Microbial producer of acid urease for its application in biocementation

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Abstract

Introduction. A popular biocementation method, microbially initiated precipitation of calcium carbonate, is accompanied by the release of ammonia and ammonium ions into the environment. The aim of the present study was selection of a producer of acid urease for its application in biocementation based on the calcium phosphate formation.

Materials and methods. Isolation, selection, identification, and characteristics of bacterial producer of acid-tolerant urease were conducted. Sequencing the 16S rRNA gene of isolate was done for its identification. Bone meal served as a source of calcium for biocementation using inactivated cells of bacterial producer of acid-tolerant urease. Assessment of sand biocementation was provided by the change of its water permeability.

Results and discussion. Selection of a bacterial strain that synthesized acid urease was carried out among bacteria isolated from acidic soil. The strain with the highest urease activity was identified by rRNA gene amplification and sequencing as *Staphylococcus saprophyticus* AU1. The physiological properties of the strain were studied. The maximum growth rate of strain AU1 was 0.15 h^{-1}, the maximum accumulation of biomass was 6.9 g/l of dry biomass, and the maximum urease activity was 8.1 mM hydrolyzed urea/min. The highest urease activity of *Staphylococcus saprophyticus* AU1 was found in the pH range from 4.5 to 5.5, and it gradually decreased with pH increasing. To ensure environmental biosafety, the use of inactivated bacterial cells that retain urease activity has been proposed. To receive completely inactive cells, they were treated with 0.5% sodium dodecyl sulfate solution for 90 minutes. Biocementation of sand was conducted using inactivated cells of *S. saprophyticus* AU1 and acid hydrolysate of bone meal, which has calcium in the form of phosphorus-containing compounds. The water permeability of biocemented sand was $2 \cdot 10^{-5}$ m/s, which makes it possible to use biocementation of this type to strengthen the soil to reduce its liquefaction, for example after an earthquake, or to control dust erosion for prevention of atmospheric pollution.

Conclusions. The main advantage of the proposed method for biocementation is the diminishing of urea consumption up to 75% and, thereby, reduction of emissions of ammonium and ammonia into the environment. In addition, the problem of bone waste disposal is solved and the cost of materials for biocementation is reduced.

Introduction

Traditional cement is widely used in construction and for grouting due to its relatively low cost and high strength. Cement production is growing worldwide at 0.8–1.2% per year, and its consumption is expected to increase to 3.7–4.4 billion tons in 2050 (Benhelal et al., 2013). The disadvantage of cement is the high viscosity of the suspension, which limits its use. In addition, cement production requires large amounts of electricity (approximately 2.6% of global energy consumption) and is accompanied by the release of 0.73 – 0.99 tons of CO_2 per ton of cement produced, which accounts for 8.0 – 8.6% of global anthropogenic carbon dioxide emissions (Aprianti, 2017; Miller et al., 2018; Nie et al., 2022). CO_2 emissions from cement production are expected to increase by up to 260% between 1990 and 2050 (Cuzman et al., 2015) and global emissions will be 2.34 billion tons of CO_2 in 2050 (Mishra et al., 2022).

It is known that the release of greenhouse gases into the atmosphere causes global warming, while climate change is recognized as one of the world's most important environmental problems. The increasing trend of carbon dioxide emissions worldwide and in leading countries is directly related to the production of cement, which is considered the second most consumed material in the world after water (Avramenko et al., 2022; Imbabi et al., 2012).

In recent years, active research has been conducted in the world to obtain an alternative material – biocement, based on the use of bacteria producing urease, which in the presence of urea and calcium ions form insoluble calcite crystals that adhere to a solid surface. The main advantage of biocement over conventional cement is the low viscosity of the biocement solution, which allows it to penetrate into small pores and microchannels of the soil and microcracks in rocks and concrete. Due to the activity of bacteria producing urease, urea hydrolysis occurs, which leads to an increase in pH and the release of carbonate ions:

$$(NH_2)_2CO + 2H_2O + \text{urease} \rightarrow 2\ NH_4^+ + 2\ OH^- + CO_2 + \text{urease} \quad (1)$$

