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Engineered bioclogging in coarse sands by using fermentation-based bacterial biopolymer formation

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Abstract. Sealing of leakage in waterfront or water-retaining structures is one of the major issues in geotechnical engineering practices. With demands for biological methods as sustainable ground improvement techniques, bioclogging, defined as the reduction in hydraulic conductivity of soils caused by microbial activities, has been considered as an alternative to the chemical grout techniques for its economic advantages and eco-friendliness of microbial by-products. This study investigated the feasibility of bioaugmentation and biostimulation methods to induce fermentation-based bioclogging effect in coarse sands. In the bioaugmentation experiments, effects of various parameters and conditions, including grain size, pH, and biogenic gas generation, on hydraulic conductivity reduction were examined through a series of column experiments while Leuconostoc mesenteroides, which produce an insoluble biopolymer called dextran, was used as the model bacteria. The column test results demonstrate that the accumulation of bacterial biopolymer can readily reduce the hydraulic conductivity by three-to-four orders of magnitudes or by 99.9-99.99% in well-controlled environments. In the biostimulation experiments, two inoculums of indigenous soil bacteria sampled from waterfront embankments were prepared and their bioclogging efficiency was examined. With one inoculum containing species capable of fermentation and biopolymer production, the hydraulic conductivity reduction by two orders of magnitude was achieved, however, no clogging was found with the other inoculum. This implies that presence of indigenous species capable of biopolymer production and their population, if any, play a key role in causing bioclogging, because of competition with other indigenous bacteria. The presented results provide fundamental insights into the bacterial biopolymer formation mechanism, its effect on soil permeability, and potential of engineering bacterial clogging in subsurface.

Keywords: bioclogging; insoluble biopolymer; bacteria; fermentation; biosealing

1. Introduction

Leakage in waterfront or water-retaining structures, such as embankments, dams, and levees, is one of the major issues in geotechnical engineering practices for the risks of high drainage costs, saltwater intrusion, contaminants migration, and structural damages (Blauw *et al.* 2009, Foster *et al.* 2000). Injection of fine clay particles or chemical compounds as grouts has been widely used to seal and repair such leaks (Eklund and Stille 2008, Ouyang and Daemen 1996). However, use of chemical grouts for sealing are often expensive and not eco-friendly (Achal and Kawasaki 2016, Farah *et al.* 2016). With increasing demands of sustainable ground improvement materials and techniques, biological methods have received increasing attention for modifying the engineering properties of soil. It is proven that microbial activities and their by-product in soils can modify various physico-chemical and engineering properties of soils, including stiffness, shear strength, permeability and erosion resistance (Chang and Cho 2014, DeJong *et al.* 2010, Ham *et al.* 2018, Kwon *et al.* 2017, van Paassen *et al.* 2010).

Bioclogging, defined as the reduction in hydraulic conductivity of soils caused by microbial activities, has been considered as an alternative to the chemical grout techniques for its economic advantages and eco-friendliness of microbial by-products (Blauw *et al.* 2009, Ivanov and Chu 2008). The bioclogging can be achieved by filling the pore spaces of soils by microbial products, such as bacterial cells, biofilms, and extracellular polymeric substances (EPS) (Abbasi *et al.* 2018, DeJong *et al.* 2013, Taylor and Jaffé 1990).

There are numerous types of microbial EPS to be potentially used for bioclogging, such as dextran from *Leuconostoc mesenteroides* (Abbasi *et al.* 2018, Kwon and Ajo-Franklin 2013, Noh *et al.* 2016, Ta *et al.* 2017), xanthan from *Xanthomonas campestris* (Lee *et al.* 2017, Qureshi *et al.* 2017), and gellan from *Sphingomonas elodea* (Chang *et al.* 2016). These are produced by lactic acid fermentation, a metabolic reaction that the six-carbon sugars are utilized by microorganisms to produce lactic acid. The production of lactic acid for EPS secretion unavoidably involves the pH change in the environment, but bioclogging in different pH conditions remains poorly examined. Additionally, the metabolic activity of microorganisms generates gases, such as methane, nitrogen and carbon dioxide, which needs to be taken into account. However, the effect of biogenic gases on hydraulic conductivity reduction have been poorly understood. Majority of previous studies with EPS-

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producing microorganisms was conducted under well-controlled environments (e.g., uniform grain sizes, ideal microbial growth temperature, pressure, and pH conditions); accordingly, the lack of understanding on EPS production and its role in hydraulic conductivity reduction still warrants further investigation. For the practical application of the EPS-producing bacteria for bioclogging, it is essential to understand behaviors of bacteria under such various environments.

In this study, we explored feasibility of using fermentation-based bacterial biopolymer accumulation to induce bioclogging in sands. In details, we experimentally evaluated both bioaugmentation and biostimulation strategies to induce bioclogging in various conditions. For the bioaugmentation method, *Leuconostoc mesenteroides* was selected and used as a model bacterium, which is a facultative anaerobic bacterium that produces an insoluble polysaccharidic biopolymer, called dextran in sucrose-rich environments. During cultivation of *L. mesenteroides* in multiple packed sand columns, variations in hydraulic conductivity were measured over the courses of microbial growth and dextran secretion. Bacterial biopolymer formation was stimulated in two different sands, coarse sand and fine sand, and with and without phosphate buffer solutions to examine the effects of grain size and pH on bioclogging. Furthermore, as bacterial fermentation processes typically produce gases, mostly carbon dioxide, effect of biogenic gas bubbles on bioclogging effectiveness was identified by controlling pore fluid pressures during the cultivation. For the biostimulation method, we attempted to stimulate indigenous soil bacterial consortiums sampled from two embankment sites to induce fermentation-based bioclogging. The presented results provide fundamental insights into the bacterial biopolymer formation mechanism, its effect on soil permeability, and potential of engineering bacterial clogging in subsurface.

2. Materials and methods

2.1 Host sands tested

In this study, two kinds of silica sands were used as the host sand to evaluate effect of grain size on bioclogging: fine sand (Ottawa F110, U.S. Silica, Frederick, MD, USA, D_{50} = 142 μm) and coarse sand (Ottawa 20/30; U.S. Silica, Frederick, MD, USA, D_{50} = 720 μm) (Fig. 1). The basic index and physical properties of these sands are listed in Table 1. Before use, fine sand was sieved using the standard test sieve No. 170 (the opening size of 90 μm) to remove the fine particles. All sand samples were pre-rinsed with deionized water (DIW) and oven-dried at 110°C, then, the dried sand samples were autoclaved at 120°C for 20 min.

