Influence of Microbial Activities in Reducing Erodibility of Sand

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ABSTRACT: Microbe-induced reduction of soil erodibility, since natural, is expected not to disrupt the natural environmental system. Although the role of bacteriogenic EPS in aggregating soil particles is widely recognized but the impact of various environmental parameters e.g., groundwater velocity and nutrient availability on bacteriogenic EPS in reducing the erodibility of soil is not very clear. In this study, a species of EPS producing soil bacteria *Bacillus megaterium* RB-05 isolated from a naturally cemented intertidal silt site was used to investigate the influence of flow velocity and nutrient availability on microbially mediated reduction of erodibility of sand. Durations of nutrient availability and media circulation velocity were observed to influence the bacterial population, amounts and composition of EPS found within sand specimens. Drained shear strength of loose sand samplers was found to increase due to EPS-related interparticle aggregation. EPS produced under fluvial activities seems to be more capable in aggregating sand grains as well as reducing erodibility of sand. Results of model sand erosion test further strengthen this conclusion.

KEYWORDS: Extracellular polymeric substance (EPS), *Bacillus megaterium* RB-05, Erodibility, Flow velocity, Nutrient availability.

1. INTRODUCTION

Erosion of soil particles due to currents or wave action causes severe damage of coastal infrastructure and natural habitat. Further, internal and surface erosion are two important failure modes of earth structures. Although erosional resistance of soil partially depends on the topographic position, slope steepness and the amount of disturbance, soil properties are the most important parameters and shear strength is the most prominent soil properties on which erodibility of soil depends (Morgan, 2005). Soil often fails due to erosion mainly due to changes in stress and reduction in shear strength (Zhang and Chang, 2012; Ke and Takahashi, 2015). Erosional resistance of soil can be improved by improving the shear strength of soil through the aggregation of erodible soil using a variety of materials such as lime, Portland cement and polyacrylamide. Since, these man-made materials are toxic or caustic, traditional techniques of preventing soil erosion can pose a threat to natural environmental system (Nugent et al., 2010). Therefore, a process to be used to enhance the resistance against erosion of soil should not disrupt the natural environmental system.

Soil environments often dominated by a great number of microorganisms which can produce profuse amount of extracellular polymeric substances (EPS). EPS are high molecular-weight polymers comprised mainly of sugar residues produced by a microorganism outside of their cell wall, protein and lipid molecules, and dead cells (Fleming et al., 2010). Microbes secrete EPS primarily to survive themselves against predation and drying. They also produce EPS in response to various environmental stress and/or to get ecological advantage. In aqueous environments, microorganisms often stick themselves to solid surfaces and to each other by forming biofilm matrix using EPS (Wingender et al., 1999). Because of their viscous nature (Asthaputre and Shah 1995), surface tension (Satputea et al., 2010), and cohesion and adhesion to the substratum (Mayer et al., 1999) EPS aggregates soil particles in harsh environments, e.g., formations exposed to the scouring action of rapidly moving groundwater (Battin et al. 2003; Godinho and Bhosale, 2009). Microbes capable of producing copious amounts of EPS are relatively endemic compared to the strains capable of producing mineral precipitates (Wingender et al., 1999). Also, EPS-producing microbes can persist on minimal nutrient and moisture levels available in many subsurface environments. Additionally, since EPS are found widely, known to be harmless and are often edible, they can provide sustenance to subterranean organisms (Maier et al., 2009) and can be considered eco-friendly. EPS-related soil aggregation therefore may not require introduction of non-endemic organisms and sustaining them with nutrients at elevated levels considered detrimental in many environmental settings (USEPA, 2011). Therefore, utilization of naturally occurring, non-pathogenic endemic microorganisms capable of producing copious amounts of EPS in reducing soil erosion could be a better alternative to conventional erosion prevention techniques often use toxic materials. Besides, microbially mediated reduction of erosion susceptibility of soil could be an attractive alternative since they are environment-friendly and cost effective.

It is known that microbially mediated stabilization of sediments is due primarily to biogenic EPS related soil particle aggregation (Dade et al., 1990; de Brouwer et al., 2005; Gerbersdorf et al., 2008). Amounts of EPS produced, and their compositions depend on the types of microorganisms and their states of growth, substrate characteristics (soil or rock hosting the biomass), and physical (e.g., temperature, groundwater velocity), chemical (e.g., pH, salt content) and electrochemical (e.g., groundwater ion concentration) particulars of the environment (Okabe et al. 1992; Stoodley et al., 1999; Velasco et al., 2006). EPS related soil particle aggregation is, therefore, not the sole parameter responsible for improving erosional resistance of soil. A variety of environmental parameters, mentioned above, also have significant role in reducing the erosion susceptibility of soil. Consequently, feasibility of EPS related soil particle aggregation in reducing erodibility of soil requires establishment of the interdependence of these factors. Although, the mechanism involved in increasing erosional resistance of soil is known but the role of these factors and/or their interdependency in reducing erosion susceptibility of soil is not very clear. Further, no report was found in the literature on in situ utilization of EPS producing microbial species to assess the efficacy of biogenic EPS in reducing the erodibility of cohesionless soil.