Carbon dioxide in water turns into carbonate ion and in the presence of calcium ions and high pH forms insoluble calcium carbonate (Stocks-Fischer et al., 1999):

$$CO_2 + H_2O \rightarrow H_2CO_3 \quad (2)$$

$$H_2CO_3 \rightarrow H^+ + HCO_3^- \quad (3)$$

$$HCO_3^- \rightarrow H^+ + CO_3^{2-} \quad (4)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3\downarrow \quad (5)$$

Thus, biocement is a mixture of at least three components: (1) an inorganic component that forms calcium carbonate, (2) a component that changes the pH and initiates the precipitation of calcium carbonate, and (3) the urease enzyme or living microbial cells with urease activity, or non-living microbial cells but retaining enzymatic activity and catalyzing a change in pH to values at which precipitation of calcium carbonate occurs. The inorganic component is soluble calcium salts that are converted into an insoluble substance. The component that changes the pH and supplies CO_2 for carbonation is urea, which is hydrolyzed by the catalytic action of microbial cells with urease activity or enzyme urease.

Several hundred scientific articles are published annually on the topic of biocementation, which indicates a growing interest in this direction of biotechnology development (Chu et al., 2012; Ivanov et al., 2019a; Omoregie et al., 2020). One of the reasons preventing the large-scale application of this biotechnology is the release of toxic

ammonia gas into the atmosphere during the biocementation process and the possible release of ammonium ions into groundwater and surface water (Gowthaman et al., 2020; Ivanov et al., 2019a).

When carrying out traditional microbially initiated biocementation, the pH of the environment, in which biocementation occurs, reaches values of 8.5 – 9.5 due to the release of ammonium ions and toxic ammonia. It is known that 99% of the released nitrogen will be in the form of ammonium ions NH_4^+ at a pH value of 7.3, but if the pH value exceeds 7.5, there is a sharp change towards the formation of NH_3 gas (Gowthaman et al., 2021). To prevent soil contamination with ammonium ions, several methods have been proposed that provide additional treatment for their removal, in particular the addition of zeolite (Keykha et al., 2019), the retention of ammonium ions near the cathode during electrobiocementation of soil (Keykha and Asadi, 2017), precipitation of ammonium ions in the form of struvite (Mohsenzadeh et al., 2021; Yu et al., 2020), washing the biocemented sand column to remove water-soluble ammonium (Lee et al., 2019), and adsorption of the removed ammonium with sulfuric acid (Ivanov and Stabnikov, 2017). But the problem of ammonia emissions remains unresolved. A promising highly environmentally friendly alternative for reducing the release of toxic ammonia into the environment may be biocementation based on calcium phosphate formation (Ivanov et al., 2019a). Calcium phosphate, similar to calcium carbonate, is a promising structural material with sufficient strength characteristics. The use of calcium phosphate compounds for soil improvement has significant advantages such as (a) calcium phosphates are non-toxic and environmentally friendly materials. They are known to be major constituents of bones and vertebral teeth, including most human hard tissues (Toshima et al., 2014), what does it prove their safety in soil application; (b) the solubility of phosphorus-containing calcium compounds depends on the pH of the environment, therefore, at an acidic pH in the soil, they can be in an insoluble state (Ivanov et al., 2019a; Kawasaki and Akiyama, 2013); (c) calcium phosphate formed in the soil can serve as phosphorus fertilizer for crop plants (Akiyama and Kawasaki, 2012). Biogrouting of soil can be carried out based on the precipitation of calcium phosphate compounds to form insoluble and strong hydroxyapatatite crystals (Akiyama and Kawasaki, 2012; Yu and Jiang, 2018) from inorganic (Dilrukshi et al., 2015; 2016) or organic phosphates (Roeselers and van Loosdrecht, 2010). So, calcium phosphate is considered to be a promising structural material with fairly high strength properties.

The production of calcium phosphate can occur according to equation:

$$4\ Ca^{2+} + 6\ CaHPO_4 + 8\ OH^- \longrightarrow Ca_{10}(PO_4)_6(OH)_2\downarrow + 6\ H_2O \qquad (6)$$

Precipitation by this method can occur due to enzymatic hydrolysis of urea, which increases the pH from 4.4 to 7.0. An economic advantage of the hydroxyapatite application is the possibility of using bone meal, obtained from cattle bones and partially used as a component of feed or fertilizer. However, about half of the bones remaining after meat processing are usually burned in cement kilns.