2.2 Bacterial culture and growth medium

2.2.1 Model bacterium for bioaugmentation

In this study, *Leuconostoc mesenteroides* strain NRRL-B523 (ATCC 14935), was selected as the model microorganism for bacterial biopolymer production. *L. mesenteroides* is a gram-positive and facultative anaerobe

Table 1 Physical and index properties of the tested sands

Properties	Fine sand (Ottawa F110)	Coarse sand (Ottawa 20/30)
D_{50} (μm)	142.5	840
D_{10} (μm)	86.97	640
C_u	1.79	1.41
C_c	1.02	0.97
USCS	SP	SP

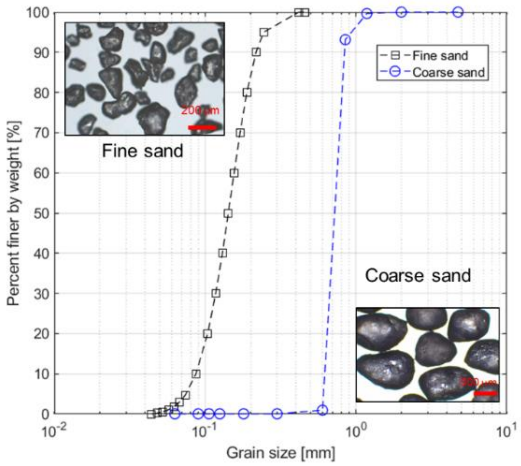


Fig. 1 Grain size distributions of the sands used

that can produce dextran, an insoluble polysaccharidic biopolymer, in anoxic conditions through the lactic acid fermentation (Lappan and Fogler 1992, Stewart and Fogler 2001). When sucrose-rich nutrient fed, *L. mesenteroides* decomposes sucrose into glucose and fructose, and produces enzyme called dextransucrase via fermentation in anoxic conditions. By the enzyme reaction, glucose is linked by glucosidic bonds and transformed into one class of polysaccharides, referred to as dextran. In particular, dextran with low molecular weight, produced by *L. mesenteroides*, has a low solubility in water, thus it precipitates and shows a gel-like texture (Jeon *et al.* 2017). This insoluble dextran has proved to have a high potential to induce bioclogging in soils (Kwon and Ajo-Franklin 2013, Noh *et al.* 2016, Stewart and Folger 2001). In addition, this bacterium is edible and has a low pathogenicity profile (Biosafety level 1), which is one advantage as to the non-native microorganism for bioaugmentation. The bacteria are generally coccoid with the average diameter of 1 μm, and they are non-motile. They can thus be transported through pores by advective flows. *L. mesenteroides* can grow but does not produce dextran when cultured in growth media containing glucose and fructose only. These characteristics of *L. mesenteroides* offer a good control on biopolymer accumulation and bioclogging. The inoculum for the bioaugmentation experiments were prepared by incubating a 1-mL frozen stock culture in a defined nutrient solution. The defined growth medium contained sucrose (40 g/L) as a carbon source, yeast extract (10 g/L) as a nitrogen source and vitamins, and 0.1 M of potassium phosphate buffer (potassium phosphate monobasic, KH_2PO_4, 13.6 g/L;

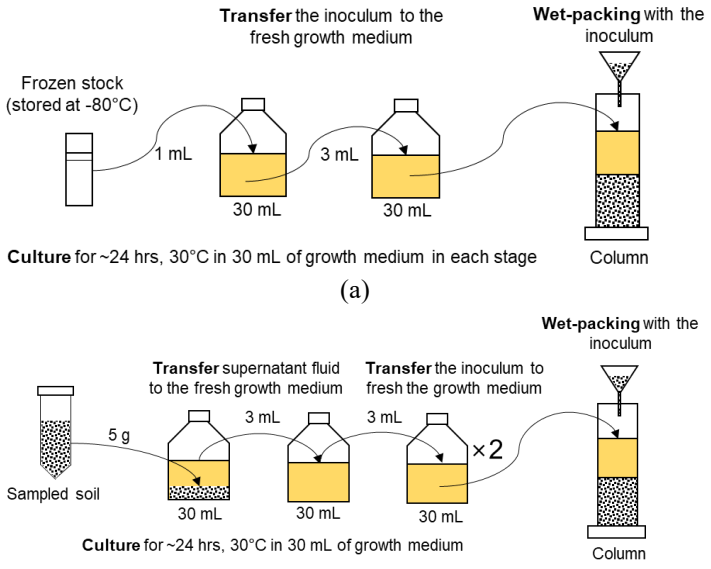


Fig. 2 Inoculum preparation for (a) bioaugmentation and (b) biostimulation experiments

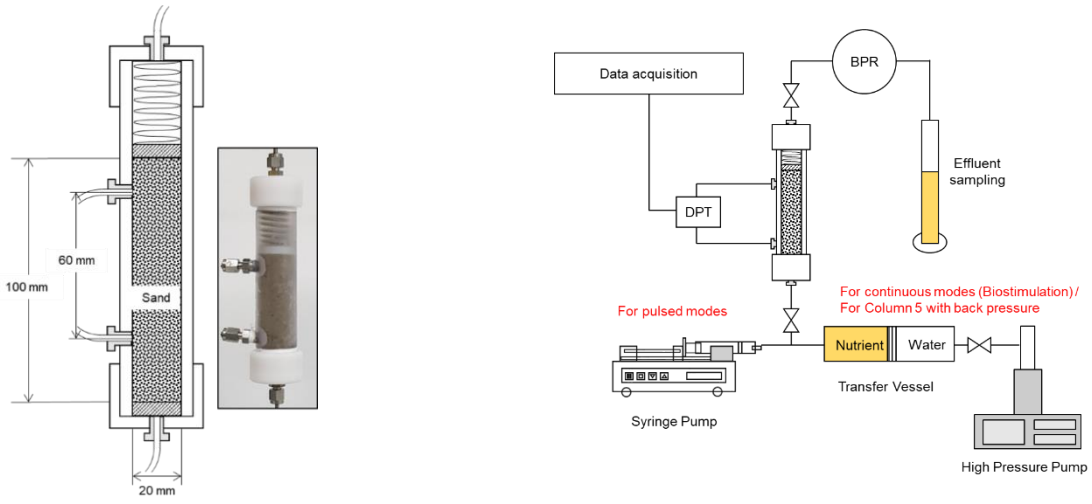


Fig. 3 (a) Schematic diagram of (a) polycarbonate column, and (b) experimental setup. DPT indicates the differential pressure transducer, and BPR denotes the back-pressure regulator

potassium phosphate dibasic, K_2HPO_4, 17.4 g/L). The pH values of the fresh growth media were approximately 7.0 with and without the phosphate buffer. After 24 h of aerobic incubation at 30°C, 30 mL of this culture was used for bioaugmentation experiments (Fig. 2(a)).

