Extracellular polymeric substance of Rhodococcus opacus bacteria effects on calcium carbonate formation

Aleksandra Szcześ [1], Magdalena Czemierska [2], Anna Jarosz-Wilkołazka [2], Edyta Magierek [3], Emil Chibowski [1], Lucyna Hołysz [1]

[1] Department of Physical Chemistry - Interfacial Phenomena, Faculty of Chemistry, Lublin, Poland

[2] Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland

[3] Institute of Agrophysics Polish Academy of Science, Lublin, Poland

Corresponding author: aszczes@poczta.umcs.lublin.pl (Aleksandra Szcześ)

Abstract: An extracellular substance extracted from the bacterial strain *Rhodococcus opacus* was used as a template for calcium carbonate precipitation from $CaCl_2$ and Na_2CO_3 solutions at 25 and 37 °C. Obtained crystals were characterized using scanning electron microscopy (SEM), X-ray diffraction (XRD) and Raman spectroscopy. Kinetics of precipitation was investigated by conductivity measurements.

The obtained results show that the used biopolymer does not affect the crystal structure but acts as a nucleation centers leading to the formation of calcite crystals with the regular size. This effect is concentration dependent i.e. the EPS concentration increase causes the crystal size decreases, and is greater if calcium chloride solution if mixed with EPS 15 min before the addition of sodium carbonate solution. The temperature increase strengthens this effect.

Keywords: calcium carbonate, exopolymer, mineralization

1. Introduction

Carbonate mineralization induced by microbes is a commonly known phenomenon and intensively described in the literature (Ferris et al., 2003; Braissant et al., 2003; Hammes et al., 2003; Dick et al., 2006; Lian et al., 2006; Braissant et al., 2007; Rodriguez-Navarro et. al., 2007; Kremer et al., 2008; Bains et al., 2015; Phillips et al., 2016). However, the mechanism of this phenomenon is still not fully and precisely explained. In the literature three main mechanisms of bacterially mediated mineralization occur: mineralization as a by-product of microbial metabolism, mineral formation on cell walls due to the ion exchange through the cell membrane and the extracellular macromolecules mediated mineralization (Lian et al., 2006). It was shown that bacterial strains with a negative zeta potential and specific functional group present on the microbial walls are capable to control calcium carbonate formation through the ability of divalent cations binding that makes them good crystal nucleation centres (Hammes et al., 2003; Lian et al., 2006). Extracellular polymeric substances (EPS) produced by microorganisms such as bacteria may also influence mineral precipitation by trapping calcium ions (Kawaguchi and Decho, 2002; Braissant et al., 2003; Ercole et al., 2007; Bains et al., 2015; Szcześ et al., 2016). These EPSs are polymeric macromolecules mainly composed of proteins, polysaccharides, glycoproteins and nucleic acids (Lee et al., 2014; Liu et al., 2014; Czemierska et al., 2017).

Calcium carbonate is abundant mineral in the world occurring as different polymorphs: amorphous calcium carbonate, calcium carbonate hexahydrate, calcium carbonate monohydrate, vaterite, aragonite and calcite, as ordered according to its decreasing solubility (Xue et al., 2009). In the literature it was shown that bio-organic macromolecules, such as proteins and polysaccharides can affect the formation and stabilization of calcium carbonate crystals (Huang and Zhang, 2012; Ma et al., 2012; Szcześ et al., 2016, Sadowski et al., 2016). Essentially, organic biomolecules may act by two ways:

as a template for mineral precipitation and/or inhibiting the transition from metastable to stable phases (Huang and Zhang, 2012). The specific functional groups of organic molecules produced by microbial cell, especially acidic groups such as carboxyl and phosphate groups, may bind cations, such as Ca^{2+}, and hence act as the crystal nucleation sites (Hood et al., 2014; Szcześ et al., 2016).

