Effects of biofilm on gas permeability of unsaturated sand

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ABSTRACT

Microbial activities may sustainably improve earthen structures like landfill covers. Typically, after consuming nutrient, bacteria produce hydrated exopolysaccharide to form biofilm which may cause bio-clogging in soil. Previous studies mainly focused on combined effects of biofilm and nutrient on water permeability of saturated sand. This study investigates biofilm effects on gas permeability of unsaturated sand at different degrees of saturation using a flexible wall permeameter. Soil with water ('Water'), only nutrient ('Nutrient') and mixture of bacteria and nutrient ('Combined'), were examined with three replicates. Statistical analysis and scanning electron microscope (SEM) were adopted for further interpretation. Compared to 'Water', gas permeability of 'Combined' and 'Nutrient' were consistently lower. The differences increase with decreasing saturation. At around 5% degree of saturation, gas permeability of 'Combined' and 'Nutrient' were 22% and 14% lower than 'Water', respectively. Statistical analysis reveals that gas permeability reduction in 'Combined' and 'Nutrient' were significant at significance level less than 0.05 (probability>95%). The reduction was attributed to pore clogging by nutrient precipitation (for 'Nutrient') or both biofilm and nutrient precipitation (for 'Combined'), as evident by SEM results. Biofilm effects on reducing soil gas permeability can be overestimated if nutrient effects are ignored.

KEYWORDS: Gas permeability, biofilm, unsaturated sand, *Bacillus subtilis*

INTRODUCTION

Bacterial activities in soil may significantly affect soil properties (Rowe, 2005; DeJong, *et al*., 2013; He, *et al*., 2013; Dashko & Shidlovskaya, 2016). This can provide new opportunities for sustainable soil improvement like using biofilm, which is estimated to form in 99% of bacterial population (Dalton & March, 1998). Biofilm refers to aggregates of bacterial cells encased in a self-produced matrix of exopolysaccharides (EPS) (Flemming & Wingender, 2010). Typically, EPS comprises of polysaccharides, proteins, nucleic acids, humic acid and water to form hydrated slime, which facilitates bacterial attachment on material surface. For soil properties, EPS may cause bio-clogging in soil pores and reduce soil permeability. Reducing gas and water permeability is important for performance of earthen structures like cover systems for landfills and mining wastes (Bouazza & Rahman, 2007; Ng & Coo, 2014).

Biofilm effects on water permeability of saturated sand and silty sand were studied by some previous researchers (Dennis & Turner, 1998; Proto *et al*., 2016). Results showed that water permeability is reduced by several orders of magnitude after nutrient solution was continuously supplied to non-sterile 1-D soil column for biofilm formation. 1-D refers to the one dimensional flow in the soil specimen while non-sterile refers that native bacteria already present in the soil, nutrient and water were not properly killed or removed. Vandevivere & Bayeye (1992) emphasized the importance of soil

sterilization to better isolate and understand the behaviour of a particular bacteria strain. However, contribution from nutrient effects was not separated. Nutrient itself normally consists of organic matters (e.g. carbohydrate and peptide), which can clog soil pores (Kaiser & Guggenberger, 2003; Mikutta *et al*., 2004) and may affect soil permeability. Considering only nutrient effects provides a reference to determine whether interaction between bacteria and nutrient causes beneficial or adverse effects. In unsaturated soil, rather than water permeability, gas permeability is also important for soil covers to estimate and minimize harmful landfill gases like methane and hydrogen sulphide. In general, gases are produced by bacteria due to metabolism, while biofilm (or exopolysachharide) is also formed as a metabolic product. Different types of gas (e.g. carbon dioxide, methane) produced mainly depends on the type of bacteria and nutrient and oxygen availability (Lengeler *et al*., 1999). The efficiency of biofilm formation is normally controlled by the type of soil and nutrient (Ma *et al*., 2017) and regulation of gene expression (Shemesh & Chai, 2013). Vargas-García *et al*. (2002) studied the biofilm formation and gas production of the bacteria *A. vinelandii* in nutrient solution with sufficient oxygen. Results show that around 1.1 grams of carbon dioxide generated can produce 0.9 grams of exopolysaccharide. When soil water content decreases, more gas flow paths in soil are created to increase gas permeability. Since biofilm consists of nearly 95% of water (Costerton *et al*., 1982; Sutherland, 2001),

decreasing soil water content may dehydrate the biofilm and thus reduce bio-clogging. Whether biofilm would improve the gas permeability even at low soil water content for potential engineering application is questionable.

In this study, biofilm effects on gas permeability of soil at different degrees of saturation were quantified using flexible wall permeameter. Three test series with three replicates and sterile preparation were performed. Statistical analysis and scanning electron microscope (SEM) are adopted to assist interpretation.