2.2.2 Bacterial consortium for biostimulation

The natural soil samples were collected from two embankment sites located in Gochang, Jeollabuk-do, Korea and Cheongju, Chungcheongbuk-do, Korea, respectively. The soil sampling tools were pre-autoclaved and washed with 70% ethanol to prevent possible contamination during the sampling. The collected soil samples were stored at ~4°C to minimize further microbial activity. The strains of the soil bacterial community in these soil samples were identified by using the new generation sequencing (NGS) method. Meanwhile, a small volume of the natural soil less than ~5 g was poured in the liquid growth medium, and aerobically cultured for ~24 h at 30°C. Herein, we used the same growth medium used for *L. mesenteroides*. This culture was transferred three times to the fresh growth medium at 1:10v/v ratio to be used as an inoculum for biostimulation

2.3 Experimental apparatus: Column setup

The experimental setup was designed to measure the change in hydraulic conductivity (or permeability) during bacteria cultivation in a sand-pack column. As shown in Fig. 3, a polycarbonate column with the internal diameter of 20 mm, the height of 140 mm and the internal volume of 43.96 mL was fabricated. This column was equipped with a differential pressure transducer (DPT; Omega Engineering, INC, Norwalk, CT, USA) at two fluid ports to estimate the

hydraulic conductivity of the sand-pack column. The hydraulic conductivity was calculated based on Darcy's law, assuming a laminar flow, as follows

$$Q = K \cdot i \cdot A = K \cdot \frac{\Delta h}{\Delta L} \cdot A \quad (1)$$

where Q is the flow rate (L^3T^{-1}) during the nutrient refilling, i is the hydraulic gradient between the two ports in the column (-), A is the cross sectional area of the column (L^2), K is the hydraulic conductivity (LT^{-1}), Δh is the head difference between the two ports in the column ($ML^{-1}T^{-2}$), which was measured using DPT, and ΔL is the distance between the two ports (L). Two fluid ports connected to the DPT were located at 2 cm above the bottom of column to obtain hydraulic conductivity variations within a soil matrix. Filter papers and porous plates were placed at the top and bottom of the sand specimen to prevent the loss of soil particles. The packed sand specimen was confined with one spring to minimize any possible volume change during fluid flows and by biogenic gas production.

One low-pressure syringe pump was used to inject fresh growth media to the column as nutrient supply for the bioaugmentation experiments, except for the column with back-pressure. One high-pressure syringe pump associated with a polycarbonate transfer vessel was also used to inject fresh growth media for the biostimulation experiments and for the bioaugmentation tests with high back-pressure. All wetting parts including fitting, the tubes and the columns were also autoclaved and the transfer vessel was sterilized by 70% ethyl alcohol prior to use.

2.4 Experimental procedures

Each sand column was prepared as follows. (i) The bacterial inoculum was transferred to the fresh nutrient solution at 10% v/v ratio, and this bacterial solution was poured into the column. (ii) Sterilized and oven-dry sand was water-pluviated into the column to achieve a fully water-saturated condition, minimizing air trapping among soil particles. Each layer of the pluviated sand with a fill thickness of ~1-2 cm was hand-tamped. The final height of the sand columns was ~10 cm, and the porosity was ~0.35-0.41 with the pore volume of ~12-13 mL. (iii) Upon the preparation of the sand column, the initial hydraulic conductivity (K_o) was measured by injecting ~10 mL of the fresh nutrient solution, minimizing the loss of bacterial cells as it is in an early stage of bacterial cell attachment. (iv) Thereafter, the sand column was left for ~3 days under no flux condition for the initial attachment of bacterial cells on sand particle surfaces and stabilization of the inoculated sand columns (Abdel Aal *et al.* 2010). After the period of cell attachment, various scenarios of bioaugmentation and biostimulation experiments were investigated as follows (Table 2).

2.4.1 Bioaugmentation experiments

The bioaugmentation experiments were conducted by cultivating the model bacteria *L. mesenteroides* in the soil columns. After 3 days of the cell attachment period of the inoculated sand column, a refilling process was carried out to supply fresh nutrients to bacteria. The fresh nutrient solution of 60 mL was fed; and this volume is approximately five times of the pore volume of the sand column, which is expected to effectively displace the previously injected liquid. The flow rate during this refilling process was controlled to be within the range of 1-4 mL/min using the low-pressure syringe pump, which corresponds to the Reynolds number of 0.05-0.2.

Table 2 Detailed conditions of column experiments

Test #	Bacteria	Soil	Test condition (Refilling strategy)	Testing period
Column 1	*Leuconostoc mesenteroides*	Fine sand	Pulsed mode (60 mL at an interval 72-96 h)	26 days (phase 1) / 17 days (phase 2)
Column 2		Coarse sand		
Column 3	*Leuconostoc mesenteroides*	Coarse sand	Pulsed mode, without buffer (60 mL at an interval 72-96 h)	65 days
Column 4			Pulsed mode, with buffer (60 mL at an interval 72-96 h)	
Column 5			Pulsed mode, with buffer 300 kPa of backpressure (60 mL at an interval 72-96 h)	66 days
Column 6	Biostimulation (soil from Gochang)	Coarse sand	Constant mode (0.1 mL/min)	47 days
Column 7	Biostimulation (soil from Cheongju)	Coarse sand	Pulsed mode (60 mL at an interval 72-96 h)	28 days

Upon the completion of the nutrient injection, the flow was stopped until the next refilling period, and the column was left for another 2-3 days, such that the bacteria in the sand consumed the nutrients. Such a nutrient refilling was repeated at an interval of 48-96 h to accumulate biopolymer in a porous medium and induce hydraulic conductivity reduction. During such refilling periods, the pressure difference was measured to monitor the permeability variation over the courses of microbial cultivation and biopolymer accumulation. This injection strategy with periodic refilling periods is referred to as a pulsed mode. Throughout a series of the column experiments using the model bacteria, the effects of sand grain sizes, pH control, and biogas generation were investigated.

Effect of grain size (Column 1 vs Column 2)

Two inoculated sand columns were prepared with coarse sand and fine sand, respectively. During 26 days (i.e., Phase 1), 60 mL of fresh nutrient medium without 0.1 M phosphate buffer was supplied into the column. Thereafter, the growth medium containing additional 0.1 M phosphate buffer was fed to promote dextran production (i.e., Phase 2). Both Column 1 and Column 2 last 43 days.

Effect of pH control (Column 3 vs Column 4)

Using Columns 3 and Column 4, the effect of pH control on bioclogging was evaluated. In Column 3, the growth media without 0.1 M phosphate buffer was used, and its initial pH was 6.95. In Column 4, the media with 0.1 M phosphate buffer was injected, of which the initial pH was 6.94. Herein, coarse sand was used in the both columns. The fermentation of *L. mesenteroides* typically

lowers the fluid pH to less than 5, because of the formation of organic acids (Naessens *et al.* 2005). However, this is off the optimal pH of dextransucrase. As the phosphate buffer maintains the fluid pH in the column at the optimal range for bacterial growth and biopolymer formation, the fluctuation of pH during the experiment can be effectively controlled.

