BIOFILM FORMATION AND CALCIUM PRECIPITATION IN *BACILLUS LICHENIFORMIS* AND *ALCALIGENES FAECALIS* FOR SOIL IMPROVEMENT

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ABSTRACT: Calcium precipitation and biofilm formation in halophilic bacteria were investigated in the current study. The strain SMK (*Bacillus licheniformis*) was isolated from plant rhizosphere and and AQ-1 (*Alcaligenes faecalis*) from marble crushing soil in Lahore. Bacterial growth, biofilm formation and $CaCO_3$ precipitation of both strains was determined under varying stress (pH, temperature, agitation and salinity). Soil aggregation was investigated under salt stress (NaCl and $MgSO_4$). Results showed that pH 8.0, temperature 42°C, 1.5 M salt (NaCl and $MgSO_4$) stress without agitation was favorable for biofilm formation. Both strains were able to precipitate $CaCO_3$ in media specially at margins of colonies in AQ-1. Effervescence of crystals with addition of concentrated HCl showed confirmatory results. Stereomicroscopic analysis of aggregates in inoculated soil and sand showed precipitation under salt stress. SMK (*Bacillus licheniformis*) showed high aggregation in soil. From the results, it was concluded that these indigenous strains have potential for sustainable agriculture by improving disturbed soil.

Key words: Calcium precipitation, *Bacillus licheniformis, Alcaligenes faecalis,* biofilm formation and salt stress.

(Received 18-11-2016 Accepted 29-05-2017)

INTRODUCTION

Omnipresent nature of microbes in soil cannot be ignored for the improvement of geotechnical properties of soil (Dejong *et al*., 2014). Microbial induced carbonate precipitation (MICP) involves the use of a biochemical process that results in sustainable soil, improvement by decreasing water permeability and increasing soil strength (Harkes *et al*., 2010). Presence of calcium carbonate in soil plays an important role on soil in maintaining soil pH and nutrient availability (Maulood *et al*., 2012).

To offset the loss caused by soil salinity, the engagement of soil microbes in liming process may be a promising approach to restore the natural balance. This process of soil cementing due to MICP is called bio grouting which has been reported for sand (Sidik *et al*., 2015). Biofilm formation, carbonate precipitation and salt tolerance are the mechanisms which offer unconventional and eco-friendly techniques to improve soil structure and crop yield in saline soil. Taking the increased amounts of CO_2 in the atmosphere as a matter of serious concerns, the biological mitigation approaches must be considered to sequester CO_2 in the form of stable calcium carbonate (Sharma *et al*., 2008). This will not only reduce the level of global warming but other side also affects, such as drought and salinity, etc (Sedjo and Sohngen, 2012). Carbonate precipitation along with biofilm formation in microbes is helpful for long term alleviation and outflow of CO_2 in the environment by facilitating long term CO_2 storage (Vahabi *et al.,* 2013 and Mitchell *et al*., 2009). Carbonate precipitation is applied for CO_2 sequestration, removal of heavy metals and biodegradation and remediation in construction materials and conservation (Silva-Castro *et al*., 2015). The present study was carried out to determine the potential of halophilic microbes from soil to precipitate carbonate and biofilm formation which may be helpful for alleviation of soil salinity.

MATERIALS AND METHODS

Characterization of the bacterial isolates: The SMK (*Bacillus licheniformis*) isolated from plant rhizosphere and AQ-1 (*Alcaligenes faecalis*) isolated from soil near marble crushing site in Lahore were finally screened from ten selected bacterial isolates. For isolation, one gram soil was serially diluted (10^{-5}) and spread on (50 µL) L-agar plates supplemented with 1M sodium chloride and incubated at 37ºC for 24 hours (Gerhardt *et al*., 1994). The colonies were further purified by sub culturing on 6 % sodium chloride containing L-agar plates. Both isolates were primarily characterized following Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Biochemical characterization of the bacterial isolates was done by catalase test, methyl red test, Oxidase test, Indole test, Voges proskauer test, DNAase test, Sudan III test and Motility test using Sulfide Indole Motility agar (Tittsler and Sandholzer, 1936). Furthermore, isolates were identified by 16S rRNA gene sequencing by sending bacterial strains to First BASE Laboratories (Sdn. Bhd. Shah Alam, Selangor, Malaysia). The nucleotide sequences were searched for homology in the GenBank

database at National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) using Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene sequences of strains were submitted to NCBI GenBank and accession numbers of isolate were obtained. From the aligned sequences a phylogenetic tree was constructed using MEGA 6.0 software using neighbor joining method (Saitou and Nei., 1987) with a bootstrap value of 1000.