Urease (urea amidohydrolase, EC 3.5.1.5) is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide and is widely distributed in the natural world, including plants, animals, and microorganisms. Ureases used in biocementation processes currently have optimal reaction pH values in the range from neutral to alkaline, and are not only labile and prone to deactivation in an acidic environment, but also do not even catalyze the reaction of urea hydrolysis, especially if the ambient temperature is higher than 25°C.

More often, biocement is produced by precipitation of calcium carbonate at an alkaline pH value. However, the use of acid urease may be beneficial under certain environmental

conditions where the initial pH is low, for example when using calcium bicarbonate as a calcium source or when producing calcium phosphate.

The aim of the present study was to select a microbial producer of acid urease for biocementation using calcium phosphate.

Materials and methods

Urease-producing bacterial strain and its cultivation

Urease-producing bacterium (UPB) strain *Bacillus* sp. VUK5 (Stabnikov et al., 2013a; 2014) was used in this study. Strain of *Bacillus* sp. VUK5 is an alkaliphilic, halotolerant, aerobic, and spore-forming Gram-positive rod. The strain was grown on a nutrient medium with the following composition: Tryptic Soy Broth (Difco Laboratories, Detroit, Michigan, USA); urea, 20 g; NaCl, 100 g; $NiCl_2 \cdot 6H_20$, 24 mg; phenol red, 10 mg; distilled water 1 l (TSB medium). The indicator phenol red was added to the medium to detect the development of urease-producing bacteria: its color is yellow at pH 6.8, but gradually changes to red if the pH rises to 8.2 and becomes bright pink (purple) at pH higher than 8.2. All components of the medium, except urea, were sterilized at 121°C for 15 minutes. A concentrated urea solution, 100 g/l, was sterilized by filtration through a 0.22 μm nitrocellulose filter to prevent urea decomposition during heat sterilization and 20 ml/l was added to sterile media (TSB). 2 ml of a concentrated solution of trace elements was added to a sterile TSB medium. The concentrated solution of trace elements had the following composition: $ZnSO_4 \cdot 7H_2O$, 0.1 g; $MnSO_4 \cdot H_2O$, 0.085 g; H_3BO_3, 0.06 g; $CoCl_2 \cdot 6H_2O$, 0.02 g; $CuCl_2$, 0.004 г; $Na_2MoO_4 \cdot 2H_2O$, 0.04 г; $FeCl_2$, 0.3 г; deionized water to 1 l. pH was adjusted to 2.0 by 1N HCl (Stabnikov et al., 2013a).

Selection of a bacterial strain that synthesizes acid-tolerant urease

Acidic soil taken under pine trees, into which urea was previously added, serves as an inoculum to obtain an enrichment culture of bacteria producing acid urease. 10 g of soil was added to 30 ml of 1.5% NaCl solution, mixed thoroughly and allowed to settle. The liquid fraction served as the starting material for obtaining an enrichment culture. Enrichment cultures were grown on a TSB nutrient medium with initial pH 5.8. An indicator bromocresol purple was added to the medium to show the pH change: at pH 5.4 to 6.8 the medium has yellow-green color, and when urea hydrolysis occurs and the pH increases, the color changes to purple, indicating that pH > 6.8. Enrichment culture, in which the color changed faster and was more intense, was used for the selection of pure cultures producing acidic urease.

Characteristics of a pure culture of acid urease-producing bacteria and its identification

The morphology of bacterial cells was observed using a Zeiss EV050 scanning electron microscope, UK. Gram staining was performed using a Gram stain kit.