MATERIALS AND METHODS

Soil, bacteria and nutrient

Toyoura sand was adopted and its properties (Ishihara, 1993) are summarized in Table 1. An aerobic rihizobacterium *Bacillus subtilis* (NCIB 3610) is a non-pathogenic model bacterium typically used for biofilm studies (Vlamakis *et al*., 2013). It was chosen, as opposed to other pathogenic model bacteria like *P. aeruginosa* and *E. coli* (O'Toole *et al*., 2000) because it is commonly found in soil to act as a biofertilizer to promote plant growth (Morikawa, 2006a; Vlamakis *et al*., 2013). The bacterial solution with 0.7 optical density was used, which corresponds to approximately 7×10^9 cells per ml (Hsueh *et al*., 2015). The nutrient was prepared by dissolving Lennox broth, 30 gL^{-1} glycerol and 6.3 gL^{-1} $MnCl_2$ in Milli-Q water to optimize the biofilm produced by

Bacillus subtilis (Morikawa *et al*., 2006b; Shemesh & Chai, 2013). Following the guideline in W.H.O. (2004), dry soil, water and nutrient were autoclaved to sterilize for 15 minutes at 121 °C to kill all microorganisms and their spores. The spore is a dormant cell type that can survive with no nutrient and return to life if nutrient becomes available (Setlow, 2006). After sterilization, the water content of soil was checked and found to be less than 0.5%.

Test program, specimen preparation and testing procedures

Three series of gas permeability tests with three replicates, namely 'Water', 'Nutrient' and 'Combined' were carried out, which represent soil mixed with only Milli-Q water (20 ml), only nutrient (20 ml) and mixture of bacteria (2 µl) and nutrient (20 ml) respectively. 'Water' served as a reference. With sterile preparation, 'Nutrient' captured only nutrient effects without biofilm formation while 'Combined' quantified effects of biofilm produced by only *Bacillus Subtilis*. Detail of test program is summarized in Table 2.

Total number of bacterial cells in the 2 µl of bacterial solution was around 2×10^7, which is approximately within the range of total cells of different bacteria types found in soil (10^5 – 10^9 cells per gram of soil) (Richter & Markewitz, 1995). Mixing 20 ml of nutrient solution with the dry soil mass was to achieve initial degree of saturation at

70%, which was the optimum condition for aerobic microbial activity (Mitchell & Santamarina, 2005). Soil compaction was performed in a class-II biosafety cabinet, which provided a sterile working environment. A cylindrical specimen having relative density of 90% with height of 20 mm and diameter of 70 mm was compacted in two layers, using similar length to diameter ratio in Zhan *et al*. (2014). Then, all specimens were incubated in a sterile chamber at 30±0.2 °C (Shemesh & Chai, 2013) for 14 days and allowed for oxygen exchange.

The initial degree of saturation of all specimens was 70%. After every 2 days of incubation and drying in the sterile chamber for 14 days, the gas permeability of each specimen was measured using a flexible wall permeameter at 25±1 °C.. The method of measuring gas permeability was in accordance with the procedures suggested by the Canadian Society of Soil Science (Carter & Gregorich, 2007). A constant pressure gradient of less than 3 was considered in this study, which ensures that air flow across the specimen is laminar. Fig. 1 shows schematic diagram of the flexible wall permeameter. By controlling the pressure regulators, 20 kPa of cell pressure was applied to confine the specimen while 0.5 kPa of differential air pressure was applied through the specimen. The gas used for the experiment was ambient air with a composition of 78% N2, 21% O2 and 1% trace gases. Then, outflow rate was measured by mass flow meter while pressure transducers were installed to measure pressure at

inflow and outflow. At the end of each drying stage and permeability measurement, the mass and dimensions of each specimen were measured. The average degree of saturation was then calculated using mass-volume relationship (Ng & Menzies, 2007) for the plot of the permeability versus degree of saturation in Fig. 2. To ensure the homogeneity of water distribution, a specimen with a height of 20 mm was used to minimize the influence of non-uniform water distribution on the measured gas permeability. This approach was based on the findings reported by Merz *et al*. (2014), who studied the soil moisture profile of sand columns subjected to drying induced by surface evaporation. They found that the degree of saturation was homogeneously distributed (± 1%) in the soil column during drying, except for the top soil surface of 5 mm thickness (± 5%).

To explain the observed differences in gas permeability among 'Water', 'Nutrient' and 'Combined', SEM analysis (JEOL 6390) was carried out in this study to confirm the biofilm formation and nutrient precipitation after 14 days of incubation. At the end of all continuous measurements, the intact specimens were freeze-dried and then trimmed into smaller sections to capture the SEM images. For future studies, it is recommended to perform microstructural analysis using Environmental SEM for a continuous microstructural observation at different degrees of saturation

Data interpretations

Calculation of the gas permeability was based on Darcy's law for one-dimensional steady –state flow in isotherm condition (Vangpaisal & Bouazza, 2004). The key assumption of Darcy's law requires that the flow is laminar. Moreover, any diffusion process was ignored in the experiment. Gas permeability of each replicate was interpreted with specimen variation using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) in post hoc analysis (Gomez & Gomez, 1984). Significance level (p) was 0.05 according to the "rule of thumb" (Kumar *et al.*, 2011). This determines whether the gas permeability of either two conditions are significantly different at a particular degree of saturation.

