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Micromorphological and Biocalcification Effects of *Sporosarcina pasteurii* and *Sporosarcina ureae* in Sandy Soil Columns

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ABSTRACT

Microbial calcium carbonate, by bridging sand particles, can play an important role in sand dune stability. A study was carried out on the cementation of sand grains and infilling of pore spaces by $CaCO_3$. Two bacterial species (*Sporosarcina pasteurii* and *Sporosarcina ureae*), three reactant concentrations (0.5, 1 and 1.5M), and six reaction times (12, 24, 48, 96, 192 and 288 hours) were tested in factorial experiment. Bacterial inocula and reactant solutions were added daily to sandy soil columns (6.5 cm height and inner diameter of 7.7 cm), while precipitation of $CaCO_3$ being investigated within 0-1.5, 1.5-3, 3-4.5 and 4.5-6 cm intervals. Chemical and micromorphological analyses revealed that $CaCO_3$ formation, inorganic C sequestration, and depth of cementation were more profound for *S. pasteurii* as compared with *S. ureae*. Both microbial $CaCO_3$ precipitation and inorganic C sequestration increased with increase in reaction time from 12 to 288 hours. Increase in reactant concentration also caused an increase in $CaCO_3$ precipitation (by 12%). Micromorphological observations showed a high degree of calcite crystals' bridging, coating on sand particles and as well infilling of pore spaces. *S. pasteurii* is thus recommended for being used in stabilization of sand dunes; due to its significant effects on $CaCO_3$ deposition and as well on sand grain cementation.

Keywords: Biomineralization, Inorganic C sequestration, Sand dune fixation, Ureolytic bacteria.

INTRODUCTION

Biocalcification is the process of calcite formation resulting from metabolic activities of autotrophic and heterotrophic organisms. Using metabolic pathway of ureolytic hydrolysis through nitrogen cycle, some of the heterotrophic bacteria are of the potential of providing high concentrations of $CaCO_3$ in short periods of time (Castanier *et al*., 1999; De Muynck *et al*., 2010). *Bacillus*, *Sporosarcina*, *Sporolactobacillus*, *Clostridium* and *Desulfotomaculum* genera are among the most urease producing bacteria in soils (Ivanov and Chu, 2008). One mole of urea is hydrolyzed intracellularly into 2 moles of ammonia and 1 mole of CO_2 [Equations (1) and (2)] through urease enzyme activity (Hammes *et al*., 2003). Soil pH increases as a result of this enzymatic reaction, and $CaCO_3$ [Equations (3) and (4)] will be precipitated from CO_3^{2-} and Ca^{2+} ions (Siddique and Chahal, 2011). Meanwhile, produced CO_2 is trapped in $CaCO_3$ preventing CO_2 emission into atmosphere.

$$CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \rightarrow NH_3 + CO_2 + H_2O \quad (2)$$

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$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$ (3)

$CO_3^{2-} + Ca^{2+} \rightarrow CaCO_3$ (4)

Landi *et al*. (2003) demonstrated that the soils of the prairies and of the forests have sequestered biogenic C from plant respiration and residues in the form of pedogenic carbonates and Ca is the rate-limiting factor to be precipitated with CO_2. Microbial activity helps inorganic sequestration of C in $CaCO_3$ form. Geological sequestration of CO_2 and its conversion into carbonate solid phases were studied by Dupraz *et al*. (2009) using *Bacillus pasteurii*. In a study of factors controlling urease enzyme and microbial deposition of $CaCO_3$ using *Sporosarcina pasteurii* previously known as *Bacillus pasteurii* (Yoon *et al*., 2001), Okwadha and Li (2010) found that CO_2 sequestration rate caused by ureolytic hydrolysis was directly proportional to microbial $CaCO_3$ formation.

Calcium carbonate precipitation induced by bacterial activity has extensively been employed to increase stability of structures in civil engineering. Ghosh *et al*. (2006) used thermophilic and anaerobic bacteria within cement-sand mortar/concrete to develop bioconcrete material. Results of the study indicated that compressive strength of concrete and cement-sand mortar increased significantly, due to the development of crystals within the pores of cement sand matrix. Effects of pure *Bacillus sphaericus* and a mixed culture of ureolytic bacteria on microbial $CaCO_3$ deposition and durability of mortar and concrete were studied by De Muynck *et al*. (2008a, b). They reported that deposited calcite on the surface of samples by bacteria decreased capillary water uptake as well as permeability towards gas. Carbonate precipitation induced by microbial activity using *Bacillus pasteurii*, an endospore forming soil microorganism, takes place with the highest urease activity, Sarda *et al*. (2009) on brick samples to increase the strength and durability.

Microbial deposition of calcium carbonate has also been employes to improve physical properties of soils and other porous media. Plastic syringe columns (60 mL) containing 100 grams sterile sand treated by *Bacillus pasteurii* were used by Stocks-Fischer *et al*. (1999) to study plugging of porous media. XRD and SEM analysis showed that calcite was precipitated in between sand particles. Using urease enzyme in glass columns (H: 30 and ID: 2.5 cm) packed with a mixture of sand and glass beads, Nemati and Voordouw (2003) created calcite cementation, which in turn caused a 98% decrease in permeability. Porosity and permeability decrease induced by *Sporosarcina pasteurii* in sand columns has also been reported by Cunningham *et al*. (2011) and Whiffin *et al*. (2007).