In the present study, a species of minimally sustained, EPS producing, endemic soil bacteria *Bacillus megaterium* RB-05 was isolated, identified and characterized in the laboratory. RB-05 was isolated from a naturally cemented intertidal silt site on the bank of river Rupnarayana near Kolaghat, West Bengal, India. RB-05 was then grown within saturated sand samples in the laboratory. The impact of flow velocity and nutrient availability on the microbial population growth and amounts of EPS and its components such as carbohydrate, protein and lipid were assessed. The role of these parameters and their interdependency in reducing erodibility of loose sand was also investigated in this study. A series of drained triaxial tests were performed to assess the microbe-induced improvement of shear strength. Model erosion tests of soil slope were further performed in the laboratory to demonstrate the proof-of-concept for the reduction of erodibility of loose sand due to microbial activities. Detachment and removal of soil particles from

the soil matrix due to seepage flow, in this research, demonstrates the erodibility of the soil.

2. MATERIALS AND METHODS

2.1 Microorganism

2.1.1 Site Description and Collection of Soil Samples

The microorganism used in the current study was isolated from a naturally cemented silt site in the southern part of West Bengal located in eastern India (Figure 1). Soil samples were collected from the bank of the river Rupnarayan near Kolaghat, India following USEPA guideline SESDPROC-300-R1 (USEPA, 2007). At the sampling location the top 1 m of the newly deposited silt was removed before pushing a thin-walled polyvinyl chloride (PVC) tube (76 mm diameter, 2.5 mm wall thickness, 2 m length) manually into the deposit to 3 m depth for obtaining the soil sample. Soil samples were stored in sterilized containers and taken to the laboratory for microbial, geotechnical, and mineralogical characterization.

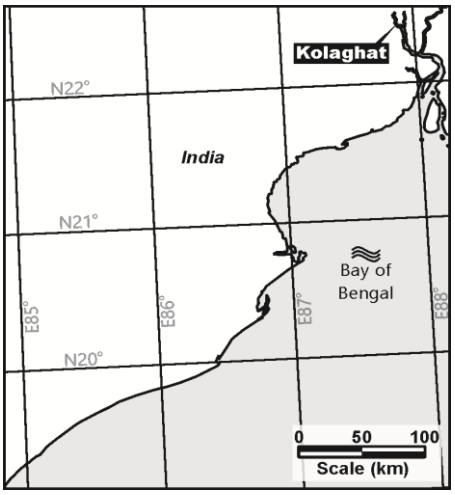


Figure 1 Site location

2.1.2 Isolation and Identification of Bacteria

Bacterial strain designated as RB-05 was isolated from soil samples by serial dilution technique. About 1 g of air-dried soil was aseptically mixed with 100 ml of sterilized distilled water by vortexing and kept undisturbed for 1 h. The bacteria-containing supernatant was extracted and serially diluted to 10^{-9}. Subsequently, 1 ml of diluted supernatant was transferred on nutrient agar media plates (Ghatak et al., 2015) and incubated at 30 °C for 2 to 3 days. Single colonies of bacteria were isolated by observing the differences in colony texture of the microbial strains in the agar plates and transferring the selected colonies to new plates until obtaining one distinctive colony. The culture was then transferred on the same solid medium and refrigerated at 4 °C for further use.

The isolated strain was then identified by 16S rDNA sequencing (Ghatak et al., 2015). The data were aligned and compared with those present in the GenBank nucleotide database using BLAST software package (Pearson and Lipman, 1988) to find the closest homologs. Thereafter, a phylogenetic tree was constructed in MEGA 3.1 software package using neighbourhood joining method and maximum parsimony approach (Kumar et al., 2004). An agar free liquid nutrient medium (Ghatak et al., 2015) was used for growing and maintaining the isolated bacterial strain. Liquid bacterial culture of RB-05 was kept refrigerated at 4 °C for further use in the present study.

2.1.3 Nutrient Media

Glucose-containing mineral salt (GMS) media was used as nutrient media in the present study for bacterial growth characterization, EPS production and sample preparation. Agar-free liquid GMS media was prepared by dissolving 20 g glucose, 3 g NH_4Cl, 0.14 g KH_2PO_4, 2.2 g K_2HPO_4, 0.14 g NaCl, 0.6 g $MgSO_4$, 2.3 mg $ZnSO_4$, 17 mg $MnSO_4$, 10 mg $CuSO_4$, 4 mg Na_2MoO_4, 10 mg EDTA, 0.4 mg $NiCl_2$, and 6.6 mg NaI in a litre of distilled water. The composition of GMS media approximates the amounts of mineral salts found in groundwater (Bohn et al., 1985). Glucose has been used in these compositions as sugar source because glucose is a predominant carbon source in many soil types (Panikov, 1995). The viscosity of the GMS medium measured with an Ostwald viscometer (Plummer, 1987) was also comparable to that of water. The pH of the medium was adjusted to 7.5 with 1 N HCl. The media was then sterilized by autoclaving at 103 kPa and 121 °C for 15 min.