RESULTS AND DISCUSSION

Gas permeability at different degrees of saturation

Fig. 2 shows gas permeability of each condition at different degrees of saturation. It is expected that the biofilm at the initial state (degree of saturation = 70%) has yet to form. Hence, the first gas permeability measurement was conducted after the 2 days of incubation and drying, with a degree of saturation of around 60%. The variation of experimental results in Fig. 2 is likely due to the heterogeneity of biofilm formation

(Flemming & Wingender, 2010) and nutrient precipitation. Statistical analysis was carried out to consider the variation and to determine the significance of difference between 'Water', 'Nutrient' and 'Combined', which is presented in later section. At a given degree of saturation, gas permeability of 'Combined' and 'Nutrient' were consistently lower than that of 'Water'. When the soil was at relatively dry condition (around 5% degree of saturation), the gas permeability of 'Combined' and 'Nutrient' were 22% and 14% lower than that of 'Water' respectively. The lower permeability of 'Nutrient' is attributed to nutrient precipitation as verified by SEM analysis. When the soil specimens were subjected to drying, decreasing water content in soil dissolved less nutrient such as organic matters. The nutrient precipitated and could clog the pores. Mikutta *et al*. (2004) showed that the same amount of dissolved organic matters can clog more pores in dry condition, compared to the moist condition. Therefore, ss indicated in the figure, gas permeability difference between 'Nutrient' and 'Water' increased with decreasing saturation. For 'Combined', in addition to the precipitation of nutrient, the further reduction of gas permeability was due to biofilm. When nutrient is present, bacterial cells secret adhesins to attach surface and connect other cells (Lemon *et al*., 2008). The bacteria then become immobile and consume the nutrient to produce EPS with high molecular mass (e.g. 1000 kDa) to surround themselves. Normally, EPS is highly viscous (Flemming & Wingender, 2010) and more viscous

than hydrated biopolymer gels like guar gum (Isobe *et al*., 1992). High viscosity of EPS with immobile cells ensures that biofilm firmly attaches on particle surface and causes bio-clogging. Also, Epstein *et al*. (2011) showed that biofilm of *Bacillus subtilis* acts as a protection mechanism for bacteria to resist gas penetration, such as anti-biocide. Consequently, the gas permeability of 'Combined' was the lowest after the biofilm formed in soil. With other beneficial effects like decomposing harmful contaminants (Morikawa, 2006a) and mitigating soil drying (Roberson & Firestone, 1992), biofilm can potentially be applied as soil barriers to reduce harmful gas emission. In terms of mechanical properties of soil, according to the study by Perkins *et al*. (2000), presence of biofilm in soil has negligible effects on the soil shear strength and stiffness. However, biofilm from rhizobacteria can facilitate the plant growth (Vlamakis *et al*., 2013) and may affect the properties of vegetated soil. The interaction between rhizobacterium and vegetation should be considered in case of studying vegetated soil. An example like *Bacillus stubtilis*, can clog the pores with roots as biofilm formation is required to colonize the plant root after first contact. (Beauregard *et al*., 2013; Allard-Massicotte *et al*., 2016).The difference of gas permeability between 'Combined' and 'Water' increased as degree of saturation decreased. It implies that dehydration of biofilm still maintained the function of reducing permeability of unsaturated soil during drying. However, the difference of gas permeability between 'Combined' and 'Nutrient'

was almost constant with degree of saturation. This suggests that biofilm formation was inhibited. Since the incubation chamber provided sufficient oxygen through air-ventilation, slow rate of biofilm formation was mainly due to the limited amount of nutrient provided and decrease of soil water content. The consumption of nutrient by bacteria always involves oxidation and reduction reactions. In this study, organic carbon in the nutrient is oxidized to provide energy for microbial activities. As suggested by Proto *et al*. (2016), the organic carbon can be simply estimated as 50% of organic components in nutrient by weight. The organic carbon content provided was only around 0.5% of total soil dry mass. It is noted that some bacteria can use other substances like carbon dioxide for oxidizing organic carbon in anaerobic environment (Thauer, 1998). But, the production of cell biomass is less than that produced by aerobic respiration (Lengeler *et al*., 1999), which may suggest that biofilm formation would be lower in anaerobic environment. Typical examples of oxidation and reduction with regards to nutrient can be found in Nealson & Saffarini, (1994) and Vepraskas & Craft (2016).More importantly, organisms also require water to transport chemicals and regulate environment for metabolism (Mitchell & Santamarina, 2005). During soil drying, less water was available for solute transport and the metabolic waste of bacteria became more concentrated. The metabolism of bacteria and the biofilm formation were thus further inhibited. Hence, the difference between ‘Combined’ and ‘Nutrient’ was

nearly constant at all degrees of saturation.

