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# Microbial Phytase-Induced Calcium-phosphate Precipitation – a Potential Soil Stabilization Method

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**ABSTRACT.** Two hypotheses were tested: (*1*) microbial dephosphorylation of phytate in the presence of $Ca^{2+}$ ions will result in the precipitation of hydroxyapatite-like crystals and (*2*) precipitation of calcium-phosphate crystals on and between sand-like particles can cause cementation. A growing culture of the dimorphic phytase-active yeast *Arxula adeninivorans* was introduced into a column filled with quartz particles and subsequently a liquid growth medium amended with calcium phytate was pumped through the column resulting in increased strength and stiffness of the quartz particle matrix. Environmental scanning electron microscope analysis combined with energy-dispersive X-ray measurement revealed cementation of the quartz particles by calcium-phosphate crystals. This microbial mineralization process could provide a novel approach to improving the mechanical properties like strength and stiffness of sandy soils.

*Abbreviations*

| | | | |
|---|---|---|---|
| *A.a.* | *Arxula adeninivorans* | EDX | energy dispersive X-ray (analysis) |
| CaPh | calcium phytate | ESEM | environmental scanning electron microscope |
| *CBS* | *Centraalbureau voor Schimmelcultures* | YNB | Yeast Nitrogen Base |

Microbial metabolic activities often contribute to selective cementation processes by producing relatively insoluble organic and inorganic compounds. Microbially induced biomineralization, such as calcite ($CaCO_3$) and calcium hydroxyapatite [$Ca_5(PO_4)_3OH$] precipitation, has been described in a wide variety of environments (Boquet *et al.* 1973; Schmittner and Giresse 1999; Belzer *et al.* 2006; Canaveras *et al.* 2006; Kremer *et al.* 2008). The formation of calcium hydroxyapatite has received considerable attention as a result of its importance in biological calcification. Calcium phosphates are the main constituent of teeth and bones and have been found in pathological mineral deposits in the form of urinary and kidney stones.

The importance of microbially induced cementation has been widely recognized in petroleum, geological and civil engineering (Dejong *et al.* 2006; Ivanov and Chu 2008). There is a growing interest in methods to improve the mechanical properties of sandy soils using microbially induced biochemical reactions in the subsurface (Whiffin *et al.* 2007). These methods simulate natural diagenetic processes that transform loose sand to sandstone. It has been documented that cracks in rock formations, especially in oil reservoirs, could be remediated by microbial mineral precipitation (Jack 1991).

Phytic acid salts or phytates (*myo*-inositol hexakisphosphates) are abundant plant constituents. They typically account for 60–90 % of total seed phosphorus. Phytate is a natural by-product of fermentation processes and a waste product in the fermentation industry.

Phytases, a specific group of phosphatases, catalyze hydrolysis of the phosphate ester bonds of phytates (by the addition of water) (Cosgrove 1966; Mullaney *et al.* 2000). They have been found in various eukaryotic and prokaryotic microorganisms (Lassen *et al.* 2001; Lim *et al.* 2007). Extracellular phytases from several filamentous fungi and yeasts have been studied in detail. The dimorphic yeast *A.a.* can utilize phytate as a sole C and P source by secreting high level of phytase into its environment (Sano *et al.* 1999).

This paper aims to explore the potential use of microbiologically induced formation of calcium-phosphate crystals (Schmittner and Giresse 1999) to improve the stress-strain-strength properties of cohesionless sandy soils.

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## MATERIALS AND METHODS

*Strain and growth conditions*. *Arxula adeninivorans* CBS 7350 was obtained from the *CBS* (Utrecht, The Netherlands) and was grown at room temperature in (*1*) liquid minimal medium, (*2*) on solid medium plates prepared according to Lambrechts *et al.* (1992), and (*3*) in CaPh amended minimal medium in batch culture; samples for $A_{600}$ were taken at various time points over 26 h. To solid medium, 1 % $Ca^{2+}$-phytate (*myo*-inositol hexakisphosphate, calcium salt; *Sigma-Aldrich*, St. Louis, MO) was added as a sole C and P source, pH being adjusted to 6.5. Agar plates contained 2 % Bactoagar (*Difco*, USA). Cell growth at 25 and 30 °C in CaPh amended minimal medium was monitored by measuring the $A_{600}$ using an UV-1601 spectrophotometer (*Shimadzu*, Japan).

