ACTA ABP BIOCHIMICA POLONICA

Vol. 63, No 2/2016
343–351
http://dx.doi.org/10.18388/abp.2015_1172

Regular paper

Environmental parameters conditioning microbially induced mineralization under the experimental model conditions*

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Microbially induced calcium carbonate precipitation is one of the biomineralization types closely dependent on the parameters of the microenvironment. Minerals are precipitated as a product of environmental and bacterial cell interactions, however, this system has very little control *via* microorganisms. The aim of research was to determine the influence of abiotic factors (pH, temperature, agitation speed of bacterial culture and calcium ion source) on the mineralization induced by *Arthrobacter sulfureus*, *Bacillus muralis* and *B. atrophaeus* strains under the standard laboratory conditions. Because of the key role of urease in biomineralization, processes occurring in environments with and without the urea were compared. For this purpose, cultivation of bacteria (*A. sulfureus*, *B. muralis* and *B. atrophaeus*) was carried out in B4 liquid medium for 5 days with various environmental parameters (pH 6–9; temperature 25–44°C; speed of agitation 0–180 rpm, different calcium sources). It was noticed that the pH and the speed of agitation clearly affect the amount of the calcium carbonate that formed. Our observations suggest that the highest precipitation rate takes place in alkaline pH between 8–9, with shaking at 180 rpms. Among studied sources of calcium ions (calcium acetate, calcium chloride and calcium nitrate), calcium acetate demonstrated the strongest potential in the biomineralization process. Moreover, work presented here demonstrates that the correlation between cultivation temperature and biomineralization process cannot be clearly evaluated. The morphology and size of calcium carbonate minerals was strain-specific, although affected by the presence of urea in the surrounding solution.

Key words: microbially induced mineralization, calcium carbonate, abiotic factors, urease

Received: 07 August, 2015; revised: 24 September, 2015; accepted: 06 October, 2015; available on-line: 19 February, 2016

INTRODUCTION

Biomineralization (synthesis of crystalline structures by living organisms) is a common phenomenon, occurring in different natural environments such as soil, sedimentary and metamorphic rocks, oceans, seas and saline lakes (Rivadeneyra *et al.*, 2004; Baskar *et al.*, 2005). According to Lowenstam & Weiner (1989) biomineralization was classified as a biologically controlled and induced process. This division is based on the degree of biological control over the biominerals' secretion system by organisms. In a biologically controlled mechanism, biomineral precipitation happens due to the cellular activities of the organisms, producing a macromolecular matrix outside the cell, within or on which inorganic particles are grown (Young & Henriksen, 2003). In this type of biomineralization, the gene responsible plays an effective role in deciding on the structure and composition of the mineral particles. The secreted minerals are unique to the bacterial species and are independent of environmental conditions (De Muynck *et al.*, 2010; Sarayu *et al.*, 2014). In contrast, biologically induced biomineralization is closely dependent on parameters of the microenvironment. Minerals are precipitated as a product of the interaction between environmental and biological activity and the system has very little control via microbial cells over the minerals that are secreted. Moreover, any change in the microenvironment will have an effect on the biominerals that are precipitated. An example of this type of biomineralization process is the formation of calcium carbonate ($CaCO_3$) by microorganisms, mainly bacteria (*Bacillus subtilis*, *B. cereus*, *B. sphaericus*, *B. megaterium*, *Pseudomonas putida*, *Sporosarcina pasteurii*, *Myxococcus xanthus*, *Arthrobacter* sp., *Micrococcus* sp.) (Hammes *et al.*, 2003; Rivadeneyra *et al.*, 2004; Baskar *et al.*, 2005; Dhami *et al.*, 2013). However, the precise role of microbes in the carbonate precipitation process still remains unclear (Konhauser & Riding, 2012). Boquet *et al.* (1973) suggested that almost all bacteria are capable of $CaCO_3$ formation. Several authors concluded that this phenomenon occurs as a by-product of different bacterial processes such as photosynthesis, urea hydrolysis, sulfate reduction and anaerobic sulphide oxidation (Castanier *et al.*, 1999; Knorre & Krumbein, 2000; Baskar *et al.*, 2006). As a result of these metabolic processes, there is an increase in the pH value of a microenvironment, which favors $CaCO_3$ precipitation.

Calcium carbonate precipitation mediated by microorganisms is governed by several key factors. According to De Muynck *et al.* (2010), there are four main factors in calcium carbonate formation: (1) the calcium concentration, (2) the concentration of dissolved inorganic carbon, (3) pH, (4) the availability of nucleation sites. Dhami *et al.* (2013) and Hammes & Verstracte (2002) also mention $[Ca^{2+}]/[CO_3^{2-}]$ ratio and bacterial species in the saturation index as a factor. It is unknown how abiotic factors directly determining bacterial growth may affect the biomineralization process.

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*The results were presented at the 6th International Weigl Conference on Microbiology, Gdańsk, Poland (8–10 July, 2015).
Accession numbers: KM036067 (*A. sulfureus* W4/124), KM036069 (*B. atrophaeus* II/39/3), and KM036074 (*B. muralis* I/2/3)
Abbreviations: LB, Luria-Bertani medium; EDTA, ethylenediaminetetraacetic acid

Hence, the question has to be raised: is there a connection between environmental conditions (such as temperature, pH, speed of agitation and calcium ions source), bacterial species, and microbially induced calcium carbonate precipitation?

The aim of this research was to determine the influence of environmental parameters (pH, temperature, agitation speed of bacterial culture and calcium ion source) on the mineralization induced by *Arthrobacter sulfureus*, *B. muralis* and *B. atrophaeus* strains in the experimental model conditions. Moreover, in relation to the hypotheses generated by researchers on the key role of urease activity, biomineralization process was compared in environments with and without the urea.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study were *Arthrobacter sulfureus* W4/124, *B. atrophaeus* II/39/3, and *B. muralis* I/2/3. The strains were maintained in the Luria-Bertani medium (LB) supplemented with 15% (v/v) glycerol and stored at –80ºC. Pre-cultures of bacteria were conducted in 30 mL of LB medium at 30ºC with constant shaking at 150 rpm for 24–48 h. When the cells reached late exponential growth phase, the bacterial cultures were used for the following experiments.