2.1.4 Bacterial Growth Characterization

Bacterial growth of the isolate RB-05 was observed by inoculating them in GMS media. Bacterial culture was transferred in a 100 ml flask (Borosil, India) containing 50 ml of GMS media, sterilized beforehand by autoclaving. The inoculated medium was placed in a shaker incubator (Orbitek LE, Scigenics, India) for shaking at 120 rpm over 24 hours at 30 °C. Bacterial growth characteristics was studied under pH between 3 and 10, temperature between 20 °C and 40 °C and incubation time of up to 6 days. A variety of sugar sources, viz., maltose, sucrose, glucose, galactose, xylose, or lactose and nitrogen sources, viz., ammonium chloride, ammonium nitrate, glycine, ammonium acetate, glucosamine or ammonium sulphate were used in the optimization exercise. The optical density (OD) of bacteria-inoculated mineral salt media, used as a proxy for bacterial population size, was determined periodically in a spectrophotometer (Sistronics 106, India) at 600 nm.

2.1.5 EPS Production and Extraction from GMS Media

RB-05-inoculated GMS media was incubated at 30 °C and 120 rpm for 24 h and cell-free EPS solution was extracted by centrifuging the media using laboratory centrifuge instrument (Remi, India) at 12000 rpm for 20 min at 4 °C. A portion of the solution was used to estimate the carbohydrate, protein, and lipid contents of EPS. The other portion of the cell-free EPS solution was mixed with double volume of cold acetone (95% v/v), stored at 4 °C for 24 h without disturbance and centrifuged at 12000 rpm for 10 min at 4 °C. Wet EPS was collected from centrifuge tubes and lyophilized to obtain dry EPS powder for the estimation of total EPS quantity. GMS media was prepared and incubated similarly as described earlier but without bacterial culture to confirm that the polymeric products were bacteriogenic.

2.2 Sand

A type of silica (97% quartz and 3% feldspar and others) sand was used in this study (Figure 2). Microscopic analysis indicated that the sand was subangular in shape. The sand had coefficient of uniformity, C_u = 1.99, a median particle size (D_{50}) of 0.55 mm and grain specific gravity of 2.65. It was classified as poorly graded sand, SP, based on the Unified Soil Classification System (ASTM, 2006). The maximum and minimum void ratios determined per ASTM D4254 and D4253 were 0.83 and 0.54, respectively. The sand was sequentially washed with an acidic solution 0.25 N HCl over 12 hours followed by a basic solution 0.25 N NaOH over 12 hours. Thereafter, the sand grains were thoroughly rinsed with de-ionized water until its pH reached a value of 7. They were then sterilized by autoclaving. Grain size distributions of Kolaghat silt and acid washed sand are also shown in Figure 2 for comparison.

2.3 Soil Sample Preparation

To assess the influence of flow velocity and nutrient availability on the amount, composition, and chemical characteristics of bacteriogenic EPS, sand samples were prepared within sterilized polypropylene containers (Tarsons, the Netherlands). Loose sand

samples – 70 mm in length, 40 mm in width and 55 mm in height – were prepared about 15.6 kN/m^3 dry densities by pluviating sterilized sand grains under autoclaved GMS medium mixed with centrifuged bacterial biomass of about 0.6 g (Datta et al., 2019). To avoid contamination, all the samples were prepared within a laminar air flow hood and kept inside an incubator at 30 °C in a sterile environment. The samples were incubated for 12 h duration to allow the bacterial population to get attached to the surfaces of sand particles through EPS (Martinez et al., 2013). Thereafter, freshly prepared bacteria-free GMS medium was continuously circulated over the samples at fluxes of up to 0.006 mm/s inside the incubator. These velocities are comparable to those of groundwater movement through coarse grained soils. Microbial growth, changes in pH, amounts of EPS and its components such as carbohydrate, protein and lipid content produced on sand particles were monitored periodically at an interval of 24 h up to 48 h.

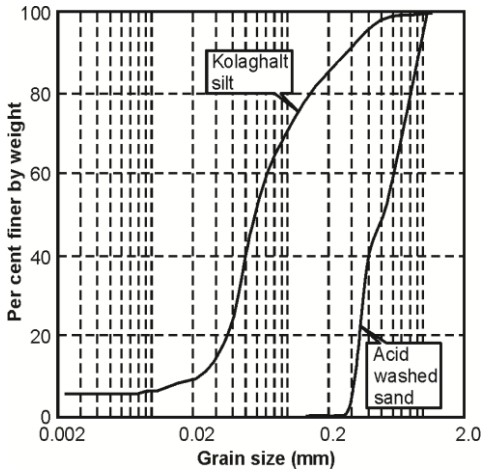


Figure 2 Grain size distribution data

2.4 Bacterial Growth and Acidity

For estimating bacterial population size within soil samples, 5 g of bacteria-inoculated sand was extracted periodically and vortexed with 5 ml of sterilized distilled water at room temperature. Bacterial population within the supernatant was estimated from the OD of the supernatant as described earlier. Acidity of the supernatant was also recorded using a digital pH meter (Oakton, Eutech Instruments). These measurements were done in triplicate to assess repeatability of results.