In the previous study using vapour diffusion method, we found that the extracellular polymeric substance produced by bacterial strain *Rhodococcus opacus* deposited on the mica surface may control calcium carbonate precipitation (Szcześ et al., 2016). In this study, mineralization experiments in the presence of extracellular polymeric substance produced by bacterial strain *Rhodococcus opacus* were conducted by the rapid mixing of calcium chloride and sodium carbonate solutions. The purpose of this research was to identify the organic template ability to bind calcium ions and the effect on calcium carbonate precipitation at room temperature 25 °C and at temperature 37 °C. The room temperature is also an optimal growth temperature for the bacterial strain used in these studies and in the further research we plan to precipitate calcium carbonate in the living cultures of *R. opacus* at this temperature. As bacteria have also an effect on pathological formation of minerals such as gallstones and kidney stones (Olavi and Çiftçioglu, 1998; Dhami et al., 2013), physiological temperature (37 °C) was chosen.

2. Materials and methods

2.1 Extracellular substance production and purification

Rhodococcus opacus was obtained from the collection owned by the Department of Biochemistry, Maria Curie-Sklodowska University, Poland. The strain was stored at 4°C in an agar medium consisted of yeast extract 0.04, malt extract 0.1, glucose 0.04 and agar 0.2 in g/dm^3. Production of *Rhodococcus opacus* was carried out as described previously in (Czemierska et al., 2016). Shortly, the cultivations of the bacteria were carried out for 7 days on a rotary shaker (130 rpm, 26°C) in 3-L Erlenmeyer flasks containing 1.5 dm^3 of liquid medium consisted of 30 g glucose, 3 g KH_2PO_4, 7.5 g K_2HPO_4, 0.75 g NH_4Cl, 0.15 g NaCl, 0.75 g $MgSO_4$ and 0.75 g yeast extract (Kurane et al., 1994). All the components of medium were purchased from Avantor, Poland. To remove bacterial cells after the incubation the culture solution was centrifuged twice at 9200 rpm for 30 min. Then the supernatant was concentrated about five times using a reverse osmosis process, centrifuged twice at 9200 rpm for 30 min and filtered using a Durapore membrane (0.45 µm diameter of pore; Millipore). Two volumes of cold ethanol (95%) were added to the filtrated solution, and then the mixed solution was left to stand at 4 °C for 72 h. After this time, the solution was centrifuged at 9200 rpm for 30 min and *precipitate I* was dissolved in distilled water and left overnight at 4 °C. Then the supernatant was again precipitated by adding one volume of cold ethanol (95%) and incubated for next 72 h at 4 °C. After centrifugation (9200 rpm, 30 min), *precipitate II* was resuspended in a distilled water and combined with the *precipitate I*. Thus obtained the combined precipitate was dialysed for 3 days at 4 °C to remove ethanol, and after dialysis it was freeze-dried using a lyophilisator (Labconco, USA). Then the obtained powder of the total exopolymer was obtained and used in the mineralization experiment.

2.2 Crystallization experiments

A 20 cm^3 of 0.05M $CaCl_2$ (p.a., Avantor Performance Materials Poland S.A.) solution was mixed with appropriate volume of exopolymeric substance dispersed in water and immediately 20 cm^3 of 0.05 M Na_2CO_3 (p.a., Avantor Performance Materials Poland S.A.) solution was added. The mixture was stirred with a glass stirrer for 1 hour. After that, the reaction products were centrifuged, washed with water and dried in a desiccator at room temperature. The precipitation was carried out at 25±1 °C and 37±1 °C. The final exopolymeric substance concentrations during precipitation were 80 mg/dm^3 and 160 mg/dm^3. As a reference system calcium carbonate was precipitated without additives by mixing 20 cm^3 of 0.05 M $CaCl_2$ solution with 20 cm^3 of 0.05 M Na_2CO_3 solution and stirring for 1 h at a constant speed. After this time period the reaction products were centrifuged, washed with water and dried in a desiccator at the room temperature.

To study the influence of addition time of Na_2CO_3, firstly $CaCl_2$ solution was mixed with EPS of total concentration 80 mg/dm^3 and after 15 min stirring the sodium carbonate was added to the

system. Then the precipitation was carried out like in other systems. The rate of the calcium carbonate formation was estimated from the plot of conductivity changes as a function of time.

