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Micrographical, minerological and nano-mechanical characterisation of microbial carbonates from urease and carbonic anhydrase producing bacteria

Navdeep Kaur Dhami [a], Abhijit Mukherjee [a,*], M. Sudhakara Reddy [b]

[a] Department of Civil Engineering, Curtin University, Bentley 6102, Australia
[b] Department of Biotechnology, Thapar University, Patiala 147004, Punjab, India

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ABSTRACT

Microbial carbonates are emerging as sustainable alternative cementing materials. We explore carbonate mineralisation by three bacterial isolates: *Sporosarcina pasteurii*, *Bacillus pumilis* and *Bacillus megaterium* that produce two enzymes urease and carbonic anhydrase. In vitro $CaCO_3$ precipitation by bacterial isolates under three different energy sources: urea, $NaHCO_3$ and CO_2 was monitored quantitatively and qualitatively. Cell viability in different treatments was studied through fluorescent microscopy. Morphological and chemical constituents of the crystals formed by different bacterial isolates were analysed by scanning electron microscopy, X-ray diffraction and Energy dispersive X-ray spectrum. In a first attempt we estimate the mechanical properties of microbial products through nanoindentation and correlate them with the established techniques such as SEM and XRD. The biochemical processes produced a mixture of two forms of carbonate, calcite and vaterite, in different proportions. We demonstrate that it is possible to engineer the mechanical properties of the carbonates by controlling the biochemical processes.

1. Introduction

The diversity of hierarchical micro-/nano-structures present in Nature is a leading source of inspiration for engineers and material scientists for designing materials with targeted properties. Nature synthesises these materials in ambient conditions (thus requiring very little energy) from organic matters and low viscosity solvents (generally water) with little risk of negative environmental consequences. These materials follow an optimal topological arrangement to satisfy multifunctional requirements (Chen et al., 2012). Moreover, natural materials are renewable, recyclable, and often self-healing. Among the emerging processes biomineralization of carbonates, especially microbially induced calcium carbonate precipitation (MICP) is gaining acceptance as a sustainable cementing material in construction (Achal et al., 2015; Dejong et al., 2013; Dhami et al., 2013c). MICP closely mimics the natural carbonate formations such as microbialites, limestone and corals. The role of microbes in creating these strong and durable geological formations has been unravelled (Castanier et al., 1999) and successfully emulated in the laboratory (Dhami et al., 2012a, 2014a; Stocks-Fischer et al., 1999). More recently, the ability of MICP in bringing some of the benefits of natural processes to engineered constructions has been demonstrated (Dejong et al., 2013; Phillips et al., 2013; Ramachandran et al., 2001). MICP was found to resist the deterioration of concrete in freeze thaw, sulphate attack and drying shrinkage (Ramachandran et al., 2001). In case of reinforced concrete MICP retards moisture and chloride ion diffusion and corrosion of steel (Dhami et al., 2012a). Several progresses have been made in unravelling the biochemical processes (Bains et al., 2015; Cacchio et al., 2012; Castanier et al., 1999; Dhami et al., 2014b), improving the bacterial strains (Achal et al., 2009b; Bergdale et al., 2012), use of industry by-products (Achal et al., 2009a; Dhami et al., 2012b), and process optimisation (Okyay and Rodrigues, 2015). The range of increase in compression strength of cement mortar and concrete reported by various investigators varies from negligible to about 35% (Dhami et al., 2013d, 2013c). Field applications of the technology are beginning to emerge (Dejong et al., 2013; Zhu and Dittrich 2016). However, to have wide engineering application it is essential to reliably know the mechanical properties of the carbonates. In this paper, we present a methodology for estimation of these properties and

* Corresponding author.
E-mail address: abhijit.mukherjee@curtin.edu.au (A. Mukherjee).

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demonstrate the potential of the biochemical process of tailoring them according to engineering needs.

Nature performs biomineralisation following several alternative pathways in varying environments. Following these different routes and conditions it should be possible to generate biominerals with targeted properties. Some of these pathways have been successfully replicated in the laboratory. Several microbial routes have been found to produce carbonates in nature but amongst all, bacteria producing two different enzymes urease (UA) and carbonic anhydrase (CA) have been found to have great potential for production of carbonates in lab. Such bacteria can utilise different substrates such as urea, sodium bicarbonate and carbon dioxide for production of carbonates. While the UA route is most well researched for field applications, CA promises to sequester atmospheric carbon dioxide and therefore, give additional sustainability benefits. Moreover, in our prior research we demonstrated that various carbonate polymorphs can be produced by choosing different pathways and environments (Dhami et al., 2013a, 2014b) (Dhami et al., 2013a, 2014b). This paper reports interconnection between mineralisation pathways and corresponding morphologies along with mechanical properties of the biominerals. Carbonate mineralisation by three bacterial isolates: *Sporosarcina pasteurii* (high UA producing), *Bacillus pumilis* (high CA producing), *Bacillus megaterium* (both UA and CA producing) have been reported. This is the first study on analysis of mechanical properties of microbial crystals through different routes by nanoindentation.