Experimental system. Two types of experimental systems were designed: with and without 2% urea to accelerate urease production. The medium and urea solutions were sterilized separately. Urea was added after sterilization by means of filtration through a sterile 0.22 μm Milipore filters. Regarding the study of the $CaCO_3$ precipitation, bacterial cultures were carried out in 60 mL of liquid medium B4, containing calcium ions at concentrations ranging from 2.2 to 2.7 g/L (Lee, 2003). The B4 medium was inoculated with 3% (v/v) of bacterial pre-culture and incubated for 5 days at variable process parameters (pH of culture medium, temperature, speed of agitation, and type of calcium source). Process parameters depending on the studied factors are shown in Table 1.

Qualitative urease activity assay. Urease activity of studied strains was determined on Christensen's Urea Agar Base (Merck). A fresh culture of bacteria was inoculated onto Christensen medium slants and incubated at 30ºC for 72 h. The reaction was recorded as positive after the appearance of a deep pink color (Atlas, 2010).

Analytical methods. Cell growth was determined by the plate counting method at regular time intervals of 24 h for 5 days of incubation. The results are expressed as a mean value of three replicates and presented as log cfu/mL in a graphic form.

The soluble Ca^{2+} was measured using ethylenediaminetetraacetic acid (EDTA) titration method (APHA, 1989; Stocks-Fischer *et al.,* 1999). Insoluble Ca^{2+} (as $CaCO_3$) was derived as the difference between the total initial Ca^{2+} concentration and the concentration of soluble Ca^{2+}.

Analysis of calcium carbonate crystals. Calcium carbonate crystals and bacterial cells were separated using Miracloth filtration materials (Calbiochem), washed and dried at 37°C for 48 h. The morphology of crystals was analyzed using optical microscopy (Olympus CX41). The crystal size was determined using Cell^B Image Acquisition Software (Olympus).

Statistical analysis. All statistical analyses were carried out using 3-way ANOVA at the confidence level of $p < 0.05$. The results of the experiments were presented as the arithmetic mean of three assays.

Kinetics of the calcite precipitation were described by a modified sigmoid logistic curve. The exponential logistic equation:

$$y = \frac{a}{1 + e^{-b(x-c)}} + d$$

where a is the range of y variation Ca^{2+}; b is μ (precipitation rate, h^{-1}); x is time (h); c is the time at the maximum (dy/dt); d is the initial concentration of Ca^{2+} at time zero and was used for calculation of the rate of calcium carbonate formation (Stocks-Fischer *at al.,* 1999). The regression analysis was performed with Origin 2015, v. 9.2 software (OriginLab Corporation, USA).

In order to compare bacterial growth curves, the Gompertz equation in conjunction with DMFit ver. 2.1. Excel add-in program, was used (www.ifr.ac.uk/Safety/DMfit/default.html., Baranyi & Roberts, 1994). The Gompertz parameter values were used to calculate maximum specific growth rates (μ_{max}), lag phase durations (T_{lag}) according to Zaika *et al.* (1998).

RESULTS AND DISSCUSION

The bacterial strains of *A. sulfureus* W4/124, *B. atrophaeus* II/39/3 and *B. muralis* I/2/3 used in this study were isolated from historical building materials (brick, mortar, and plaster coated paint). Taxonomic position of the examined strains was defined by 16S rRNA gene sequencing. The nucleotide sequences of genes were deposited in the GenBank Database, at the National Center for Biotechnology Information (NCBI) under the accession numbers KM036067 (*A. sulfureus* W4/124),

Table 1. Environmental conditions used in study of the biomineralization process

Analyzed factor	Parameters of bacterial cultivation			
	pH of medium	Temperature of incubation [°C]	Speed of agitation [rpm]	Source of calcium ions
pH	6, 7, 8, 9	30	150	calcium acetate
Temperature	7	25, 30, 37, 44	150	calcium acetate
Speed of agitation	7	30	0, 100, 180	calcium acetate
Source of calcium ions	7	30	150	calcium acetate calcium chloride calcium nitrate