The water used was from a Milli-Q plus water purification system (Millipore, USA) with a resistivity of 18.2 MΩ cm.

2.3 Precipitate characterization techniques

The morphology and crystal form of the precipitated calcium carbonate crystals were investigated by scanning electron microscopy ((Quanta 3D FEG, FEI), Raman spectra (inVia Reflex instrument, Renishaw, UK) and X-ray diffraction (Empyrean, PANalytical). Phase composition of the precipitated calcium carbonate was calculated by using the software provided with the apparatus based on Rietveld's method (Rietveld, 1969; Scrivenera et al., 2004). The X-ray diffraction of powder samples results in a pattern characterised by reflections at certain positions. Their height, width and position can be used to determine many aspects of the material's structure. Ritveld's method consists in matching the experimental pattern with a theoretical pattern.

2.4 Determination of Ca^{2+} adsorption to exopolymeric substance via zeta potential measurements

The exopolymeric substance was dispersed in a 1mM NaCl solution and containing the EPS concentration equal to 250 mg/dm³. To obtain different concentration of calcium ions, $CaCl_2$ solutions of different concentrations were added and the mixture was stirred vigorously by hand for 30 seconds. Then, after 15 min the electrophoretic mobility was measured using Zetasizer Nano (Malvern, UK) and the zeta potential was calculated from Smoluchowski equation. All measurements were repeated 3-5 times. The time after which the zeta potential was measured was chosen on the basis of the preliminary results of conductivity measurements versus time (results not shown here) in the system containing EPS and calcium ions.

3. Results and discussion

Characterization of extracellular polymer from *Rhodococcus opacus* using infrared spectroscopy and X-ray photoelectron spectroscopy (XPS) showed that the acidic molecule contained carboxyl, hydroxyl, ammonium and phosphate groups (Czemierska et al., 2016). It suggested that it would be able to bind metal ions, e.g. calcium ions, and thus these as the crystal nucleation sites could facilitate the calcium carbonate formation. In order to confirm this hypothesis the changes in the zeta potential of extracellular polymeric substance dispersion due to the adsorption of calcium ions were measured and they are presented in Fig. 1.
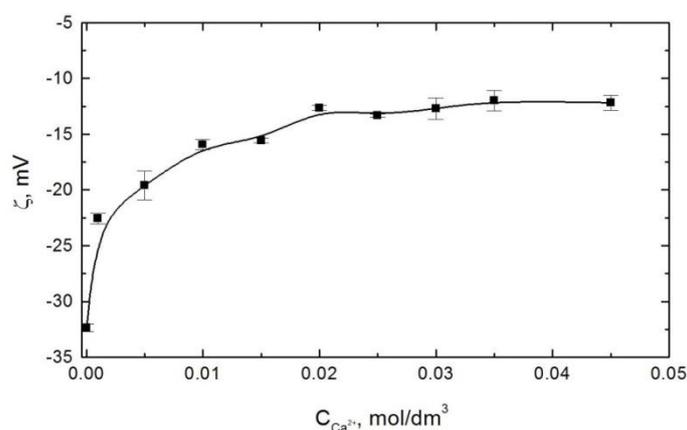
Fig. 1. Zeta potential changes of the EPS as a function of calcium ions concentration (the pH of the solution is 7.5)

The value of zeta potential of exopolymeric substance solution without calcium ions is around -32.0 mV and tends towards less negative values with increase in Ca^{2+} concentration until it reaches plateau at calcium ion concentration equals to 0.02 M. This is caused by adsorption of calcium ions to the functional group of exopolymeric substance and confirm calcium ions binding capacity for EPS produced by *Rhodococcus opacus* bacteria strain.