Effect of gas generation (Column 4 vs Column 5)

Bacterial fermentation generates carbon dioxide as well as organic acids. This gas bubble generation can cause de-saturation of the sand column by displacing water (Or *et al.* 2007). Expectedly, we observed that gas bubbles were generated by bacterial activities, and those were entrapped in pores in Columns 1-to-4. As the gas bubbles displace bacterial solution in sands, the sand pack is filled with less bacterial solution, which can result in slower and less bacterial growth and EPS generation. Thus, the Column 5 experiment was carried out with application of 300 kPa of back-pressure. This increased the solubility of biogenic carbon dioxide gas and minimized the bubble generation in the column. Herein, the growth medium for Column 5 was maintained the same with Column 4, and coarse sand was used in the both columns.

2.4.2 Biostimulation experiments

In field-scale implementation to induce bioclogging, two classes of injection strategies can be considered. One method is a pulsed injection mode, in which a certain volume of the nutrient solution is rapidly injected and paused to allow bacterial activities for couple of days. After the bacterial activities to a certain extent, the injection of nutrient solution can be carried out again to facilitate further clogging. Such a injection-pause cycle can be repeated until the target performance is achieved. The other strategy is a continuous injection mode, in which the nutrient solution is continuously injected at a certain flow rate. This continuous injection can continue until the target performance is achieved.

The biostimulation experiments were performed on two natural soil bacterial consortiums and with two injection strategies. The inoculum based on Gochang soil was inoculated to Column 6, and the continuous injection mode was applied, where the growth medium was continuously injected at the flow rate of 0.1 mL/min. The inoculum based on Cheongju soil was inoculated to Column 7, where the nutrient refilling was carried out in a pulsed mode as the same with Columns 3-to-5. In the both columns, coarse sand was used as the host sand. The growth medium containing 0.1 M of phosphate buffer as the same with Columns 3-to-5 was fed to the both columns.

2.5 Post-experiment analysis

2.5.1 Environmental scanning electron microscopy (ESEM) images of biopolymer-associated sand grains

At the end of the column experiments, the sand grain samples were collected from Columns 4 and 5 to observe the produced biomasses, including biopolymers, colonized on sand grain surfaces. Without even air-drying, the wet sand grain samples were placed on a stub and imaged using the environmental scanning electron microscopy (ESEM). The images were taken in a high vacuum condition using the ESEM equipment (Quanta 650, FEG Co, Eindhoven, The Netherlands) at 10-20 kV.

2.5.2 Effluent sampling and product analysis

During the periodic refiling, the fluid samples were acquired to measure the variations of pH and cell density from the effluents. Upon sampling, the pH was measured immediately with the pH meter (Orion Star A215, Thermo Fisher Scientific, USA). After the measurement of pH, the total microbial population in the effluent samples were identified using the quantitative polymerase chain reactions (qPCR) assay. Each effluent sample of 2 mL was centrifuged for 10 min at 5000g, and its pellet was collected to extract DNA using DNeasy Blood & Tissue (QIAGEN, Hilden, Germany). Thereafter, the qPCR experiments were conducted with QuantStudio 3 real-time PCR system (Thermo Fisher Scientific, MA, USA) using TaqMan detection chemistry (Applied Biosystems, CA, USA) (Kim *et al.* 2018). This qPCR assay was applied to the effluent samples obtained from Columns 3 and 4.

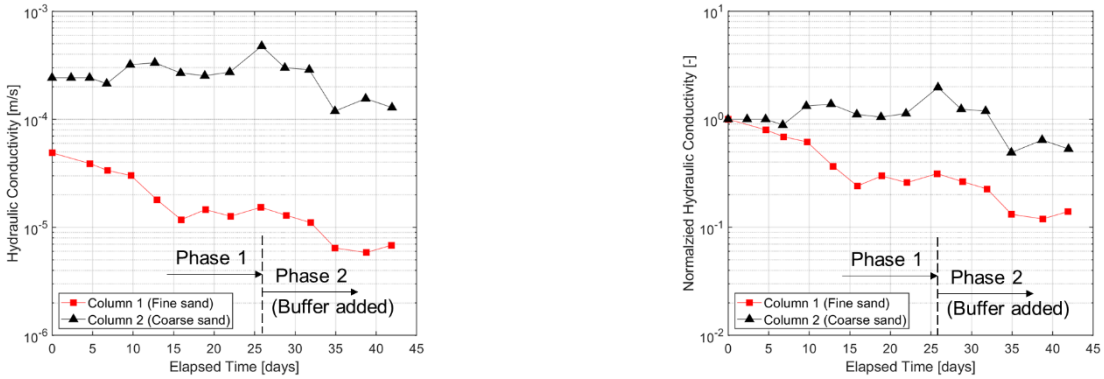
2.5.3 Quantification of organic matter mass using the loss on ignition method

The loss on ignition (LOI) method was applied to determine the organic matter contents in the biopolymer-associated sand samples for Columns 3, 4, and 5. Upon termination of the column experiments, the columns were dismantled, and the sand grains at the middle region were sampled. These sand samples were oven-dried at 110°C for 24 h to remove the moisture. Thereafter, 5 g of dried biopolymer-containing sand samples were kept in a furnace under 375°C for 16 h. Thereafter, the mass of the leftover matter was measured. The organic matter content (OM) was calculated, as follows

$$OM = \frac{w_{\text{pre-ignition}}\,(g) \;-\; w_{\text{post-ignition}}(g)}{w_{\text{post-ignition}}(g)} \tag{2}$$

2.5.4 Batch tests for biopolymer production

For quantification of biopolymer saturation accumulated in the column during the bioaugmentation experiments, the mass of biopolymer (dextran) produced by *L. mesenteroides* was measured via batch tests. One culture group was cultured in the growth medium without the phosphate buffer, which corresponds to Column 3; the other culture group was grown in the growth medium with the phosphate buffer, which corresponds to Column 4. Four samples per group were cultured, and the mass of biopolymer produced were averaged. In each sample, the inoculum of *L. mesenteroides*, which was aerobically grown under an ambient condition for 24 h was transferred to the fresh growth medium at the ratio of 1:10 v/v. Thereafter, the inoculated bacteria were aerobically cultured under ambient condition (room temperature, no shaking) for approximately one week, which gave sufficient time for biopolymer formation. This grown culture was poured on the filter papers (GF4-90, CHMLAB Group) with a 2.7 μm pore size placed on a porcelain Buchner funnel, and the gel-like biopolymers were separated by draining the culture liquid with vacuum. Thereafter, the biopolymers filtered on the



(a) (b)

Fig. 4 Changes in (a) hydraulic conductivity and (b) normalized hydraulic conductivity of sands with different grain sizes.

filter paper were dried for ~24 h at 60°C in an oven, and hence the mass of dried biopolymers was measured. The dry masses of biopolymers produced under the corresponding growth conditions were used to compute the amounts of biopolymers accumulated in the columns, assuming that these accumulated proportional to the refilling times and the total amounts of sucrose.