2.5 EPS Extraction from RB-05-inoculated Sand Samples

EPS was also extracted from RB-05-inoculated sand samples. To do this, a portion of it was recovered periodically from bacteria-inoculated samples and oven dried at 70 °C. Thereafter 20 g of dry bacteria-inoculated sand was vortexed with 25 ml sterilized distilled water for 30 min to obtain the supernatant. The supernatant was recovered and centrifuged at 12000 rpm for 20 min at 4 °C to get the cell free EPS solution. As described earlier, the cell free solution was used to estimate the carbohydrate, protein, and lipid contents of EPS.

2.6 Chemical Characterization of EPS

Carbohydrate content of the EPS was estimated spectrophotometrically. Towards this, 1 ml of cell free EPS solution was mixed with 4 ml of Anthrone reagent and heated within a boiling water bath for 15 min. The mixture was allowed to settle to room temperature and its OD was measured at 630 nm using a spectrophotometer. The carbohydrate content was estimated by comparing the OD with a standard calibration curve prepared for similar solutions containing known amounts of glucose (Morse, 1947).

Protein content of the EPS was also estimated spectrophotometrically following Lowry et al. (1951). Towards this, 1 ml cell free EPS solution was mixed with 3 ml of alkaline copper sulfate solution (pH 8.5) before storing the mixture at 30 °C for 10 min. Thereafter 0.5 ml of Folin's reagent was added to the mixture before incubating it again at 30 °C for 30 min. OD of the mixture was then measured at 660 nm. The protein content in the EPS was estimated by comparing the OD with a Bovin serum albumin standard curve.

Lipid content of the EPS was estimated by solvent extraction (Conrad et al., 2003). 10 ml of cell free EPS solution was mixed with 15 ml of an organic solvent prepared by mixing chloroform and methanol in 1:2 (v:v) ratio. The mixture was shaken for 24 h at 21 °C in a shaker incubator operated at 200 rpm and centrifuged at 4000 rpm at 21 °C. The supernatant was collected and dried at 70 °C. The weight loss was taken as an estimate of lipid content.

2.7 X-ray Diffraction (XRD) Study

Oven dried samples of Kolaghat silt at 70 °C, weighing 1 ± 0.5 g, were placed inside the sample holder of an X ray diffractometer (XRD-I X´Pert, PANalytical BV, the Netherlands) for mineralogical assessment. The diffractometer was operated at a scanning speed of 2° /min using 0.154 nm Cu-Kα radiation to obtain data covering 20° to 70° 2θ range.

2.8 Scanning Electron Microscopy (SEM)

Scanning electron microscopic analysis was conducted on bacteria-inoculated sand particles. For SEM, 1 to 2 mg of sand sample oven dried at 70 °C was coated with thin gold layer by plasma sputtering. Those were then characterized with a 5 to 20 kV electron beam under high vacuum in a scanning electronic microscope VEGA$_{LSV}$ (TESCAN, Czech Republic) to obtain an image of the sample surface.

2.9 Triaxial Compression Tests

To assess the efficacy of microbial metabolic products on soil shear strength a series of isotropically consolidated drained triaxial compression tests were conducted on loose cylindrical sand samples having 37 mm diameter and 74 mm height. Bacteria-inoculated and bacteria-free triaxial samples were prepared by pouring sterilized acid-washed sand within 50 ml GMS media mixed with or without 0.3 g centrifuged biomass inside the laminar air chamber. These samples were then incubated for 24 h at 30 °C in a sterile environment keeping fully saturated. After 24 h, the incubated samples were consolidated under a cell pressure of 100 kPa, 200 kPa and 300 kPa and subsequently sheared monotonically at a constant strain rate of 0.12 mm/min. The post-consolidation relative densities of these triaxial samples were 40% ± 2%.

2.10 Model Soil Erosion Test

Several experiments such as Pinhole Test (Sherard et al., 1976), Erosion Function Apparatus (Briaud et al., 2001), Hole Erosion Test (Wan and Fell, 2004), Slot Erosion Test (Wan and Fell, 2004) and Jet Erosion Test (Hanson, 1991) had been designed and used by the researchers to evaluate erosional resistance of soil. Among those, Hole Erosion Test (HET) is a simple, fast, and economical way to test non-plastic soils for assessing the erosion potential against piping (Farrar et al., 2007). To demonstrate the effectiveness of microbe-induced sand particle aggregation in reducing erodibility, a few simple erosion model tests were performed in the laboratory. The experimental setup adopted in this research (Figure 3) is somewhat similar as the HET (Wan and Fell, 2004) and sufficient to demonstrate the erosion phenomenology.

