Consolidation of Sand Particles by Aggregates of Calcite Nanoparticles Synthesized by Ureolytic Bacteria under Non-Sterile Conditions

Salwa M. Al-Thawadi*

Department of Biology, University of Bahrain, Sakhir, Kingdom of Bahrain

*salthawadi@uob.edu.bh

***Abstract*- BioCementation technology depends on the consolidation of sand particles by using pure microorganisms (*B. pasteurii*) under complete sterilization conditions during the cellular growth. In this study, an enrichment culture of ureolytic bacteria was used to precipitate aggregates of calcite in-situ under non-sterile conditions. Those bacteria were enriched in the presence of 3 M urea. Soil sample was collected from agricultural fields in Sakhir, Bahrain. Then they were used to precipitate calcite in-situ and consolidate sand particles in the presence of high concentration of 1 M equimolar urea and calcium ions. Continuous feeding of cells was applied. Optical microscope, SEM, EDS, XRD and XRF examinations revealed that this impure bacterial culture resulted in precipitating aggregates of calcite nanoparticles in spherical arrangement in-situ which consolidate packed sand column. Natural competence between bacteria in the enrichment culture did not affect their ability to precipitate calcite nanoparticles and strengthen the sand without interfering with the hydraulics of the treated sand. Therefore, cost of BioCementation technology can be lowered due to the enrichment of local bacteria under non-sterile conditions.**

Keywords- Biocementation; Calcite Nanoparticles; Ureolytic Bacteria; Urease; Sandstones

I. INTRODUCTION

BioCementation or BioGrout is a technology by which microorganisms control the precipitation of calcium carbonate between the sand particles producing high strength. It could be used for strengthening valuable regions (biodunes), against continuous progressive erosion of the coastline and for instabilising underwater slopes (slope liquifaction) by creating cemented zone that is no longer be able to liquefy [1].

Sporosarcina pasteurii is widely used as a source of urease enzyme for BioCementation reaction (Equ. 1). It produces intracellular urease which is close to 1% of the cell dry weight [2]. A study done on clogging sand column by using *S. pasteurii* showed inconsistency in urease production by the desired microbe [2]. *Bacillus sp.* MCP11 (DSM 23526) is a new strain of urease positive bacteria offers specific advantages over the existing strain (*S. pasteurii*) [3]. Those advantages were: higher urease specific activity, higher stability (robust), higher tolerance to high concentration of ammonium, and more consistent in urease production.

$$CO(NH_2)_2 + 2H_2O + Ca^{2+} \xrightarrow{urease} 2NH_4^+ + CaCO_3 \quad (1)$$

The production of urease active bacteria is one of the main costs for applying BioCementation technology [4], therefore cost-efficient process for urease production is needed. The isolation of pure ureolytic bacteria for BioCementation will add to the cost of this technology. Therefore, this study aims at designing a method to selectively enrich highly urease positive bacteria from local environment to be used to cement sand particles. This use of enrichment culture in consolidating the sand particles will develop an industrially suitable cost-effective bacterial process for BioCementation technology especially if the whole process was performed under non-sterilized conditions. Furthermore, the shape and type of calcium carbonate crystals will be examined by Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDS), X-ray Diffraction (XRD) and X-ray Fluorescence Spectroscopy (XRF).

II. MATERIALS AND METHODS

A. Enrichment of Ureolytic Bacteria under Non-Sterile Conditions

Potential microbial calcite precipitating bacteria with high urease activity (33 mM urea hydrolysed.min^{-1}, measured by conductivity), specific activity (11 mM urea hydrolysed.min^{-1}.OD^{-1}, the amount of urease activity per unit biomass at OD600 nm) were enriched from agricultural fields in Sakhir, Kingdom of Bahrain under non-sterile conditions. One gram of soil was placed in 50 ml of growth medium (250 ml shaking flasks, at 30^{o}C, for 36 h). Urease activity and specific urease activity measurement was examined as in [3]. The enrichment medium consisted of 20 g.L^{-1} Yeast extract (YE), 5 M urea, 152 mM ammonium sulphate and 100 mM sodium acetate.