The nearly full-length 16S rRNA gene was amplified by polymerase chain reaction (PCR) with forward primer 27F and reverse primer Universal 1492R (Lane, 1991). Purified PCR products were sequenced using an ABI PRISM 3730xlDNA sequencer and a ready-made ABI PRISM BigDye Terminator Cycle Sequencing reaction kit. Primers 27F, 530F, 926F, 519R, 907R and 1492R were used to sequence both strands of the 16S rRNA gene:

27F (5'-AGAGTTTGATCMTGGCTCAG-3'); 530F (5'- GTGCCAGCMGCCGCGG-3'); 926F (5'-AAA CTY AAA KGA ATT GAC GG-3'); 519R (5'-GWATTACCGCGGCKGCTG-3'); 907R (5' -CCG TCH ATT CMT TTR AGT TT-3'); 1492R (5'-ACGGYTACCTTGTTACGACTT-3'). The resulting PCR products were purified and sequenced using an ABI PRISM3730xl DNA capillary analyzer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator Cycle Sequencing ready-reaction kit (Applied Biosystems). The forward and reverse sequences obtained on the ABI 3730 XL analyzer for AU1 were aligned using BioEdit version 7.1.9. The sequences were finally assembled to obtain the full-length sequence, and the full-length sequence was compared to other sequences available in the NCBI Genbank database using BLAST (http://blast.ncbi.nlm.nih.gov).

Microscopic analysis

Gram staining was performed using a standard set of dyes. Scanning electron microscopy (SEM) was performed using a Zeiss EV050, UK. Samples were fixed in 2% glutaraldehyde for 2 hours, washed three times with 0.1 M cacodylate buffer for 20 minutes, and stepwise dehydrated in 50, 70, 85, and 95% (v/v) ethanol solutions for 10 minutes, dried in a Polavon E3100 vacuum dryer, Quorum Technologies, UK, and then sputter coated with Au-Pt using an Emitech SC7620, Quorum Technologies, UK.

Estimation the concentration of bacterial cells by plating on solid nutrient media

The number of colony-forming units (CFU) in the experiment with inactivation of *Staphylococcus saprophyticus* AU1 cells by treatment with dodecyl sulfate was determined by seeding 0.1 ml of a suspension of bacterial cells from its ten-fold sterile dilutions on a Petri dish, which were filled with agar. The number of CFUs was counted after incubation of Petri dishes at 30°C for 2 days.

Determination of bacterial biomass concentration

Bacterial biomass concentration was determined using a calibration graph based on optical density. Optical density measurements were carried out using a photoelectrocolorimeter at 590 nm and the biomass concentration was determined using a calibration graph. The content of absolutely dry biomass (ADB) of bacteria was determined by the standard method after filtering and drying the sample at a temperature of 105°C to constant weight.

Determination of physiological characteristics of acid urease producer

The specific growth rate was determined using the equation:

$$\mu = (\ln X_1 - \ln X_0)/(t_1 - t_0), \quad (7)$$

where X_1 and X_0 are biomass concentrations at time t_1 and t_0, respectively.

pH determination

pH was measured on a pH meter Eutech instruments pH 2700. A pH meter with a measurement range from -2 to 20 pH having 0.002 pH accuracy.

Determination of urease activity

Urease activity was determined using a TDS-3 portable conductometer: the amount of released ammonium was determined according to the calibration graph by changing the electrical conductivity of the solution, μS/cm, due to hydrolysis of urea under the action of the urease enzyme (Stabnikov et al., 2022). The molar concentration of NH_4^+ (Y) correlated linearly (R^2 = 0.999) with the change in the electrical conductivity of the solution (ΔX) in μS/cm over 5 minutes. Urease activity (UA) was defined as the amount of ammonium formed in 1M urea solution per minute.

Materials for biocementation

Sand. River sand was sifted through a metal sieve with a pore opening diameter of 0.5 mm (Fig. 1a).

Bone meal (Manufacturer: ODO "Lisichansk Gelatin Plant") with a wet content of 5%, protein 6.8%, lipids 1.8%, calcium 29.3%, phosphorus 13.4% (Fig. 1b).

a **b**

Figure 1. Sifted sand (a) and bone meal (b).

Analysis of particle size distribution of sand and bone meal

Particle size distribution and average size were measured using a Bettersizer S3 Plus particle size analyzer (Bettersize Instruments, Dandong, China). For each sample, 3 measurements were taken to determine the average particle size. The maximum sizes of 10% (D10), 50% (D50) and 90% (D90) of all particles were determined.