To investigate the effect of exopolymeric substance obtained from bacterial strain *R. opacus* on calcium carbonate precipitation $CaCl_2$ and Na_2CO_3 solutions of a concentration equal to 0.05 M were selected. SEM technique was used to learn about morphological structure of the obtained deposits. Figs. 2 and 3 show SEM images of $CaCO_3$ precipitated at 25 and 37 °C for the duration of 1 h, in the absence and presence of the additive, respectively.
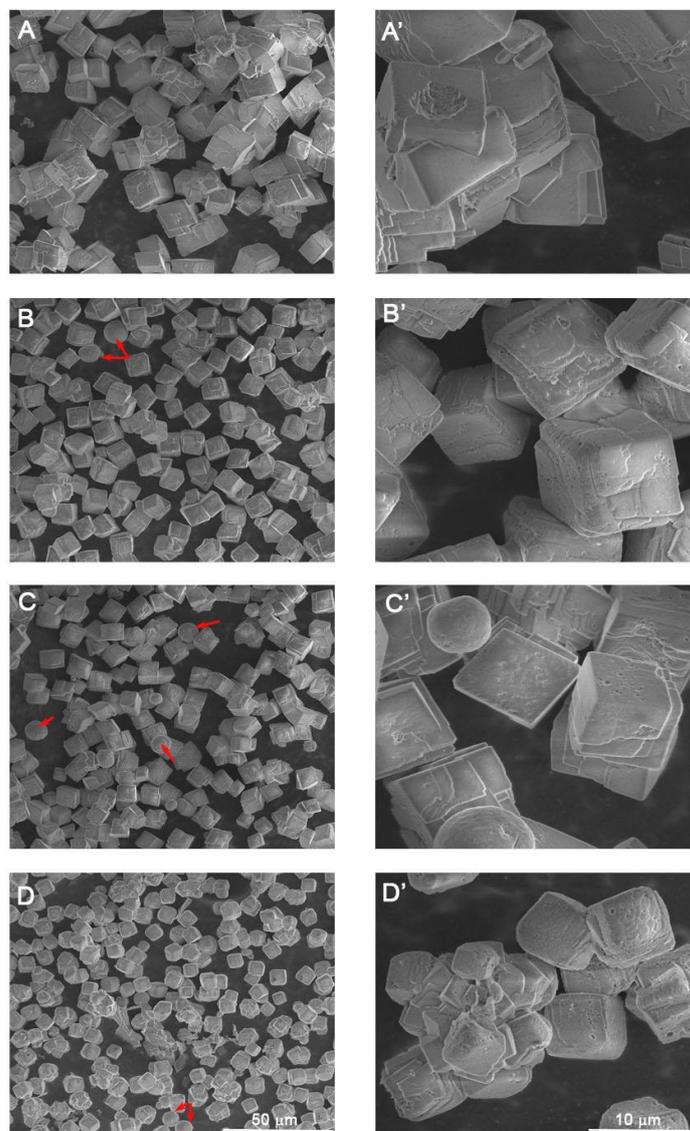
Fig. 2. SEM images of calcium carbonate precipitated from $CaCl_2$ and Na_2CO_3 solutions at 25 °C: (A-A') without the presence of biopolymer, (B-B') in the presence of biopolymer 80 mg/dm³, (C-C') in the presence of biopolymer 80 mg/dm³ added 15 min before the Na_2CO_3 solution addition, (D-D')) in the presence of biopolymer 160 mg/dm³ (the size scale is placed in the pictures)

From Figure 2 A-A' it is seen that in the reference system (without EPS) at 25 °C irregular cubic crystals, which are characteristic for calcite, are formed. If the precipitation was carried out in the presence of EPS of its total concentration equal to 80 mg/dm³, mainly rhombohedral $CaCO_3$ particles of a smaller size appear together with some small amount of the spherical ones (marked with red arrows (Fig. 2B). Spherical crystal shape is characteristic for vaterite. Doubling the EPS concentration causes the appearance of several spherical particles among rhombohedral ones (Fig. 2D and D'). The crystal size decreases with the EPS concentration increase. As a consequence of the 15 min time delay of Na_2CO_3 solution addition to the mixture of $CaCl_2$ and EPS, the obtained deposit is a mixture of rhombohedral