Determination of significance of difference between the conditions by statistical analysis

Fig. 3 compares the gas permeability of each conditions (mean ± standard error, n=3) by statistical analysis with consideration of specimen variation. Three degrees of saturation (15%, 30% and 45%) corresponding to about 4, 8 and 12 days of incubation were selected. As shown in the figure, only the gas permeability of 'Combined' was significantly different from that of 'Water' with $p<0.05$ at 45% degree of saturation, which was 18% lower than 'Water'. At both 15% and 30% degree of saturation, the gas permeability of 'Combined' and 'Nutrient' were significantly lower than 'Water' at $p<0.01$ and $p<0.05$, respectively. This suggests that presence of biofilm or only nutrient can successfully reduce gas permeability of soil at a probability of 99% and 95%, respectively.

When gas permeability of 'Combined' was compared to that of 'Nutrient', no significant difference was found for all degrees of saturation ($p>0.05$). This implies that the effect of biofilm on gas permeability reduction can be over-estimated if nutrient effects are not considered. The overestimation could possibly be higher in previous studies (Vandevivere & Baveye, 1992; Dennis & Turner, 1998; Proto *et al*., 2016) in

which nutrient solution was continuously supplied to induce biofilm. It should be careful that biofilm is less biodegradable than nutrient, which raises an issue about durability. Moreover, different bacteria require specific nutrient to trigger signalling compounds and increase biofilm formation (Morikawa *et al*, 2006b; Shemesh & Chai, 2013). Considering only nutrient effects is important to comprehensively understand biofilm effects.

Determination of microstructure of specimens by SEM

Fig. 4(a), 4(b) and 4(c) show the microstructure of 'Water', 'Nutrient' and 'Combined' obtained using SEM at the end of all tests, respectively. For 'Water', there were no substances possibly clogging soil pores. For 'Nutrient' case, nutrient like organic matters precipitated due to absence of soil water, which then adsorbed on the particle surface. As shown in Fig. 4(b), the precipitated nutrient connected two adjacent particles and the outline of particles was unobstructed. This is similar to SEM images given by Zang *et al*. (2016), which studied the precipitation of organic polymers in sand. For 'Combined' shown in Fig. 4(c), irregular layers and folds of biofilm covered the particle surfaces and made the outline of particles non-uniform. These features of biofilm are consistent with the SEM images of biofilm on sand, as reported by Jean *et al*. (2004). Fig. 4(d) shows the presence of both biofilm and nutrient for the case of

‘Combined’ by SEM image. Aside from folds of biofilm, substances with crystalline structure were also found on the particles. This originated from inorganic minerals (e.g. $MnCl_2$) in nutrient, implying the nutrient precipitation. Such SEM image verifies the existence of both nutrient and biofilm in ‘Combined’. After 14 days, biofilm was found to be preserved as degradation of EPS requires a complex of enzymes produced by diverse microbes (Wolfaardt *et al*., 1999). The preservation of biofilm maintained the gas permeability reduction at low water content in soil. Since structure similar to biofilm did not exist in ‘Nutrient’, it confirmed that sterile preparation successfully achieved negligible contamination in ‘Nutrient’ and its effects were only due to the nutrient precipitation.

Fig. 5 shows SEM image of biofilm produced by *Bacillus subtilis* in soil at the end of the tests (14 days). The length of most bacterial cells was 1-2 μm similar to SEM provided by Morikawa (2006a) and Morikawa *et al*. (2006b), which studied biofilm of *Bacillus subtilis* in solution. This indicates that the biofilm was only produced by *Bacillus subtilis* after sterile specimen preparation. Effects caused by other microorganisms in ‘Combined’ were negligible. In this study, identification of each component is based on the morphology and SEM images reported by previous studies. Other direct alternatives such as Energy Dispersive Spectroscopy (EDS) may be used to provide further confirmation for each component.