Bone meal dissolution was carried out according to (Gowthaman et al., 2021). Bone flour, 50 g, was added under stirring to 200 ml of distilled water, and 60 ml of hydrochloric acid, 2 M HCl (5 ml per minute) was gradually added. The hydrolysis process lasted 80 minutes. The remaining bone meal was filtered off and the resulting solution was used in further studies.

Determination of water permeability of biocemented sand

To determine the water permeability of the treated sand samples, 0.1–0.2 l of tap water was supplied by gravity from 1 l of a container with water at a practically constant hydraulic pressure of 0.5 m of water. This measurement was close to ASTM D2434-68 (2006)

"Standard Test Method for Permeability of Granular Soils". The hydraulic permeability of sand, P, in a sand core was calculated according to equation:

$$P = V/(t \cdot A), \text{ m/s}, \quad (8)$$

where V is the volume of water, which was used to determine the water permeability of a sand column, m^3; t is the time for which water passes through the sand, s; A is the cross-sectional area of the column, m^2.

Biocementation of sand using inactivated *Staphylococcus saprophyticus* AU1 cells with urease activity

Sifted river sand was added to columns with a volume of 50 ml (height 7 cm, diameter 3 cm) in an amount of 60 g and was carefully compacted. The prepared columns were mounted on laboratory stands (Fig. 2).

Figure 2. Sand columns for biocementation.

A suspension of inactivated bacterial cells, 20 ml, was slowly supplied into the column, the effluent outlet from the column was closed and kept for 2 hours for cell adsorption. The culture liquid was separated from the column by gravity, the sand in the column was washed with 20 ml of distilled water, and biocementation was carried out by feeding 20 ml of biocementation solution for 24 hours (one bioprocessing cycle).

Acid hydrolysate of bone meal, which has calcium in the form of a phosphorus-containing compound, was used as a source of dissolved calcium in the biocementing solution. To reduce the water permeability of sand, the biocementation cycle was repeated 8 times. As a control, a column into which a calcium solution was supplied, but without urea was used.

Statistical analysis

The experiments were carried out in triplicates. Statistical processing of the experimental results was carried out using special programs for personal computers. Data are presented as mean ± standard deviation.

Results and discussion

Isolation and identification of a strain producing acid urease

A tenfold dilution of enrichment culture with a sterile 1.5% NaCl solution was distributed onto solidified liquid medium TBS in Petri dishes and incubated at 30°C for 2 days. Single colonies, the color of the medium near changed from yellow-green to purple, were isolated into pure cultures and tested for urease activity when grown on a liquid medium under aerobic conditions in a batch cultivation mode in flasks on a shaker at room temperature for 4 days (Fig. 3).

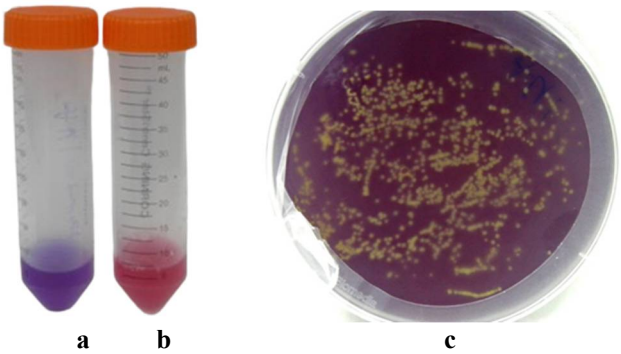


Figure 3. Growth of an acid urease producer in a liquid medium (a, left tube) and on solid medium in the presence of the bromocresol purple indicator (c); growth of *Bacillus* sp. VUK5 in a liquid medium with phenol red is shown (b).

Urease activity of strains isolated from an enrichment culture is shown in Figure 4.

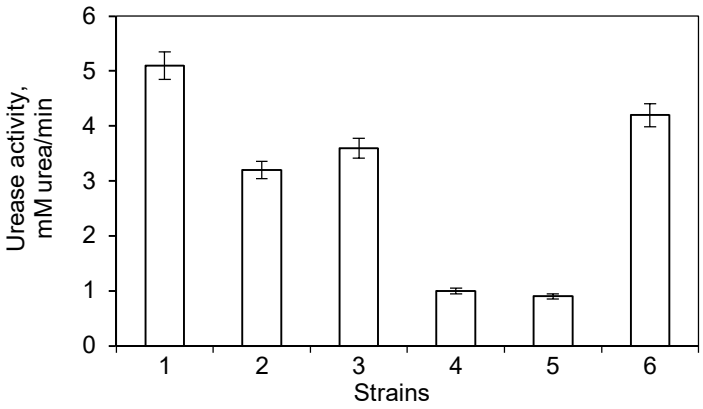


Figure 4. Urease activity of isolated strains producing acidic urease.