Role of microbial deposition of calcium carbonate on soil physical properties (porosity, permeability, and as well hydraulic conductivity) has been investigated by several researchers (Whiffin *et al*., 2007; Cunningham *et al*., 2011; Sarmast *et al*., 2011a, b). Vast areas in arid and semi-arid ecosystems are covered by sand dunes, the stabilization of which is of great importance. Besides, micromorphological observations, demonstrating microbial calcite morphology and pattern that help bridge and cement particles are lacking in literature. Despite the fact that genesis of calcium carbonate in calcareous soils of Iran is reported in the literature (Khormali and Nabiollahi, 2009; Farpoor *et al*., 2011; Moazallahi and Farpoor, 2012; Owliaie, 2012), however, limited information is presently available on biocalcite formation in soils and sediments in Iran. The present research was conducted to:

1). Study the levels, reaction time, and depth of secondary carbonates.

2). Compare *Sporosarcina pasteurii vs. Sporosarcina ureae* microbial activity on calcium carbonate formation.

3). Determine the micromorphology of bridging and cementing particles.

MATERIALS AND METHODS

The research was conducted in the framework of a factorial, completely randomized design experiment of 3 replicates. Effects of two bacterial species

(*Sporosarcina pasteurii* and *Sporosarcina ureae*), 3 concentrations of urea and $CaCl_2$ mixture (0.5, 1 and 1.5M), along with 6 time intervals (12, 24, 48, 96, 192 and 288 hours) were studied on microbial calcium carbonate formation.

Soil Sample

Soil sample was taken from sand dunes of Jupar area in Kerman Province. Air dried soil sample was sieved through a 2 mm sieve and routine soil physicochemical properties determined. Pipette method was employed for particle size distribution analysis of the soil (Gee and Bauder, 1986). Electrical conductivity of the saturated extract (Rhoades, 1982), and pH of the saturated paste (Mclean, 1982) were determined applying Jenway EC and pH meters. Calcium carbonate equivalent (CCE) was investigated through back titration (Nelson, 1982). Gypsum content was measured through acetone method (Nelson and Sommers, 1982). No gypsum was detected during chemical analyses and through morphological observations. Table 1 shows physicochemical properties of the soil sample used in the experiment.

Soil Column Parameters

Plastic columns with heights of 6.5 and inner diameters of 7.7 cm were packed with 500 grams of sterile sandy soil to study the depth of bacterial activity. The daily flows including inoculum, fixation flow of 50 mM $CaCl_2$ and cementation flow (urea + $CaCl_2$) were added uniformly and continuously to the columns. The sandy soil columns were incubated at 28±2˚C. Flow rates were adjusted according to the hydraulic conductivity of the sandy soil (10^{-2}-10^{-3} cm sec^{-1}).

Microorganisms and Growth Conditions

Sporosarcina pasteurii (PTCCi 1645: previously known as *Bacillus pasteurii*; Yoon *et al*., 2001) and *Sporosarcina ureae* (PTCCi 1642) were obtained from Iran Research Organization of Science and Technology. Both species are gram positive, endospore forming, of the potential of being grown in alkaline environments, and bear urease activity. Bacterial inoculums, using appropriate medium cultures (Table 2), were prepared at 30˚C, being shaken for 24 hours and at 140 rpm. The pH of the media was

Table 1. Selected physicochemical properties of the soil studied.

Particles (Percent)							Texture	EC (dS m^{-1})	pH	CCE [a] (%)	Gypsum
Sand					Silt	Clay					
Very Coarse	Coarse	Medium	Fine	Very Fine							
22.3	35.5	19.4	8.7	1.1	6	7	Sandy	0.7	8.4	8.8	-

[a] Calcium Carbonate Equivalent.

Table 2. Composition of culture media.

Bacteria	Culture	Composition	Content (g L^{-1})
S. pasteurii	Tryptic Soy Broth	Peptone from casein	15
		Peptone from soymeal	5
		NaCl	5
S. ureae	Nutrient Broth	Peptone	5
		Meat extract	3

adjusted to 8 using 1M KOH prior to being autoclaved with no urea addition. Filter sterilized urea was added following autoclaving. Estimation of bacterial density was performed through second tube (6×10^{8} cell mL^{-1}) of McFarland Barium Sulfate Standard test tubes (Cappuccino and Sherman, 1987).