SUMMARY AND CONCLUSIONS

To investigate effects of biofilm and only nutrient on gas permeability of unsaturated sand, three different conditions were tested: soil with water ('Water'), nutrient ('Nutrient') and mixture of bacteria and nutrient ('Combined'). SEM images proved that owing to successful sterile preparation, negligible contamination was achieved in 'Nutrient' while the biofilm and its effects were only contributed by *Bacillus subtilis.*

Gas permeability of 'Combined' and 'Nutrient' were consistently lower than 'Water' for all degrees of saturation. At around 5 % degree of saturation, 'Combined' and 'Nutrient' were 22% and 14% lower than 'Water', respectively. Precipitation of nutrient causes the permeability reduction in 'Nutrient' while both nutrient precipitation and biofilm formation in 'Combined' reduce the permeability. Interaction between immobile bacterial cells and viscous EPS allows biofilm to firmly adsorb on particle surface to cause bio-clogging. The difference of gas permeability between 'Combined' and 'Water' increased with decreasing degree of saturation, which implies that permeability reduction by biofilm is maintained during soil drying. However, the difference between gas permeability of 'Combined' and 'Nutrient' was almost constant with saturation since the biofilm formation was inhibited with decreasing soil water

content. The results of this study does not necessarily change even if different bacteria concentrations are used. This is because as long as biofilm forms successfully, the gas permeability of 'Combined' is the lowest, followed by 'Nutrient' and then 'Water'. Further research works are still needed before any practical application can be considered.

Statistical analysis considering specimen variation supports that gas permeability of 'Combined' and 'Nutrient' was significantly lower than 'Water' ($p<0.05$). However, there was no significant difference between 'Combined' and 'Nutrient' ($p>0.05$), implying that biofilm effects can be overestimated without consideration of nutrient effects.

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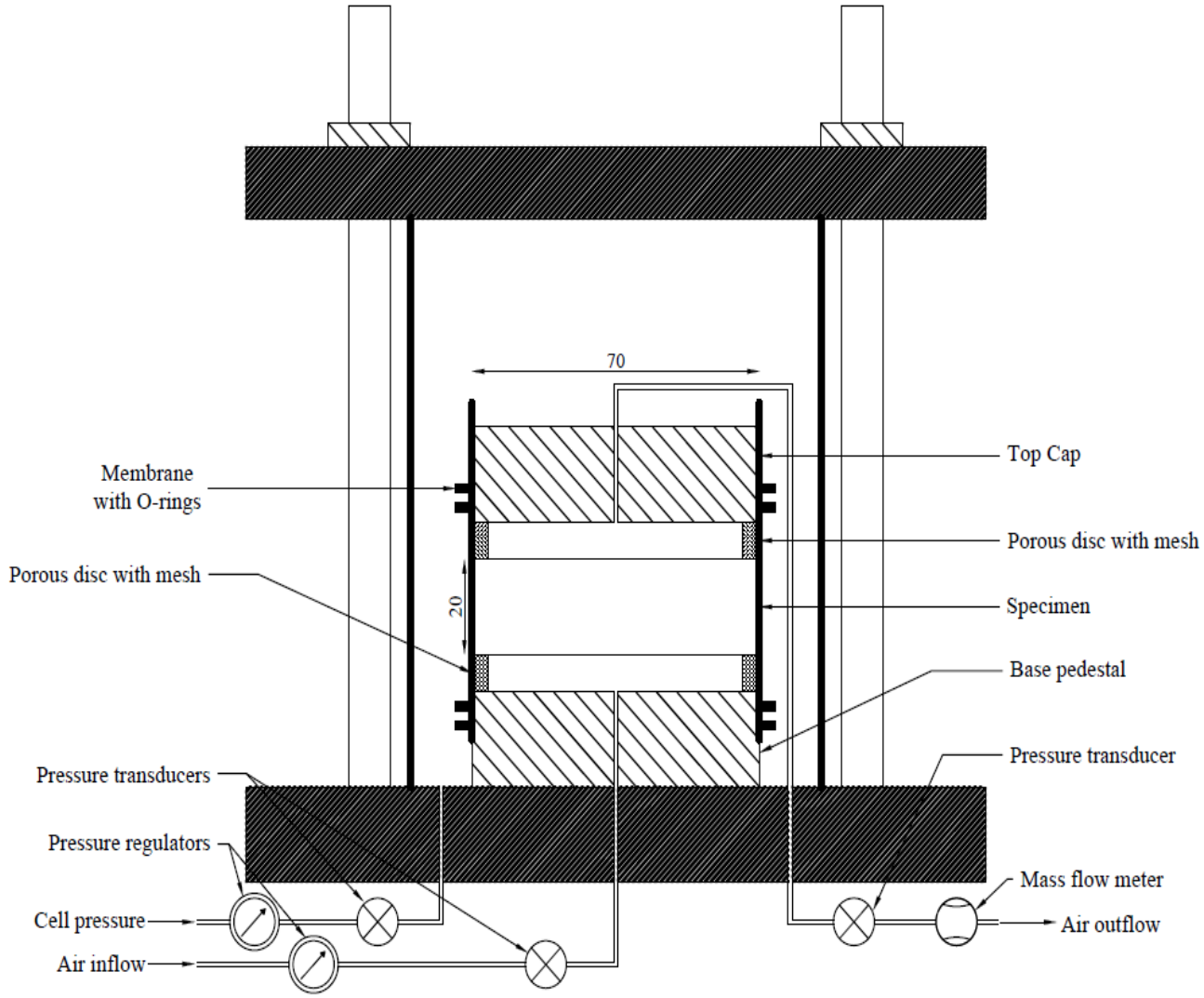


