$Caco_3$ Precipitation in Selected Bacteria Mixture by Activated Microbial Induction

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Abstract**—**Activated microbial induction of $CaCO_3$ precipitation (AMICP) is a niche with innovative approaches that involves the exploration of bacterial attacks towards the alteration/transformation of the physical properties of soils. A handful have been documented on this technology in developed countries however, much is yet to be understood in developing African countries like South Africa, just as vermicomposting and waste water treatment technologies are gaining grounds. It is widely known that AMICP by urea hydrolysis in natural soils is possibly affected by contacts between ureolytic and non-ureolytic bacteria, the study explored a designed and experimental assessment of the relations between ureolytic and non-ureolytic bacteria and their interactive effects on AMICP. Through existing studies an artificial leveled groundwater medium was injected with model species of bacteria i.e., ureolytic species *Sporosarcina pasteurii* and the non-ureolytic species *Bacillus subtilis*. The control treatment was inoculated with a pure culture of *S. pasteurii* under measurements of pH, optical density (OD), development of NH_4-N, dissolved calcium (DC) and dissolved inorganic carbon (DIC). Outcomes revealed DC precipitated as $CaCO_3$ slower in the control mixture than in the mixed culture irrespective of unfavorable conditions in the mixed culture, i.e., lower concentrations of pH and CO_3^{2-}. Higher density of bacterial cells in the mixed culture resulted from *B. subtilis* showing significant higher growth rate than *S. pasteurii*. Previous authors indicate that the presence of the non-ureolytic bacterial species, *B. subtilis*, stimulate AMICP process through supply of nucleation sites in the form of non-ureolytic bacterial cells.

Keywords**—***Natural soils, Bacillus subtilis, Sporosarcina pasteurii, Bacteria, Microbial attack*

I. Introduction

As recorded by [1] biomass in all major types of soils consist of prokaryotes with an estimated average of 2.2×10^8 cells/cm^3 of soil in the top 10 m. The outcomes of prokaryotes' metabolic reaction interact with different soil composition which could alter the properties of the soil. As such, bacterial activity can be utilised towards the beneficial, sustainable and environmentally friendly transformation of soil properties for soil enrichment, enhancement, stabilisation and modification for agrian and engineering purposes [1]. This also involves the AMICP as a fast growing biogeochemical treatment for soil which successfully precipitates $CaCO_3$ in the soil and consequently improves its strength and stiffness thereby lowering water permeability [2], [3]. Presently, the application of AMICP in the developed world is vast in areas of environmental protection, contaminated site remediation, geoenvironmental and geotechnical application, structural restoration and architecture etc., [4-7]. A number of soil bacteria can trigger $CaCO_3$ precipitation via diverse metabolic processes namely; autotrophic and heterotrophic processes. However, [6] recorded that the most effective biogeochemical process for AMICP entails the microbial hydrolysis of urea catalysed by the microbial enzyme- urease (urea amidohydrolase, EC 3.5.1.5). The enzymatic hydrolysis of urea as reported by [8] is roughly 10^{14} times faster than the spontaneous reaction, with urea-hydrolysing microorganisms i.e., eukaryotes and prokaryotes being universal in natural soils and triggering the process of urea hydrolysis in soils globally [9]. From (1) urea is firstly hydrolysed to carbamate and ammonia as noted by [9]. Carbamate is then rapidly hydrolysed to yield carbonic acid and ammonia as seen in (2) which in turn is hydrolysed as expressed in (3) and (4) having equilibrium constants of pK_1 6.3 and pK_a 9.3 respectively [10].

$$CO(NH_2)_2 + H_2O \rightarrow \text{urease} \rightarrow NH_3 + NH_2COOH \quad (1)$$
$$NH_2COOH + H_2O \leftrightarrow H_2CO_3 + NH_3 \quad (2)$$
$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \quad (3)$$
$$NH_3 + H_2O \leftrightarrow NH4^+ + OH^- \quad (4)$$
$$Ca^{2+} + HCO_3^- + OH^- \leftrightarrow CaCO_3 \downarrow + H_2O \quad (5)$$

It is clear that from these expressions a net increase in soil pH is expected, and in the presence of DC the process could produce precipitates of $CaCO_3$ so long the medium is oversaturated, with regard to $CaCO_3$ as represented in (5). Moreover, it is clearer that AMICP is a complex process carefully stabilised by certain parameters: (a) DIC (b) pH (c) abundant nucleation sites and (d) DC concentration [11]. As recorded by [10] the first three parameters are directly affected by urea-hydrolysing (ureolytic) microbial action represented in (1) to (4) and by bacterial cell abundance allowing bacteria nucleation sites. In terms of soil enrichment, two main steps are used in the execution of AMICP: (a) bio-augmentation, where a particular ureolytic bacterial strain is introduced to the treatment site with urea, nutrients and calcium; and (b) bio-activation/stimulation, where local ureolytic bacteria are

Manuscript received March 0, 2016; revised March 0, 2016; submitted for review March 18, 2016.