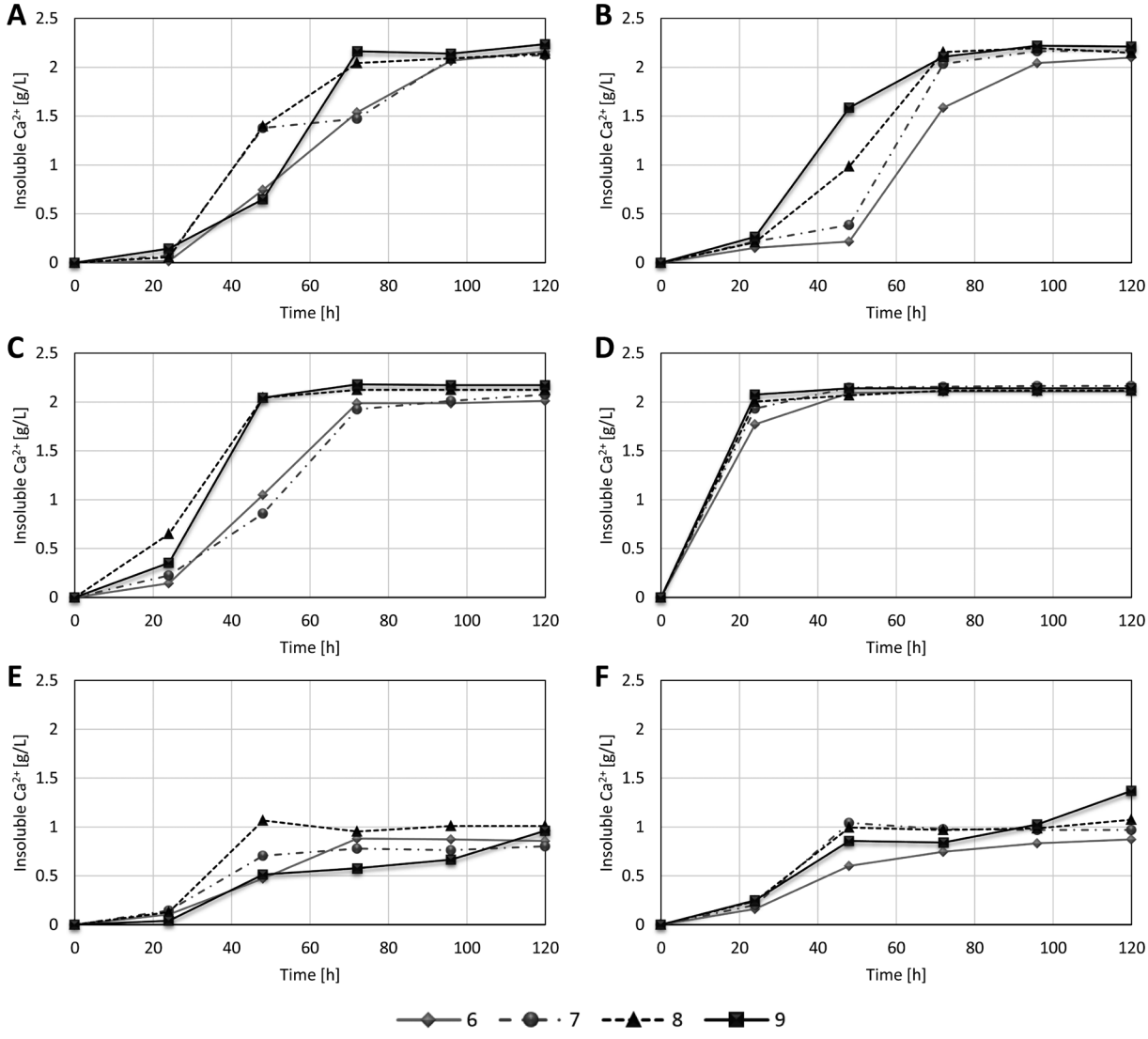

Figure 1. Time courses of mineralization induced by *B. muralis* (**A–B**)*; A. sulfureus* (**C–D**), and *B. atrophaeus* (**E–F**) in various pH of a given medium. Medium without urea (**A, C, E**) and supplemented with urea (**B, D, F**).

KM036069 (*B. atrophaeus* II/39/3), and KM036074 (*B. muralis* I/2/3). All studied strains: *A. sulfureus, B. muralis* and *B. atrophaeus,* revealed urease activity on Christensen's Urea Agar Base. Enzymatic hydrolysis of urea is a well-known process of biomineralization and is a straightforward model for studying microbial calcium carbonate formation. During this process, hydrolysis of urea is caused by an enzyme — urease (urea amidohydrolase; EC 3.5.1.5). One mole of urea $CO(NH_2)_2$ is hydrolyzed intracellularly to one mole of ammonia NH_3 and one mole of carbamate NH_2COOH, which in turn hydrolyzes into one mole of ammonia NH_3 and carbonic acid H_2CO_3. These products subsequently equilibrate in water to form biscarbonate and two moles of ammonium and hydroxide ions. An increase in alkalinity causes a shift in the biscarbonate equilibrium resulting in a carbonate ion formation, and in the presence of soluble ions precipitating as calcium carbonate (Hammes *et al.*, 2003; Dhami *et al.*, 2013). Due to the key role of urease in biomineralization, the assay under the experimental model conditions was conducted with and without urea in the environment.

Kinetics of calcite precipitation and bacterial growth in various pH of the surrounding solution

The biomineralization phenomenon was observed for all studied strains in the experimental system, both with and without urea in the environment. After 120 h of cultivation in the presence of 2.3 g/l of soluble Ca^{2+}, insoluble calcium carbonate of between 2.0 to 2.3 g/l was obtained in precipitation induced by *A. sulfureus* and *B. muralis* and 0.8–1.4 g/l by *B. atrophaeus.* However, the pH clearly affects the amount of formed calcium carbonate and the rate of the precipitation process (Fig. 1). For all studied bacteria, the highest precipitation rate was observed in alkaline pH ranging between 8–9, while the lowest in pH about 6. There were no statistically significant differences in the kinetics of the mineralization process in the medium whether with or without urea. *A. sulfureus* which in the presence of urea was characterised by three times as high precipitation rate, (0.42–0.55 h^{-1}), was the exception. Interestingly, when it comes to *A. sulfureus*, $CaCO_3$ formation was observed within the first 24 h of the process, during the logarithmic phase