*Cementation of glass beads*. A 50 mL culture of *A.a*. cells grown overnight at room temperature under continuous shaking in CaPh amended minimal medium, was pumped with a peristaltic pump at a rate of 0.5 mL/min through two 60 mL polypropylene columns filled with ≈150 μm glass beads (*Sigma-Aldrich*) (Fig. 1A). After this treatment, which allowed the yeast cells to attach to the glass beads matrix, 2 L of CaPh

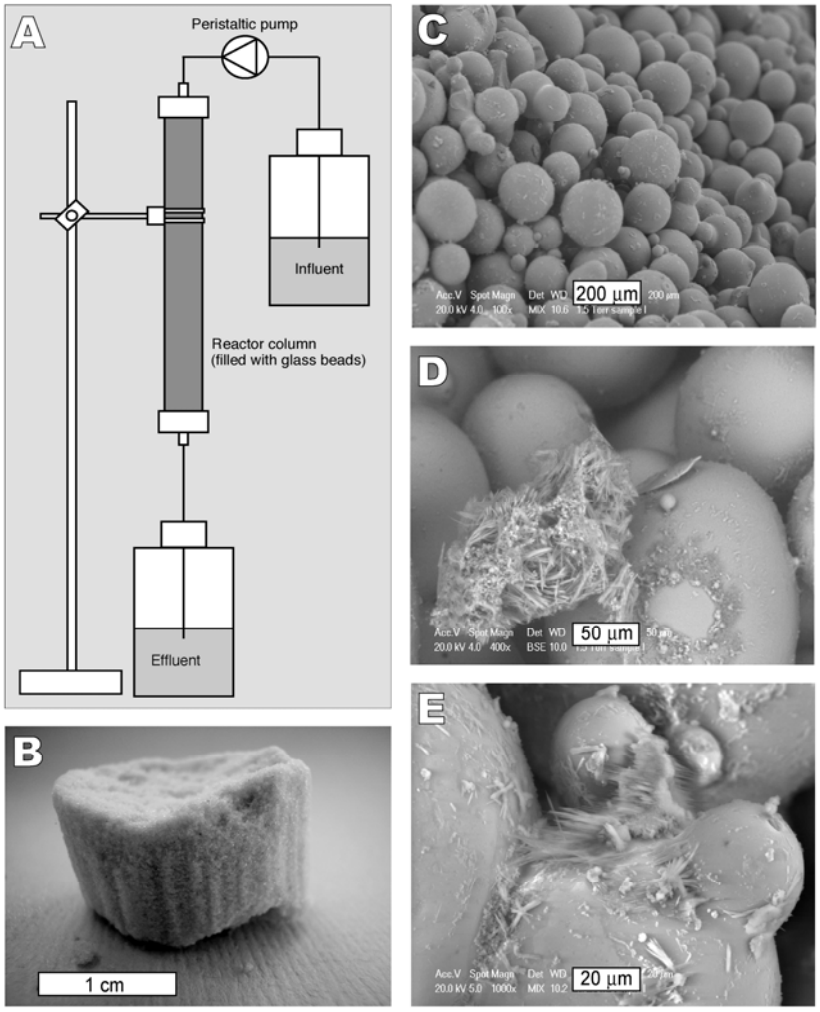


**Fig. 1.** A growing culture of *A.a*. was introduced in a column filled with glass beads (**A**); subsequently a liquid growth medium amended with CaPh was pumped through the column resulting in increased strength and stiffness of the glass beads matrix (**B**); ESEM analysis revealed cementation of the quartz particles by aggregates of needle-like crystals (**C**–**E**).

suspension (5.6 mmol/L) amended with 1 g/L YNB (*Invitrogen*, USA) was pumped (0.5 mL/min) through the glass bead packed columns. A parallel column, inoculated with *A.a*. and flushed under identical conditions with 2 L glucose solution (1 %) amended with 1 g/L YNB, served as a negative control. After a 3-d incubation at room temperature, the columns were flushed with 2 L of demineralized water (2 mL/min) and dried at room temperature.

*ESEM and EDX*. Cemented glass beads were examined with an ESEM (*Philips Electron Optics*, The Netherlands). The elemental composition of the calcium-phosphate crystals was determined by using a digital controlled EDX system, fitted to the ESEM. The samples were not coated because ESEM allows the examination of specimens in the presence of gases.

## RESULTS AND DISCUSSION

The average growth curves, determined from triplicates, showed a higher growth yield at 30 than at 25 °C (Fig. 2). Sano *et al.* (1999) showed that *A.a*. strains produce high level of phytase while growing actively at 40–50 °C, but these temperatures are irrelevant for field applications.