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provided with a substrate designed to activate/stimulate $CaCO_3$ precipitation [10]. On one hand, onsite bio-augmentation process does not have a 100% success rate since it is based on the introduction to the soil of large quantities of monoclonal bacterial mixtures with indeterminate survival and reproduction chances as these bacteria are often vulnerable to predation by eukaryotes and unable to succeed with native microorganisms. On the other hand, bio-activation/stimulation, enhances the growth of a particular union of native soil microfauna through the influence of specific growth conditions [12]. Although, [13] explains that a downside to this approach is that the first soil concentration of ureolytic bacteria could hinder the rate of ureolytic AMICP in the site to be treated. Furthermore, [10] stated that another factor influencing bio-activation is the cellular regulation over express urease which includes three regulation processes namely; (a) constitutive, whereby urease is constantly expressed by the organism; (b) inducible, whereby urease is expressed in response to the presence of urea over a particular threshold concentration; and (c) repressible, whereby urease expression is inhibited in response to the presence of nitrogen-rich compounds, including high concentrations of urea [9]. Out of these regulators, the constitutive regulation is considered the most promising for a successful AMICP process even though, the commonest regulation type is the repressible expression as recorded by [9]. The interactions within the microbial community affect the geochemistry and the microbial ecology of their environment, and the presence of non-ureolytic bacteria in the soil has been revealed to affect the parameters controlling $CaCO_3$ precipitation in different ways: heterotrophic bacterial metabolism, for instance, has been shown to induce $CaCO_3$ dissolution under aerobic conditions due to the mineralisation of organic carbon and the consumption of ammonium [14]. Conversely, as reported in [10] the electronegativity of the bacterial cell surface supports complexation of dissolved metals with the complexes possibly acting as nucleation sites for mineral precipitation and as such, hasten $CaCO_3$ precipitation. To date, most of the research on AMICP as recorded by [10] has revolved around ureolytic bacteria focusing on the catalysis of urea hydrolysis, the efficiency of calcite production and the transformation of soil physical properties by model bacteria. However, few studies of ureolytic AMICP have been done using mixed bacterial cultures in the lab [13] and others have been done onsite by activation/stimulation of native ureolytic bacteria [5] with little or no attention been paid to the effect on the system of the non-ureolytic bacteria present in the experimental setting. However in this exploration, possible conflicting effects of ureolytic and non-ureolytic bacteria on $CaCO_3$ precipitation is unveiled from a simple, two-species batch activity. An AMICP process comprising of two model bacteria, the ureolytic species *Sporosarcina pasteurii* and the non-ureolytic species *Bacillus subtilis*, was used to understand the chemical and biological progression of the $CaCO_3$ precipitation process in soil. The two-species model system were explored so as unravel some of the possible relationships between bacteria of two different unions and their eventual impact on AMICP.

II. CONCEPTUAL AND EXPERIMENTAL APPROACH

A. Bacteria and Conditions of Growth

For the *Ureolytic bacterium*: a pure culture of *Sporosarcina pasteurii* (DSMZ 33) was grown under agitation of 100 rpm at 30ºC in Nutrient Broth (NB) supplemented with 2% w/v urea (333 mM) until the exponential phase of growth was reached [10]. Subsequently, the bacteria were harvested by centrifuging 16,100 g at 6 min, resuspended in a sterile $CaCO_3$ precipitation medium. The process was done twice to prepare the inoculum of *S. pasteurii*. The final concentration of *S. pasteurii* for all treatments described was roughly 10^7 m/L bacteria. In the case of the *Non-ureolytic bacterium:* the inoculum of the model gram-positive bacterium, *Bacillus subtilis* (DSMZ 6397), was prepared as earlier done [10]. The final concentration of *B. subtilis* in each of the mixed treatments was 10^7 m/L bacteria. *B. subtilis* is often used as a biotic control for AMICP activities, as it does not trigger $CaCO_3$ precipitation and does not upset the concentrations of DC as observed by [10].