Table 2. Parameters of bacterial growth and calcium carbonate precipitation

Bacterial strain	Factor	Factor value	Growth parameters				Biomineralization parameters			
			B4 without urea		B4 with urea		B4 without urea		B4 with urea	
			T_{lag} [h]	μ_{max} [h^{-1}]	T_{lag} [h]	μ_{max} [h^{-1}]	μ_{CaCO3} [h^{-1}]	Y [%]	μ_{CaCO3} [h^{-1}]	Y [%]
B. muralis	pH	6	25.5	0.44	0	0.24	0.08	94.4	0.13	96.1
		7	0	0.24	0	0.40	0.12	90.8	0.17	93.0
		8	0	0.18	0	0.35	0.17	92.5	0.12	94.4
		9	23.9	3.06	26.1	0.27	0.18	91.6	0.12	96.9
A. sulfureus		6	0	0.32	0	–	0.13	83.8	0.42	87.3
		7	0	0.40	0	–	0.10	83.7	0.49	84.9
		8	0	0.68	0	–	0.17	66.4	0.54	68.7
		9	0	0.69	0	–	0.18	80.8	0.55	83.8
B. atrophaeus		6	18.9	2.09	20.9	0.15	0.11	32.3	0.09	32.5
		7	0	0.13	12.9	0.16	0.16	28.8	0.85	29.2
		8	0	0.26	0	0.21	0.87	38.5	0.22	38.9
		9	20.6	2.09	0	0.23	0.05	41.7	0.05	40.1
B. muralis	Temperature [°C]	25	0	0.23	0	0.18	0.02	88.8	0.02	79.1
		30	0	0.18	0.6	0.22	0.03	93.3	0.04	90.5
		37	8.3	0.21	0	0.16	0.08	93.6	0.06	97.6
A. sulfureus		25	0	0.12	0	–	0.03	85.0	0.76	98.3
		30	0	0.10	0	–	0.04	89.5	0.76	98.3
B. atrophaeus		25	0	0.28	10.2	0.22	0.07	61.3	0.02	58.4
		30	19.8	0.17	3.3	0.22	0.05	61.7	0.01	58.1
		37	0	0.42	8.0	0.31	0.05	49.5	0.30	39.2
		44	0	0.33	0	0.9	0.12	37.7	0.76	35.8
B. muralis	Agitation speed [rpm]	0	4.5	0.08	31.8	0.21	0.08	22.5	0.02	26.0
		100	0	0.19	18.1	0.26	0.03	30.0	0.02	22.3
		180	0	0.33	20.5	0.32	0.10	94.5	0.01	93.4
A. sulfureus		0	24.7	0.15	0	–	0.04	5.1	0.09	95.6
		100	0	0.15	0	–	0.04	12.3	0.35	98.5
		180	0	0.21	0	–	0.04	95.6	0.40	99.3
B. atrophaeus		0	22.2	0.06	0	0.03	0.03	18.8	0.45	4.8
		100	29.5	0.05	0	0.08	0.03	24.6	0.06	15.8
		180	18.4	0.20	11.8	0.16	0.09	45.7	0.07	38.5
B. muralis	Calcium source	calcium acetate	8.6	0.14	14.3	0.17	0.15	90.4	0.10	95.2
		calcium chloride	0	0.26	22.6	0.13	0.02	8.0	0.04	14.5
		calcium nitrate	0	0.14	11.9	0.26	0.09	4.2	0.05	11.7
A. sulfureus		calcium acetate	0	0.22	0	–	0.04	95.7	0.43	99.7
		calcium chloride	0	0.17	0	–	0.05	22.5	0.70	99.7
		calcium nitrate	4.8	0.07	0	–	0.08	42.2	0.56	99.4
B. atrophaeus		calcium acetate	23.1	0.22	20.8	0.24	0.06	34.5	0.11	32.6
		calcium chloride	15.1	0.28	7.1	0.09	0.06	20.0	0.03	19.7
		calcium nitrate	7.3	–	0	–	0.25	35.2	0.05	5.4

T_{lag}, lag phase durations; μ_{max}, maximum exponential growth rates; μ_{CaCO3}, precipitation rate; Y, precipitation efficiency; (–) as a result of decrease in the number of viable cells it was impossible to indicate the value of μ_{max}

of the bacterial growth. After this time, the increase in the amount of calcium carbonate was lower, and what is more, a sudden decline in the total number of viable cells was detected. For the rest of the strains, the maximum amount of precipitated $CaCO_3$ was obtained after 48 h (*B. muralis* and *A. sulfureus*) and 72 h (*B. subtilis*), however, the increase of insoluble $CaCO_3$ was noted up to 120 h into the process. There was no significant difference in bacterial growth profiles and specific growth rates (Table 2). However, the microbiologically induced $CaCO_3$ precipitation appeared to be correlated with the growth of the studied strains.

According to Hammes & Verstraete (2002) and Al-Thawadi (2011) the pH of the environment is an important factor determining both, precipitate and solubility of the calcium carbonate. Both, the high efficiency and rate of the calcium carbonate precipitation are related to the metabolic activity of the cells, as well as the specific urease activity. The effect of pH between 6–7 is minor, as the bacterial cells protect the enzyme from acidity. Moreover, at neutral pH, the predominant carbonate species in the environment are biscarbonate (HCO_3^-) rather than carbonate (CO_3^-) (Alhour, 2013). The activity of urease increased between pH 7.5–8.0 and reached a maximum at pH 8.0–9.0, whereas at above pH 9.0 it was slowly decreasing. The optimal pH for calcium carbonate precipitation induced by the studied strains is consistent with results reported by Stocks-Fischer *et al.* (1999) and Okwadha & Li (2010). According to the authors, biomineralization occurs at pH between 8.3 and 9.0, where urease activity remains high. It is worth noting that alkaline pH has not always had a positive influence on microbiologically induced mineralization. With respect to microorganisms which do not show urease activity, alkaline pH can even inhibit calcium carbonate precipitation.

Effect of temperature on biomineralization process and bacterial growth

In the first step of this research, the growth ability in the temperature range of 25, 30, 37 and 44°C on B4 medium was determined. Bacteria *B. atrophaeus* were able to grow in the widest temperature range 25–44°C, while *B. muralis* did not reveal the ability to grow only at the highest temperature. For *A. sulfureus,* growth was only observed between 25–30°C. For further study of the biomineralization process under the model conditions, the