Biofilm formation: Qualitative analysis of biofilm formation was done by preparing Congo red supplemented brain heart infusion broth (BHI) following previous modification by Hassan *et al*., (2011). Plates were supplemented with different combinations of glucose (2 %) with 1.5 M NaCl or $MgSO_4$. Both strains were streaked on plates and incubated for 24 hours at 37°C. The plates were recorded as black colonies for positive and red colonies for negative results. Optimal condition for bacterial growth and biofilm was determined (in terms of tightly bound cells) following procedure of Qurashi *et al*., (2012). Optimum pH, temperature and salt concentrations for bacterial growth and biofilm formation were determined. For this the inoculum (100 µL) from fresh culture (cell densities adjusted to OD_{600nm} 0.3) of each isolate was added in sterile L-broth (10 mL) with different molar concentrations (0, 0.5, 1, 1.5, 2 and 2.5) of either NaCl or $MgSO_4$ and incubated at non shaking conditions. For determining optimum pH, each salt supplemented media was adjusted at different pH values (pH 6, pH 7, pH 8) at incubation temperature of 37ºC. Similar experiments were performed for different incubation temperatures i.e., 4ºC, 37ºC and 42ºC at pH 7.0 (Fig. 2). To study the effect of shaking on bacterial strains, cultures on respective conditions of pH or temperature were incubated for 120 hours (optimized) under shaking (160 rpm-shaker-Lab tech Daittanlabtech Co. ltd) conditions in glass test tubes. To study the effect of different abiotic surfaces (borosilicate and polystyrene tubes) on biofilm, biofilm was checked at optimized culture conditions. Results of biofilm (tightly bound cells) are reported as a normalized values (OD 570 nm / OD 600 nm) following Qurashi *et al*., (2012).

Characterization and analysis of Calcium carbonates crystals: For analysis of calcium carbonates crystals deposition, cultures (25 mL) were incubated on acetate supplemented agar media (Boquet *et al*., 1973) at 28˚C for 21 days in the incubator in plastic petriplate. Plates were wrapped with parafilm to avoid loss of moisture. After 21 days of incubation, precipitation of $CaCO_3$ crystals on bacterial growth streaks was analysed under stereomicroscope at 40 X magnification. The crystals were separated by digging media with sterile scalper on glass slides and stained using methylene blue and observed under stereomicroscope. The experiment was also repeated in acetate supplemented broth media (Boquet *et al*., 1973), inoculated with fresh culture (24 hours-37˚C) to make final cell densities (0.5 OD 600 nm) of both isolates. Cultures were incubated at 30˚C for 21 days in shaking incubator at 160 rpm at 28℃ temperature. After 21 days of incubation, $CaCO_3$ crystal formation was recorded at the bottom of each test tube. The deposited crystals were collected by filtration using Whatman filter paper. The crystals were washed with water and stained with methylene blue and observed under stereomicroscope at 40 X magnification. The verification for the presence of $CaCO_3$ crystals was done by addition of conc. HCl on $CaCO_3$ crystals obtained from growth streaks and brisk effervescence was recorded for positive results.

Soil and Sand Aggregates: To test the efficacy of bacterial strain to show aggregation in soil and sand under salt stress, sterilized and sieved garden soil and sand samples were kept in glass petriplate (14 gram per plate) each supplemented either with 1 M NaCl or $MgSO_4$ (per gram weight of soil) and inoculated with fresh bacterial culture (OD 0.5 600 nm per gram weight of soil). Plates were covered and samples were incubated at 28°C for 15 days in the incubator. Plates were watered when soil was about to dry. After 15 days samples were weighed for wet weight. For dry weight analysis, aggregates were dried for 24 hours at room temperature and collected by using sieves of mesh size 18 and weighed again. Microscopic analysis of soil aggregates was done by using stereomicroscope at 40 X magnification.

Statistical analysis: Statistical data analysis was performed using IBM SPSS statistics 21 soft ware for one way analysis of variance using tukeys post hoc test between means of three replicates. Standard errors of the means were calculated and the error bars are shown in each figure.

RESULTS AND DISCUSSION

The colony characteristics of isolate SMK showed pale yellow color, irregular margins and gummy surface while cells were non-motile, endospores former and gram positive rods. Positive results for catalase and methyl red tests and negative results for oxidase, indole utilization tests and Voges-Proskauer tests were recorded. The isolate showed good growth on 6 % sodium chloride containing LB-agar plates and positive results for Sudan 3 staining. Isolate AQ-1 showed white colony, round shape, umbonate elevation and mucoidy texture. Cells were gram-negative diplobacilli, capsulated and non-spore forming motile. Positive results were recorded for methyl red test, voges proskauer and catalase test while negative test for DNAase test. The results of 16S rRNA gene sequences (Fig. 1a-b) showed that isolate SMK was