Experimental Treatments

Inoculum solution (36 m L^{-1}) was added to the sandy soil columns by use of dropper and then immediately, followed by addition of 142 ml of 50 mM $CaCl_2$ as fixation fluid. Following the fixation fluid being fully added, the cementation fluid a mixture of 72 ml of (0.5, 1 and 1.5M equimolar of $CaCl_2$ and 72 mL of urea) was flushed through (Whiffin *et al*., 2007). The fixation and cementation fluids were made to pass through the sandy soil columns under a constant head. Time intervals of 12, 24, 48, 96, 192 and 288 hours were applied to different columns. Finally, and at due time for each treatment, samples were removed from incubator, and addition of daily flows stopped to end bacterial activity with the measurements being carried out immediately for the replicates. Selection of treatments was based on Whiffin (2004) followed by changes applied according to pre-experiments performed prior to the ongoing research.

Measurements and Analyses

To remove the soil samples, the columns' covering were cut off first. Calcium carbonate equivalent was determined at 4 depths separated by a micrometer (Vernier Caliper). The thickness of the cemented layer was also determined, making use of micrometer. Since CCE in *Sporosarcina ureae* treated samples showed no significant differences with depth, concentration, and time factors, micromorphological observations were conducted only on the samples treated by *Sporosarcina pasteurii.* Two depths (0-3 and 3-6 cm) were selected for thin section preparations (horizontal cuttings) using guidelines described in Murphy (1986). An Olympus, BH_2 petrographic microscope was used for thin section descriptions (Stoops, 2003) in plain (PPL) and cross Polarized (XPL) lights and then photographed by a digital Sony camera.

The ratio proposed by Okwadha and Li (2010) was employed to evaluate the sequestrated CO_2. Urea hydrolysis and microbial deposition of $CaCO_3$ are stoichiometrical reactions indicating why the level of sequestrated CO_2 is a direct proportion of the level of microbial $CaCO_3$ precipitation. The concentration of CO_2 is therefore calculated using the following simple ratio: $44/100= x/y$, where x is the level of sequestrated CO_2 and y the amount of precipitated $CaCO_3$ (Okwadha and Li, 2010).

MSTAT-C software was employed for ANOVA analysis, and Duncan test ($P< 0.05$) for a mean comparison of the data.

RESULTS AND DISCUSSION

Microbial $CaCO_3$ Formation and CO_2 Sequestration

The results obtained indicated that bacterial species, reactant concentration, reaction time, depth intervals as well as the mutual effects of these factors significantly influenced the formation of calcium carbonate ($P< 0.01$). Mean calcium carbonate formation were recorded 12.2 and 9.2% for *S. pasteurii* and *S. ureae*, respectively. The difference in $CaCO_3$ precipitation between the two bacterial species could be attributed to different capabilities of these species to provide varied types of urease enzymes (McCoy *et al*., 1992). Comparing different strains of bacteria, Hammes *et al*. (2003) also reported that the level of urease enzyme was different among various strains.

Calcium carbonate precipitation significantly increased for *S. pasteurii* as reactant concentration increased, but no statistically significant difference was observed for *S. ureae*. Mean calcium carbonate contents were 11.0, 12.3, and 13.4%, for reactant concentrations of 0.5, 1.0 and 1.5M, in *S. pasteurii* columns *vs.* 9.1, 9.3, and 9.3% for *S. ureae* (Figure 1-a). Nemati and Voordouw (2003), and also Okwadha and Li (2010) have reported that reactant concentration increase microbial calcium carbonate formation. On the other hand, Hammes *et al*. (2003) reported that due to the significant effect of calcium ions on urease enzyme activity, calcium carbonate precipitation by *S. pasteurii* increases as a result of increased reactant concentrations. They speculated that calcium ions could better facilitate trans-membrane transport or improve the intracellular signaling process. Tobler *et al*. (2011) also mentioned that *S. pasteurii* is capable of tolerating high concentrations of Ca^{2+}. Whiffin (2004) reported that *S. pasteurii* gains the necessary energy for calcium metabolism from the ATP produced during urea hydrolysis. No data for such mechanisms have been reported for *S. ureae*.

Lack of significant differences among 12, 24, and 48 hour treatments in both *S. pasteurii* and *S. ureae* species (Figure 1-b) might be due to inadequate microbial fixation time. The significant differences among 96, 192, and 288 hour treatments observed for *S. pasteurii* might likely be due