B. $CaCO_3$ Precipitation Media

The $CaCO_3$ precipitation media reported by [10] were based on an artificial groundwater solution (AGW) representing coastal aquifer with the following compositions: $MgCl_2$ (1 mM), $MgSO_4$ (1 mM), $NaHCO_3$ (2.56 mM), NaCl (14.35 mM), $CaCl_2$ (2.43 mM) and KCl (0.32 mM) with total ionic strength (31.5 mM). The two distinct precipitation media prepared include: (a) full-strength medium, NBU, in which AGW was supplemented with 7 mM urea and 13 g/L NB providing *B. subtilis* with all necessary nutrients but limiting growth of *S. pasteurii* due to low concentration of urea; and (b) one-third strength medium- 1/3 NBU which contained AGW supplemented with 7 mM urea and 4.34 g/L NB which restricted the growth of *B. subtilis* due to lower nutrient availability. To avoid untimely $CaCO_3$ precipitation the pH of the AGW was adjusted to 6.5 using 1N HCl prior to the introduction of urea and NB. Subsequent to NB inclusion to the medium, the pH increased to roughly 7.4 due to the chemical properties of the NB. The Media were filter-sterilised using through 0.2 μm sterile filters [10].

C. $CaCO_3$ Precipitation Treatments

Assessing the effect of non-ureolytic bacteria on AMICP involved the injection of NBU and 1/3 NBU media with both bacterial species having the treatments denoted as NBps and 1/3 NBps, respectively. The control treatment consisted of NBU injected with *S. pasteurii* alone and denoted as NBp treatment. Each treatment was prepared in duplicate; the initial volume of each replicate was 200 mL and were injected without shaking in corked 250 mL flasks at ambient temperatures for a period of up to 10 days. Over the testing activity, aliquots from each treatment were analysed at scheduled times.

D. Chemical Analysis

The samples studied were filtered via 0.22 µm filters upon harvesting. Concentrations of DC and ammonium concentrations measured by ion exchange chromatography (Dionex 500; eluent: 20 mM methanesulfonic acid, flow rate: 1.0 mL/min; column type: cation separation – IonPac - CS12A, 4×250 mm) [10]. The standard measurement error was 0.002 and 0.006 mM for Ca^{2+} and NH_4^+, respectively. The pH was measured upon sampling with a pH probe having an error measurement of 0.01. For DIC measurements, filtered samples were put in glass vials holding H_3PO_4 which was already rinsed using helium for 10 min to avoid equilibration with atmospheric CO_2. The DIC content was then obtained from an IRMS (isotope ratio mass spectrometer) Delta Plus XP (Thermo Scientific, NY, USA), utilizing Gas Bench II [10]. Ten solutions of $NaHCO_3$ (concentrations ranging from 3.91 to 7.15 mM) were used for calibration with 0.01 mM standard error measurements.

E. Biological Analysis

As recorded by [10] growth in bacterial was determined in reference to OD via absorbance measurement at a wavelength of 600 nm. Colony-forming units (CFU) of the two species counted on two distinct growth media namely; NB agar and NB agar supplemented with 20 g/L urea (333 mM). Considering that *S. pasteurii* cannot grow on NB agar outside urea site or high concentrations of ammonium salts, CFU counts on NB agar plates indicated the concentration of *B. subtilis*, whereas CFU counts on NB-urea agar plates represented the total concentration of bacteria in the mixed cultures (treatment NBps and 1/3 NBps). Therefore, the comparison of the CFU values between the two plate types distinguished the bacterial species in the mixed culture. Subsequent to the injection of the media, CFUs were counted at designated times of 17th and 40th h of the activity.

F. Measurements of Zeta Potential Value

Measurement of the zeta potential value involved growing and harvesting of monoclonal cultures of *S. pasteurii* and *B. subtilis* described earlier. Subsequently, each bacterial culture was resuspended in an AGW-based medium augmented with 7 mM of urea. The pH was then adjusted to one of three different values: 7.40, 7.90 and 8.40, with 3N NaOH [10] while each treatment was prepared in triplicate. The zeta potential value was measured using the 90Plus particle size analyser by Brookhaven Instruments (Holtsville, NY) as per [10] with every measurement redone five times under 0.52 mV standard error of measurement.

III. DISCUSSION OF FINDINGS

The average of treatment duplicates were plotted for the measured Ca^{2+}, NH_4^+, pH and OD. The standard deviations were characteristically small. Figure 1a-f therefore shows the smooth curves of the activity series.

A. Dissolved Ca^{2+} (DC)

Reduction in concentration of DC was recorded in the activity plausibly due to $CaCO_3$ precipitation. The mixed culture with NBps treatment as recorded by [10] experienced the quickest exhaustion of DC having roughly 2.40 mM of Ca^{2+} depleted over the first 80 h of the activity as shown in Figure 1a. Correspondingly, a close 100% precipitation of $CaCO_3$ is shown in Figure 2b. Slower calcium exhaustion was completed after 123 h in the case of the control NBp treatment. The Ca^{2+} exhaustion rate for the 1/3 NBps mixed culture treatment was closely related to the NBp control treatment having close to 100% precipitation of $CaCO_3$ in about the same time.