Bacillus licheniformis (accesson number KR052006) while AQ-1 as *Alcaligenes faecalis* (accession number KR052007). Qualitative analysis of biofilm in BHI medium supplemented with congo red and combinations of glucose, $MgSO_4$ and NaCl showed that SMK showed non mucoidy colonies in all combinations while red colonies in the presence of NaCl. Isolate AQ-1 showed red colonies in BHI medium supplemented with either salt. However, addition of glucose resulted in dark-brown colonies in both isolates. Quantitative analysis of biofilm formation of strain AQ-1 was high at 1M $MgSO_4$ and 0.5 M NaCl stress when temperature or pH extremes were not present and strain SMK showed highest biofilm at 1.5 M NaCl stress however, the response of $MgSO_4$ was variable In general, response of AQ-1 for biofilm was higher in $MgSO_4$ supplemented media while SMK showed higher biofilm under NaCl stress (Fig. 2). Biofilm formation was high at extreme conditions of pH and temperature. Results of biofilm formation at non shaking conditions showed high biofilm in borosilicate tubes when compared to polystyrene tubes (Fig. 3). Calcium precipitation was recorded at 21 days of incubation and staining of crystals with methylene blue showed variable morphology and noticeable bubbling on the addition of concentrated hydrochloric acid (HCl). Deposition of crystals was high in strain AQ-1 as compared to SMK. Moreover, the deposition of crystals was concentrated near margins of the colony in AQ-1 on solid medium (Fig. 4).

Efficacy of inoculation to aggregate soil and sand samples under salt stress (supplemented with 1 M NaCl or $MgSO_4$) (Fig. 5) showed that aggregation improved under salt stress compared to non-inoculated control. Response of strain SMK was better as compared to AQ-1. Strain AQ-1 increased aggregation (17 %) in sand while in soil only 5 % aggregation improved under $MgSO_4$ stress. Strain SMK improved (49 %) aggregation in soil and improved (44 %) in sand the presence of NaCl stress as compared to non-inoculated control. However, in $MgSO_4$ treated samples, strain SMK increased 32 % aggregation in sand and 20 % in soil as compared to non-inoculated treatments.

The bacterial strains named AQ-1 and SMK were characterized and finally identified up to genus level. In the previous study, isolates were identified as *B. licheniformis* (Ibrahim *et al*., 2013) and *Alcaligenes faecalis* (Kumar *et al*., 2012). Among the molecular tools used in research, 16S rRNA gene sequencing is the commonly used technique for bacterial identification and is becoming a conventional and superior to traditional approaches in bacterial identification (Stamatoski *et al*., 2016). Results of the initial qualitative estimation of biofilm, showed that SMK showed non mucoidy colonies in all combinations. This change in colony morphologies is associated with stress tolerance and might suggest a tendency of cells to form biofilm and in line with recent study where the presence of sodium chloride and glucose improved the biofilm formation (Zamarren˜o *et al*., 2009; Rode *et al*., 2007; Freeman *et al*., 1989). The cellular adaptations and changes in growth style i.e. from free floating to sessile stage (i.e., biofilm) are direct reflections of the visible differences observed in colony morphology (Sousa *et al*., 2013). Further quantification of biofilm in glass tubes showed that biofilm formation was high at high salinity, glass test tubes, non-shaking conditions and extremes of pH (6.0 or 8.0) and temperatures (4°C and 42°C). Results are in line with previous findings showing biofilm growth depends on many factors (Wang *et al*., 2015). In the previous study, the strain *Alcaligenes faecalis* GPA-1 had been reported to be alkaliphilic, showing good growth at pH extremes 9.0 to10.0, temperature 37°C and salt stress of 3 % (Veetil *et al*., 2012). While studying the effect of various agitation rates and incubation time on protease production in halo tolerant *Bacillus licheniformis* TD4; 250 rpm and 24 hours were found optimum for production of enzymatic activity (Suganthi *et al*., 2013). Effect of temperature and incubation time in stimulating biofilm formation has been studied in bacteria (Nyenje *et al*., 2013). Biofilm formation in marine microbes have been reported to offer several benefits to upkeep biological activities in fluctuating environmental conditions (Dang and Lovell., 2016). Microorganisms in free floating stage have fewer chances of survival under severe conditions (Welch *et al*., 2012). It is important to speculate that these halophilic bacteria with ability to form biofilm and precipitate $CaCO_3$ in media thus playing a significant role in CO_2 sequestration efficiently (Silva-Castro *et al*., 2015). Involvement of exopolysaccharide in making biofilm architecture and calcium carbonate precipitation has been reported previously (Decho, 2010). On the addition of concentrated acid (HCl) in precipitated crystals placed on glass slides, visible bubbling appeared. It has been reported to be efficient in mineral precipitation by many workers (Marvasi *et al*., 2012). It is significant to note that strains used in the present study started to precipitate minerals after 7^{th} day, contrary to previous study while *Bacillus marisflavi* M3 has been reported to precipitate in liquid medium supplemented with organic matter after one month of growth i.e 30th days (Silva-Castro *et al*., 2015). Several factors were considered to be responsible for effective precipitation mechanisms among which fluctuating pH around bacterial cell, cell membrane, cell wall, and exopolysaccharides contributed significantly for this mechanism (Silva-Castro *et al*., 2015). EPS material was loosely or tightly attached to the cell and results in entrapment of calcium ions at a given pH (Bhaskar and Bhosle, 2005). The entrapped calcium thus resulted in the development of $CaCO_3$. The presence of negative charge on micro-organisms helped to scavenge the of divalent cations i.e, Ca^{2+}, Mg^{2+}, at cell surfaces offering unique