As an experimental aim, wedge shaped sand models having slope of 30° were prepared within sterilized polypropylene containers (Tarsons, the Netherlands). The sand wedges were constructed, similar as triaxial samples, by pluviating sand grains under sterilized GMS medium mixed with centrifuged bacterial biomass of about 7.4 g (Figure 3). Samples were prepared carefully to maintain the slopes and, also the dry densities of approximately

15.6 kN/m^3 after placement. The vertical end of the sand wedge was supported by autoclaved glass marbles, about 10 mm in diameter, retained behind sterilized, stiff, and permeable high-density polyethylene (HDPE) mesh used for uniform flow distribution. Similar sand models were also prepared without using biomass for control.

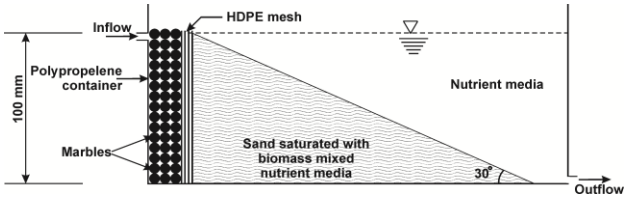


Figure 3 Schematic of sand erosion model test setup

After construction, the models were covered with autoclaved polythene with a few holes plugged with sterilized cotton to preclude contamination and ensure oxygen availability and placed inside a sterilized incubator for incubation over 24 h at room temperature. The sand models were maintained in a fully submerged condition under nutrient media to keep it completely saturated throughout the incubation period keeping the inlet and outlet pipes closed.

Thereafter, wedge shaped samples were subject to the internal erosion test under constant hydraulic head. Towards this, sterilized distilled water was allowed to run from a reservoir to the container via sterilized tubes at a constant rate of 0.006 mm/s through the sand slope for 8 h. Erosion severity is often directly estimated by the mass of eroded particles (Ke and Takahashi, 2015). Therefore, at the end of each test, eroded soil particles were collected at the base of the model box and oven dried to determine the soil loss. Subsequently, the surface profile of sloped soil samples was also carefully observed after termination of each test since visual observation has been extensively considered as a qualitative tool for internal erosion characterisation (Ouyang and Takahashi, 2015). Immediately after the model erosion test, photographs were also taken to facilitate visual surveying of the erosion severity. Masses of eroded sand and soil profiles of bacteria-inoculated and bacteria-free models were compared for assessing the influence of bacterially mediated soil aggregation on the erosion susceptibility. All the tests were conducted in duplicate.

3. RESULTS AND DISCUSSIONS

3.1 Characterization of Intertidal Silt

Due to relative lack of access into the intertidal silt site at Kolaghat (Figure 1), no in situ tests were carried out. However, resistance encountered during pushing in of thin-walled PVC tube soil sampler to 3 m depth below ground surface, the representative standard penetration test (SPT) blow counts are expected to be between 0 and 2. Bacterial colony count has been found to be between 9.3×10^9 and 1.5 ×10^{17} at a depth of about 0.3 m and decreases with the depth of soil. Mineralogical characterization of the extracted soil sample collected from intertidal silt site is presented in Figure 4. Microbial strains isolated from soil samples collected from this site were also found to produce EPS, calcite, and struvite. Grain size distribution of Kolaghat silt is shown in Figure 2.

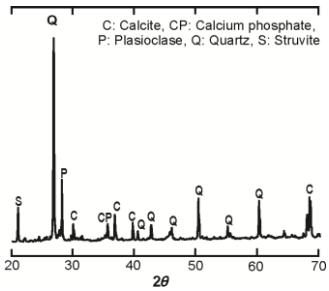
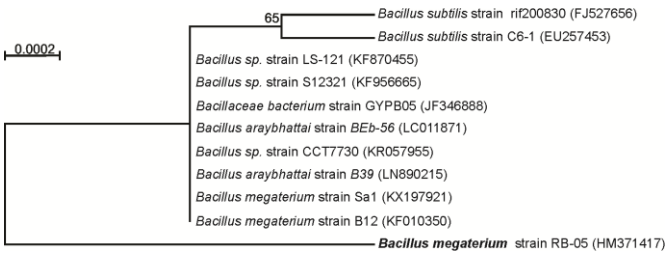


Figure 4 XRD data of Kolaghat silt

3.2 Isolation and Identification of Bacteria

The isolated strain has been identified as *Bacillus megaterium* RB-05 (GeneBank accession No: HM371417) by our research group (Figure 5). The selected bacterial species are known as an endemic soil residing bacteria. Table 1 lists a few characteristics obtained from microbiological investigation of the selected strain.