Fig. 1. Schematic diagram of a flexible wall permeameter for measuring gas permeability of soil (unit: mm)

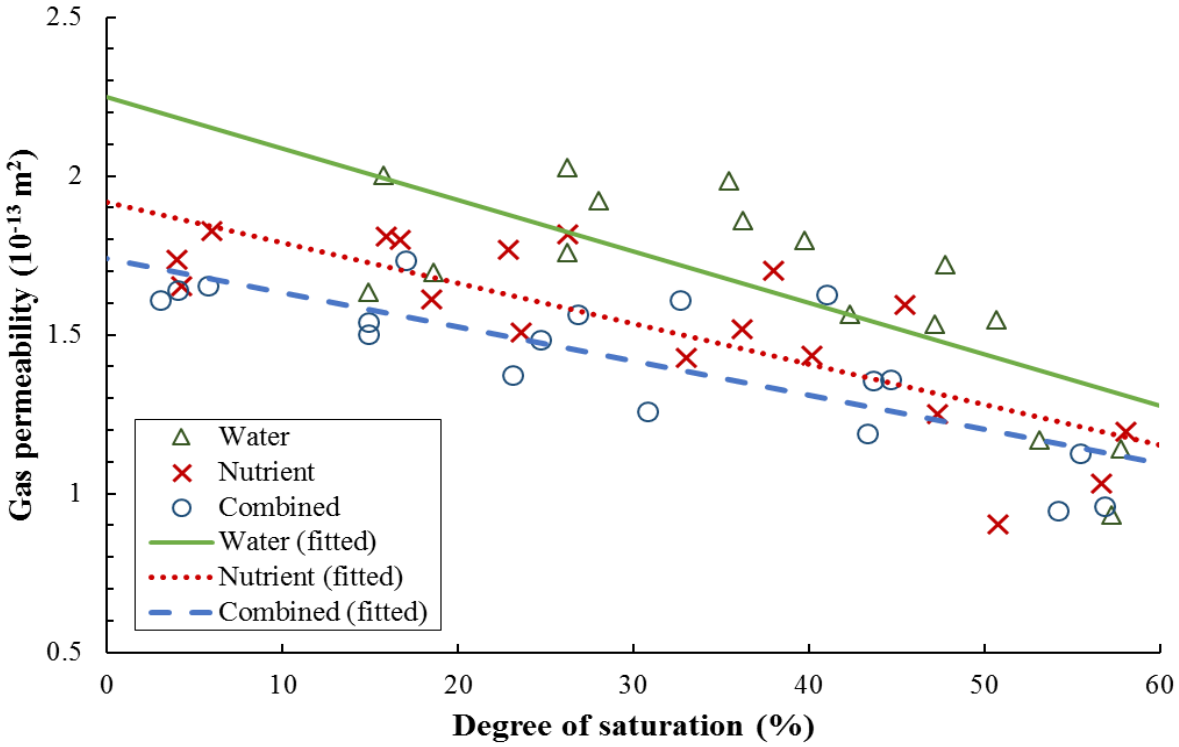


Fig. 2. Variation of gas permeability of each condition at different degrees of saturation

