Influence of medium composition on biomineralization and morphology of newgrowths

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Abstract. The research presents rational (from the point of view of processability) types of bacteria, nutrient media, biochemical agents and their quantities for biogenic mineral formation under specified conditions. The principles of the influence of metabolism processes of microorganisms on the change in the alkalinity of medium are established. The dependences of morphological parameters (size, shape) of mineral components formed during biomineralization in simulated media on the type of nutrient in the crystallization medium are established. The authors formed a phenomenological model of the processes occurring as a result of the bacterial activity of various strains, nutrient components and precursors of crystallization initiation as stages of structure formation during the production of building materials.

1. Introduction

Bacterial biomineralization is a promising tool of nature-related technologies, which allow biogenic synthesis of minerals capable of influencing the structure formation of the structure formation of composite materials and, as a result, the change of their physical and mechanical properties [1]. The establishments of mechanisms and principles of crystal formation, taking into account the generic nature of the applied bacteria and their derivatives for the synthesis and modification of processes and products of technogenic mineral formation, type and concentration of nutrient components and precursors participating in biochemical reactions, will identify the most promising types of bacterial cultures and suitable crystallization conditions.

Despite the existing works on bacterial biomineralization [2-6], in order to establish a general picture of biomineralogenesis in technogenic systems of building materials, it is necessary to develop principles for the control of mineral formation processes of composite building materials using microorganisms and their derivatives for the synthesis and modification of products and processes of technogenic mineral formation taking into account the medium for the formation of newgrowths, as well as the specifics of the technological stages implemented in production conditions.

In this regard, the purpose of the research was to establish principles of the influence of bacterial activity on chemical and mineralogical processes in model systems, i.e. without the presence of crystallization seeds in the form of concrete mixture components (binder, aggregates, additives), depending on the type of microorganism and its metabolism, the composition of the nutrient medium and biochemical agents, taking into account existing technological conditions for the formation of a matrix of cement-based building material.
2. Materials and methods

2.1. Strains and inoculation procedure
During the research the strains of gram-positive spore-forming bacteria from the All-Russian collection of microorganisms of Institute of Biochemistry and Physiology of Microorganisms named after G.K. Scriabin, Russian Academy of Sciences of *Bacillus Lysinibacillus sphaericus* (VKM B-509), *Bacillus megaterium* (VKM B-40), *Bacillus pumilus* (VKM B-23), *Sporosarcina pasteurii* (VKM B-513), since under natural environmental conditions they are involved in biomineralization processes, mainly calcium carbonate. The bacterial strains from the surface of slant agar were transferred to a sterile meat-peptone broth (PMB) and then they were cultivated in a laboratory shaker at a temperature of 37 °C and a speed of 120 rpm.

2.2. **pH** measurement
In order to establish the range of changes in pH level and evaluate the processes of urea hydrolysis by bacterial cultures, the initial pH value was set at level 7 by adding HCl. The pH measurement of the solutions was carried out by the potentiometric method, both before the experiment and during it, with daily intervals.

2.3. **Microscopic culture examination**
The localization and morphology of crystalline newgrowths was studied using light microscopy method (laboratory microscope Axio Scope.A1, A-Plan 20 / 0.45 and A-Plan 63×/0.1 lenses, C-mount adapter 0.63×; ProgRes SpeedXTcore5 camera) with periodic photofixation in order to justify the mechanism of crystal formation, to observe the nucleation and growth of crystals. In order to characterize the micromorphology of the synthesized crystals and their aggregates, the authors used a high resolution scanning electron microscopy (TESCAN MIRA 3 LMU microscope), which included an X-MAX 50 Oxford Instruments NanoAnalysis energy dispersive spectrometer for electron probe microanalysis.

2.4. **Biomineralization experiments**
CaCl₂ was used as a source of calcium ions. The following ranges of precursors were used to determine rational concentrations during the synthesis: for CaCl₂ — 5 and 10 g / l, for CH₃N₂O — 20 and 30 g / l. In order to find the optimal conditions for the crystallization initiation process, 4 variants of the medium were used, differing from each other by the variation of the concentrations of precursors and the type of nutrients. The composition of model solutions is presented in Table 1.

