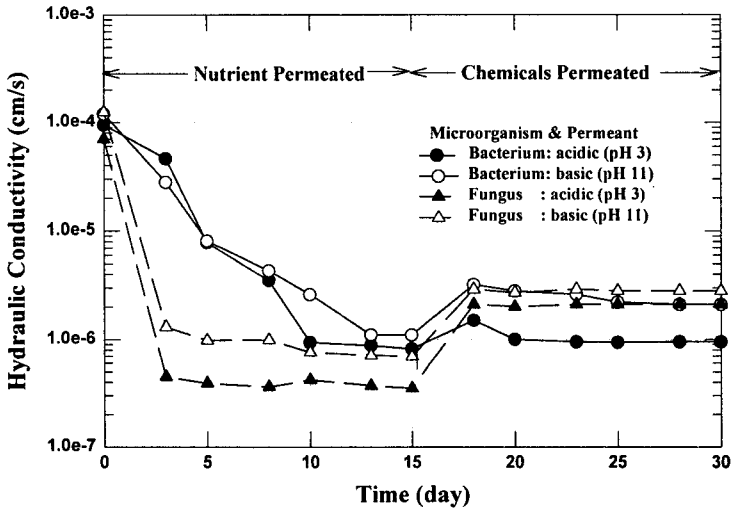


Figure 3. Hydraulic conductivity change of specimens permeated with nutrient solution and chemical solution

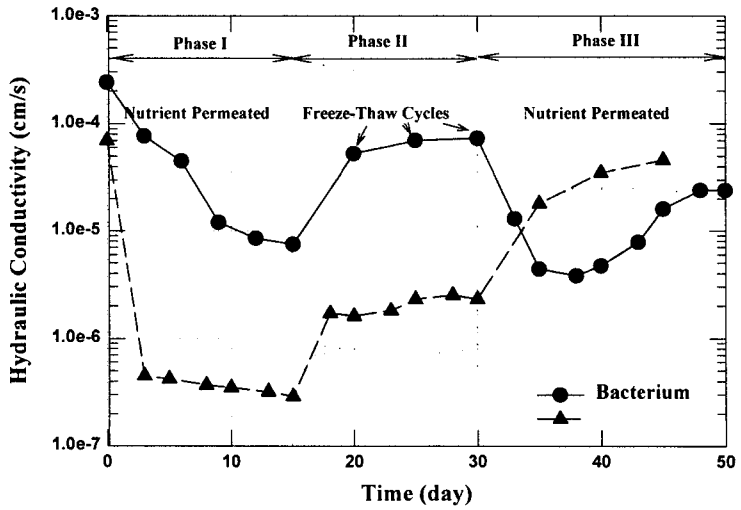


Figure 4. Hydraulic conductivity changes with freeze-thaw cycles

CONCLUSIONS

In this research, bacterium (*Azotobacter chroococcum*) or fungus (*Aureobasidium pullulans*) was inoculated into soils such as sand or residual soil to observe permeability reduction due to biofilm formation in the soil pore. After carrying out the laboratory scale permeability tests under several conditions, the following conclusions were made.

1. As a result of SEM observation of biofilm, it can be observed that bacterium forms several layers of mesh forms of biofilm between soil particles as well as soil surface. The mesh layers effectively clog the soil pores, which resulted in permeability reduction. In the case of fungus inoculum, increased thickness of biofilm formed on soil surface completely plugs the soil pore.

2. Residual soil of initial permeability in the range of $1.6\times10^{-4}\sim1\times10^{-4}$ cm/s was inoculated with *A. chroococcum*. Permeability was decreased as much as $1/10\sim1/100$ and maintained constant. Specimen inoculated with *A. pullulans* showed permeability reduced to $1/10\sim1/100$ of the initial value. By the comparison of culturing time required for permeability reduction to reach the least permeability, soil specimen inoculated with *A. pullulans* showed much less time compared to that of *A. chroococcum*. When substrate supply was discontinued and resumed, bacterium showed better recovery rate of biofilm than fungus.

3. Resistance to chemical substances of biofilm formed on soil surface was evaluated. If acidic permeant (0.1N HCl) or basic permeant (0.1N NaOH) is permeated, no drastic permeability increase was observed with the soil specimen inoculated with *A. chroococcum* when acidic liquid was permeated while basic liquid permeation increase permeability significantly. When acidic or basic liquid was permeated into the soil inoculate with *A. pullulans*, permeability was increase approximately 30 % for both cases. This imply that fungus is much sensitive to chemical substances than bacterium.

4. Durability of biofilm to temperature change was evaluated by repeated freeze-thaw cycles with residual soils inoculated with bacterium or fungus. Initial permeability of residual soil mixed with *A. chroococcum* was 1.7×10^{-5}cm/s, which increased to 7.3×10^{-5} cm/s by three freeze-thaw cycles. Porosity was also increased from 0.58 to 0.59 implying that biofilm was impaired. If substrate was provided to the soil, however, initial permeability and porosity were almost recovered. Residual soil mixed with *A. pullulans* showed the permeability increase by freeze-thaw cycles from 5.7×10^{-6} cm/s to 2×10^{-6} cm/s. Porosity of the specimen was also increased from 0.53 to 0.57. This means that bio-barrier formed by fungus can be impaired by the adverse environment, such as low temperature or non-neutral permeant.

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