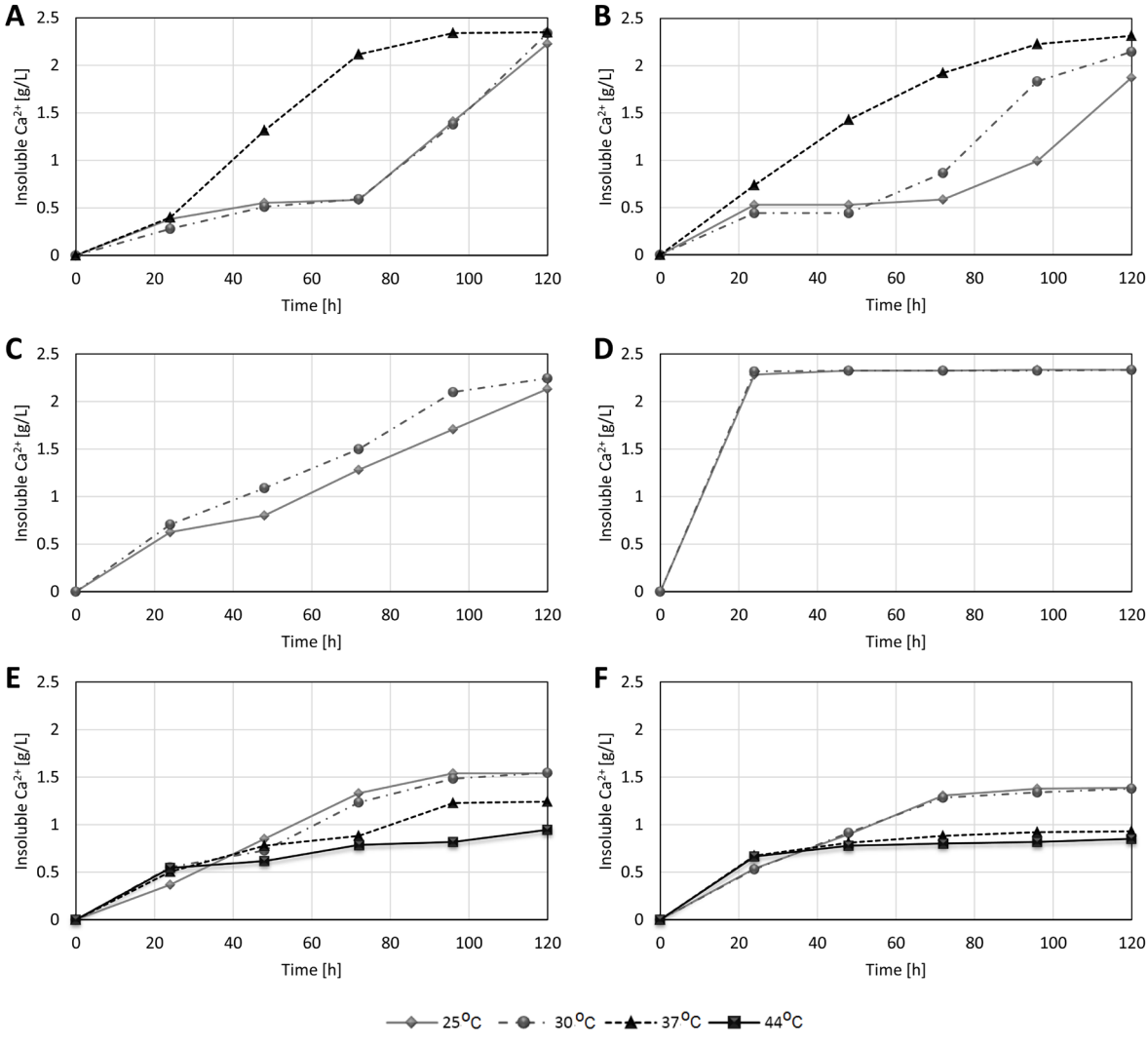

Figure 2. Time courses of mineralization induced by *B. muralis*
(**A–B**); *A. sulfureus* (**C–D**), and *B. atrophaeus* (**E–F**) in various temperatures of cultivation. Medium without urea (**A, C, E**) and supplemented with urea (**B, D, F**).

