IOP Conf. Series: Materials Science and Engineering **427** (2018) 012022 doi:10.1088/1757-899X/427/1/012022

Biomineralization of calcium carbonate controlled by biomolecules produced by *Bacillus* sp.

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Abstract. In this study, culture supernatant of Bacillus subtilis KP7 growing on liquid LB medium consisted of 2 % of molasses was used for calcium carbonate synthesis. Synthesis of the calcium carbonate structures was performed in a reactor by mixing $CaCl_2$ and Na_2CO_3 solutions at 25°C. The calcium carbonate particles were characterized by optical microscopy, FTIR and XRD analysis. In addition, the size distribution and the value of zeta potential were determined. It was observed that properties of obtained calcium carbonate depend on the supernatant amount. The smallest crystals of pure calcite with disordered structure were obtained when the concentration of the cell-free supernatant was 5 % (v/v).

1. Introduction

Calcium carbonate is an extremely important material both in the fundamental research and industry due to their properties, such as non-toxicity, high porosity, and surface area [1]. In nature, calcium carbonate is a biomaterial consisted of both mineral (c.a. 95 %) and organic (c.a. 5 %) phases [2]. The organic content involved in biomineralization was found to be mostly biomolecules containing the functional groups such as carboxylic, phosphate, sulfate, hydroxyl or amino [3]. Those biomolecules can act as nucleation sites, and control the nucleation and further growth of the crystals to desired shape and

morphology. During the last decades a wide range of organic additives or templates have been used in the precipitation of calcium carbonate to obtain product with desired properties [1]. A very interesting issue seems to be the application of molecules produced extracellularly by microorganisms as agents controlling the growth of calcium carbonate. It is well known that in nature microorganisms can induce the mineralization of calcium carbonate via three mechanisms: as a result of microbial metabolic pathways, ions exchange through the cell membrane, and extracellular polymeric substances (EPS) mediated mineralization [4]. These EPS may influence mineral formation by trapping calcium ions and inhibit the transformation of metastable to thermodynamically stable phases [5-7].

The purpose of this study was to investigate the influence of cell-free supernatant on the course of calcium carbonate precipitation. The application of cell-free supernatant to control the morphology of calcium carbonate allows to develop a new eco-friendly technology of inorganic materials production. The culture supernatant contains not only the EPS (protein, polysaccharides, and enzymes) but also the other metabolic products, such as biosurfactants and other ions, which can affect the shape, size and polymorphs of calcium carbonate. It is happened because in living organisms not only the proteins but also the content of reaction fluid (ions, polysaccharides, amino acids, and other metabolic products) takes part in biomineralization of calcium carbonate.

2. Materials and Methods

Bacillus subtilis KP7 was obtained from a collection owned by the Department of Industrial Microbiology and Biotechnology, University of Lodz. Production of bio-products by Bacillus sp. was carried out as described previously in Paraszkiewicz et al. [8]. The culture was grown on a media containing 2 % of beet molasses. The strain was cultivated aerobically for 96 h in a shaker (120 rpm) and at a constant temperature, 30 °C. Next, to remove the bacterial cells from the media the culture solution was centrifuged at 10.000 x g for 20 min and then filtered using a cellulose nitrate membrane filter (0.22 μm diameter of pore; Sartorius). The surface tension of the supernatant was measured using a ring method (type K100, Krüss). The pH of supernatant was found to be 8.0±0.1.

Calcium carbonate was synthesized using a semi-batch method at 25 °C. Firstly, 125 ml of calcium chloride (purity > 99 %, Across Organics) and di-sodium carbonate (p.a., Avantor) solutions at 0.01 M were separately prepared. Then, the calcium chloride solution was placed in a reactor vessel equipped with a mechanical stirrer (type OS20 S, ChemLand, Poland). The reaction was started when the Na_2CO_3 solution was dozing (Masterflex L/S, Cole Parmer) to the reactor vessel with constant rate, 150 ml/min. The mixture was stirred at a constant speed for 1 h. After that the precipitated calcium carbonate was removed by centrifugation (MPW, Poland) at 4000 rpm for 10 min. The precipitate was washed several times with deionized water and once with ethanol (96 %, POCh). Next, the solid was dried at 40 °C and kept in a desiccator for further analysis. In the experiment with biomolecules, the calcium ions were contacted with cell-free supernatant at concentration 2.5 and 5 % for 24 h.

The shape of calcium carbonate was characterized by optical microscopy (AxioImager M 1, Zeiss). The polymorphs of the precipitate was analysed by X-ray diffraction (D8 ADVANCE, Bruker, Massachusetts, USA). The size of crystals was analysed using a laser diffractometer (Mastersizer2000, Malvern, UK). The zeta potential of the crystals was measured using a Zetasizer2000 apparatus (Malvern, UK).