For the biostimulation experiments, such batch experiments are not feasible because the exact species and their microbial reactions were unknown. Thus, the mass of organic matters, which was seen as gel-like insoluble biopolymers, was measured at the end of column experiments. The sand columns were dismantled and divided into four sections when the column experiments were terminated. The biopolymer-accumulated sands from each section was thoroughly rinsed with deionized water using the standard test sieve (Sieve No. 60; 250 μm of aperture), to separate biopolymers from the sand grains. Then, the detached biopolymers mixed with water were poured onto the filter papers, and then the filtered and dry mass of biopolymers was measured for each section.

3. Result and analysis

3.1 Effect of grain size (Column 1 vs Column 2)

Fig. 4 shows the variations in hydraulic conductivity K of sands caused by bacterial biopolymer accumulation by *L. mesenteroides* in Columns 1 and 2, in which the host soil was the fine sand in Column 1 and the coarse sand in Column 2, respectively. During the first 26 days (Phase 1), the growth medium without phosphate buffer was supplied into the columns; and thereafter, 0.1 M of phosphate buffer was added to the growth medium to control pH and promote dextran production (Phase 2).

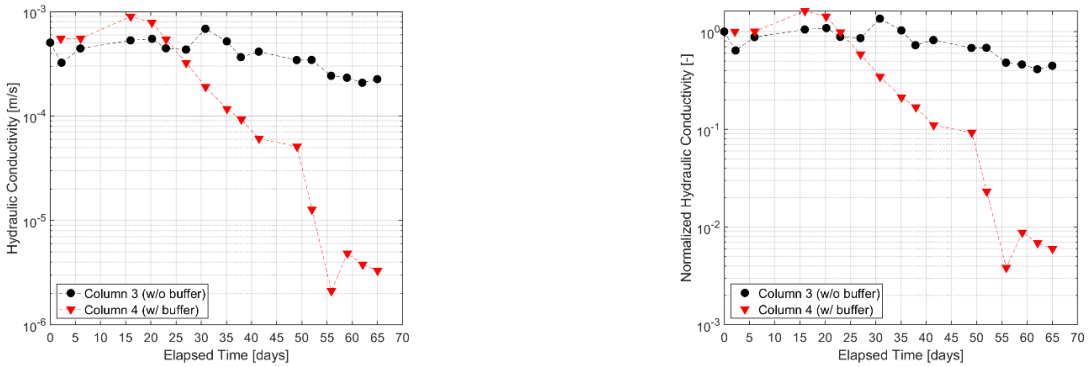
In Phase 1, which lasted 26 days, K of fine sand (Column 1) was reduced by ~76% from 4.9×10^{-5} m/s to 1.5×10^{-5} m/s. However, in the coarse sand (Column 2), no significant reduction in K was observed for the first 26 days (Phase 1). This indicates that the K reduction rate differed with the grain size. The both sands are fairly uniformly sized with the mean grain size of 140 μm for the fine sand and 840 μm for the coarse sand, respectively. Therefore, the mean pore sizes of the fine sand in Column 1 and the coarse sand in Column 2 are estimated to be approximately ~10 μm and ~60 μm, respectively (i.e., 10% of D_{10}). It is presumed that the fast rate in K reduction observed in Column 1 is attributable to the smaller mean pore size of the fine sand. Contrary to that, noticeable K reduction in the coarse sand presumably requires the greater amount of insoluble biopolymer than that in the fine sand, owing to the larger mean pore size.

In Column 1, the K decreased gradually to ~1.2–1.5×10^{-5} m/s during the first 16-18 days, and it reached a plateau for the following 10 days, as shown in Fig 4. However, upon the onset of phosphate buffer addition (Phase 2), the hydraulic conductivity begun to decrease further to ~5.8×10^{-6} m/s as pH of the pore fluid was controlled to be from an acidic condition to a weak acidic condition. The K value of the fine sand (Column 1) decreased and converged to ~5.8×10^{-5} m/s after ~43 days of the experimental run, which corresponds to the K reduction by ~88%. Meanwhile, the K value of the coarse sand (Column 2) was also further reduced from ~3×10^{-4} m/s to 1.3×10^{-4} m/s during Phase 2, partly due to the controlled pH and thus the promoted biopolymer production. This was the K reduction by ~50%, which was still significantly less reduction than in the fine sand.

In general, microbial fermentation processes produce various metabolic by-products, such as organic acids and/or carbon dioxide gas, and thus decrease the pH of pore fluids (Silverman and Munoz 1974). Therefore, pH reduction during microbial fermentation processes is inevitable. However, the results demonstrate that the phosphate buffer can serve to provide additional nutrients and at the same time to keep the pH condition suitable for *L. mesenteroides* to grow and produce more dextran. It is worth noting that in both the fine and coarse sand columns, additional dextran production and further reduction of K were achieved by the addition of phosphate buffer. Such pH buffer effect is further examined in the following section.

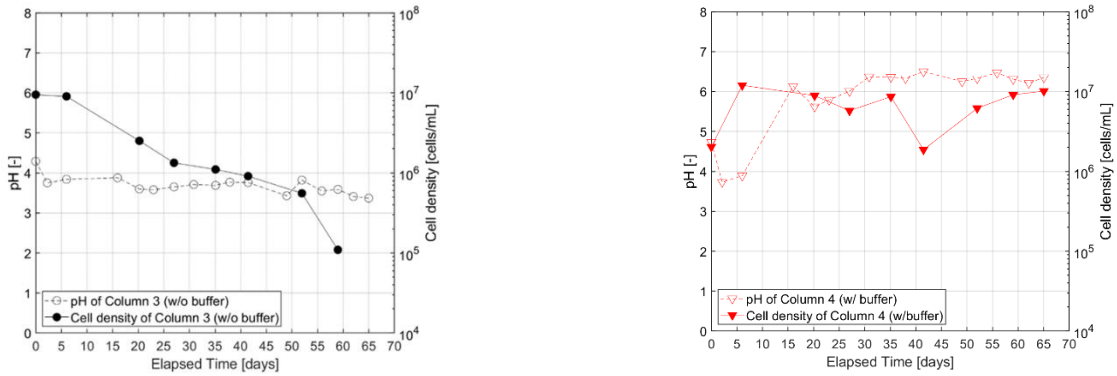
3.2 Effect of pH (Column 3 vs Column 4)

Fig. 5 shows the temporal reductions in hydraulic conductivity K of the coarse sands in Columns 3 and 4 during 65 days of experimental runs, in which the effect of



(a) (b)

Fig. 5 Changes in (a) hydraulic conductivity and (b) normalized hydraulic conductivity of sands with different pH



(a) (b)

Fig. 6 pH and cell density analysis with effluent of (a) Column 3 and (b) Column 4

Table 3 Organic matter contents (OM) using the LOI method

Sample #	OM (mg/g of sand)			
	1	2	3	Avg.
Column 3	7.21	6.61	7.12	6.98
Column 4	19.15	19.39	19.02	19.19
Column 5	26.66	24.69	25.82	25.72

the addition of addition of 0.1 M phosphate buffer was examined. The growth medium without 0.1 M phosphate buffer was fed to Column 3, and the growth medium with 0.1 M phosphate buffer was fed to Column 4, respectively.