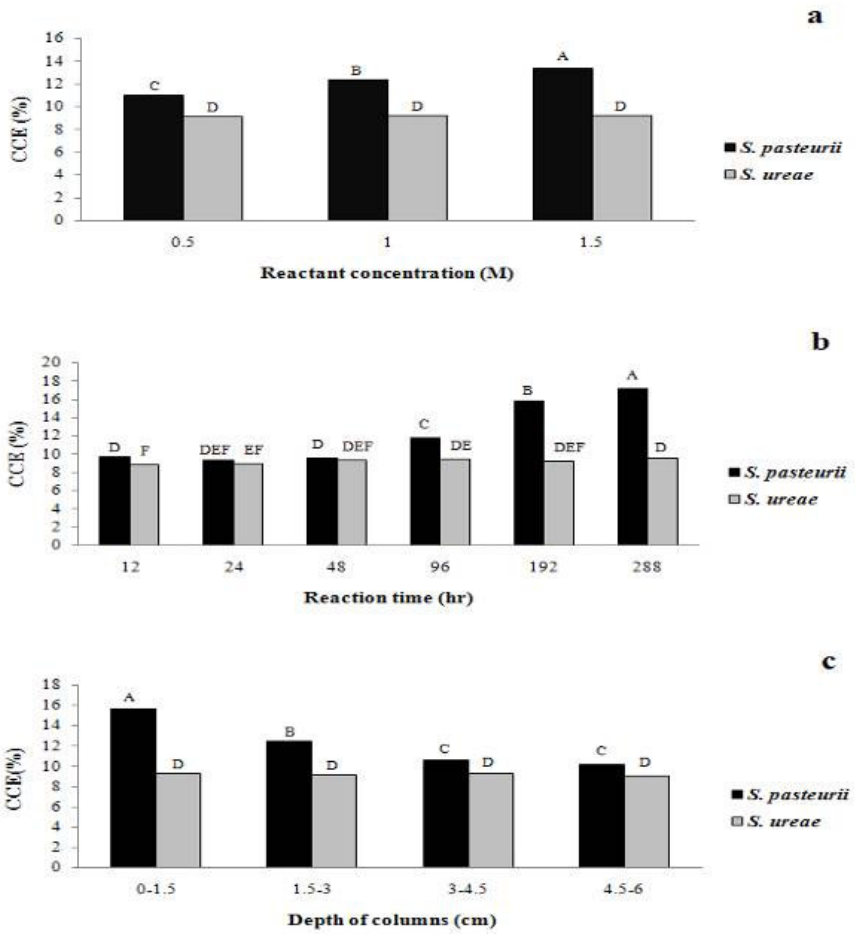

Figure 1. Effect of reactant concentration (a), reaction time (b), depth (c) and bacterial species on Calcium Carbonate Equivalent (CCE).

to daily addition of inoculums and reactants, as also reported by Whiffin (2004). Low differences among time interval treatments were recorded for *S. ureae*, which might be attributed to less urease activity of this bacterial species. McCoy *et al*. (1992) reported that although *S. ureae* and *S. pasteurii* are closely related, the kind and activity of urease enzyme in the two species are different.

Calcium carbonate content in the top 0-1.5 cm was significantly higher than that in the 4.5-6 cm for *S. pasteurii* treated columns (15.6% as compared with 10.2%), which shows a significant decreasing trend of calcium carbonate content with depth (Figure 1-c). The change in calcium carbonate precipitation with depth for *S. ureae* was not significant (from 9.3 to 9.1%). Whiffin *et al*. (2007) and Achal *et al*. (2009a, b) also found similar results for *S. pasteurii* in sand columns. Different amounts of calcium carbonate precipitates in the four depths of soil columns by *S. pasteurii* may have also been caused by limitation in availability of nutrients, reactants and oxygen flow due to higher activity and adsorption of bacteria in the surface layer of the soil, thus sealing the upper pore spaces (Day *et al*., 2003). Besides, facultatively anaerobic *S. pasteurii* growth is normally higher in the presence of oxygen, explaining higher microbial formation of calcium carbonate in the surface layers (Day *et al*., 2003; Achal *et al*., 2009a, b).

Carbon dioxide consumption also showed an increasing trend with reaction time, reactant concentration, and depth reduction in *S. pasteurii*. The close relationship between sequestrated CO_2 and precipitated microbial calcium carbonate was also observed by Okwadha and Li (2010).

Micromorphological Studies

Micromorphological observation of samples treated with *S. ureae* showed no visible calcium carbonate cementation and induration. This supports the earlier results of no significant relationship among different treatments for *S. ureae*. Therefore, a complete detailed micromorphological analysis for *S. ureae* was not performed in this study. Figure 2-a shows a thin section of the blank sample (sandy soil with no treatments). Plagioclase, pyroxene, quartz, biotite, igneous microlites, and opaque particles were observed in the blank sample.

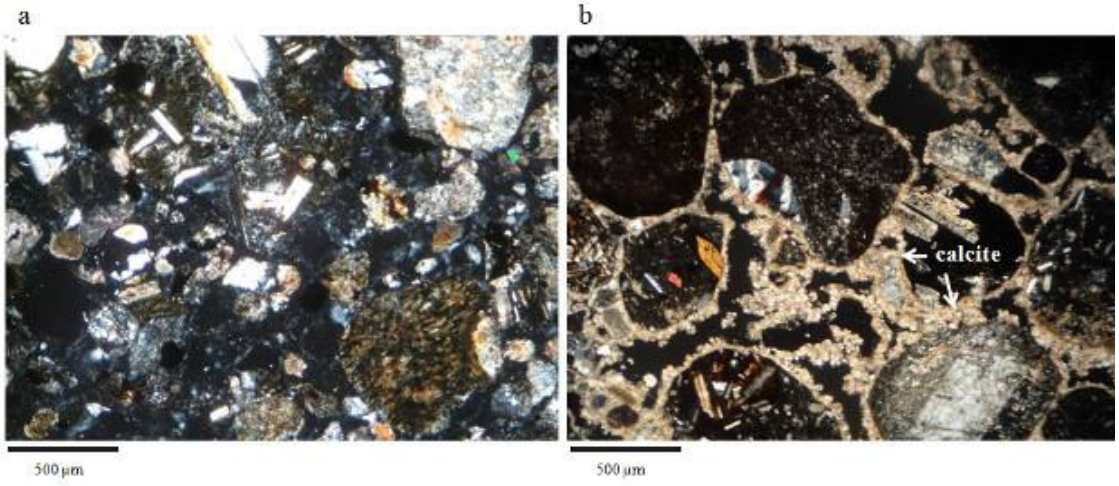

Figure 2. Thin sections of blank sample at 0-3 cm depth (a) and sample treated by *S. pasteurii* (b) in 288 hours, 1.5M reactant concentration and 0-3 cm depth (both samples in XPL).