**Table 1.** Variants of combinations of nutrients and precursors for modeling the crystallization medium

| Bacteria                        | Cell concentrations | Water, l | Peptone 30 | Glucose 10 | Yeast Extract 5 | CH₃N₂O 20 | CH₃N₂O 30 | CaCl₂ 5 | NaCl 10 | NH₄Cl 10 |
|---------------------------------|---------------------|----------|------------|------------|----------------|-----------|-----------|---------|---------|---------|
| *Lysinibacillus sphaericus* VKM B-509 / | +       | +        | +          | +          | +              | +         | +         | +       | +       | +       |
| *Bacillus megaterium* VKM B-40 / | +       | +        | +          | +          | +              | +         | +         | +       | +       | +       |
| *Bacillus pumilus*              | +       | +        | +          | +          | +              | +         | +         | +       | +       | +       |
| *Sporosarcina pasteurii* VKM B-23 / | +       | +        | +          | +          | +              | +         | +         | +       | +       | +       |

Thus, in order to assess the contribution of parameters to the biomineralization process, the following combinations were formed: “type and amount of culture broth - type and amount of nutrient component - type and number of precursors”. The control was crystallization medium without the use of a bacterial inoculum.
3. Results

3.1. Growth kinetics of bacterial culture
According to the obtained data and the construction of kinetic growth curves of bacterial cultures (Figure 1), the following growth phases were identified:

1. Lag phase: it began after the introduction of the bacterial culture into the nutrient medium and was up to 2 hours for B-509, B-40, B-513 cultures, for B-23 was up to 4 hours. At that time, the population did not increase, but there was an increase in cell volume.

2. Transition phase: for bacteria B-509, B-40, B-513 it was observed from 2 to 4 hours, for B-23 - from 4 to 6 hours.

3. The exponential phase for strains B-513 and B-509 was recorded from 4 to 16 hours, for strain B-23 it was from 6 to 24 hours, for strain B-40 - from 4 to 24 hours.

4. The stationary phase: it occurred in strains B-513 and B-509 after 16 hours of cultivation, in strains B-23 and B-40 after 24 hours.

![Figure 1. Kinetic parameters of growth of bacterial cultures, CFU](image)

According to the kinetics of bacterial growth in the MPB medium, bacterial strains are ranked by growth rate in the following sequence: Bacillus megaterium (VKM B-40) → Lysinibacillus sphaericus (VKM B-509) → Sporosarcina pasteurii (VKM B-513) → Bacillus pumilus (VKM B-23).

3.2. Kinetics of pH measurement
The specificity of clinker mineral hydration processes is the increased alkalinity of the cementing system (pH = 12–14). On the other hand, microbial biomineralization by urolitic bacteria is possible only at alkaline pH values. This contributes to the formation of carbonate ions and negatively charged functional groups on the cell surface. Therefore, at the initial stage, the nature of the pH change during the incubation of various types of bacteria in various media was studied.

It was found that the change in the concentration of urea in the culture medium leads to changes in the level of pH and the productivity of bacteria (Figure 2). During the experiment, a significant difference in pH values was established between the control and the studied groups with a bacterial inoculum and precursors. Thus, the pH of the experimental group increased to 9–11 with an increase in incubation time. However, the pH value in the control group remained almost unchanged and remained at about 7. In addition, the alkaline component was influenced by a nutrient medium (peptone or yeast extract). A comparative assessment of the change in pH during the incubation period of bacteria allowed establishing that a composition with the addition of a yeast extract, which was pH = 10–11, was characterized by a higher final pH level of the medium compared to the same indicator.
for the composition with peptone — pH = 8–9.5.

Due to the fact that the model system was subjected to analysis in the absence of concrete mixture components, it is planned to establish the principles of the influence of the pH of a binder on the resistance of the microbiota under the conditions of the cement mixture at the next stage of work.

3.3. Crystal morphology

The crystals formed as a result of induction have a complex, irregular shape, arising as a result of the interaction of crystallization precursors and bacteria (Figure 3 c, e, f, g, h).

The obtained particles are of different sizes; idiomorphic crystals are observed in individual samples. According to morphology, the formed crystals are characterized as cubic (Figure 4 c), polygonal lamellar (Figure 3 b, d, e, f, h), spherical (Figure 3 c, e, f, g, h). Many crystalline formations along their contour are uneven, with visual signs of growth structures. The morphology of crystals formed as a result of biomineralization differs depending on the type of applied nutrient components (peptone and yeast extract). Spherical crystals are characteristic of a medium with the addition of yeast extract (Figure 3 a, b, c, d; Figure 4 a, b); irregular polyhedrons are formed as a result of interactions of peptone and precursors (Figure 3 e, f, g, h; Figure 4 d).