Notes: Bootstrap values (1000 replicates) are shown on nodal branches; scale represents % substitutions per nucleotide position; GenBank accession numbers are in parentheses

Figure 5 Phylogenetic tree from 16S rRNA sequencing

Table 1 Characteristics of *Bacillus megaterium* RB-05

Isolate	RB-05
Cell morph.	Rod shaped
Cell size	~5 μm long, ~2 μm in dia
Gram stain	Gram positive
Respiration	Aerobic
Spore formation	Endospore forming

3.3 Bacterial Growth and EPS Characterization

RB-05 was noted to grow satisfactorily in mineral salt medium containing different carbon (maltose, sucrose, glucose, galactose, xylose, or lactose) and nitrogen (ammonium chloride, ammonium nitrate, glycine, ammonium acetate, glucosamine, or ammonium sulfate) sources with pH between 3 and 10 and temperature between 20 °C and 40 °C. RB-05 grew optimally after 2-day incubation at 35 °C within glucose and ammonium chloride containing mineral salts medium having pH 7.5.

This strain was noted for its capability of producing copious amount of EPS. After 24 h of incubation in agar-free liquid GMS medium, RB-05 produced 1.1 g of dry EPS. Crystalline or amorphous solids or semisolids were not found in the absence of live bacteria confirming that the polymers found were bacteriogenic. RB-05 EPS was found to contain 50% carbohydrate, 20% protein, 18% lipid and 12% miscellaneous materials, e.g., dead cells, nucleic acids and ionic materials attached to the EPS (Figure 6). RB-05 was noted to be minimally sustained and precipitate calcite through enzymatic urea hydrolysis process (Rivadeneyra et al., 1991).

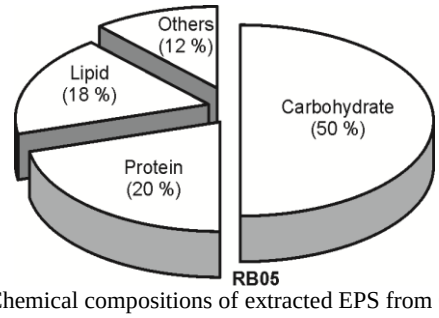


Figure 6 Chemical compositions of extracted EPS from GMS media

3.4 Influence of Flow Velocity on Bacterial Growth and Acidity

In the absence of nutrient media circulation, pH were found to decrease but OD to increase with incubation duration (Table 2). These observations are probably because of the production of bacteriogenic intermediate metabolic products which are acidic in

nature and/or due to the onset of death phase resulting from nutrient depletion since there is no continuous flow of nutrient media. Whereas with increasing flow velocity, OD was found to increase significantly (Figure 7) indicating increased microbial growth and/or metabolic activities resulting from continuous nutrients availability and increased environmental stress. On the other hand, marginal increase in soil pH with continuous media circulation may have been due to continued nutrient supply and washing out of bacteriogenic acidic intermediate metabolic products (Datta et al., 2019).

Table 2 pH and OD at zero media velocity

Incubation duration (h)	pH	OD at 600 nm
24	6.154	0.454
48	5.362	0.553

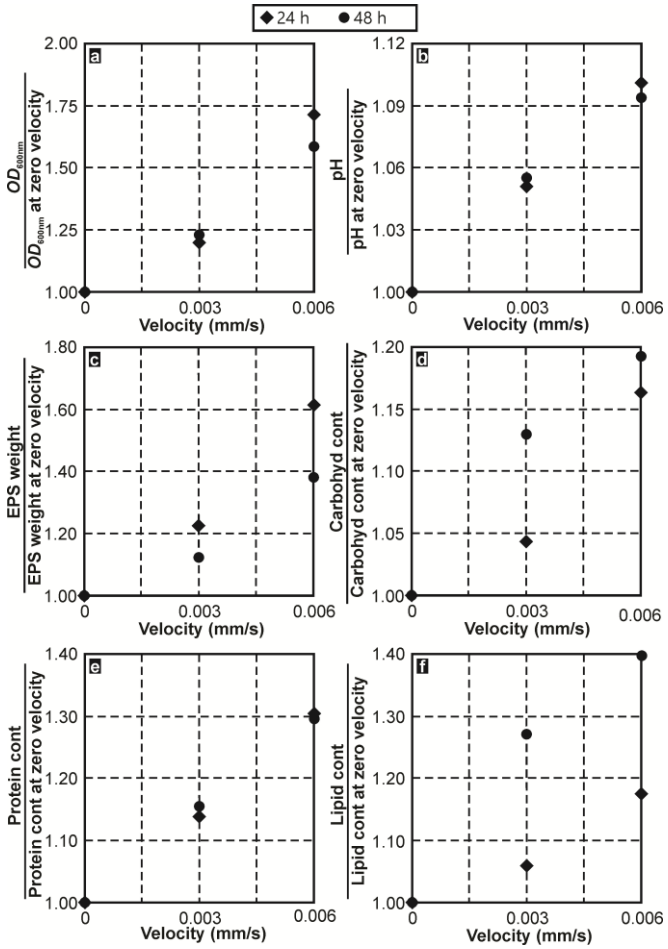


Figure 7 Influence of duration of nutrient availability and media circulation velocity on (a) OD, (b) pH, (c) EPS, (d) carbohydrate, (e) protein, and (f) lipid content