3. Results and discussion

The morphologies of calcium carbonate precipitated in the presence of cell-free supernatant are presented in figure 1. From figure 1a-b it can be seen that in the control sample synthesized without

biomolecules a mixture of rhombohedral calcite and spherical vaterite crystals were formed. Addition of various amount of cell-free supernatant produced by Bacillus subtilis KP7 resulted in formation of only one phase, calcite crystals with some deformation on the surface. From literature it is known that biomolecules can affect the morphology of crystals by presence of some irregularities (e.g. terraces and depletion) on the surface, and distribution of the crystals' edges [1,7]. The crystals' structure was more distorted when more supernatant were added to the reaction system.

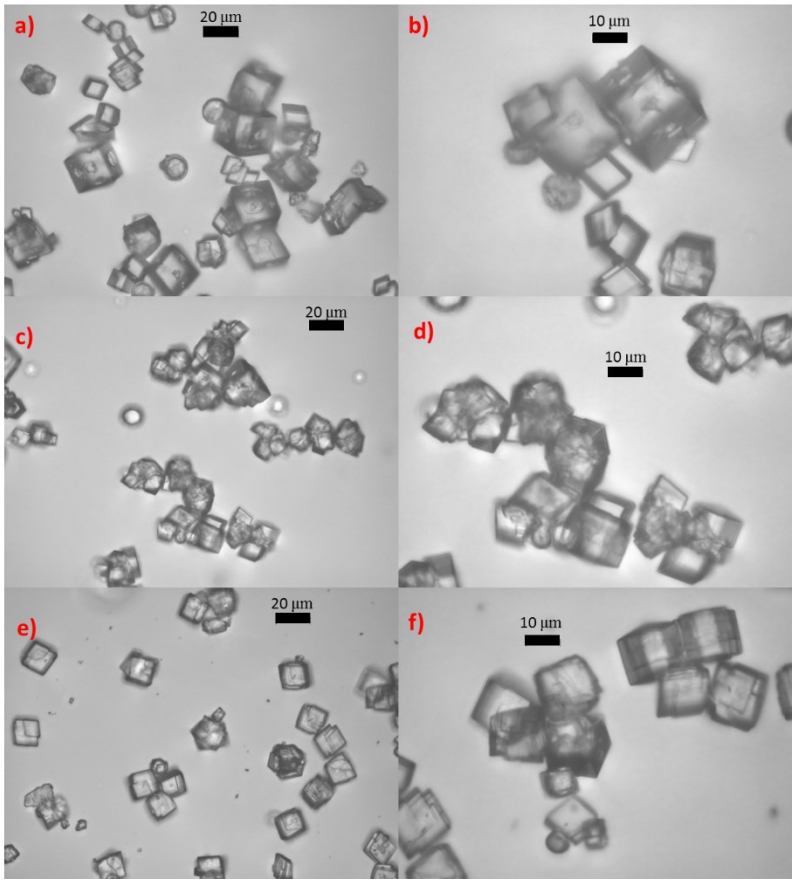

Figure 1. Optical images of calcium carbonate crystals obtained in the presence of biomolecules produced by *Bacillus subtilis* KP7. (a-b) control sample (without biomolecules), (c-d) 2.5 %, (e-f) 5 %.

The polymorphs of obtained crystals were analysed by X-ray Powder Diffraction technique, and the XRD patterns are shown in Figure 2. The presence of calcite indicates the peaks located at 2θ values of 23.0; 29.4; 36.1; 39.4; 43.1; 47.5; 48.5°. Whereas, the formation of vaterite in the system is evidenced by peaks at 24.9, 27.2; 32.9; 43.1; 50.2° (JCPDS 00-033-0268). Based on the data presented in Figure 2, the phase composition of precipitates was calculated using the Rietvield method. It was found that the control sample contains 15.1 % of vaterite and 84.9 % of calcite. The sample synthesized with 2.5 % of the supernatant is consisted of 1.2 % of vaterite and 98.8 % of calcite. While addition of 5 % of the supernatant leads to the formation of pure calcite. These data suggest that biomolecules present in

the supernatant favours the formation of calcite crystals in the reaction medium. It is worth to mention that the supernatant contained not only the EPS but also (bio)molecules (fermentable sugars, D- and L-lactic acid, short-fatty acids, phenolic compounds and products of sugar caramelization) delivered from the molasses [9]. All those biomolecules can possess functional groups which can bind calcium ions and can be involved in the mineralization of calcium carbonate in nature. This interaction can affect the nucleation rate as well as the crystals growth [1].