In our previous work, we reported the synergistic role of bacterial CA with UA in MICP in case of *Bacillus megaterium* (Dhami et al., 2014b). Dual participation of UA and CA in calcification has also been reported in *B. simplex* and *B. sphaericus* by Achal and Pan (2011) and Komala and Khun (2014). Several carbonate polymorphs of $CaCO_3$ are formed under different conditions including three anhydrous forms: calcite (rhombic), aragonite (needle like) and vaterite (spherical), two hydrated crystalline phases, monohydrocalcite ($CaCO_3{\cdot}H_2O$) and ikaite ($CaCO_3{\cdot}6H_2O$), and various amorphous phases (ACC) (Gebauer et al., 2010; Rieger et al., 2007). The strength and durability of the carbonate depends on the type and structure of the precipitated $CaCO_3$ polymorphs (Rodriguez-Navarro et al., 2012). The formation of different polymorphs by bacteria has been reported to be dependent on various factors like composition of growth medium, type of substrate, temperature, pH, saturation index, $[Ca^{2+}]/[CO_3{}^{2-}]$ ratio and bacterial species (Gorospe et al., 2013; Rodriguez-Navarro et al., 2012). Rodriguez-Navarro et al. (2012) reported that the fate of final calcium carbonate polymorph formed during biomineralisation in a particular situation depends on the amount of dissolved organic carbon (DOC) due to bacterial activity. At a low DOC, formation of calcite is favoured while a high DOC promotes stabilization of vaterite. Local environment too plays an important role in determining the texture, strength and mechanical properties of biominerals (Kim et al., 2011; Zamiri and De, 2011). In this paper, we correlate the morphologies and the mechanical properties of microbial carbonate polymorphs formed in different environments with varying carbon sources.

2. Materials and methods

2.1. Microbial strains and culture conditions

Three different microbial strains were used in the present study (Table 1). *Sporosarcina pasteurii* (SPs) is a well-known and well researched strain that produces high amount of urease (UA) and is most commonly applied for MICP in materials. *Bacillus pumilis* (BPm), on the other hand, is known for high carbonic anhydrase (CA) production with great potential for sequestration of CO_2. The third strain, *Bacillus megaterium SS3* (BMg), has been found to produce both the enzymes in significant amounts by the present authors (Dhami et al., 2014b). Standard nutrient broth (5.0 g peptone, 1.5 g beef extract, 1.5 g yeast extract and 5.0 g sodium chloride per litre) was used to culture the bacterial isolates. The aim of this part of the experiment is to observe the effect of different routes (inducing UA and CA) on production of urease and carbonic anhydrase enzymes in the three isolates. For this purpose, specific media and supplements for enhancing production of target enzymes have been used (Table 1). The tests were divided into three groups: In group 1 for UA production, nutrient broth with 2% urea and 10 μM $NiCl_2$ was used and the pH was maintained at 8.0. In group 2 for inducing the production of CA, 10 μM zinc sulphate and 25 mM $NaHCO_3$ were supplemented in the nutrient broth media with pH 8.0 (Dhami et al., 2014b). In group 3, 99.5% pure CO_2 at constant rate of 20 ml/min was passed for 1 min in closed flask with Nutrient broth media (at initial pH 10) supplemented with $ZnSO_4$ to achieve final pH of 8.0. All the sets were inoculated with 5×10^7 cells/ml of specified bacterial cultures (SPs and BMg in set 1 with urea; BPm and BMg in set 2 and 3 with $NaHCO_3$ and direct CO_2). The sets were prepared in 500 ml Erlenmeyer flasks containing 100 ml of the specified media. All the flasks were prepared in triplicates and incubated at 37 °C in an orbital shaker at 120 rpm for 120 h.

Changes in pH were determined up to 24 h by withdrawing the culture broths at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72, 96 h. UA and CA activity were also determined by collecting the culture broths after 24, 48, 72, 96 and 120 h.

2.2. In vitro $CaCO_3$ precipitation

To evaluate the effect of different treatments and routes on calcium carbonate precipitation, all the sets described in Section

Table 1
The bacterial strains and media.

Identifier	Strain used	Media and supplements	Enzyme induced	Route
SPU	*Sporosarcina pasteurii ATCC 11859* (standard UA producing culture (Stocks-Fischer et al., 1999)	Nutrient broth +2% Urea + 10 μM $NiCl_2$—pH 8.0 (Dhami et al., 2014a,b)	UA	Ureolytic
BPN	*Bacillus pumilis* (Wanjari et al., 2011) (standard CA producing culture)	Nutrient broth + 25 mM $NaHCO_{3+}$ 10 μM $ZnSO_4$—pH 8.0 (Dhami et al., 2014a,b)	CA	CO_2 sequestration
BPC		Nutrient broth + CO_{2+} 10 μM $ZnSO_4$—pH 8.0	CA	CO_2 sequestration
BMU	*Bacillus megaterium SS3* (Dhami et al., 2014a,b) (Both UA and CA producing lab isolate)	Nutrient broth + 2% Urea + 10 μM $NiCl_2$—pH 8.0	UA	Ureolytic
BMN		Nutrient broth + 25 mM $NaHCO_{3+}$ 10 μM $ZnSO_4$—pH 8.0	CA	CO_2 sequestration
BMC		Nutrient broth + 99% CO_2 1 min + 10 μM $ZnSO_4$—pH 8.0	CA	CO_2 sequestration