It can be clearly seen that the K reduction rate in Column 4 was significantly greater than that in Column 3. In both Columns 3 and 4, there was no significant K reductions until approximately 35 days and 20 days elapsed, respectively. Thereafter, K of each column started to fall after 35 days for Column 3 and after 20 days Column 4, respectively. In Column 4 with the pH buffer, the K value decreased quickly from 5.5×10^{-4} m/s to 3.3×10^{-6} m/s by more than two orders of magnitudes. This corresponds to the K reduction by more than 99%. In Column 3 without the pH buffer, approximately ~50% of K reduction was observed. These two contrasting results between Columns 3 and Column 4 clearly demonstrate the importance of pH controls in utilization of bacterial fermentation processes to induce bioclogging. This result is also consistent with the result of Column 2. In Column 2, the K further decreased when the pH buffer was fed.

Table 3 shows the organic matter contents measured for Columns 3 and 4. Herein, the organic matter produced by bacteria implies the biomass, including cells and biopolymers. The organic matter content (OM) was determined to be ~7.0 mg/g of sand in Column 3 and 19.2 mg/g of sand in Column 4. This also supports the difference in K reduction observed in the both columns.

Fig. 6 shows the variations in cell density and pH of the effluent samples, measured by qPCR method and by using a pH meter. The initial pH values of the fresh growth media were controlled to be ~7.0 for both Columns 3 and 4. In Column 3, upon the inoculation the pH dropped to and was maintained at 3.6-3.8 over the course of the experiment. Such lowered pH values in the both columns were because of the production of lactic acids and carbon dioxide during microbial fermentation by *L. mesenteroides*. On the other hand, the pH in Column 4 was maintained at ~5.5-6.5 with the continued refilling, mainly due to the addition of 0.1 M phosphate buffer.

This pH difference between Columns 3 and 4 led to the difference in cell density. The cell density in Column 4 was kept at the range of 10^6-10^7 cells/mL. Whereas, in Column 3, the initial cell density was ~10^7 cells/mL, but soon it decreased continuously to ~10^5 cells/mL. This result is consistent with the previous study by Wolf and Fogler (2001), where the optimal pH for the exponential growth of *L. mesenteroides* is reported to be ~6.8.

Moreover, it is known that the highest yield of dextran-

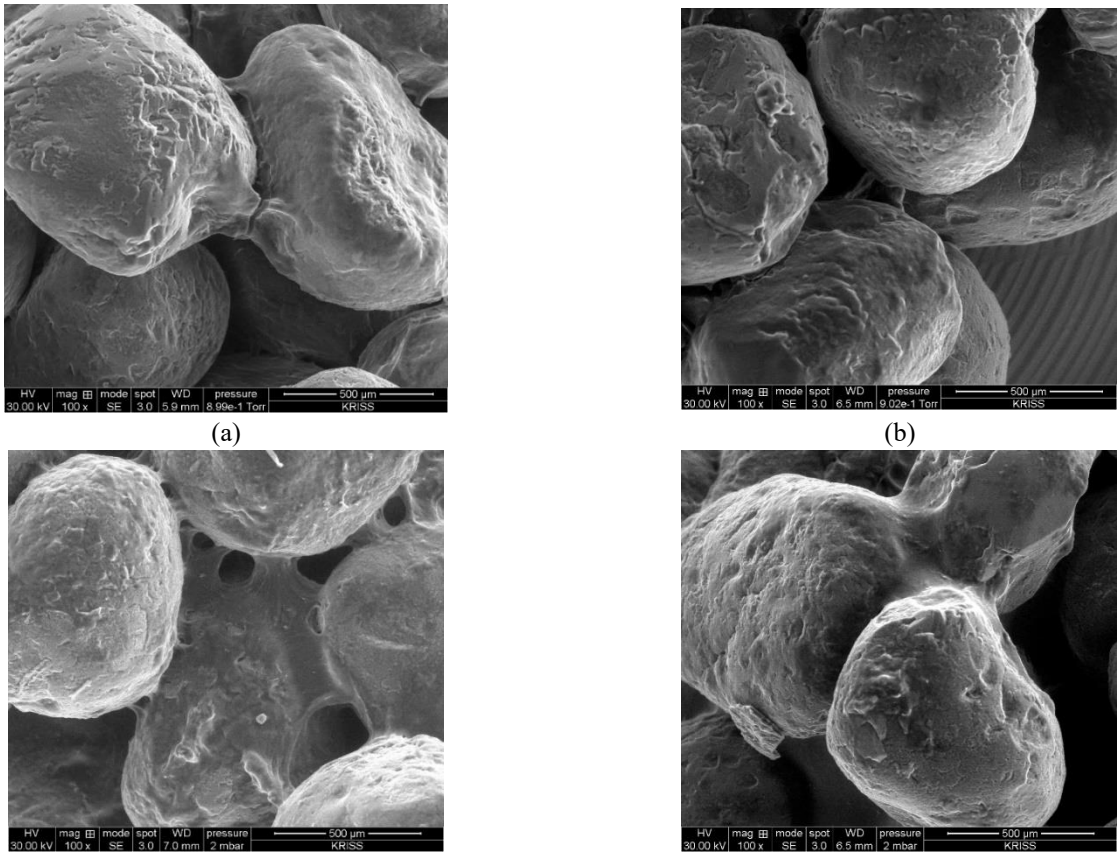


Fig. 7 ESEM images of grains colonized by biopolymer in (a), (b) Column 4 and (c), (d) Column 5

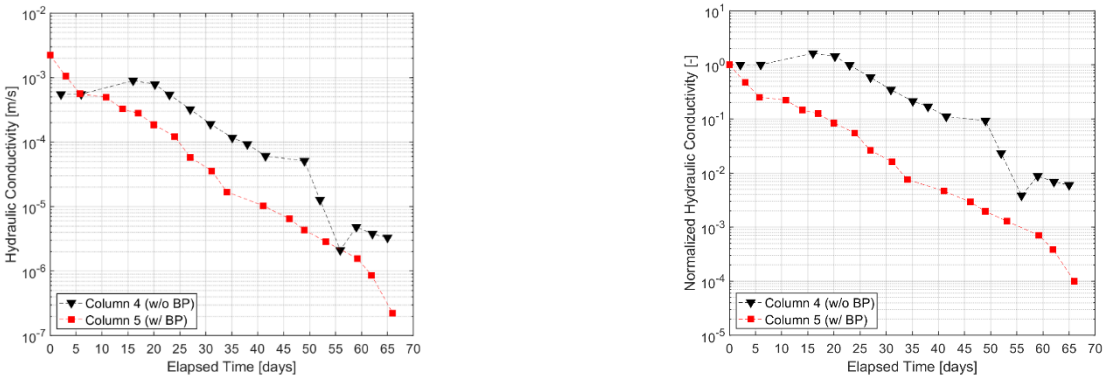


Fig. 8 Changes in (a) hydraulic conductivity and (b) normalized hydraulic conductivity of sands with different backpressure

producing enzyme (i.e., dextransucrase) occurs in the pH range from 5.5 to 7.0, due to the consistent proton motive force as a regulator of enzyme production in such pH (Otts and Day 1987). This implies the higher yield of dextransucrase in Column 4 than in Column 3. Accordingly, the strong acidic condition with pH < 4 in Column 3 had negative impacts on the cell density and the enzyme activity, which reduced the amount of dextran produced and consequentially caused the little *K* reduction.