precipitation site for $CaCO_3$ crystals (Ercole *et al*., 2007). Biofilm formation and mineral deposition by *S. pasteurii*

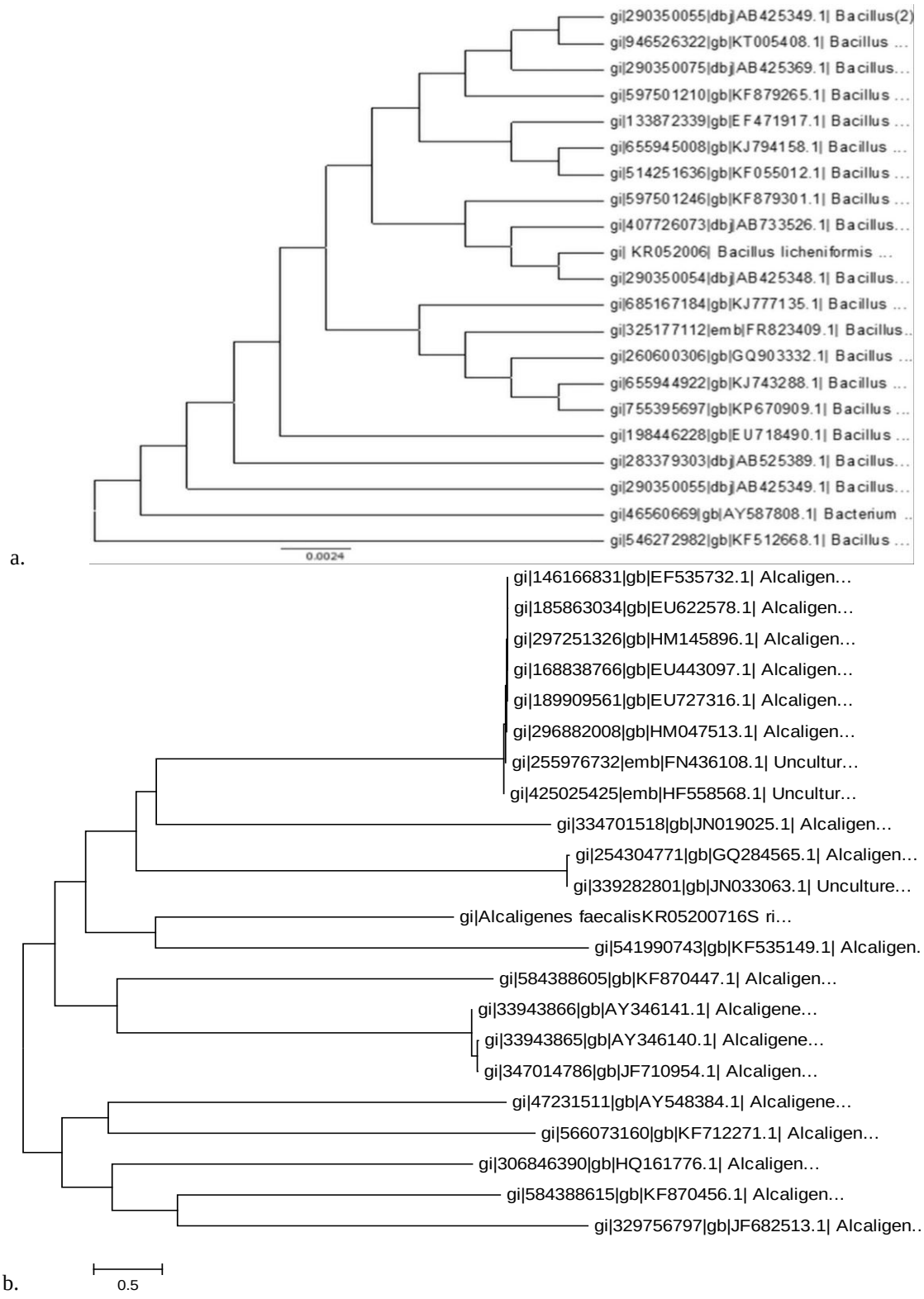

Figure 1 a:. Evolutionary relationship of isolate *Bacillus licheniformis* SMK with related taxa using the Neighbor-Joining method b: Evolutionary relationships of strain AQ-1 *Alcaligenes faecalis* with other taxa using neighbor joining method.