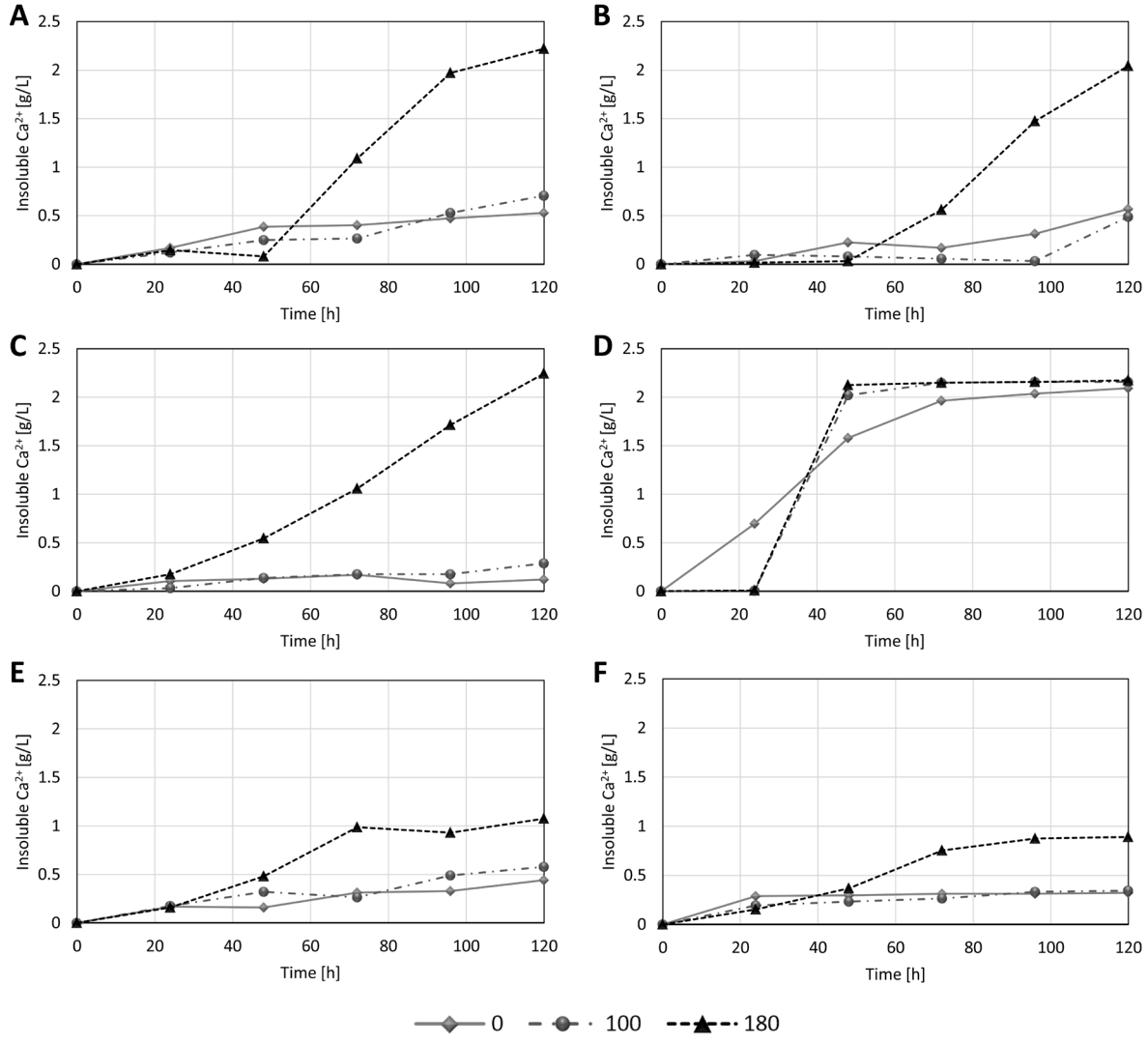

Figure 3. Time courses of mineralization induced by *B. muralis* (A–B); *A. sulfureus* (C–D), and *B. atrophaeus* (E–F) at various speeds of agitation.
Medium without urea (**A, C, E**) and supplemented with urea (**B, D, F**).

temperatures were selected at which the growth of microorganisms was observed.

On the basis of the results, a correlation between incubation temperature and the biomineralization process cannot be clearly evaluated (Fig. 2). The highest efficiency of this process was noted for *B. atrophaeus* and *A. sulfureus* at 25–30°C, whereas for *B. muralis* it was at 37°C. For the latter strain, the temperature did not significantly influence the amount of calcium carbonate precipitate, but at higher temperatures, the process was three times faster, and the value of μ equaled 0.08 h^{-1} (Table 2). The aspect deserving much interest is that, for *B. muralis,* independently of the incubation temperature, the growth rate was similar and ranged between 0.18 to 0.23 h^{-1}, however at 37°C, it reached its maximum. For *A. sulfureus* bacteria, medium supplementation with 2% of urea resulted in the highest efficiency, as well as the most active calcium carbonate precipitation. Among the rest of the studied strains, no statistically significant differences were observed during the processes which were held in media with or without urea in the environment (Fig. 2).

Temperature seems to have an effect on calcium carbonate precipitation. Nevertheless, the role of temperature as an environmental factor is still secondary in comparison to pH. However, the optimum temperatures have a positive effect on bacterial precipitation of calcite, increasing the ability of the strain to form crystals. Baskar *et al.* (2006) found that 25°C was optimum for mineralization mediated by *B. thuringiensis* and *B. subtilis*. At lower temperatures (5–15°C), carbonate precipitation started only after 15 days of incubation. As Ferris *et al.* (2003) and Nemati *et al.* (2005) pointed out, calcite precipitation rates did not vary significantly at different temperatures. Although in lower temperature the time required for biomineralization process to occur was longer due to urea hydrolysis rates, e. g. at 20°C generated a longer lag time before critical supersaturation when compared to 30°C. Regardless of the applied temperature, calcite was the predominant polymorph, with aragonite and vaterite also present in trace amounts. On the other hand, as indicated by Weiss *et al.* (2014) when studying chemical nonbiological precipitation of $CaCO_3$, the temperature determined the formation of different calcium carbonate polymorphs. It was observed that lower temperatures (25–30°C) were favorable for vaterite formation, whereas calcite and aragonite were the preferred