B. Microscopic Examination of Calcium Carbonate Precipitation

BioCementation experiments on microscopic slides were carried out for the enrichment culture. The preparation of microscopic samples was done as in Al-Thawadi & Cord-Ruwisch [3]. Calcium carbonate crystal formation was examined (immediately and after 24 hours) by a compound microscope (BX51) fitted with a digital Camera.

C. Sand Compaction in a 10 cm Packed Sand-Column

SiO_2 sand (125-500 μm) was dry packed in a 60 ml plastic syringe (10 cm long, diameter of 3 cm). Three replicates were prepared. The packed sand-columns were tapped for about 3 min with a rubber hammer to give an even bulk density. They were then up-flushed with 3-void volumes of deionized water with flow rate of 103 $ml.h^{-1}$ and the stoppers were inserted to maintain a confining pressure.

D. Experimental Running

The packed sand-columns were up-flushed with bacterial enrichment culture, urease activity of 33 mM urea $hydrolysed.min^{-1}$ and a specific urease activity of 11 mM urea $hydrolysed.min^{-1}.OD^{-1}$. Four-void volumes of the bacterial culture were up-flushed at a flow rate of 103 $ml.h^{-1}$. Cementation solution (1 M, equivalent concentration of 1M calcium/urea solution) was up-flushed into the columns continuously. Three sand-columns were prepared for consolidation experiment. Then, the columns were kept for 24 hours at room temperature for the reaction to complete and sent to unconfined strength (UCS) measurement.

E. SEM, EDS, XRD and XRF Examinations

Subsequent to UCS measurement for the consolidated sample, the samples were subjected to SEM examination (SEM-Zeiss Evols 10). Sand particles of one of the consolidated sample were placed on aluminium stubs using "Carbon Tabs". The stubs were then placed in a dust proof container and allowed to dry completely at room temperature, overnight before being coated with a 20 nm layer of Gold in a Balzers Union Ltd. "sputter coater". EDS Microanalysis of $CaCO_3$ crystals was carried out using Bruker AXS Quantax and Esprit 1.8 software. SEM samples for X-ray analysis (EDS) were not coated with gold but otherwise treated the same way.

XRD analysis was conducted using a Bruker D8 x-ray diffractometer. A diffraction pattern was obtained using monochromated copper K-alpha radiation and slits of 6 mm at the x-ray tube. A VÅNTEC-1 area detector was used for data collection. The spectrum obtained was collected in the 15–90° 2θ range. XRD analysis was performed in Materials Research Laboratories (MRL) in USA.

XRF is an effective method for analyzing the main components as well as low-level (nominally 10 ppm) contaminants in relatively thick (several micron) layers. The sample was analyzed using a Bruker S2 X-Ray Fluorescence instrument with an energy dispersive x-ray detector and operated under helium. XRF analysis was performed in Materials Research Laboratories (MRL) in USA.

III. RESULTS AND DISCUSSION

To determine the possibility of obtaining strength from using enrichment culture (mixture of bacteria) and type of $CaCO_3$ crystal formations; packed sand-columns were up-flushed with enrichment culture, followed by up-flushing of cementation solution (calcium/urea). Then, the developed strength was measured by UCS and the crystals were examined by SEM, EDS, XRD and XRF.

A. Enrichment of Ureolytic Bacteria

A method to specifically enrich bacteria from most soil within a short cultivation period (24-36 h), ideally suitable for BioCementation process was developed. Selection conditions (High pH, presence of urea up to 5 M) have enriched for bacteria which were superior to the existing strain of *S. pasteurii*. Those bacteria which can degrade urea, are highly tolerant to urea and ammonia at pH 9.0 and hence ideally suited the BioCementation process.