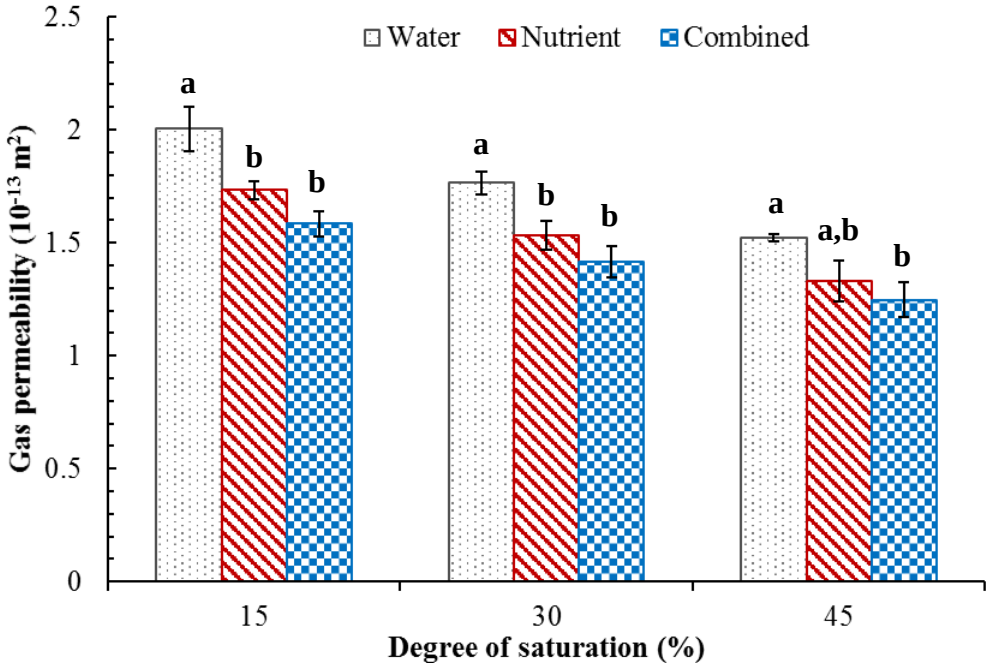
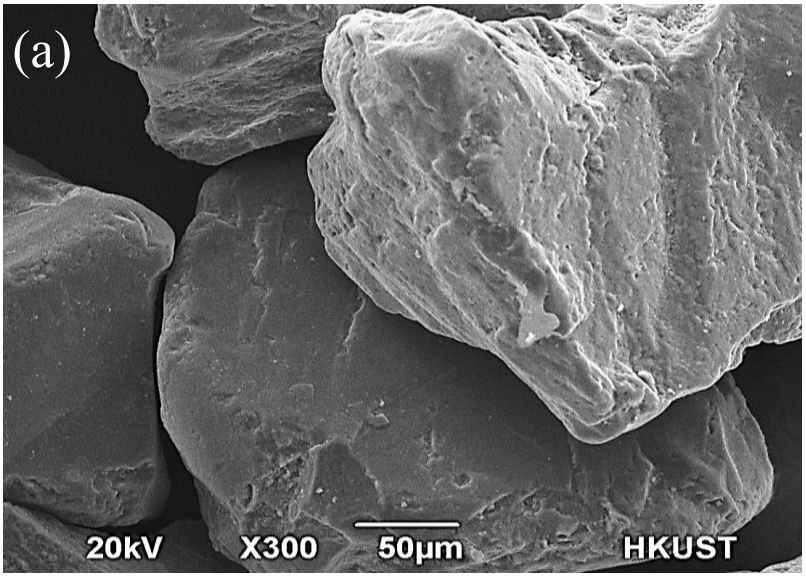
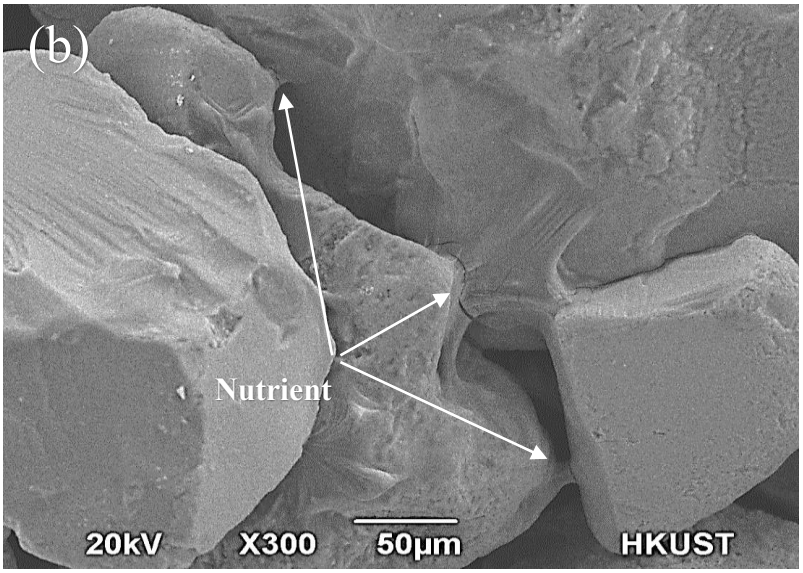
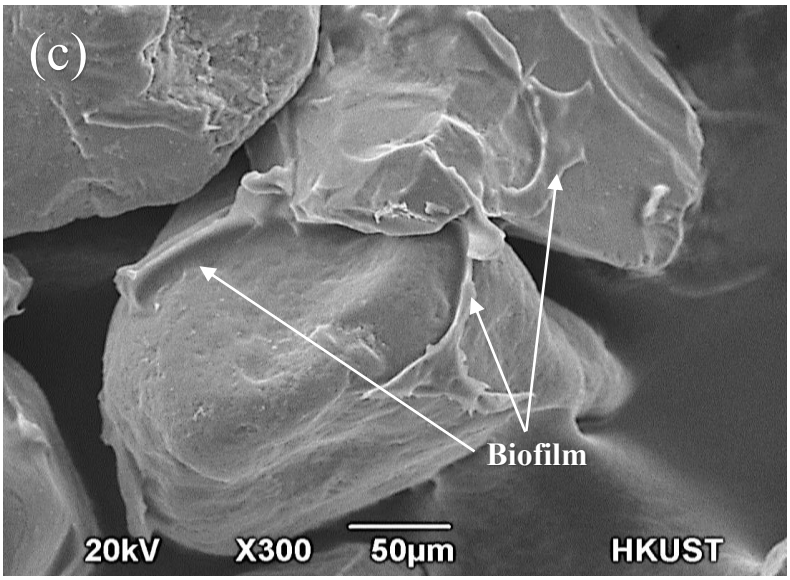