regular crystals and agglomerates of irregular rhombohedral ones (Fig. 2C and C'). Here, some single spherical crystals are also visible. Although the statistical test of crystals size was not done, based on SEM images it can be seen that in the presence of EPS obtained crystals do not differ significantly in size within each system.
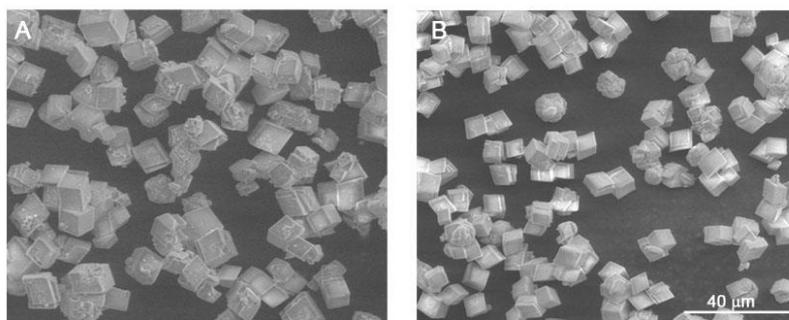
Fig. 3. Scanning electron micrograph of calcium carbonate crystals precipitated from $CaCl_2$ and Na_2CO_3 solutions at 37 °C: (A) without the presence of EPS, (B) in the presence of 80 mg/dm³ EPS. The scale bar is the same in both micrographs

Calcium carbonate precipitated at 37 °C without additives consists of rhombohedral crystals with smooth surface, as well irregular having the disordered surface (Fig. 3A). In the presence of EPS (80 mg/dm³), apart from a few cauliflower-like crystals, mainly rhombohedral ones with smooth surface have appeared (Fig. 3B). Comparing with the crystals obtained at a higher temperature, those precipitated at 37 °C are smaller.

The diffractograms and Raman spectra of the all prepared samples are shown in Figs. 4 and 5, respectively. From Figure 4 it can be seen that the reflections appear for calcite crystals only. Likewise, all Raman spectra show bands at 154 cm⁻¹, 282 cm⁻¹, 712 cm⁻¹ and 1086 cm⁻¹ indicating the existence of calcite (Gabrielli et al., 2000).
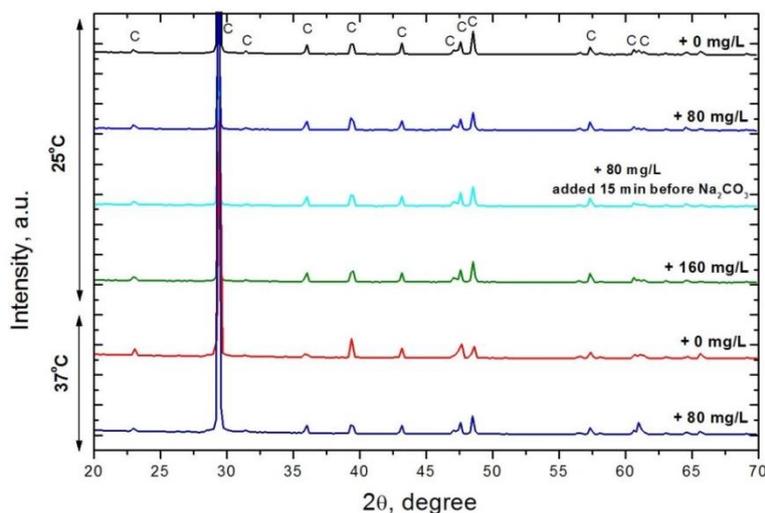
Fig. 4. XRD patterns for calcium carbonate precipitated at 25 and 37 °C without additive and in the presence of EPS at different concentrations (C) calcite

The phase composition of obtained samples was also estimated basing on their diffractograms using Rietveld's method (Table 1). It can be seen that calcite is the only one or main component in the all samples. Excluding the calcium carbonate obtained at 25 °C without additives and in the presence of EPS at concentration equal to 80 mg/dm³, in other samples very small amount of vaterite is present.