has been reported to show potential to mitigate CO_2 release in sequestration (Mitchell *et al*., 2009). Aggregation was higher in sand as well as in soil. The microbial metabolites act together with the soil elements and could influence the soil properties (Gat *et al*., 2014). Use of microbial activities can be utilized for modification of soil properties for sustainable agriculture (DeJong *et al*., 2010). Increased aggregation was direct reflection of the fact that calcification resulted in reduction of pore space during consolidation (Zamarren˜o *et al*., 2009). Calcium carbonate precipitation in sand was found to be higher than the organic soil that might be attributed due to the organic ligands and complex porosity (Sidik *et al*., 2015). Increased $CaCO_3$ precipitation by microbes might helped the soil to aggregate and offer suitable treatment for improving soil structure and porosity. Improvement in soil structure due to $CaCO_3$ precipitation has already been reported (Whiffin *et al*., 2007). Though significant, microbial $CaCO_3$ precipitation process was influenced by several factors yet to be explored at molecular level. In this context, further research work is in progress. The findings of the present study will be helpful in mitigating CO_2 in the atmosphere and global warming.

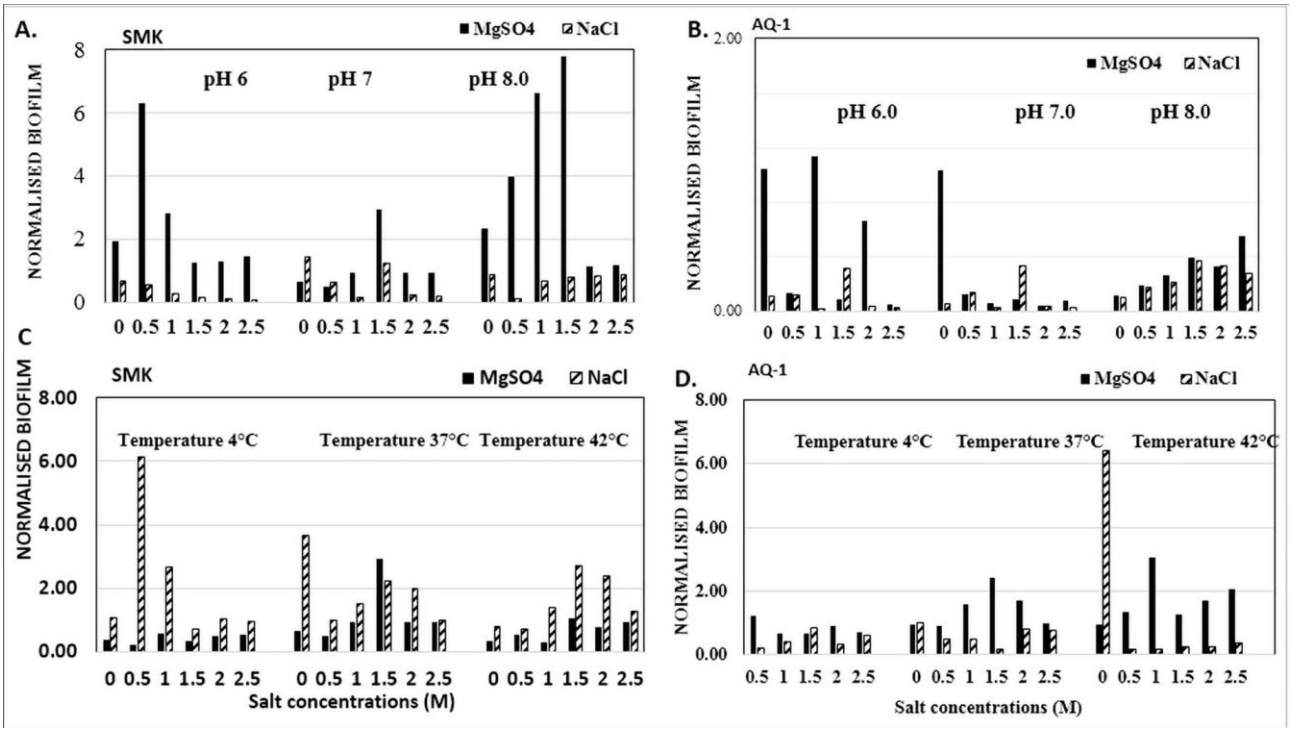

Figure 2. Effect of different pHs, temperatures and salt concentrations on bacterial biofilm formation at non shaking conditions in glass tubes