Although chemical analysis showed that there is about 8.8% calcium carbonate equivalent in this sample (Table 1), but the calcium carbonate is in a disseminated form based on micromorphological observations with no secondary calcite crystals being found.

Calcite crystals bridging sand particles were observed in thin sections of the *S. pasteurii* treated samples (Figure 2-b). Although micromorphological observations are qualitative and no quantification technique was used throughout the present study, however, the thin sections studied clearly showed the difference in relative amounts of calcite crystals among treatments.

Thin sections from 0-3 cm depth of columns showed more calcite crystals as compared with the 3-6 cm depth for all the reactant concentrations (Figure 3). The decreasing trend of microbial calcium

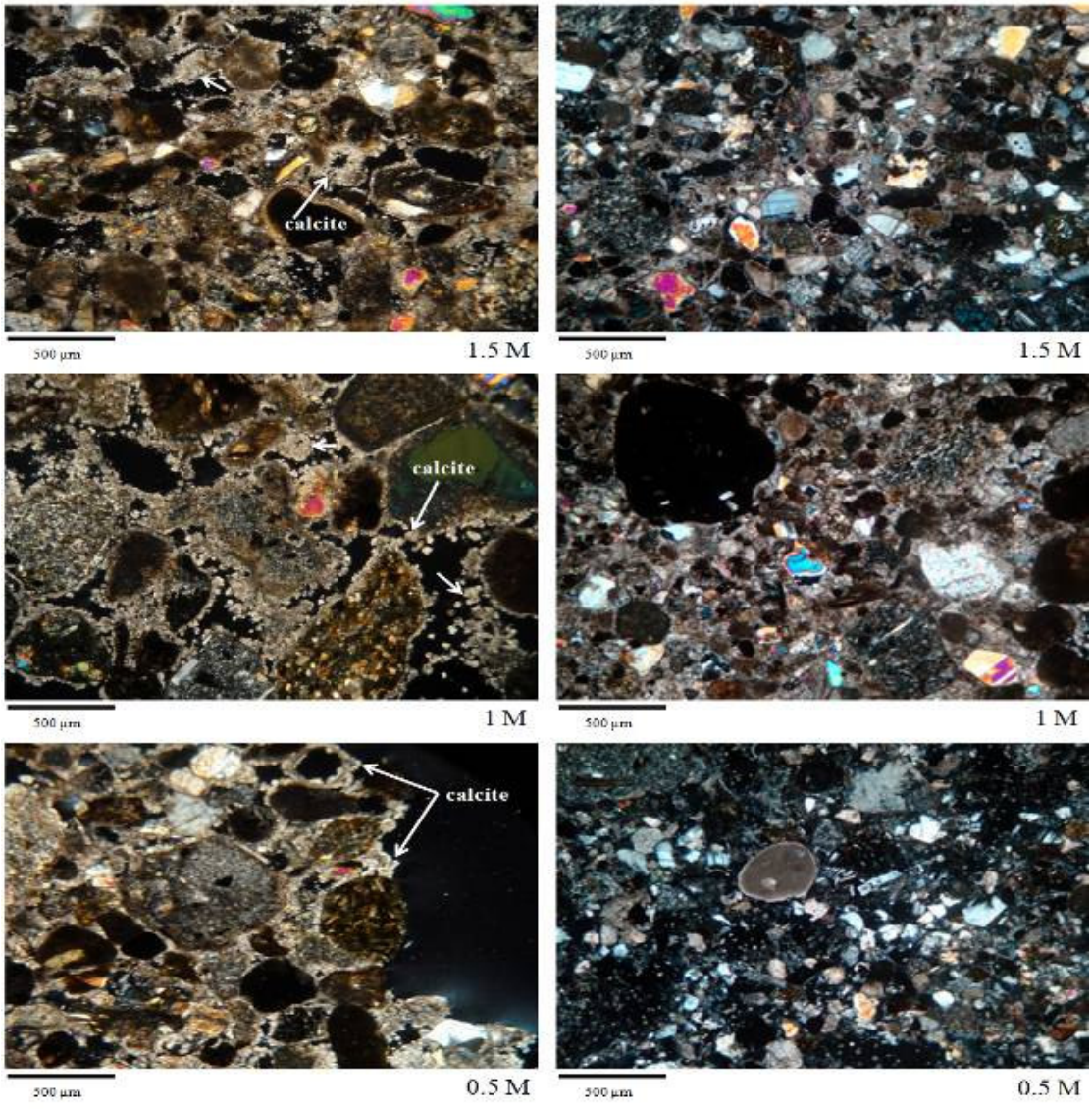

Figure 3. Thin sections (XPL) of 192 hour treatment for *S. pasteurii* in 0.5, 1.0 and 1.5M reactant concentrations showing the effect of column depth (left picture 0-3 cm and right pictures 3-6 cm).

