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Microbially induced $CaCO_3$ precipitation through denitrification: An optimization study in minimal nutrient environment

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ABSTRACT

So far, researchers investigated microbially induced $CaCO_3$ precipitation (MICP) for soil reinforcement, self-repairing concrete and Ca^{2+} removal from industrial waste streams. Reported MICP yields were mainly achieved under nutrient-rich conditions. However, creating the tested nutrient-rich conditions in intended applications is both an economical and a practical issue. Therefore, investigation of MICP in more realistic conditions is necessary. This study presents optimization of MICP through denitrification in minimal nutrient conditions. To optimize their MICP performances, we isolated two strains, *Pseudomonas aeruginosa* and *Diaphorobacter nitroreducens*, by following an application oriented selection procedure. Upon performance optimization, in 2 days, *D. nitroreducens* and *P. aeruginosa* precipitated 14.1 and 18.9 g $CaCO_3$/g NO_3-N, respectively. Repetitive $CaCO_3$ precipitation was also achieved from a single inoculum in both 2 days and 3 weeks intervals. Selected strains and the process were further evaluated for three MICP applications: (1) Ca^{2+} removal from paper mill wastewater (2) soil reinforcement, (3) crack repair in concrete. Overall, denitrification was found to be an effective process to remove Ca^{2+} from paper mill wastewater. *P. aeruginosa* and *D. nitroreducens* could be introduced as potential candidates for soil and concrete applications due to their enhanced precipitation yields, resilience and performance under minimal nutrient conditions.

1. Introduction

The metabolic activities such as sulfate reduction, iron reduction [1], urea hydrolysis [2–4], denitrification [5,6], methane oxidation [7] and photosynthesis [8] are known to give rise to microbially induced calcium carbonate ($CaCO_3$) precipitation (MICP). Boquet et al. [9] stated that $CaCO_3$ precipitation is a common as well as a circumstantial behavior in the bacterial world where most of the bacteria are able to precipitate $CaCO_3$ under proper conditions. For MICP, bacteria create substantially alkaline pH conditions and produce dissolved inorganic carbon [8,10]. Furthermore, bacterial cells act as ideal nucleation sites for formation of $CaCO_3$ crystals [4,10].

Since urea hydrolysis yields significant amount of carbonate ions and pH increase which induce the $CaCO_3$ precipitation, it was extensively investigated for applications such as soil improvement [1,11], Ca^{2+} removal from paper mill wastewater [14] and microbially self-healing concrete [15]. *Bacillus pasteurii* and *Bacillus sphaericus* were the most prominent species in those studies [2–4,11–13]. MICP through ureolysis has certain drawbacks in application. For instance, one of the purposes in sand or soil improvement applications is to decrease permeability and protect groundwater sources from leakages. However, urea hydrolysis generates ammonium, which may easily pollute these sources [6]. Anaerobic/anoxic conditions which inhibit ureolysis are also an issue in deeper parts of soil and concrete cracks. If the Ca^{2+} removal from industrial wastewater is considered, the cost of the treatment increases by addition of phosphoric acid and urea. More importantly, ammonia is produced and needed to be subsequently removed. These issues promoted attention to alternative pathways.

Oxidation of organic carbon by reduction of NO_3^-, so called denitrification, does not produce toxic by-products. Due to its highly negative standard Gibbs free energy (ΔG°) (Eq. (1)), denitrification can be expected to dominate in the presence of nitrate (NO_3^-) and organic carbon under O_2 limited conditions.

$$CH_2COO^- + 2.6H^+ + 1.6NO_3^- \rightarrow 2CO_2 + 0.8N_2 + 2.8H_2O$$
$$\Delta G^\circ = -785 kj/mol \quad (1)$$

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Table 1
Composition of the modified M9 media in different setups

Compounds		Isolation of strains & kinetic growth	Dehydration, re-activation	All $CaCO_3$ precipitation experiments
Buffer (g/L)	$Na_2HPO_4{\cdot}7H_2O$	8.5	–	–
	KH_2PO_4	3	–	–
M9 salt solution (g/L)	NaCl	0.5	0.5	–
	$MgSO_4$	0.24	0.24	0.24
	$CaCl_2$	0.011	0.011	6.325
Carbon source (g/L)	Na-Formate	6	6	6
	Methanol	4	4	–
Nitrate source (g/L)	KNO_3	0.72	1.011	–
	$Ca(NO_3)_2{\cdot}4H_2O$	–	–	1.18
Phosphorus source (g/L)	Na_3PO_4	–	0.105	0.0105