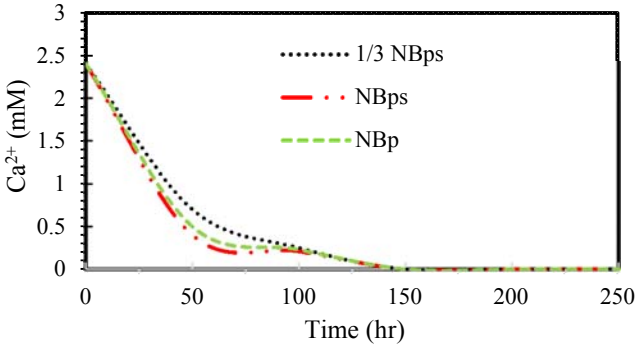

Fig. 1a. Transformation with time in DC concentration

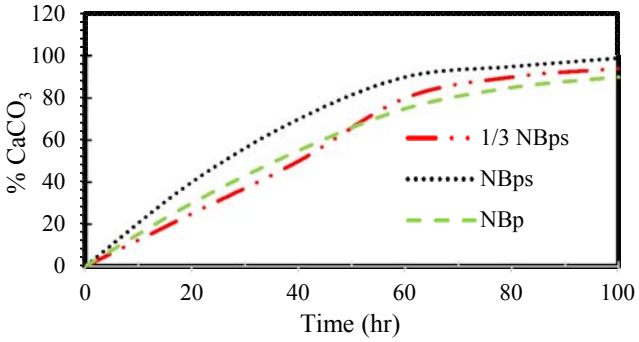

Fig. 1b. Transformation with time in % of exhausted Ca precipitated as $CaCO_3$

B. Concentration of NH_4^+

The increase in ammonium concentration as shown in Figure 1c is plausibly ascribed mainly to urea hydrolysis in conformance to a stoichiometry of 2:1 recorded by [10] which further points to the representations in (1) and (2) but also partially due to mineralisation of NB. The final ammonium concentrations for control NBp and mixed culture NBps treatments were identical having 18.43 mM while the 1/3 NBps treatment had final ammonium concentration noticeably lower at 14.64 mM as reported by [10].

C. pH

Figure 1d shows the changes in pH with respect to time. Initial pH values of the series of treatments were approximately 7.4 as obtained by [10] which may be accounted for by the pH of

the NB introduced subsequent to the 6.5 pH adjustment. In the case of the control NBp treatment, pH values increased rapidly within the first 100 h of the activity closely at pH 1 to a level of roughly pH 8.40 [10]. Nevertheless, for the mixed culture NBps treatment, a different trend was observed. Here, the pH values increased over the first 10 h of the activity to a value of 7.74. Conversely, a decline to a minimum value of 7.39 followed after 28 h. Furthermore, the pH values increased again until the 125th h and then fluctuated around a value of 8.40. Whereas, in the 1/3 NBps mixed culture treatment, pH values increased during the first 18 h of the activity to a value of 8.06 and a slight decrease to a value of 7.98 at the 28th h after which there was an increase until the 123rd h and a final value was reached fluctuating around 8.50 for the series of treatments.

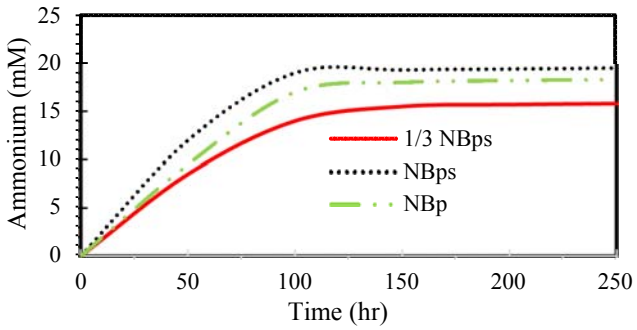

Fig. 1c. Transformation with time in ammonium

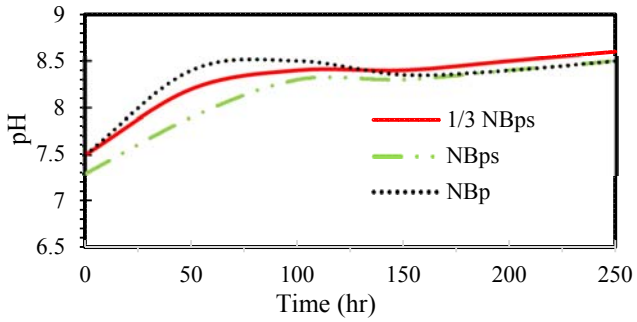

Fig. 1d. Transformation with time in pH

D. Concentration of DIC

Over the first 80 h of the activity the total concentrations of DIC were higher by an average of 2.6 mM for the mixed culture NBps treatment against the control NBp treatment. As shown in Figure 1e, the 1/3 NBps treatment had DIC concentrations similar to the NBp treatment.