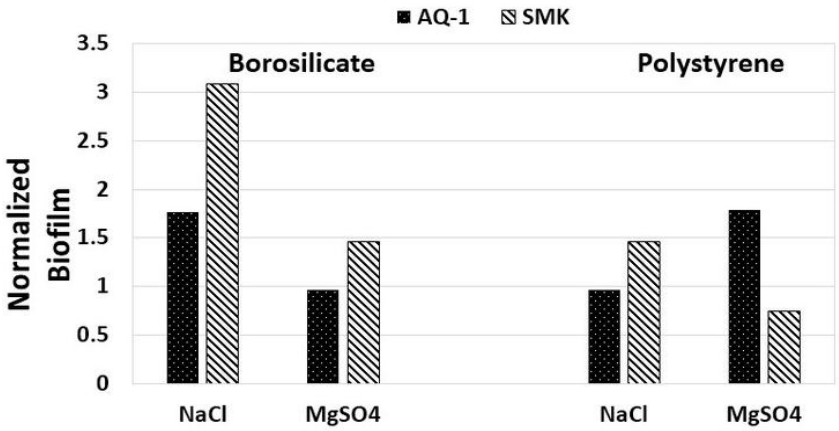

Figure 3. Effect of optimized culture conditions on bacterial biofilm formation (normalized values) at non shaking conditions using borosilicate and polystyrene tubes

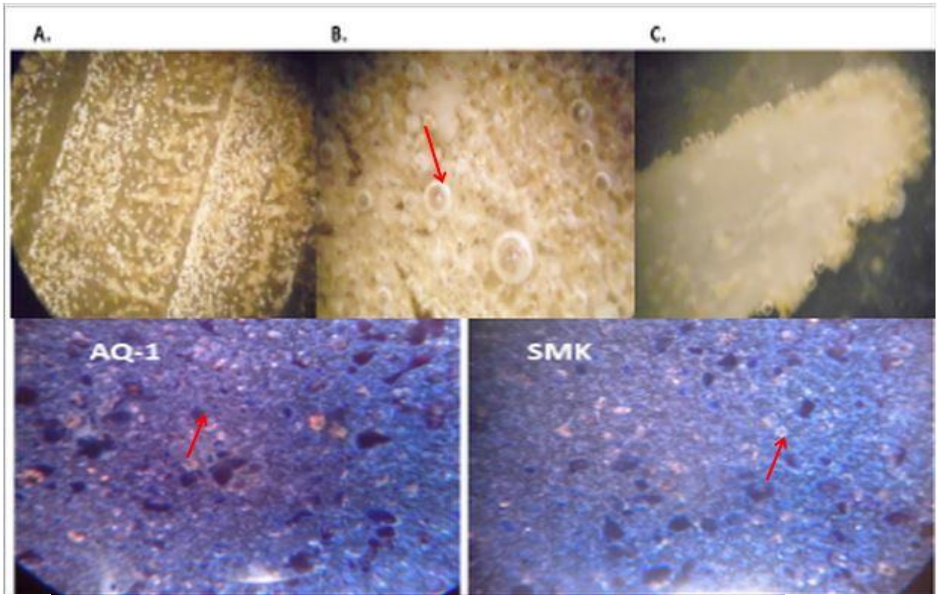

Figure 4. Deposited crystals of Carbonate in cultured colonies grown on agar plates. Staining of deposited crystals of AQ-1 and SMK with methylene blue.