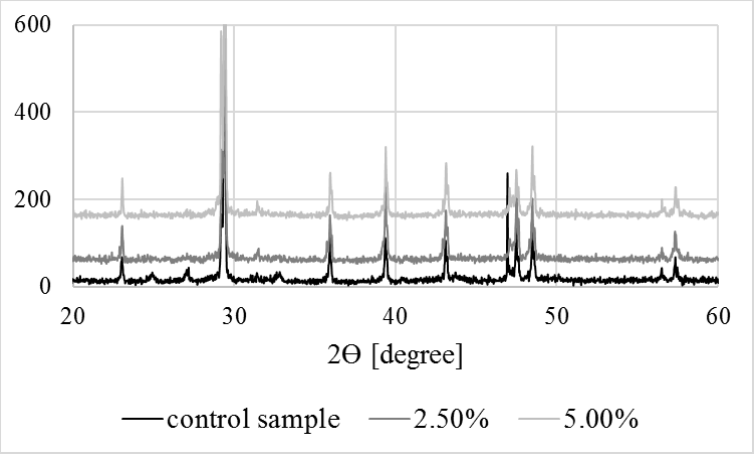

Figure 2. XRD patterns of calcium carbonate crystals obtained in the presence of bio-products produced by *Bacillus subtilis*.

The size distribution and the diameters of crystals are presented in figure 3 and table 1. It can be seen that biomolecules in the supernatant reduce the size of formed crystals. The mean diameter (d_{50}) was 27.7, 17.7 and 12.1 μm for crystals obtained at 0, 2.5 and 5 % of the supernatant concentration, respectively. The values of d_{10} and d_{90} decreased with an increase in the supernatant content in the reaction mixture from 16.8 to 6.1 and from 43.5 to 22.2 μm, respectively. We believe that the biomolecules can react not only with calcium ions but also with the crystals surface inhibiting the growth of crystals. For this reason, the small particles are formed in the presence of cell-free supernatant.

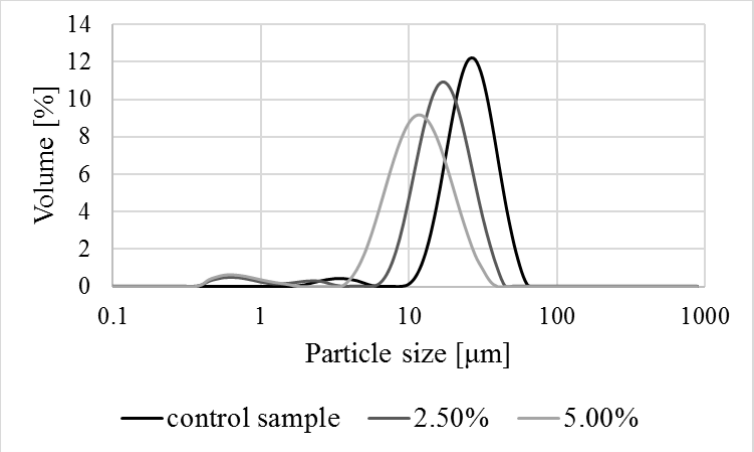

Figure 3. Size distribution of calcium carbonate crystals obtained in the presence of bio-products produced by *Bacillus subtilis* KP7.

Table 1. Effect of cell-free supernatant concentration on the crystal's size (The standard deviation is lower than 1 μm).

Concentration [%]	d_{10} *[μm]*	d_{50} *[μm]*	d_{90} *[μm]*
0	16.8	27.7	43.5
2.5	10.0	17.7	29.4
5.0	6.1	12.1	22.2

The presence of the biomolecules at the surface of the crystals was proven by zeta potential measurements and the data are presented in Table 2. It was observed that the particles precipitated without the additives possess negative value of zeta potential equals -25.6 mV. The value was changing when the supernatant was added to the reaction medium and it was -30.6 and -36.4 at 2.5 and 5 % of the supernatant concentration, respectively. We suspect that the changes in the zeta potential value results from the presence of the biomolecules at the crystals surface.

Table 2. Zeta potential (ζ) values of calcium carbonate crystals precipitated in the presence of biomolecules (The standard deviation is lower than 1 mV).

Concentration [%]	ζ *[mV]*
0	-25.6
2.5	-30.6
5.0	-36.4

Literature data showed that *Bacillus* species are microorganisms producing extracellularly various proteins (mostly enzymes), amino acids, biosurfactants (surfactin, iturin and fengicyn) and a lot of

polysaccharides [8]. The presence of biosurfactants was proven by surface tension measurements, and it was found to be 35.03 mN/m. The biomolecules possess functional groups such as carboxyl, hydroxyl, amino and aliphatic. Among them the carboxyl and hydroxyl group are suggested to play a major role in the biomineralization.

4. Conclusions

Presented data showed that the biomolecules produced by *Bacillus subtilis* KP7 growing on media containing 2 % of molasses can be used as agent to control the growth of calcium carbonate. These biopolymers can affect the morphology, size and surface properties of the crystals. It was shown that smaller crystals with some irregularities at the surface were formed when in the reaction medium the cell-free supernatant was added. This finding encourage us to further studies on this topic. We believe that using cell-free supernatant produced by *Bacillus* sp. can develop an eco-friendly technique to produce inorganic matrices for environmental application. Moreover, the application of molasses as a source of nutrition to microorganisms growth can reduce the cost of biopolymers production.

Acknowledgments

Authors wishing to acknowledge the National Centre of Science for financial support (project no. 2014/15/D/ST8/00544).

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