Fig. 3. Statistical analysis considering specimen variations of gas permeability (mean± standard error, n=3) at 15%, 30% and 45% of saturation (equivalent to 4, 8 and 12 days of incubation respectively); At a particular degree of saturation, different letters (a, b) in either two conditions represents significant difference between their gas permeability at level of $p<0.05$ (probability more than 95%)


(a)
20kV
X300
50μm
HKUST


(b)
Nutrient
20kV
X300
50μm
HKUST


(c)
Biofilm
20kV
X300
50μm
HKUST

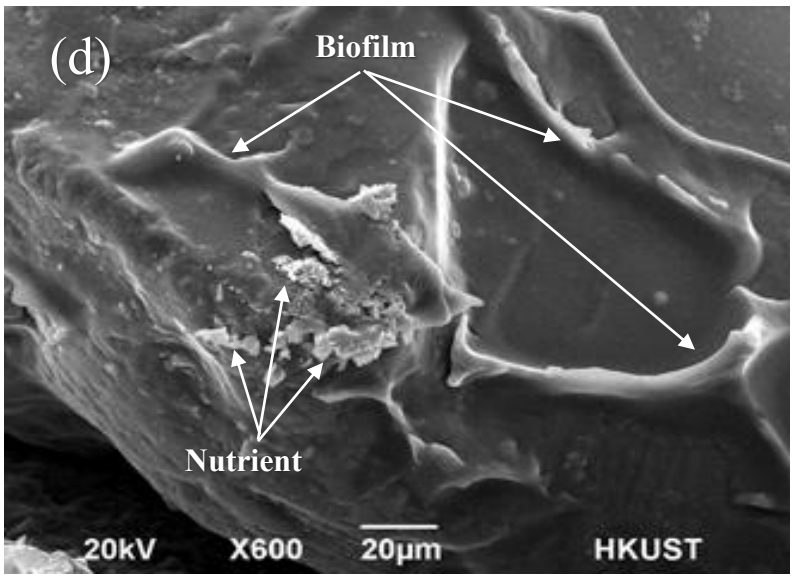


Fig. 4. Microstructure of specimens in (a) 'Water', (b) 'Nutrient' and (c) 'Combined' taken by using SEM (x300) after 14 days of incubation; (d) SEM image (x600) of 'Combined' showing presence of both biofilm and nutrient

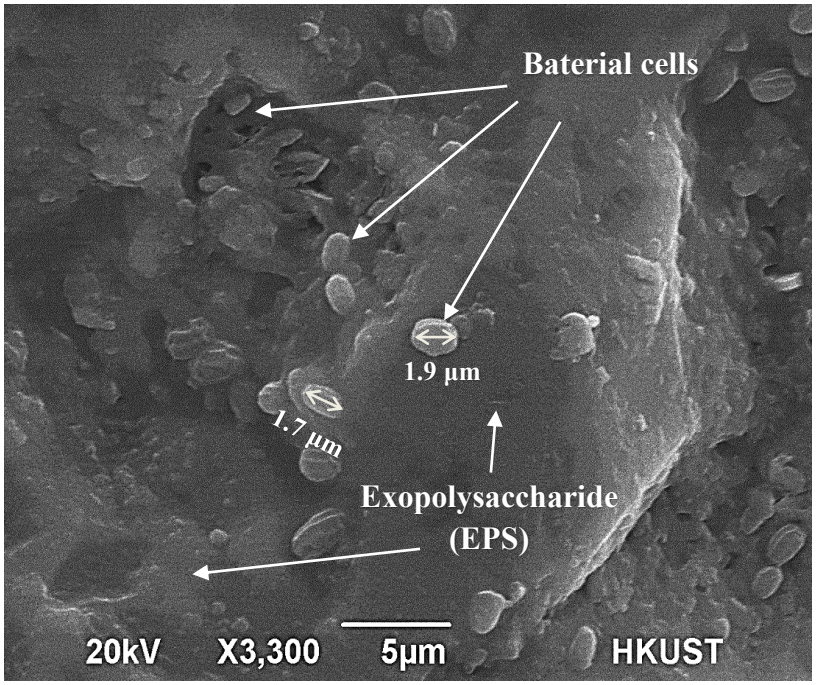


Fig. 5. SEM images of biofilm produced by *Bacillus subtilis* in soil at the end of all tests (after 14 days)