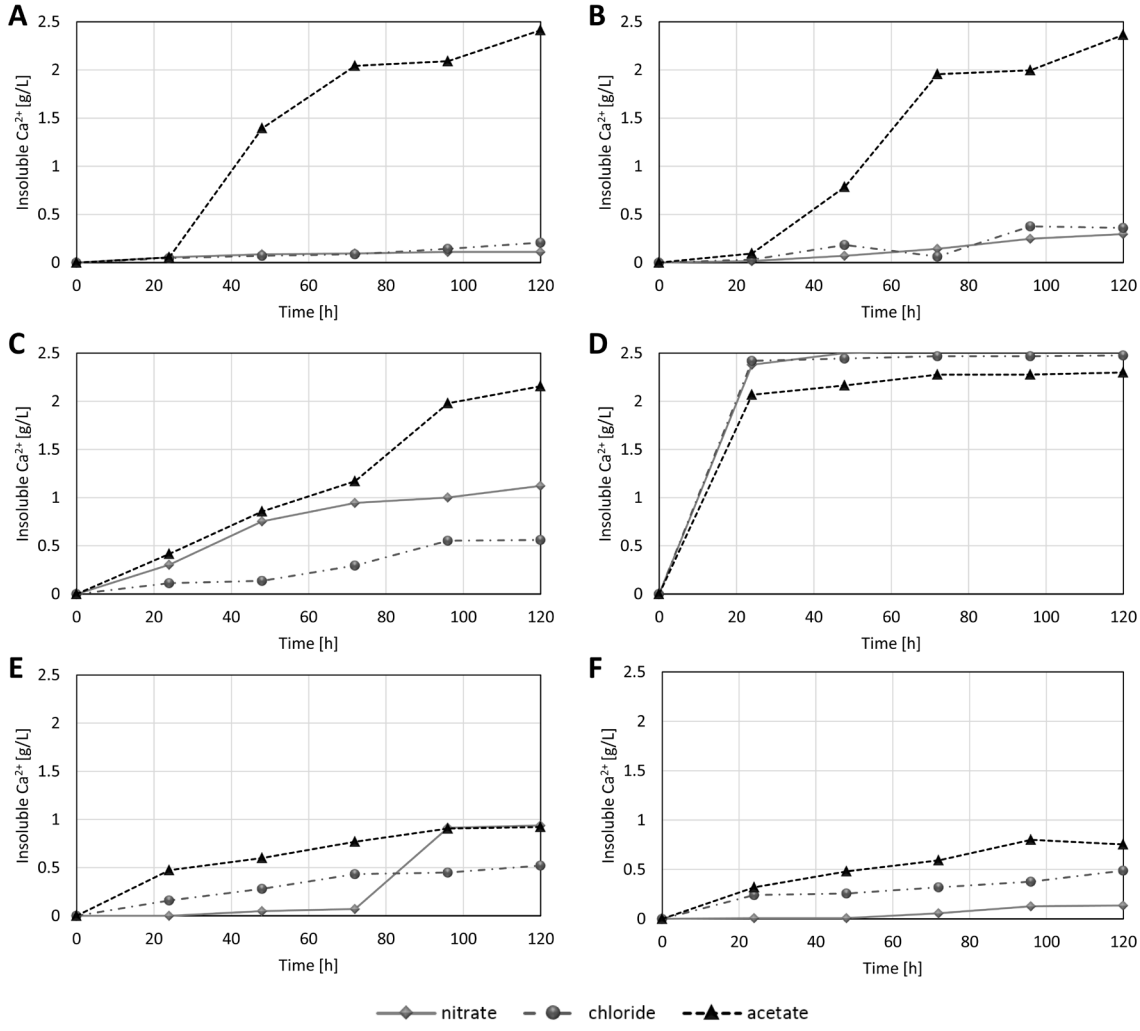

Figure 4. Time courses of mineralization induced by *B. muralis* (A–B); *A. sulfureus* (C–D), and *B. atrophaeus* (E–F) in the presence of different sources of calcium in the environment.
Medium without urea (**A, C, E**) and supplemented with urea (**B, D, F**).

polymorphs that were formed at significantly higher temperatures, even 50 and 70°C, respectively.

The impact of agitation speed on calcium carbonate precipitation

All of the strains considered in this study are aerobic microorganisms, therefore the source of oxygen and thus, indirectly, the speed of agitation has an important influence on their growth. On account of the results, it was assumed that the speed of agitation determined microbiologically induced mineralization (Fig. 3). For each analyzed strain, the lowest amount of precipitated calcium carbonate, between 0.12–0.52 g/L and 0.3–0.7 g/L, was observed in the culture without agitation or when the agitation speed had been set at 100 rpm. Increasing the agitation speed to 180 rpm, resulted in over a 60% increase in the process' efficiency for *B. muralis* and 20% for *B. atrophaeus*, in a medium with or without urea (Table 2). *A. sulfureus* was the exception. In the presence of urea it effectively formed calcium carbonate (96–99%), regardless of the agitation speed (Fig. 3). However, statistically significant differences were noted for calcium carbonate precipitation in the presence of urea. When the agitation speed was between 100–180 rpm, the precipitation rate was found to be four times as high, in comparison to the culture without stirring (Table 2).

For studied bacteria, the speed of agitation had an impact on the time courses of bacterial growth and growth rate. Although both, the biomineralization process and bacteria growth, were agitation-dependent, there was no correlation between them. For every analyzed strain, the optimum agitation speed leading to high efficiency and intensity of biomineralization was 180 rpm.

It could be inferred that for *A. sulfureus* strain, controlled mineralization was observed, rather than a microbiologically induced process. It may be evidenced by the lack of correlation between environmental factors (pH, temperature, speed of agitation) and the kinetics of the biomineralization process. What is noteworthy, is that regardless of the process variables, after 24 h of cultivation the decrease in the number of viable cells was noted. Hence, it was impossible to designate the maximum growth rate in each and every conducted experiment. The significant increase in pH due to urea hydrolysis