E. Culture Growth Patterns

Diverse bacterial growth patterns were observed for the different treatments as shown in Figure 1f after [10]. In the mixed culture NBps treatment the exponential growth phase was initiated around the 17th h of the activity whereas for the control NBp treatment, the exponential growth phase began around after the 40th h of the activity. The increase in OD for treatment 1/3 NBps is divided into two phases: the first began around the 17th h of the activity as was the OD increase in the full-strength NBps treatment while the second phase began around the 40th h as was the OD increase in the control NBp treatment.

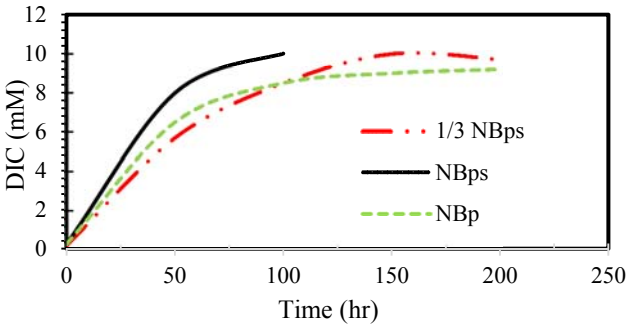

Fig. 1e. Transformation with time in DIC

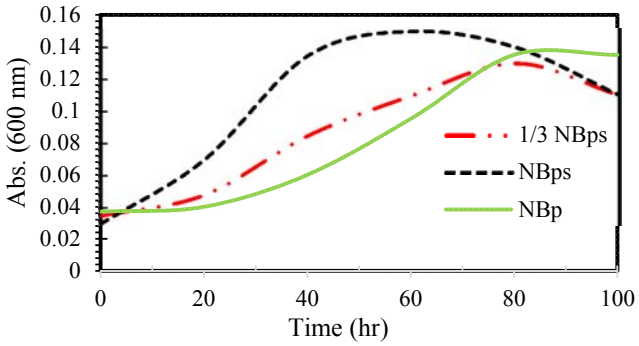

Fig. 1f. Transformation with time in OD at 600 nm

With regards to the CFUs, the count of *B. subtilis* in the mixed culture NBps and 1/3 NBps treatments increased by two orders of magnitude within the first 17 h of the activity. In the NBp treatment injected with *S. pasteurii* alone, the CFU count increased by one order of magnitude. At the 40th h of the activity, CFU counts of *B. subtilis* in the 1/3 NBps treatment had not changed substantially whereas those in the NBps treatment increased by another order of magnitude and as such, confirmed the OD measurement outcomes.

F. Zeta Potential

The zeta potential values of both bacterial species decreased with an increase in pH. *S. pasteurii* zeta potentials ranged from −19.51 mV at pH 7.40 to −23.10mV at pH 8.40. *B. subtilis* zeta potential values ranged from −22.28mV at pH 7.40 to −24.18mV at pH 8.40.

IV. Conclusions

Outcomes of the mixed culture exploration provided insight into the complexity of the relationships between the different bacteria over the ureolytic AMICP. It showed that, via the activity, the non-ureolytic bacterial species displayed a substantially higher growth rate, which caused higher bacterial density. The comparatively higher growth triggered a decrease in pH of the precipitation medium which led to a lower carbonate ion concentration regardless of the higher total DIC concentrations. However, the presence of non-ureolytic bacteria enhanced higher rate of $CaCO_3$ precipitation. For this

reason, [10] indicated that the non-ureolytic bacterium, *B. subtilis*, facilitated $CaCO_3$ precipitation via supplying more nucleation sites. Furthermore, the similarity in zeta potentials of the two bacterial species buttresses the conclusion such that, the presence of non-ureolytic bacteria can have a noteworthy influence on ureolytic AMICP. Moreover, the scaling up of ureolytic AMICP takes into account likely relationships between ureolytic bacteria and native non-ureolytic bacteria and their consequence on the process of precipitation.

ACKNOWLEDGMENT

The Authors appreciate the University of Johannesburg where the study was carried out.

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