The high level of urease activity (33 mM urea $hydrolysed.min^{-1}$) which was observed in some of the enrichment trials combined with the fact that urease activity was the key factor for successful BioCementation, demonstrated that the enrichment culture can be used for BioCementation process under high concentration of calcium/urea without the need of purification and sterilisation.

BioCementation technology is expensive mostly due to the expense of urease, medium, sterilization and others (Tables I and II). The average cost of producing 100 L bacterial culture which is needed for large scale BioCmentation process was $3166 (Table I). The cost of importing ureolytic strain will be paid only once unless the strain lost its activity. In the case of using local isolates, purification and identification of bacterial cells are time consuming, laborious and costly processes. Those processes cost an average of $876. Biocementation without importing the ureolytic bacteria (use enrichment) will reduce the cost by 11.2%, while sterility, purification and identification of the unknown bacteria will reduce the cost by 18.3%. Therefore,

BioCementation Technology was successfully reduced by 30% due to cementing the sand particles by enrichment culture through non-sterile conditions (avoiding purifying and identifying the ureolytic isolates). Therefore, using enrichment cultures in the BioCementation process could be more practical and cost effective process. The majority of BioCementation cost is due to the use of yeast extract (Table I), so a further reduction could be reached by using another cheap growth medium.

TABLE I COST OF BACTERIAL PRODUCTION (100 L) FOR BIOCEMENTAION TECHNOLOGY

Growth medium/strain	Weight required (Kg)	Price (USD)	Note
Yeast extract*	2	$1927.2	
Ammonium sulphate*	1	$536	
Sodium acetate*	0.82	$292.7	
Sterilization	—	$ 46-66	(5)**
Bacillus pasteurii ***	—	$ 354 (ATCC 11859)	For profit- $ 354 Non-profit $ 295
Total cost		$3156-3176	
Average cost		$3166	

*The supplier is Sigma-Aldrich, **A reference, *** The supplier is TTCA

TABLE II COST OF PURIFICATION OF BACTERIA FROM THE ENRICHMENT CULTURE INCLUDING PREPARATION OF GROWTH MEDIUM (200 ML)

Growth medium/others	Weight required (Kg)	Price (USD)	Note
Yeast extract*	4.0 g	$ 3.9	
Ammonium* sulphate	2.0 g	$ 1.1	
Sodium acetate*	1.7 g	$ 0.6	
Bacterial Agar*	3.0 g	$ 4.4	
sterilization	_	$ 0.092-0.13	
Petri dishes	_	$ 9.4	Amazon.com
Labour cost	_	$ 112.6	6hX18.76 USD (Bureu of labour statistics, 2010)
Identification	_	$ 390.6	fungal biodiversity centre, UK
Total cost	_	$ 522-522.73	
Average cost	_	$522.4	

B. Calcium Carbonate Crystals Precipitation Examined by Light Microscope

Spheres and rhombohedral crystals of spherical arrangement were successfully precipitated by the use of the enrichment culture (Fig. 1), similar to those produced by a pure culture of MCP11 DSM 23526 [3, 6]. It was particularly interesting to visualise the initial crystal formation which is assumed to be caused by the oversaturation of $CaCO_3$ in the presence of adequate nucleation sites. Within minutes from the initiation of the BioCementation process, spherical $CaCO_3$ deposits started to precipitate (Fig. 1).

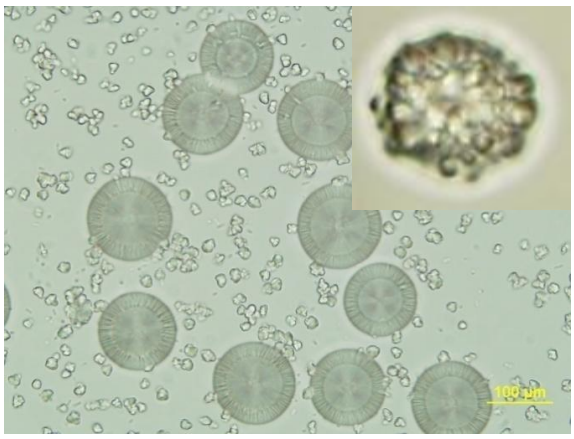

Fig. 1 Light microscopic image of calcium carbonate precipitation by impure ureolytic bacteria (Bar 100 µm)
Inset: Calcium carbonate precipitation in spherical form after maturation