After 65 days of experimental run in Column 4, the column was dismantled and the wet biopolymer-associated sand grains were sampled. These wet soil samples were imaged using the ESEM to observe the biopolymers formed and accumulated within the sand matrices, as shown in Fig. 7. In all the wet soil samples from the both columns, it was observed that the insoluble gel-like biopolymers coated and connected the sand grains, and filled the voids among grains, though the biopolymer quantity and the extent of grain-bridging or void-filling differed within the column and between the columns. Note that the ESEM applies a low vacuum to a sample, causing water evaporation, the liquid tends to migrate into corners that can reduce their surfaces in such a condition. Thereby, it is worth noting that there is possibility that liquid associated with biopolymers has migrated to grain-contacts during ESEM imaging. Nevertheless, these images show the produced and

accumulated biopolymers in sands, which caused the significant reduction in hydraulic conductivity. Our results corroborate the previous studies by Taylor and Jaffé (1990), Cunningham *et al.* (1991), Abdel Aal *et al.* (2010) and Hand *et al.* (2008), where they also observed the similar bioclogging by bacterial biomass and biofilms in porous media.

3.3 Effect of gas generation (Column 4 vs Column 5)

Fig. 8 shows the changes in hydraulic conductivity of Column 4 and 5; in Column 5, a backpressure of 300 kPa was applied, and no backpressure was applied to Column 4. We observed that the bacterial reaction generated carbon dioxide gas bubbles in the columns (i.e., Columns 1-to-4). Whereas, no gas bubbles were generated in Column 5 due to the elevated gas solubility by the backpressure. In Column 5, the K reduction occurred drastically from 2.22×10^{-3} m/s to 2.23×10^{-7} m/s during 66 days. The K reduction of two orders of magnitudes took more than 50 days in Column 4 with no backpressure. Contrarily, it only took less than 35 days in Column 5 where the backpressure was applied. Finally, the K reduction of four orders of magnitudes was achieved. The organic matter content of Column 5 was measured to be ~25.7 mg/g of sand, which was greater than that of Column 4 (Table 3). This indicates that more biopolymers were produced and accumulated in Column 5 than in Column 4, which caused the greater K reduction. Figs. 7(c) and 7(d) show the biopolymer-associated sand grains which were sampled from Column 5.

Upon each refilling of fresh growth medium into the columns, as the bacteria grew and microbial fermentation proceeded, carbon dioxide gas was generated in the columns and nucleated in the sand-packs. Such gas bubble formation displaced pore water out of the sand-packs, reducing the amount of pore water and nutrients including sucrose within the column. It is presumed that the reduced amount of nutrients and pore water in the sand-packs limited the cell growth and dextran, as previously reported in Harshey (2003) and Or *et al.* (2007). In unsaturated conditions, remaining liquid is retained as thin films on grain surfaces, and the microbial mobility and growth rate are thus significantly reduced when compared to those found in liquid media (Harshey 2003). Furthermore, in unsaturated conditions, the formation of biopolymer can be localized to water menisci at grain contacts due to the air-water surface tension, and/or to water films on grain surfaces, owing to the hydrophilic wettability of silicate sand surfaces. Thereby, the pore loci of biopolymers have the minimal effect on the K reduction, compared to the case under water-saturated conditions where biopolymers can be formed at any locations in pores, such as pore throats or pore centers (Or *et al.* 2007).

3.4 Biostimulation test results

The strains of the soil bacterial community in the collected soil samples were identified by using the new generation sequencing (NGS) method. The NGS analysis revealed that the indigenous bacterial communities from Gochang and Cheongju soil mainly included the families of

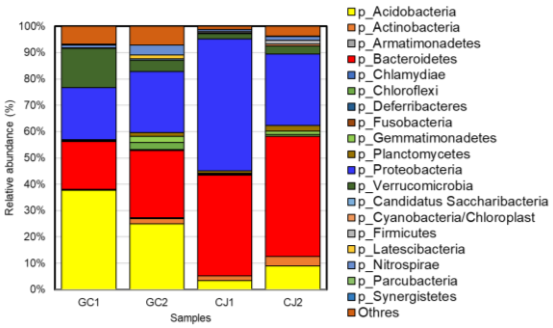


Fig. 9 The composition of bacterial population in sampled soils from Gochang and Cheongji. GC1 and GC2 are the results from the Gochang soil sample, and CJ1 and CJ2 are from the Cheongju soil sample

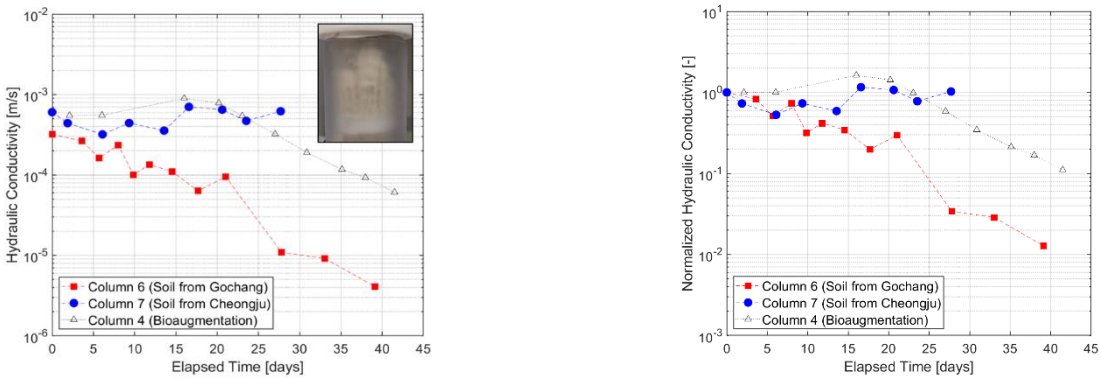
Pseudomonadaceae and *Xanthomonadaceae*, which are *Proteobacteiria* phylum, as shown in Fig. 9. In addition, the families of *Enterococcaceae*, *Lactobacillaceae* and *Leuconostocaceae* were also found in Gochang soil, which are known to be capable of bacterial fermentation.

Fig. 10 shows the K reduction as a result of stimulating indigenous soil bacteria consortiums. In Column 6 with the microbes from Gochang soil, the K decreased from 3.20×10^{-4} m/s to ~4×10^{-6} m/s over the course of 40 day-long experiment, which was the reduction by approximately two orders of magnitude. This was because of insoluble biopolymer formation. The inset figure in Fig. 10b shows the copious amount of biopolymers produced at the headspace on the top of the sand pack.