3.5 Influence of Flow Velocity on EPS and its components

Presence of EPS on and around the sand particles was seen in scanning electron photomicrographs of all bacteria-inoculated sand samples (e.g., in Figure 8) before and after exposure to fluvial activities. In the absence of media circulation, total EPS extracted from bacteria-inoculated sand as well as its components such as carbohydrate, protein and lipid contents are summarized in Table 3. Non-polar groups present in carbohydrate, protein and lipid contents of EPS are known to contribute to EPS hydrophobicity (Yu et al., 2006) which in turn helps in EPS - EPS attachment (Donlan, 2002). It is also known that protein content of EPS enhances the affinity of EPS to negatively charged surfaces (Jenkinson, 1994) and lipid content of EPS enhances the affinity of EPS to solid surfaces (Wingender et al., 1999). Since sand surface is electronegative (Iler, 1979), it therefore seems that observed amounts of carbohydrate, protein and lipid contents of EPS are likely to contribute to EPS-EPS attachment as well as attachment of EPS to sand particles.

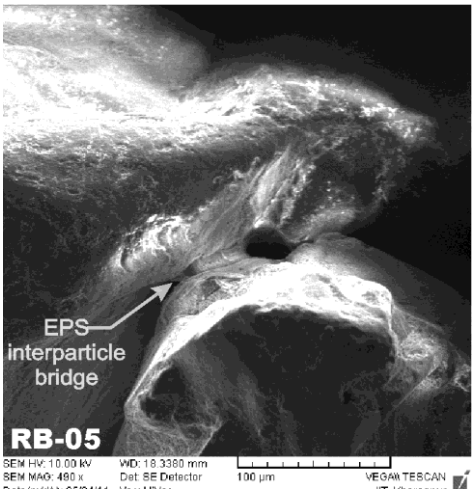


Figure 8 SEM photomicrographs of RB-05-inoculated sand at a flow velocity of 0.006 mm/s after 48 h incubation

Table 3 Amount of EPS and its components at zero media velocity

Incubation duration (h)	EPS Components (mg/g of dry sand)			EPS (mg/g of dry sand)
	Carbohydrate	Protein	Lipid	
24	0.47	0.19	0.16	1.01
48	0.56	0.24	0.18	1.23

In the absence and presence of fluvial activities, total EPS extracted from bacteria-treated sand as well as its components were found to increase with incubation duration (Table 2, Figure 7). The results presented in Figure 7 also indicated that the amounts of EPS and its components also depend on the flow velocity. Under fluvial activities, total amount of EPS increased by about 160%. With increasing flow velocities, carbohydrate and protein contents of EPS increased by about 120% and 130%, respectively. Correspondingly lipid content increased by more than 135% (Figure 7).

With increasing flow velocity, hydrophobic interaction increases with the increase in non-polar groups of EPS (Yu et al., 2006). Therefore, the rate and degree of EPS - EPS attachment is expected to increase under the fluvial activities. With increasing flow velocity, the affinity of EPS to negatively charged solid surfaces (in the present study, sand surface) also increases since the amount of protein and lipid content increases. So, the rate and degree of attachment of EPS to sand particles are also expected to increase under the fluvial activities. Data obtained in this study, therefore, clearly indicates that how various environmental parameters e.g., media circulation velocity and durations of nutrient availability influences the amount and composition of EPS and enhances erosional resistance of sand by enhancing EPS related sand particle aggregation. This attachment mechanism also explains the typical microbial response to fluvial activities in the aqueous environment.

3.6 Triaxial Compression Tests

Influence of EPS produced by RB-05 on drained strength of bacteria-inoculated saturated loose sand was assessed from triaxial test results on bacteria-treated and bacteria-free samples. Drained friction angle, ϕ', and cohesion intercept, c', were estimated by fitting a straight line at different strain levels in the s'–t space, where s' and t represent $(\sigma'_a + \sigma'_r)/2$ and $(\sigma'_a - \sigma'_r)/2$, respectively, and σ'_a and σ'_r denote the effective stresses in the axial and radial directions, respectively (Table 4). Drained triaxial test results indicated a measurable increase in shear strength for bacteria-treated

loose sand samples compared to bacteria-free samples possibly due to EPS-related sand particle aggregation (Table 4 and Figure 8). Further, for the bacteria-treated loose samples, peak deviator strengths were found to mobilize at greater strain levels compared to untreated samples (not shown in this paper). Since shear strength has a strong influence on the reduction in erosion susceptibility of soil, it is expected that the increased shear strength of bacteria-treated sand samples will be effective in reducing its erodibility.