A. Calcium Carbonate Crystals Examined by SEM, EDS, XRD and XRF

The cemented samples were examined by SEM after 24 hours from the initiation of the cementation reaction. Rhombohedral crystals in spherical arrangements were observed in (Fig. 2). Those crystals were in aggregates of more than 20 μm. Energy Dispersive X-ray (EDS) revealed that the crystals were composed of $CaCO_3$ (Fig. 3).

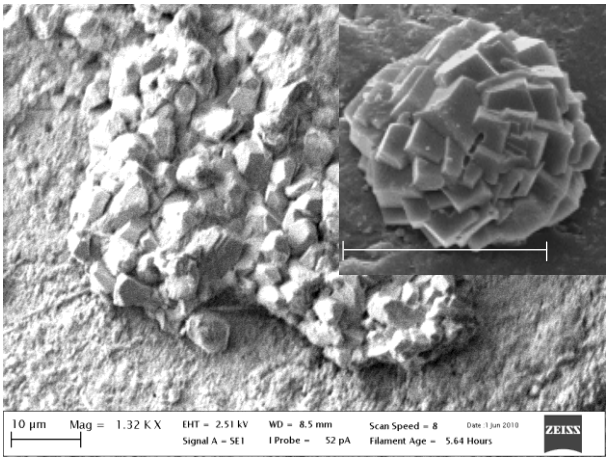

Fig. 2 SEM of calcite nanoparticles precipitation on sand particle (non-coated) sample
Inset: SEM of calcite nanoparticles precipitation on sand particle coated with gold (B, Bar: 20 μm)

Calcite, aragonite and vaterite are three crystal forms of $CaCO_3$ nucleation (same chemical formula, different structure). Calcite was proven to be precipitated between sand particles in the cemented samples by XRD analysis (Fig. 4). The samples were a match for Quartz (SiO_2), Calcite ($CaCO_3$), Graphite (C) Gypsum ($CaSO_4.2H_2O$) and Potassium Calcium Sulfate ($K_6Ca(SO_4)_4$).

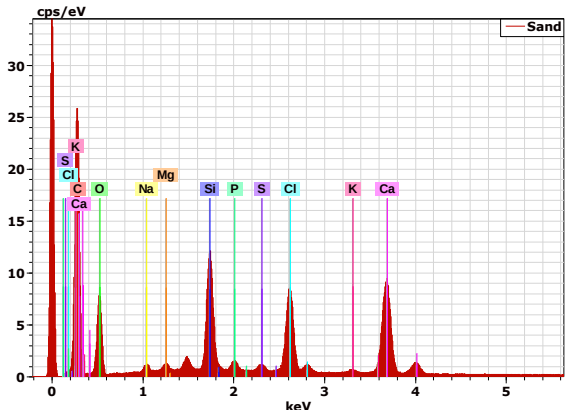

Fig. 3 EDS analysis of the cemented sand particles proving the presence of calcium carbonate crystals and other impurities such as Cl, K, S, Na, Mg, P