This corresponds to the crystal morphology presented in Figs. 3 and 4. Nevertheless, its content is equal or less than one percent and which is within standard deviation of the method.
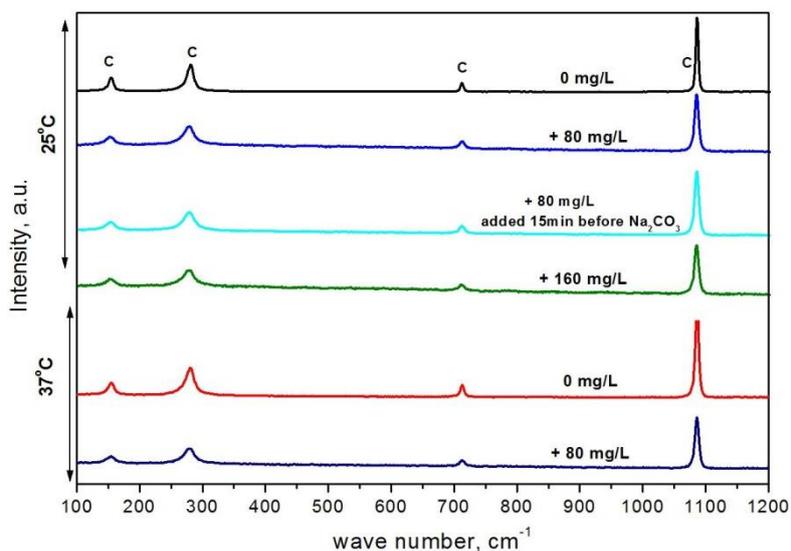
Fig. 5. Raman spectra of calcium carbonate precipitated at 25 and 37 °C without additive and in the presence of EPS at different concentration (C) calcite

Table 1. Phase composition of the precipitated calcium carbonate calculated from the diffraction patterns using Rietveld's method

Additive	25 °C		37 °C	
	Calcite, %	Vaterite, %	Calcite, %	Vaterite, %
no EPS	100% ±1%	0	99.4% ±1%	0.6% ± 0.4%
+ 80 mg/L EPS	100% ±1%	0	99.0% ± 2%	1% ±1%
+ 80 mg/L EPS 15 min before	99.4% ±0.3%	0.6% ±0.1%	—	—
+ 160 mg/L EPS	99.7% ±0.4%	0.3% ±0.1%	—	—
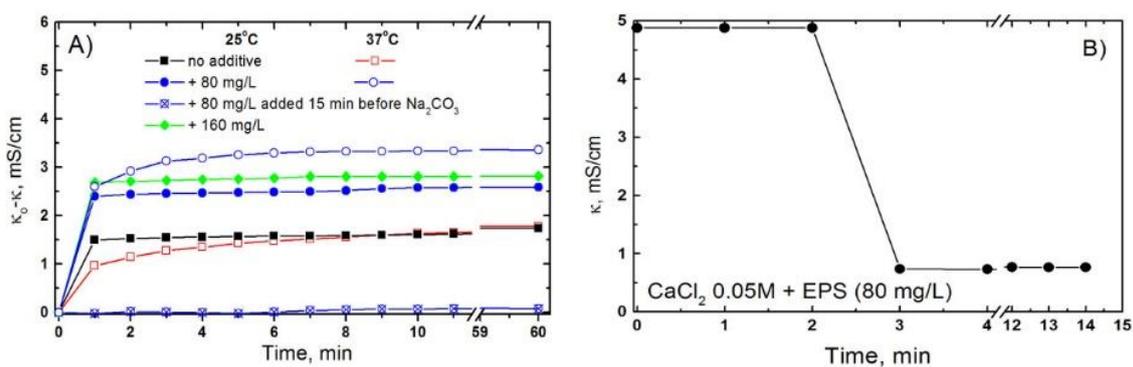
Fig. 6. A) The conductivity changes (shown as a difference between the initial and actual values) during the calcium carbonate precipitation at 25 and 37 °C, B) the conductivity changes (represented as measured) of 0.05 M $CaCl_2$ solution after EPS addition at 25 °C

To control the kinetics of nucleation and crystal growth the conductivity measurements were carried out during the calcium carbonate precipitation with and without the presence of exopolymeric substance at 25 and 37 °C. The initial conductivity measurement (κ_o) was done before the addition of sodium carbonate solution. The conductivity changes are presented as the difference between the

initial measurement (κ_0) and the conductivity (κ) measured after given time and can be seen in Fig. 6A.