Biological reduction of NO_3^- generates CO_3^{2-} and HCO_3^- ions, which are necessary for $CaCO_3$ precipitation. It has 2 times higher carbonate yield per mole of electron donor (per mole of acetate) than ureolysis [5]. MICP through denitrification has been explored as an alternative pathway in soil reinforcement [5,6]. However, all the studies conducted so far were under nutrient-rich conditions and investigated for single use performance. Not only for soil reinforcement but also for other possible applications such as crack repair in concrete and Ca^{2+} removal from industrial waste streams, these conditions are quite rare. Moreover, in these particular applications, microorganisms often expose to harsh conditions such as high temperatures, starvation, nutrient (trace elements, vitamins, yeast) deficiency and dehydration. For instance, in closed system pulp and paper industries, wastewater temperatures may increase up to 60 °C [14]. Similarly, for concrete applications bacterial agents ought to withstand temperatures around 70 °C during cement hydration [15]. Dehydration stress mostly occurs in concrete and soil applications. During the curing of mortar, water reacts with cement particles and aggregates, thus the matrix become drier. Different from concrete, in soil, MICP itself decreases the porosity and water permeability of the soil, thus the available water for bacteria [11]. Moreover, for practicality, adding bacteria in the powder form is more preferable since it is easy to transport and has longer shelf-life. Apart from case specific stress conditions, starvation occurs in all the intended MICP applications since precipitation around the bacteria severely affect the nutrient diffusion kinetics [11]. Furthermore, single inoculum might need to perform repetitive precipitation in different intervals during which nutrients are limited or unavailable. This study presents optimization of MICP through denitrification in minimal nutrient environment by using two newly isolated resilient strains, *Pseudomonas aeruginosa* and *Diaphorobacter nitroreducens*. The strains were considered for Ca^{2+} removal from paper mill wastewater, crack repair in concrete and soil reinforcement. Among them, Ca^{2+} removal from paper mill wastewater was also experimentally studied to draw the attention to a more feasible and environmentally friendly process for MICP than currently used ureolysis. The study was conducted in three consecutive steps; (1) isolation and selection of appropriate strains (2) performance optimization and repetitive $CaCO_3$ precipitation (3) evaluation of the process and strains' performances for Ca^{2+} removal from wastewater, soil reinforcement and crack repair in concrete.

2. Materials and methods

In all experiments, 125 ml PYREX® serum bottles (Corning, USA) with rubber stoppers and metal caps were used. Reactor headspaces were flushed with Argon (Ar) gas to provide anoxic conditions. Incubations were carried out at 28 °C and on 120 rpm shaker. For all experiments, apart from the ones with paper mill wastewater, liquid and/or solid minimal media (modified M9 media) (Table 1), lacking of trace elements, yeast and vitamins, were used. Modified compositions of M9 media for different experimental set-ups are given in Table 1. Unless mentioned differently, for all $CaCO_3$ precipitation experiments non-buffered M9 media containing 2 mg/L PO_4-P (Table 1) (to minimize interference due to $Ca_3(PO4)_2$ formation, $K_{sp},Ca_3(PO4)_2) \ll K_{sp},CaCO_3$) was used and pH measurements were conducted at the beginning and at the end of the experiments. Initial biomass concentrations were set equally by using flow cytometer, and given as cells/mL throughout the study. Abiotic control experiments were simultaneously conducted to confirm precipitation due to bacterial activity.

2.1. Bacterial strains: isolation, characterization and selection

The denitrifiers were isolated from soil by inoculating two batch reactors containing sterile M9 media (Table 1) with a specific C-

Table 2
Isolated strains and closest taxonomy identification[a].

Label	Species	Query Length (R–F)	Ident % (R–F)
m1	*Pseudomonas denitrificans* ATC 13,867	1071–1115	99–98
m2	*Paracoccus denitrificans* PD1222	1091–801	97–96
m3	*Pseudomonas denitrificans* ATC 13,867	398–1044	98–99
m4	*Pseudomonas stutzeri* A1501	734–1155	97–99
m5	*Diaphorobacter nitroreducens* TPSY	1096–1096	99–99
f1	*Paracoccus denitrificans* PD1222	1000–776	98–99
f2	*Pseudomonas aeruginosa* PAO1	1021–116	99–92
f3	*Paracoccus denitrificans* PD1222	1133–NA	98–NA
f4	*Pseudomonas aeruginosa* PAO1	1123–941	97–98
f5	*Pseudomonas sp.* UW4	151–160	100–98

[a] m: The species isolated by using methanol as carbon source.
f: The species isolated by using formate as carbon source.
R: Results based on 1378r-reverse primer.
F: Results based on 63f-forward primer.