carbonate cementation by *S. pasteurii* with depth was also reported by Day *et al*. (2003) using an XRD technique. They showed that maximum microbial calcium carbonate formation was in the surface layer and no calcite crystal formed below 3.8 cm depth. Bang *et al*. (2001) also came to the conclusion that microbial calcium carbonate was more capable of surface crack remediation than the subsurface cracks. This observation also supports the data from the chemical analysis. Since the growth and ureolytic hydrolysis of *S. pasteurii* is sensitive to O_2 concentration in the soil environment (Achal *et al*., 2009a, b), a decreasing trend of calcite formation and increase in porosity with depth is not surprising.

Calcite coating, infilling, and bridging were among the pedofeatures observed in thin sections of the samples treated. Verrecchia and Verrecchia (1994) have also reported biocalcite morphology, crystal habits, and microfabrics formed by calcifying bacteria. Figure 4 shows the effect of reactant concentrations on calcite crystals formed by *S. pasteurii* for the 288 hour treatment. The thin section images from 0-3 cm layer of columns treated with 1.5M reactant concentration showed a continuous cementation of $CaCO_3$ infillings as compared with only bridging and coating morphology of calcite in columns treated with 1M reactant concentration. Isolated calcite micro spars were the dominant crystals observed in thin sections treated with the 0.5M concentration. Pore spaces are also reduced increasingly with the increase of reactant concentration (Figure 4).

No significant difference was observed in calcium carbonate equivalent in the samples treated for 12, 24, and 48 hours. This is supported with the fact that no cementation was observed in the same treatments. Thin sections from the 0-3 cm depth treated for 96, 192 and 288 hours by 1.5 M reactant, however, showed an increasing trend of microbial calcite formation with time. The highest level of microbial calcium carbonate formation and the least porosity were observed in the thin section treated with 1.5M concentration for 288 hours. Coating and bridging of calcite were dominant in the 192 hour sample, but only weak coatings and bridging of $CaCO_3$ were found in the 96 hour sample (Figure 5).

Cemented Layer Thickness

The thickness of the top cemented layer was statistically significantly ($P< 0.01$) affected by bacterial, reactant concentration, and reaction time. The cemented layer created by *S. pasteurii* had a mean thickness of 16 mm which was significantly thicker than the cemented layer created by *S. ureae* (7mm). Whiffin (2004) reported that the activity of urease enzyme and composition of media culture are among the factors controlling the cementation layer.

A mean comparison of data showed that increase in reactant concentration and reaction time tend to increase cementation depth in *S. pasteurii*, but no statistically significant difference was observed for *S. ureae* (Figure 6). High activity of urease enzyme in *S. pasteurii* is extensively reported in the literature (Stocks-Fischer *et al*., 1999; Whiffin, 2004; Sarda *et al*., 2009). Although urease enzyme was not determined in the present research, but it is possible that higher activity of this enzyme could be the reason for the greater cementation depth observed in the *S. pasteurii* treated columns, also supported by data from chemical analysis.

Time intervals of 288 and 192 hour showed the greatest cementation depths of 20.9 and 18.8 mm, respectively. This difference was not statistically significant; however, treatments of 12, 24, 48 and 96 hour had significantly lower cementation depths (Table 3). Mean cemented layer thickness (Table 3) varied from 8.4 mm (0.5 M reactant concentration) to 13.1 mm (1.5 M reactant concentration). An increasing cementation depth with reactant concentration was also supported by CCE,