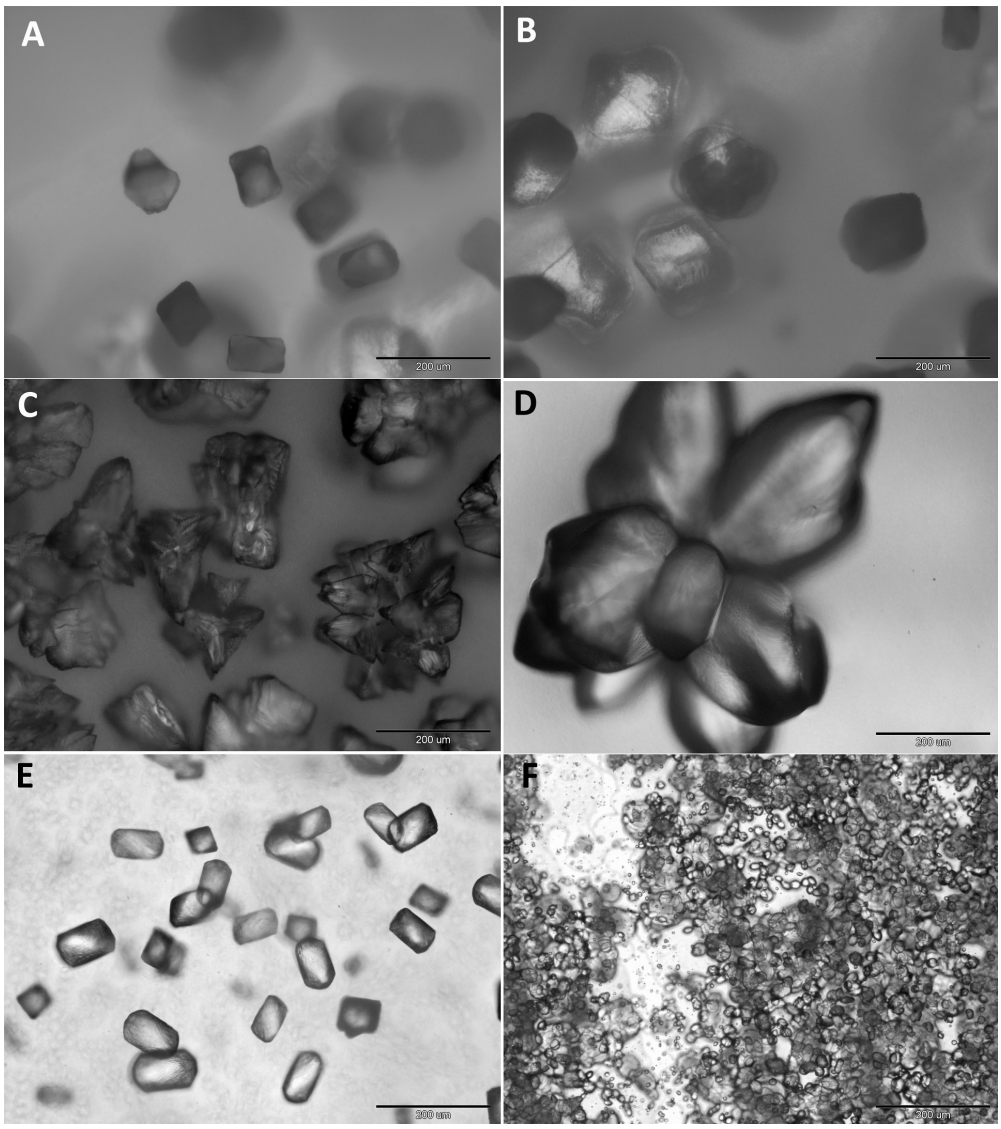

Figure 5. Morphology of calcium carbonate crystals formed by *B. muralis* (A–B), *A. sulfureus* (C–D) and *B. atrophaeus* (E–F) in medium without urea (B, D, E) and supplemented with urea (A, C, E).

could facilitate the bacterium to move on to the death phase.

Biomineralization and bacterial growth profiles in presence of different calcium sources

In order to achieve an effective mineralization mediated by bacteria, it is necessary to apply the appropriate source of calcium ions. The bacterial growth and biomineralization profiles were studied in media supplemented with different calcium sources such as: calcium acetate ($Ca(C_2H_3O_2)_2$), calcium chloride ($CaCl_2$) and calcium nitrate ($Ca(NO_3)_2$). The initial concentration of soluble calcium ions was between 2.5–2.7 g/L. The type of calcium source affected the microbiologically induced mineralization. For the *B. muralis* bacteria, the highest efficiency (90–95%) was observed when medium was supplemented with calcium acetate, both with and without urea (Fig. 4). In the presence of calcium chloride and calcium nitrate, precipitation yield was significantly lower, 8–14.5% and 4.2–11.6%, respectively. In both cases, the biomineralization process took a long time and the precipitation rate ranged between 0.2 and 0.5 h^{-1} (Table 2). Calcium acetate was also the most preferred calcium source in biomineralization mediated by *A. sulfureus* in medium without urea. The presence of calcium acetate resulted in a 70 and 50% higher process yield in comparison to calcium chloride and nitrate, respectively. Interestingly, for media enriched with urea, a high yield and constant rate of precipitation was observed, regardless of the applied type of calcium source. There was no significant difference in growth profiles among different media. However, the maximum exponential rate was observed in media containing calcium acetate. On the basis of our findings, it could be inferred that the optimal calcium source for the biomineralization process mediated by studied strains was calcium acetate. There were no significant differences in growth profiles among different media.

Our results confirm the observations by Lee (2003). The author performed biomineralization in medium containing calcium acetate and found that the maximized yield was 0.91 mg of calcite. On the other hand, results obtained by Achal & Pan (2014) revealed that the minimum calcite amount was precipitated in medium supple-

mented with calcium oxide, whereas the maximum was with calcium chloride.

Characterization of calcium carbonate crystals by microscopic analysis

For *B. atrophaeus* and *B. muralis,* rhombohedral crystals were observed, while for *A. sulfureus* complex crystalline structures such as druses were predominant. On the basis of the microscopic analysis, the correlation between environmental parameters and the crystal morphology (size, shape, color), cannot be clearly evaluated. However, the structure and size of biocrystals were dependent on the features of the bacterial strain and the presence of urea in the environment. The biggest rhombohedral crystals, of an average size of 94.6×59.5 μm (B4 without urea) and 188×177 μm (B4 with urea), were observed in cultures of *B. muralis*, while the smallest, 93.5×57.9 μm (B4 without urea) and 21.6×18.3 μm (B4 with urea), for *B. atrophaeus*. Wang and Nilsen-Hamilton (2013) assumed that the size, morphology, orientation, composition and the localization of mineral secretion are microbial species-dependent.

CONCLUSIONS

On the basis of our results, it could be stated that under the appropriate experimental model conditions it is possible to perform microbiologically induced mineralization. The study of the biomineralization phenomenon, from the point of view of basic research, has led to clarification that the environmental factors such as pH of the surrounding solution, agitation speed and type of calcium source determine biomineralization. However, a correlation between incubation temperature and this process cannot be clearly evaluated. The addition of urea to the culture medium did not significantly affect the bacterial growth and the constant rate of calcium carbonate precipitation mediated by *B. atrophaeus* and *B. muralis*. For *A. sulfureus, B. atrophaeus* and *B. muralis*, the calcium acetate was the most preferred calcium source and the pH of the surrounding solution above 8.0, and agitation speed set to 180 rpm were the most appropriate factors for the biomineralization process. Moreover, the structure and size of biocrystals depended not on the environmental conditions, but in particular on the features of the bacterial strain and the presence of urea in the environment. In the light of development of future engineering technologies involving microbially induced mineralization such as conservation of monuments, sand consolidation, and the remediation of cracked concrete, the above-mentioned results play a major role.

Acknowledgements

The authors would like to acknowledge Paulina Markowiak, MSc., for invaluable technical support, which was necessary to conduct our research.

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