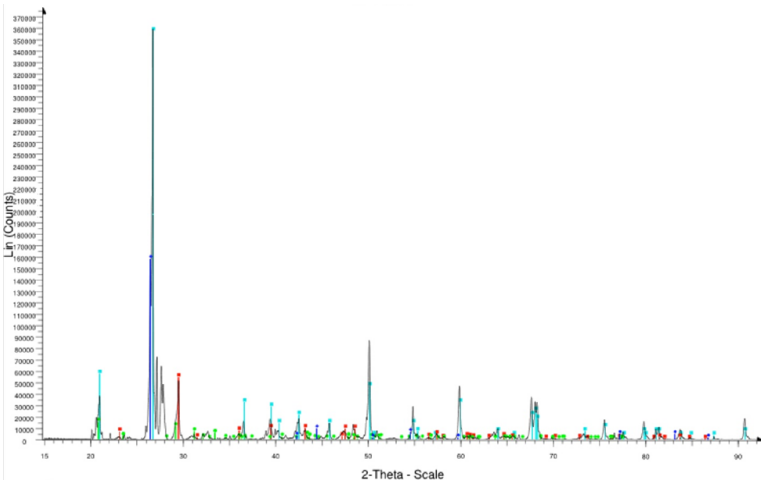

Fig. 4 XRD analysis of the cemented sand particles by enriched bacteria
Quartz (SiO_2), Calcite ($CaCO_3$), Graphite (C) Gypsum ($CaSO_4.2H_2O$)

XRD alone is not enough to determine the exact elemental analysis of the precipitated crystals; therefore XRF was needed. This analysis has confirmed the presence of calcite (high XRF counts) and a number of other complexes (low XRF counts) associated with the calcite.

According to XRF, the presence of high concentration of CaO (33.77%) among the sand particles (SiO_2) (46.8%), proves that the sand columns were cemented by the precipitation of calcite but not vaterite or aragonite (Table III). The presence of some oxides such as MgO, AL_2O_3, TiO_2, MnO, Fe_2O_3 SrO ... etc, were also determined in the BioCemented sand by XRF analysis (Table III).

TABLE III OXIDE CONCENTRATIONS (%) WITHIN XRF ANALYZED CEMENTED SAND PARTICLES

Oxides	Concentration (%)	Oxides	Concentration (%)
MgO	0.330	Cr_2O_3	0.130
AL_2O_3	1.900	MnO	0.020
SiO_2	46.840	Fe_2O_3	1.620
P_2O_5	1.160	ZnO	0.030
SO_3	3.170	Rb_2O	0.010
Cl	8.310	SrO	0.140
K_2O	2.010	ZrO_2	0.030
CaO	33.770	SnO_2	0.040
TiO_2	0.480		

As a general phenomenon, ureolytic bacteria in the presence of high concentration of calcium and urea produce two types of $CaCO_3$ precipitations; spherical deposits and rhombohedral crystals. Both spherical deposits and rhombohedral crystals were observed in a previous study [3]. Rhombohedral calcite crystals due to ureolytic bacterial activity in the absence of the spherical deposits were observed in other studies [7-9]. This alkaline localized area close to the cell surface is suggested to be due to active movement of calcium ions through Ca^{2+}/2H Pump [10]. The chemical precipitation of rhombohedral crystals was described by Warren and his colleagues [11].

Rhombohedral calcite crystals nucleate through self-assembly process which requires the combination of calcium cation and carbonate anion [12]. The actual mechanism of the formation of those rhombohedral crystals from the spheres remains still unclear. However; in the literature similar observations of agglomerates of rhombohedral calcite was found at pH of 8.5 [13] and super-saturation index of 30-40 [14].

The impure culture successfully strengthens the sand without the need of isolating a pure strain to cement the sand. Furthermore, BioCementation was successfully achieved with low sterility conditions. This success in using the enrichment culture in BioCementation reaction is very important in applying this technology in large scale trials, as the bacterial cells can be grown on site without the need of importing cells from other places.

A soft rock (1.0- 1.2 MPa) was produced by concentrating the cells in-situ through up-loading of impure cells followed by continuous uploading of cementation solution (calcium/urea). Interestingly, the obtained strength was attributed to the point-to-point contact of nanoparticles of calcite crystals in the form of aggregates, which formed bridges between the adjacent particles. The production of strength due to this type of contact was confirmed by recent studies [3, 4].