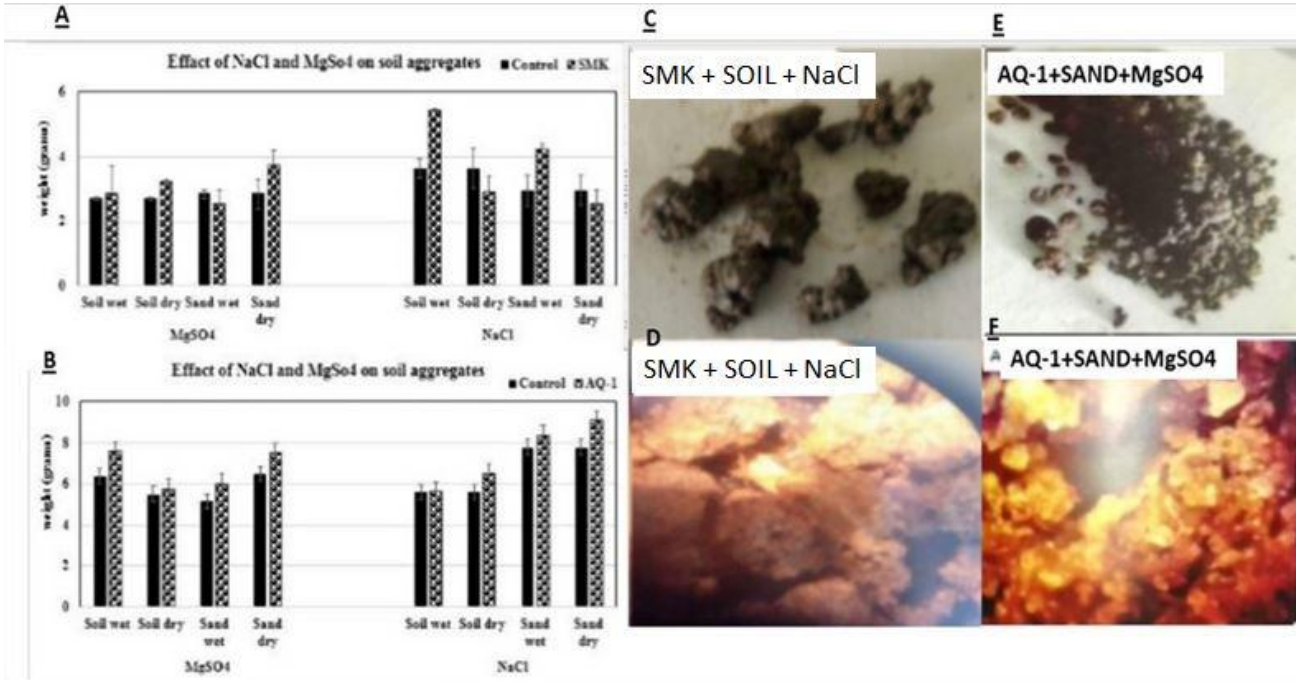

Figure 5. Effect of inoculation on soil, (A-B) Aggregate formation in sand and soil under NaCl and $MgSO_4$ stress, (C and E) aggregates formation in petri plates under NaCl and $MgSO_4$ stress, (D-F) Stereomicroscopic view of aggregates developed in soil and sand.

REFERENCES

Bhaskar P.V., N.B. Bhosle (2005). Microbial extracellular polymeric substances in marine biogeochemical processes. Curr. Sci. 88: 45–53.

Boquet E, A. Boronat, A. Ramos-Cormenzana (1973). Production of calcite (calcium carbonate) crystals by soil bacteria is a General Phenomenon. Nature. 246(5434): 527-529.

Dang, H. and C.R. Lovell (2016). Microbial surface colonization and biofilm development in marine environments. Microbiol. Mol. Biol. Rev. 80(1): 91-138.

Decho, A.W. (2010). Overview of biopolymer-induced

mineralization: What goes on in biofilms? Ecol. Eng. 36(2): 137-144.

Dejong, J., B. Martinez, T. Ginn, C. Hunt, D. Major and B. Tanyu (2014). Development of a scaled repeated five-spot treatment model for examining microbial induced calcite precipitation feasibility in field applications. Geotech. Test J. 37(3): 424–435

Dejong, J.T., B.M. Mortensen, B.C. Martinez, D.C. Nelson (2010). Bio-mediated soil improvement. Ecol. Eng. 36(2): 197-210.

Ercole, C, P. Cacchio, A.L. Botta, V. Centi and A. Lepidi. (2007). Bacterially induced mineralization of calcium carbonate: the role of exopolysaccharides and capsular polysaccharides. Microsc. Microanal. 13(01): 42-50.

Freeman, D.J., F. R. Falkiner and C. T. Keane (1989). New method for detectingslime production by coagulase negative staphylococci. J. Clin. Pathol. 42(8): 872-874.

Gat, D., M. Tsesarsky., D. Shamir and Z. Ronen (2014). Accelerated microbial-induced CaCO3 precipitation in a defined coculture of ureolytic and non-ureolytic bacteria. Biogeosci. 11(10): 2561-2569.

Gerhardt, G.G., R.G.E. Murray, W.A Wood and N.R Kreig (1994). Methods for general and molecular bacteriology, ASM, 1325. Massachusetts Ave. N. W. Washington, D. C.

Harkes, M.P., L.A. Paassen, J.L. Booster, V.S. Whiffin and M.C. Loosdrecht (2010). Fixation and distribution of bacterial activity in sand to induce carbonate precipitation for ground reinforcement. Ecol. Eng. 36(2): 112-117.

Hassan, A., J. Usman, F. Kaleem., M. Omair, A. Khalid and M. Iqbal (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz. J. Infect. Dis. 15(4): 305-311.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J. T. Staley, S.T. Williams. (1994). Bergey's manual of determinative bacteriology. Lippincott Williams and Wilkins, Baltimore, Md, USA, 9th edition,

Ibrahim, D., H.L. Zhu, N. Yusof, Isnaeni and L.S. Hong (2013). *Bacillus licheniformis* BT5.9 isolated from changar hot spring, malang, indonesia, as a potential producer of thermostable α-amylase. Trop. Life. Sci. Res. 24(1) : 71–84.