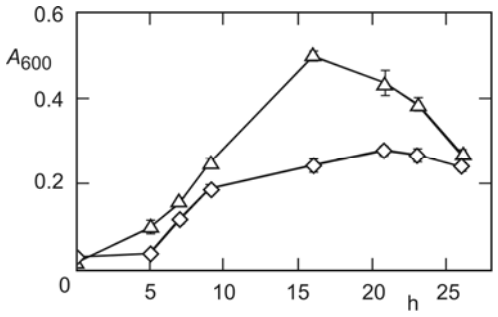


**Fig. 2.** Growth curves ($A_{600}$) of *A.a*. in minimal medium with CaPh as a sole source of P and C at 25 (*diamonds*) and 30 °C (*triangles*); *error bars* correspond to SD.

Introduction of *A.a*. in the quartz particles column and the subsequent addition of liquid growth medium amended with CaPh resulted in cementation of the quartz particles matrix (Fig. 1B). No significant cementation was observed in the negative control. ESEM analysis of cemented parts revealed aggregates of needle-like crystals in the pore space between the glass beads (Fig. 1C–E).

EDX analysis showed that the distribution of both Ca and P was associated with the presence of these crystals (Fig. 3). The uncovered areas of the quartz beads showed peaks corresponding to Si and O. The needle-like crystals gave a characteristic spectrum with major peaks corresponding to Ca, P, and O, and no significant peak corresponding to C. The Ca-to-P ratio was ≈1.55 suggesting a mixture of calcium-phosphate crystal forms such as monetite ($CaHPO_4$), whitlockite [$Ca_9(Mg,Fe^{2+})(PO_4)_6HPO_4$], and hydroxyapatite [$Ca_5(PO_4)_3OH$].

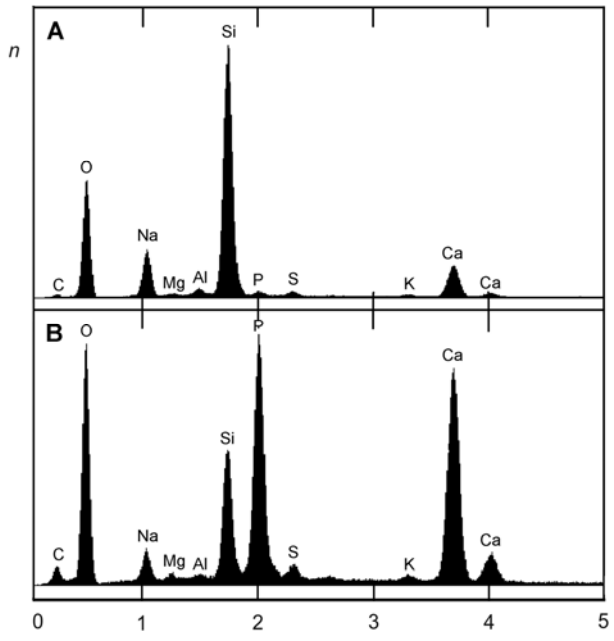


**Fig. 3.** Typical EDX spectra for small areas of quartz beads (**A**) and calcium phosphate crystals (**B**); *x*-axis – primary electron energy (keV), *y*-axis – X-ray counts (*n*).

The low solubility of CaPh at pH > 5 is a possible obstacle to its effective use as a biostabilization agent because it is difficult to introduce poorly soluble compounds into soils or sediments with small pore sizes. We observed (visual inspection) the strongest cementation in the upper half of the column, which probably resulted from gradual clogging of the pores with suspended yeast cells and CaPh particles and subsequent calcium-phosphate crystal formation.

Although phytases are produced by various groups of microbes (Lim *et al.* 2007), yeasts, which are simple mostly non-pathogenic Eukarya, with high extracellular phytase activity (Lambrechts *et al.* 1992), serve as ideal candidates to study the potential applications of *in situ* calcium-phosphate precipitation.

The most parsimonious interpretation of our results is that the degradation of CaPh complexes by phytase-active microorganisms can lead to the formation of calcium-phosphate crystals, such as hydroxyapatite [$Ca_5(PO_4)_3OH$], thermodynamically the most

stable calcium-phosphate salt. Microbially induced precipitation of calcium-phosphate crystals in cohesiveless siliciclastic soils can result in increased strength and stiffness. This process might be applicable to capture of radionuclides and metal contaminants in ground water systems, through the co-precipitation of these contaminants with calcium phosphate (Thomson *et al.* 2003). Therefore, its phytase-induced precipitation is one of the different microbial processes that should be explored for their applications in biocementation and bioremediation.

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