IV. CONCLUSIONS

The cost of BioCementation technology was reduced by using enrichment culture to cement the sand particles. Further reduction was achieved by performing the BioCementation process under non-sterile conditions.

ACKNOWLEDGMENT

The author would like to acknowledge the financial support given by University of Bahrain for this work.

REFERENCES

[1] M. Van der Ruyt, M., and W. van der Zon, "Biological in situ reinforcement of sand in near-shore areas," Proceedings of the Institution of Civil Engineers Geotechnical Engineering, vol. 162, pp. 81–83, 2009. doi: 10.1680/geng.2009.162.1.81 Paper GE-D-07-00033.

[2] V.S. Whiffin, "Microbial $CaCO_3$ precipitation for the production of biocement," Ph.D dissertation. Murdoch University, Western Australia. (2004).

[3] S. Al-Thawadi, and R. Cord-Ruwisch, "Calcium carbonate crystals formation by ureolytic bacteria isolated from australian soil and sludge," *Journal of Advance Science and Engineering Research*, vol. 2, pp. 13-26, 2012a.

[4] M. A. Van; G. A. van den Ham, M. Blauw, M. Latil, N. Benahmed, and P. Philippe, "Preventing internal erosion phenomena with the BioGrout process," in *Proc.* the 15th European Conference on Soil Mechanics and Geotechnical Engineering - Geotechnics of Hard Soils – Weak Rocks, 2011, DOI: 10.3233/978-1-60750-801-4-1079.

[5] Acsion-Industries 2002. Sterilisation. Comparison between various sterilisation methods. Viewed 26 August 2003 [Online] http://www.acsion.com/steriliz.htm. In V.S. Whiffin, "Microbial $CaCO_3$ precipitation for the production of biocement," Ph.D dissertation. Murdoch University, Western Australia. (2004).

[6] S. M. Al-Thawadi, R. Cord-Ruwisch, and M. Bououdina, "Consolidation of sand particles by nanoparticles of calcite after concentrating ureolytic bacteria in situ," *International Journal of Green Nanotechnology*, vol. 4, pp. 1–9, 2012b. DOI: 10.1080/19430892.2012.654741.

[7] S. S. Bang, J. K. Galinat, and V. Ramakrishnan, "Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*," *Enzyme & Microbial Technology*, vol. 28, pp. 404–409, 2001.

[8] S. S. Bang, and V. Ramakrishman, Microbial application in strengthening of sandy sub-bases and in remediation of concrete cracks, proposal submitted to National Research Council, USA, 2002.

[9] A. Mitchell, and F. Ferris, "The Influence of *Bacillus pasteurii* on the Nucleation and growth of calcium Carbonate," *Geomicrobiology Journal*, vol. 23, pp. 213–226, 2006.

[10] F. Hammes, and W. Verstraete, "Key roles of pH and calcium metabolism in microbial carbonate precipitation," *Reviews in Environmental Science and Biotechnology*, vol. 1, pp. 3–7, 2002.

[11] L. A. Warren, P. A. Maurice, N. Parmar, and F. G. Ferris, "Microbially mediated calcium carbonate precipitation: Implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants," *Geomicrobiology Journal*, vol. 18, pp. 93-115, 2001.

[12] Y. Guo, L. Yang, X. Yang, X. Zhang, S. Zhu, and K. Jiang, "Effect of self-assembly of sodium acrylate on the crystallization of calcium carbonate," *Macromolecular Bioscience*, vol. 3, pp. 163–168, 2003.

[13] C. Y. Tai, and F. B. Chen, "Polymorphism of $CaCO_3$ precipitated in a constant-composition environment," *AIChE Journal*, vol. 44, pp. 1790–1798, 1998.

[14] M. Dittrich, B. Müller, D. Mavrocordatos, and B. Wehrli, "Induced calcite precipitation by *Cyanobacterium Synechococcus*," *Acta Hydrochimica et Hydrobiologica*, vol. 31, pp. 162–169, 2003.