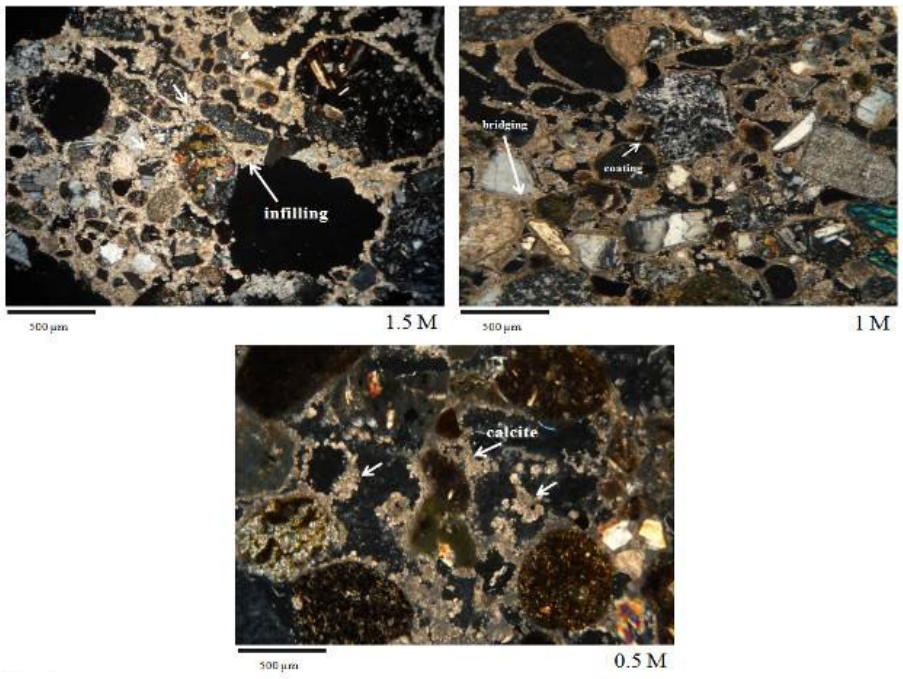

Figure 4. Thin sections (XPL) of 0-3 cm depth for 288 hour time treatment of *S. pasteurii* showing the effect of reactant concentrations (0.5, 1.0, and 1.5M).

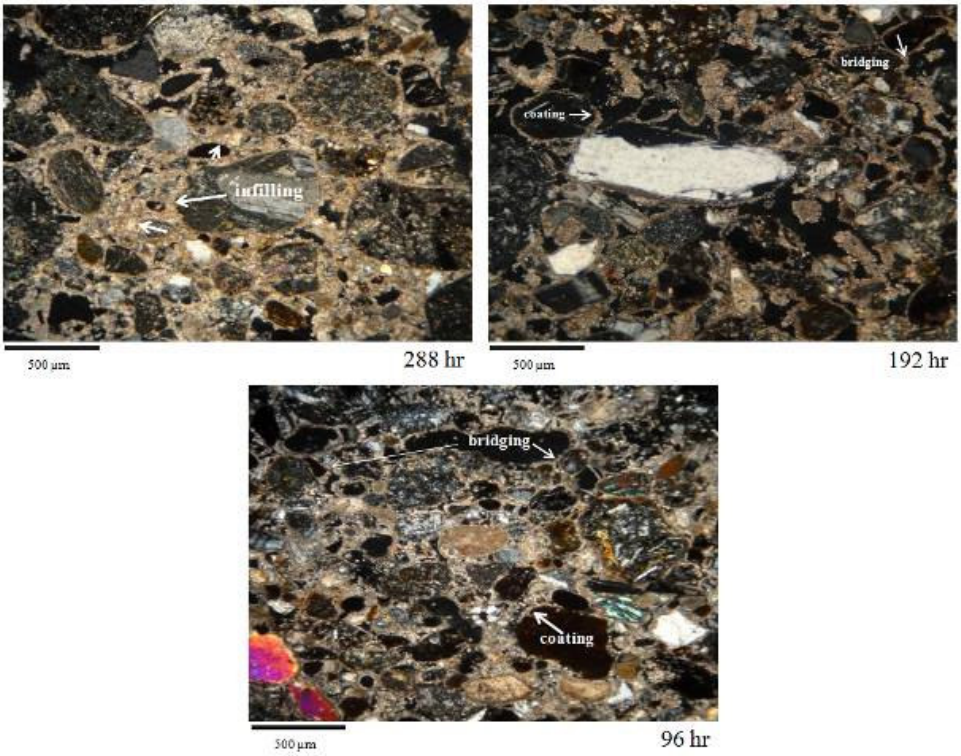

Figure 5. Thin sections (XPL) of 0-3 cm depth, 1.5M reactant concentration for *S. pasteurii* showing the effect of time (96, 192, and 288 hours).