After dismantled the column, the mass of insoluble biopolymers formed was measured by dividing the column into four sections with the regular interval. Although it was a qualitative estimation on the accumulated biomass, we observed that the biopolymers were formed more vigorously close to the inlet (the bottom region), and its amount decreased toward the outlet within the column. This is because the nutrient was injected from the bottom inlet. This result confirms that the flow direction for nutrient supply affects the spatial distribution of bacterial biopolymer accumulated, as previously observed by Taylor and Jaffé (1990). Our result highlights the importance of the strategy for nutrient injection, including flow direction.

The 16S rRNA gene sequence analysis on the effluent samples revealed that the dominant microorganisms in Column 6 (Gochang soil) were *Enterobacter* strain, which is known to be capable of fermenting sugar to produce lactic acid. We also confirmed vigorous gas bubble generation, which implies active microbial fermentation. Compared to Column 4 where the bioaugmentation was conducted with *L. mesenteroides*, the rate of K reduction in Column 6 was more rapid than that in Column 4. This indicates that the biostimulation can also be effective in inducing bioclogging in such coarse sands.

On the other hand, no or minimal change in the hydraulic conductivity was observed for 30 days in Column 7, where indigenous microbes from Cheongju soil were stimulated. This result, which is contrary to Column 6 (Gochang soil), is presumably attributed to the difference in bacterial ecology between the sampled soils. It is presumed that the population of biopolymer-producing species was



(a) (b)

Fig. 10 Changes in (a) hydraulic conductivity and (b) normalized hydraulic conductivity of biostimulation experiments.

Table 4 Mass of dextran produced by *L. mesenteroides* from the batch culture

Properties	Without buffer	With buffer
Filtered mass of dextran (g per 44 mL liquid culture)	0.1112	0.1777
Volume of filtered dextran (mL per 44 mL liquid culture)	0.0741	0.1185
Mass of dextran per 1 g sucrose (mg/g)	2.78	4.44

minimal in Column 7 (Cheongju soil), such that the supplied nutrients were mostly consumed by the other majority species. This was confirmed by 16S rRNA gene sequence analysis, in which the dominant organisms were identified to be some species included in *Enterobacteriaceae* family.

This demonstrates that effectiveness of biostimulation methods is primarily affected by the presence of proper microorganisms capable of targeting reactions in the soils to be treated. Furthermore, even though the targeting species are present, their population needs to outnumber the other species to be superior over nutrient competition.

The difference in injection strategies can affect the *K* reduction results. In this study, the effect of injection strategy however remains poorly examined because of no *K* reduction observed in Column 7. The effect of injection strategy on bioclogging efficiency warrants further investigation.

3.5 Batch test results on biopolymer production

According to our experiment conditions, the quantity of biopolymers (dextran) produced was measured as *L. mesenteroides* were aerobically cultured in the growth media with and without 0.1 M phosphate buffer. Table 3 summarizes the batch test results. When 44 mL growth medium containing 40 g/L sucrose, 10 g/L yeast extract, and 0.1 M phosphate buffer was used, the dry mass of dextran was ~0.178 g. Thus, it appears that *L. mesenteroides* produces approximately 4.05 mg/mL dextran. On the other hand, when 44 mL growth medium without the buffer was used, the dry mass of dextran was ~0.111 g; it was found that *L. mesenteroides* produces approximately 2.5 mg/mL dextran. This result again confirms that the pH control has a significant influence on dextran production.

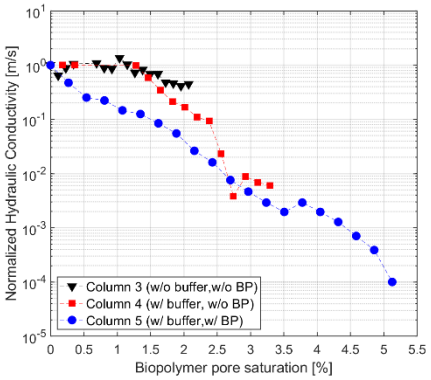


Fig 11 Changes in normalized hydraulic conductivity with dextran pore saturation in Column 3, 4 and 5

4. Discussion

The pore saturation of the produced dextran in Column 3, 4 and 5 was estimated based on the batch test results. It was assumed that all columns have the porosity of 0.4, and thus the same pore volume of 12.5 mL. It was measured that the biogenic gas bubble generation caused the displacement of approximately 4 mL pore fluid during the intervals between the refilling periods with no backpressure (Columns 3 and 4). It implies that the growth medium of ~8.5 mL was used for biopolymer production in Columns 3 and 4. Whereas, the growth medium of 12.5 mL was used in Column 5. Therefore, the total mass of dextran formed in the sand packs per each refilling is computed to be ~21 mg in Column 3, ~34 mg in Column 4, and ~51 mg in Column 5, respectively. Then the pore saturation of dextran within the sand packs (or biopolymer pore saturation) can be estimated, assuming the dextran density to be 1.5 g/mL (Noh *et al.* 2016).

Fig. 11 shows the normalized hydraulic conductivity against the biopolymer pore saturation. Overall, it appears that the biopolymer pore saturation less than 5% can cause the hydraulic conductivity reduction by more than two orders of magnitudes when pH buffer is fed. Note that we consider only the volume of solid (insoluble biopolymer), but the biopolymers present as a porous material in an aqueous phase (Jeon *et al.* 2017). It is important to note that the apparent bulk volume of dextran can be much greater than its net solid volume.

5. Conclusions

In this study, engineered bioclogging using fermentation-based bacterial biopolymer was investigated. In particular, effect of various parameters and conditions, including particle size, buffer addition, and backpressure, were examined through a series of column experiments. As a result, the hydraulic conductivity reduction was found to be more rapid in fine sand than in coarse sand, owing to the smaller pore size. The control of pH using the phosphate buffer facilitated bacterial activities and induced effective bioclogging. While fermentation inevitably accompanies carbon dioxide gas generation, gas bubble generation (or desaturation) degraded the bioclogging efficiency. When backpressure applied to increase carbon dioxide solubility and minimize free bubble generation, the hydraulic conductivity reduction appeared to be more significant. Meanwhile, the presence of indigenous species capable of biopolymer production plays a key in success of implementing the biostimulation strategy, and further their population should outnumber the other indigenous bacteria so as to be able to compete over nutrients. The presented results reveal that fermentation-based bacterial biopolymer formation can reduce the hydraulic conductivity of coarse sand by three-to-four orders of magnitudes or by 99.9-99.99% in controlled environments, which implies the hydraulic conductivity reduction to the level of silts. The presented results provide fundamental insights into the bacterial biopolymer formation mechanism, its effect on soil permeability, and potential of engineering bacterial clogging in subsurface.

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