Kumar, A., T. Veetil, J. James, S. Subash, D. Joy, L.M.S. Dev and V. Thankamani (2012). Characterization of Alcaligenes faecalis GPA-1 producing thermostable extracellular α-amylase Res. J. Biotechnol. 3(4): 19-27. ISSN: 2229-791X

Marvasi, M., K.L.Gallagher, L.C. Martinez, W.C. Pagan, R.E. Santiago, G.C. Vega and P.T. Visscher, (2012). Importance of B4 Medium in determining organomineralization potential of bacterial environmental isolates. Geomicrobiol. J. 29(10): 916-924.

Maulood, P.M., A.O. Esmail, M.S. Dohuki and D.A. Darwesh (2012). Comparison between calcimetric and titrimetric methods for calcium carbonate determination. OJSS. 02(03): 263-268.

Mitchell, A.C., A.J. Phillips, R. Hiebert, R. Gerlach, L.H Spangler and A.B. Cunningham (2009). Biofilm enhanced geologic sequestration of supercritical CO2. Int. J. GreenH. Gas. Con. 3(1): 90-99.

Nyenje, M., E. Green. and R.N dip (2013). Evaluation of the effect of different growth media and temperature on the suitability of biofilm formation by *enterobacter cloacae* strains isolated from food samples in South Africa. Molecules. 18(8): 9582-9593.

Qurashi, A.W. and A.N. Sabri (2012). Bacterial exopolysaccharide and biofilm formation stimulate chickpea growth and soil aggregation under salt stress. Braz. J. Microbiol. 43(3): 1183-1191.

Rode, T.M., S. Langsrud, A. Holck and T. Møretrø (2007). Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. Int. J. Food Microbiol. 116(3): 372-383.

Saitou, N. and M. Nei. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sedjo R and B. Sohngen (2012). Carbon sequestration in forests and soils. Annu. Rev. Resour. Econ. 4(1):127-144.

Sharma, A., A. Bhattacharya, R. Pujari and A. Shrivastava (2008). Characterization of carbonic anhydrase from diversified genus for biomimetic carbon-dioxide sequestration. Indian. J. Microbiol. 48(3): 365–371.

Sidik, W., H. Canakci. and H.J. Kilic (2015). An investigation of bacterial calcium carbonate precipitation in organic soil for geotechnical applications. IJSTC. 39(C1): 201-205.

Silva-Castro, G.A., I. Uad, A. Gonzalez-Martinez, A. Rivadeneyra, J. Gonzalez-Lopez and M.A. Rivadeneyra (2015). Bioprecipitation of calcium carbonate crystals by bacteria isolated from saline environments grown in culture media amended with seawater and real brine. BioMed. Res. Int. 1-12.

Sousa, A.M., I.Machado., A. Nicolau and M.O. Pereira, (2013). Improvements on colony morphology identification towards bacterial profiling. J. Microbiol. Meth. 95(3): 327-335.

Stamatoski, B., M. Ilievska, H. Babunovska, N. Sekulovski and S. Panov, (2016). Optimized genotyping metho for identification of bacterial contaminants in pharmaceutical industry. Acta. Pharm. 66(2): doi:10.1515/acph-2016-0011.

Suganthi, C., A. Mageswari, S. Karthikeyan, M. Anbalagan, A. Sivakumar and K.M. Gothandam (2013). Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments Gothandam. J. Genet. Eng. Biotechnol. 11: 47–52.

Tittsler, R.P and L.A. Sandholzer (1936). The use of semi-solid agar for the detection of bacteria motility. J. Bact. 31: 575.

Vahabi, A., A.A. Ramezanianpour, H. Sharafi, H. S. Zahiri, H. Vali and K.A. Noghabi (2013). Calcium carbonate precipitation by strain *Bacillus licheniformis* AK01, newly isolated from loamy soil: A promising alternative for sealing cement-based materials. J. Basic Microbiol. 55(1): 105-111.

Veetil, A.K.T., J. James, S. Subash, D. Joy, L.M.S Dev and V. Thankamani (2012). Characterization of *Alcaligenes faecalis* GPA-1 producing thermostable extracellular α-amylase. Res. Biotechnol. 3(4): 19-27.

Wang, X., G. Wang and M. Hao (2015). Modeling of the *Bacillus subtilis* bacterial biofilm growing on an agar substrate. Comput. Math Methods. Med. 1-10.

Welch, K., Y. Cai and M. Strømme (2012). A method for quantitative determination of biofilm viability. J. Funct. Biomater. 3(4): 418-431.

Whiffin, V.S., L.A. Paassen and M.P. Harkes (2007). Microbial carbonate precipitation as a Soil Improvement Technique. Geomicrobiol. J. 24(5): 417-423.

Zamarren, D.V., R. Inkpen, and E. May (2009). Carbonate crystals precipitated by freshwater bacteria and their use as a limestone consolidant. Appl. Environ. Microbiol. 75(18): 5981–5990.