Figure 2. Change in pH of the model solution depending on the composition and type of bacterial strain.
medium with yeast extract

a) Bacillus pumilus (VKM B-23)  
Cell concentrations – 5×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

b) Bacillus megaterium (VKM B-40)  
Cell concentrations – 3×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

c) Lysinibacillus sphaericus (VKM B-509)  
Cell concentrations – 5×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

d) Sporosarcina pasteurii (VKM B-513)  
Cell concentrations – 5×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

medium with peptone

e) Bacillus pumilus (VKM B-23)  
Cell concentrations – 5×10^6 CFU/ml; CaCl_2 – 10 g/l; CH_4N_2O – 30 g/l

f) Bacillus megaterium (VKM B-40)  
Cell concentrations – 3×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

g) Lysinibacillus sphaericus (VKM B-509)  
Cell concentrations – 5×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

h) Sporosarcina pasteurii (VKM B-513)  
Cell concentrations – 5×10^6 CFU/ml; CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

Figure 3. Morphology of crystals induced by bacterial cultures

In samples synthesized with the participation of the bacterial strain B-513, particles are formed in the form of cubic crystalline formations (Figure 4 c). Spherical crystals are induced in solutions with inoculums of strains of *L. sphaericus*, *B. pumilus* and *B. megaterium* (Figure 4 a, b).

Medium with yeast extract  
am) Lysinibacillus sphaericus (VKM B-509)  
Cell concentrations 3×10^6 CFU/ml, CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

Medium with peptone  
b) Bacillus pumilus (VKM B-23)  
Cell concentrations 5×10^6 CFU/ml, CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

c) Sporosarcina pasteurii (VKM B-513)  
Cell concentrations 5×10^6 CFU/ml, CaCl_2 – 50 g/l; CH_4N_2O – 20 g/l

d) Bacillus megaterium (VKM B-40)  
Cell concentrations 3×10^6 CFU/ml, CaCl_2 – 5 g/l; CH_4N_2O – 20 g/l

Figure 4. Microstructure of crystals induced by bacterial cultures

4. Conclusion

According to the combined analysis of the kinetics of bacterial growth in the MPB medium, periodic microscopic observation of the process of mineral formation, and analysis of the pH medium, bacterial strains are ranked by the intensity of formation of finely dispersed inclusions located on the bottom of the Petrie dish and on the surface of the bacterial film. Thus, in model systems, rational (from the point of view of processability) types of bacteria, nutrient media, and biochemical agents for biogenic mineral formation under simulated conditions were revealed: *B. pumilus* VKM B-23 in a medium on a yeast extract with an inoculum concentration of 3×10^6 CFU/ml, with the addition of CaCl_2 - 10 g / l; CH_4N_2O – 30 g / l; *S. pasteurii* VKM B-513 in peptone medium at an inoculum concentration of 5×10^6, with the addition of CaCl_2 - 50 g / l; CH_4N_2O – 20 g / l.

The integrated morphological assessment of the obtained crystals, taking into account the
frequency of occurrence in the research samples, made it possible to rank them in the following sequence: spherical, formed mainly in a medium with the addition of yeast extract → irregular polyhedrons, mainly in a medium with the addition of peptone.

According to the presented results, taking into account the works on the physicochemical processes of the formation of inorganic newgrowths in the presence of bacteria, the authors proposed a phenomenological model of the processes occurring as a result of bacterial activity with the participation of various types of bacterial cultures, nutrient components and precursors of crystallization initiation. It is formed as the ratio and mutual influence of the stages of biominalization and the stages of structural formation of building material, using the technology of epy introduction of precursors of microbial biominalization and a bacterial agent or association of agents in concrete mix:

I - supersaturation of the solution with crystallization initiators ↔ tempering of the concrete mixture with water, change in the chemical composition of the liquid phase;

II - nucleation (the first stage of nucleation), which follows a heterogeneous path ↔ the beginning of hydration; binder setting period; the formation of a C – S – H gel;

III - crystal formation, depending on such medium parameters as pH, concentration of the bacterial culture, concentration and type of component of nutrient medium and the crystallization initiation precursor ↔ hydration, growth of newgrowths, strength gain;

IV - aggregation of crystals, their growth while maintaining the concentration of crystallization precursors ↔ hydration, growth of newgrowths, strength development;

Further research of bacterial cultures on biominalization processes with components of cement mixture will be carried out using all of the above mentioned cultures, culture media and precursors to continue ranking and identify principles.

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