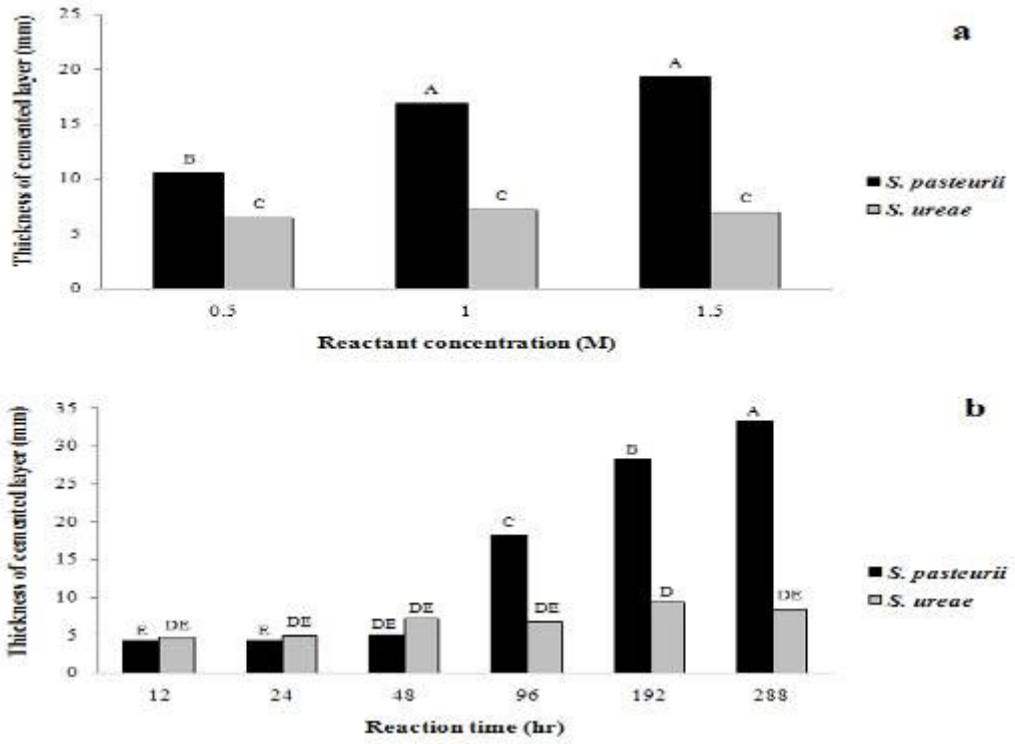

Figure 6. Effects of reactant concentration (a) and reaction time (b) on thickness of $CaCO_3$ layer in two bacteria species.

Table 3. Mean comparison data of time and concentration on thickness of cemented layer (mm). [a]

	Time treatment (hr)						
Concentration (M)	12	24	48	96	192	288	Mean
0.5	4.8d	5.1d	4.6d	7.8d	13.8c	14.5c	8.4B
1	3.8d	3.8d	7d	13.6c	18bc	25.8a	12A
1.5	5d	4.6d	6.0d	15.8c	24.6a	22.3ab	13.1A
	4.5C	4.5C	6 C	12.4B	18.8A	20.9A	Mean

[a] Same letter within each column indicates no significant difference among treatments (P< 0.05).

CO_2 sequestration and micromorphological studies, as discussed earlier.

CONCLUSIONS

In this investigation bacterial species, reactant concentration, reaction time, and depth were among the factors affecting calcium carbonate formation. In *S. pasteurii* treated columns, mean calcium carbonate precipitation increased significantly toward the upper layers with increasing reactant concentration through time. No significant calcite precipitation was observed for *S. ureae*. CO_2 sequestration and depth of cementation layer of calcium carbonate showed a similar trend with the calcium carbonate equivalent.

Micromorphological observations of thin sections from different layers of treated columns with *S. pasteurii* also supported the chemical data. Microbial calcium carbonate showed bridging, coating, and / or infillings of sand particles in thin sections, causing reduction in soil porosity. The cementation

induced by microbial calcium carbonate formation technique could likely be used in arid and semi-arid areas to stabilize sand dunes.

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اثرات *Sporosarcina pasteurii* و *Sporosarcina ureae* بر روی میکرومورفولوژی و تشکیل آهک زیستی در ستون‌های خاک شنی

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چکیده

کربنات کلسیم میکروبی با ایجاد پل در بین ذرات شن، نقش مهمی در پایداری تپه شنی ایفا می نماید. ما مطالعه‌ای روی سیمانی شدن ذرات شن و پرشدگی منافذ توسط $CaCO_3$ انجام دادیم. آزمایش به صورت فاکتوریل با فاکتورهای دو گونه باکتری (*Sporosarcina pasteurii* و *Sporosarcina ureae*)، سه غلظت واکنشگر (۰/۵، ۱ و ۱/۵ مولار) و شش زمان واکنش (۱۲، ۲۴، ۴۸، ۹۶، ۱۹۲ و ۲۸۸ ساعت) انجام شد. مایه تلقیح باکتری ها و محلول های واکنشگر روزانه به ستون های خاک شنی (ارتفاع ۶/۵ و قطر داخلی ۷/۷ سانتی متر) اضافه شدند و رسوب $CaCO_3$ در فواصل ۱/۵-۰، ۳-۱/۵، ۴/۵-۳ و ۶-۴/۵ سانتی متر مورد بررسی قرار گرفت. آزمایشات شیمیایی و میکرومورفولوژیکی نشان دادند که کارایی گونه *S. pasteurii* در تشکیل $CaCO_3$، ترسیب C غیرآلی و عمق سیمانی شدن بیشتر از گونه *S. ureae* بود. با افزایش زمان واکنش از ۱۲ به ۲۸۸ ساعت، رسوب میکروبی $CaCO_3$ و ترسیب C غیرآلی افزایش یافت. افزایش غلظت واکنشگر باعث افزایش ۱۲ درصدی رسوب $CaCO_3$ شد. مشاهدات میکرومورفولوژیکی درجه بالایی از بلورهای کلسیت را به صورت پل و پوشش روی ذرات شن و پرکننده منافذ نشان داد. با توجه به تاثیر معنی‌دار باکتری *S. pasteurii* در رسوبگذاری $CaCO_3$ و سیمانی شدن ذرات شن، استفاده از این باکتری جهت تثبیت تپه های شنی توصیه می گردد.