After total mixing of the aqueous solutions of calcium chloride and sodium carbonate the conductivity changes rapidly. The sharp change in the conductivity observed during the first minute of the precipitation process shows that nucleation and crystallization processes occur. When calcium carbonate was precipitated without additive, at both investigated temperatures, the conductivity changes tend to the same value. Overall trends in $\Delta\kappa$ evolution are similar and the effect of EPS is proportional to its concentration. At lower temperature plateau is reached already after one minute after the solutions mixing. However, the crystallization occurs too fast to allow a quantitative analysis of the data. The temperature increase extends the time required to reach a plateau which may indicate that the process of precipitation is slowed down. It's worth noticing that the biggest $\Delta\kappa$ changes appear at 37 °C for the system containing exopolymeric substance at a concentration of 80 mg/dm^3. Larger conductivity changes than in the reference system without EPS may indicate increasing number of nucleation centers leading to the formation of a large number of small crystals. This hypothesis is confirmed by SEM images showing that smaller crystals are formed at higher EPS concentration. The small $\Delta\kappa$ changes in the presence of exopolymeric substance added 15 min before the sodium carbonate solution addition are caused by earlier adsorption of calcium ions to the polymer before addition of Na_2CO_3 solution. It is confirmed by the conductivity changes of 0.05 M calcium chloride solution mixed with the exopolymeric substance of total concentration equal to 80 mg/dm^3 (Fig. 6B). Up to 2 min after EPS addition to the calcium chloride solution the conductivity value is constant. After that time the sharp conductivity decrease appears and then remains constant until the end of the measurements. This decrease in conductivity indicates calcium ions adsorption on acidic functional groups of EPS which may act as a nucleation centers for $CaCO_3$. During precipitation experiment, if $CaCl_2$ solution was mixed earlier with EPS as a result of the adsorption the local concentration of calcium ions increases on the EPS surface. Therefore after addition of sodium carbonate solution many of small $CaCO_3$ crystals are precipitated. It is clearly seen in SEM images that crystals obtained in this experiment (Fig. 3C) are smaller than that precipitated after immediate mixing of the starting solutions (Fig. 3B). It is also known that vaterite prefers to form at a high supersaturation (Szcześ and Sternik, 2016). Hence in this system some spherical crystals are seen in SEM images (Figs. 3C and C') as well as in XRD data (Table 1).

It is worth noticing that both concentration and the amount of $CaCl_2$ and Na_2CO_3 solutions used for calcium carbonate precipitation used in the experiments without EPS as well as with its addition were the same. Therefore the quantitative conductivity changes during precipitation should be the same. The obtained data show that if the exopolymeric substance was present in the system these changes are bigger although the trend is the same. The changes in the conductivity confirmed the binding of calcium ions by EPS within 3 min. However this process were conducted in 1 mM NaCl solution, whereas after the immediate mixing of the starting solutions the ionic strength was higher. It may accelerate the adsorption process. However, to confirm this hypothesis additional experiments using the solutions of lower concentration will be conducted.

4. Conclusions

In this study influence of exopolymeric substance extracted from bacterial strain *Rhodococcus opacus* on calcium carbonate precipitation from calcium chloride and sodium carbonate solutions was investigated. The obtained results indicate that the exopolymeric substance present in the solution has no influence on the crystal structure but acts as the nucleation centers thus leading to the formation of regular calcite crystals whose size depends on the EPS concentration. It was found that the EPS is capable of binding calcium ions and hence act as nucleation centres influencing calcium carbonate precipitation. This effect is concentration dependent and at 25 °C doubling of EPS concentration leads to acceleration of crystals nucleation and reduction of the crystal size. This effect is greater if calcium chloride solution if mixed with EPS 15 min before the addition of sodium carbonate solution. Increase in temperature to the physiological (37 °C) causes formation of smaller crystals in the reference system, as well in the presence of EPS. It was also shown that the exopolymer presence do not influence the crystal form and mostly calcite is formed.

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