Table 4 Drained shear strength parameters[a]

Sample	c' (kPa)	ϕ'
Untreated sand	0	33.8° ± 0.6
RB-05 Treated sand	14.2 ± 2.7	33.8° ± 0.3

Note. [a]Mean ± 95% confidence interval based on drained triaxial compression test data

3.7 Model Sand Erosion Test

Sand wedge having slope of 30º prepared with loose sand without and with minimally sustained live bacterial populations of RB-05 were exposed to water percolation over 8 h for assessing the influence of microbe-induced sand particle aggregation on erosion susceptibility. The bacteria-treated sand behaved exceptionally better than the untreated sand when subjected to continuous circulation of water through the model sand specimen. The profiles of slope surfaces before and after completion of test showed sign of piping for the slope of untreated sand (Figure 9). No such sign was observed for sand slopes treated with bacteria. Eroded sand masses further confirm these observations (Table 5). These results indicate a strong influence of EPS produced by the microorganisms used in this study in situ in reducing erosion susceptibility. Similar influence of ex situ biopolymers on erosion of sand-silt mixtures was observed by Adams et al. (2013).

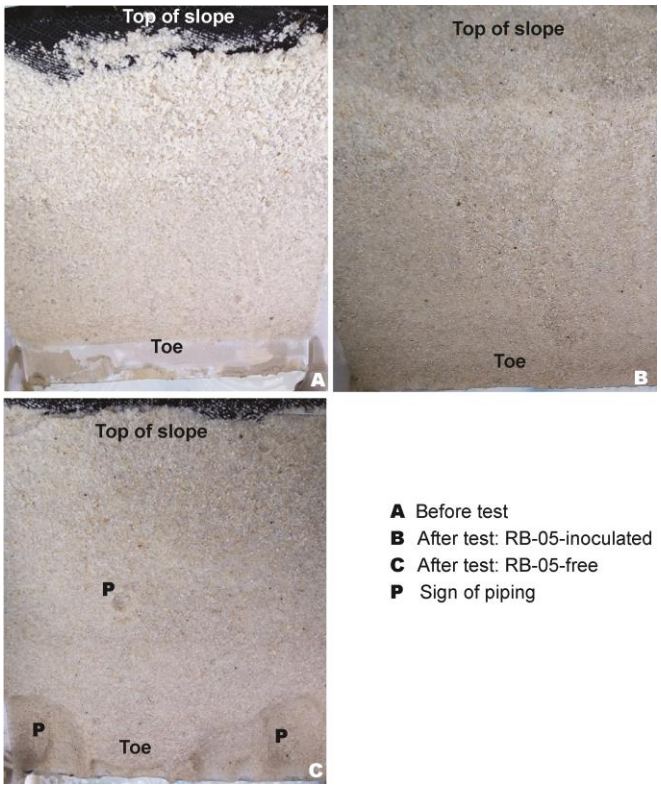


Figure 9 Top view of slope in sand erosion test

Table 5 Model sand erosion test result

Sample	Eroded Sand (%)
Untreated sand	8.4
RB-05 Treated sand	3.8

4. CONCLUSIONS

Over the past several decades, in geology, the roles of bacteriogenic EPS in aggregating soil particles are widely recognized. Feasibility of EPS related soil particle aggregation requires establishment of the impacts of various environmental parameters, e.g., pH, groundwater velocity, nutrient availability, and ecology. In the present study, clean, poorly graded sand was successfully treated in situ with live, minimally sustained bacterial populations with a single dose and continuous supply of nutrient media. Durations of nutrient availability and media circulation velocity were observed to influence the bacterial population growth, amounts and composition of EPS found within saturated sand samples. In the absence of media circulation, bacterial population growth, amounts of total EPS as well as its components were found to increase with incubation duration. Further, in the presence of continuous nutrient availability these amounts were noticed to increase with both flow velocity and incubation duration. With increasing flow velocities, amounts of EPS was found to increase by about 160%; Correspondingly, carbohydrate, protein and lipid contents of EPS increased by about 120%, 130% and 135%, respectively.

Non-polar groups present in carbohydrate, protein and lipid contents of EPS increase EPS-EPS attachment by increasing EPS hydrophobicity. Protein and lipid content of EPS enhances the affinity of EPS to negatively charged and solid surfaces respectively (Wingender et al., 1999). Since, EPS-EPS and EPS-sand particle attachment increases with increasing flow velocity it is expected that the rate and degree of EPS related sand particle aggregation increases with increasing flow velocity. EPS produced under higher flow velocity is expected to attach to sand particles more efficiently. Attachment mechanism of EPS to sand particle demonstrates the typical microbial behaviour under fluvial activities in the aqueous environment. It further explains the microbe-induced stabilization of sandy soil.

Isotropically consolidated drained triaxial test results obtained in this research indicate improved shear strength of loose soil samples due to EPS-related sand particle aggregation. To proof the concept that the bacteriogenic EPS can be used to reinforce sand particles to enhance the erosional resistance of sand, bacterial treatments were also applied to a model wedge shaped sand specimen. Sand treated in situ with live, minimally sustained bacterial populations considerably less erosion than untreated sand. After the bacterial treatment, amount of eroded sand mass decreased about 5% demonstrates the increased erodibility of bacteria-treated sand. Future work includes the interdependence of environmental parameters, chemical properties of sand and EPS on the attachment mechanisms of EPS on sand surface. Because, for microbe-induced sand particle aggregation to be a feasible erosion reducing technique, this interdependence must be clearly understood.

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