Strain AU1 with higher urease activity was selected for further studies. The strain was identified by rRNA gene amplification and sequencing. Partial nucleotide sequences were combined to obtain the complete nucleotide sequence of the 16S rRNA gene. Strain AU1 was nonsporeforming Gram-positive spherical cells. Determination of the nearest neighbor phylogenetic sequences of the 16S rRNA gene of strain AU1 using the BLAST search program against known species in the National Center for Biotechnology Information database revealed that it is a member of the genus *Staphylococcus*: related strains are *Staphylococcus saprophyticus* ZK-3(100% identity); *Staphylococcus* sp. WW60 (100% identical), and *Staphylococcus saprophyticus* T86 (100% identity). Complete nucleotide sequence of the 16S rRNA gene of strain AU1 is as follows:

GCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGATAAGGAGCTTGCT
CCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTT
CGGGAAACCGGAGCTAATACCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTG
CTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACG
ATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAACGTGTAAGTAACTGTGCACGTCTT
GACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTC
CATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
GTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCAACGCATTAAGCACT
CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAAACCTTACCAAATCTTGACATCCTTTGAAAACTCTA
GAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGC
ACTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTAT
GATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAA
TCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACGACTACATGAAGCTGGAATCGCTAGTA
ATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGA
GAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCGTCGAAGGTGGGACAAA
TGATTGGGGTGAATCTAA.

The marine bacterium *Staphylococcus saprophyticus*, a producer of alkaliphilic protease with a wide range of temperature activity (10-80 ºC) was identical (99%) to *Staphylococcus saprophyticus* ZK3 and *Staphylococcus* sp. WW60 (99%) (Uttatree and Charoenpanich, 2018). *Staphylococcus* sp. WW60 is an endophytic bacterium with strong activity against *Candida* yeasts (Das et al., 2019). A scanning electron microscope (SEM) image of strain AU1 cells is shown (Fig. 5).

Characteristics of the isolated strain of urease-producing bacteria *Staphylococcus saprophyticus* AU1

The isolated strain *Staphylococcus saprophyticus* AU1 was cultivated and its urease activity was compared with the known strain *Bacillus* sp. UK5 (Stabnikov, 2013a). Strains UK5 and AU1 were grown on the appropriate nutrient media described in section Materials and methods. The initial pH value of the medium for the UK5 strain was 7.2 and 5.8 for the AU1. Cultivation was carried out under aeration conditions under shaking 150 rpm for 3 days. The growth curves and changes in urease activity during the cultivation of these two strains are shown in Figure 6.

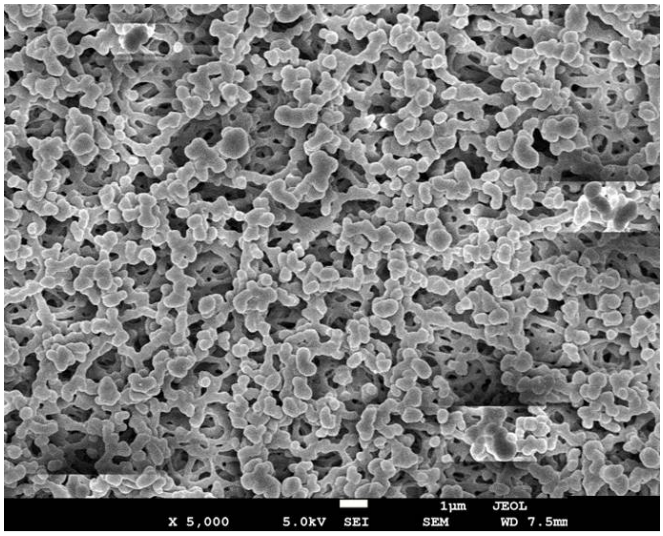


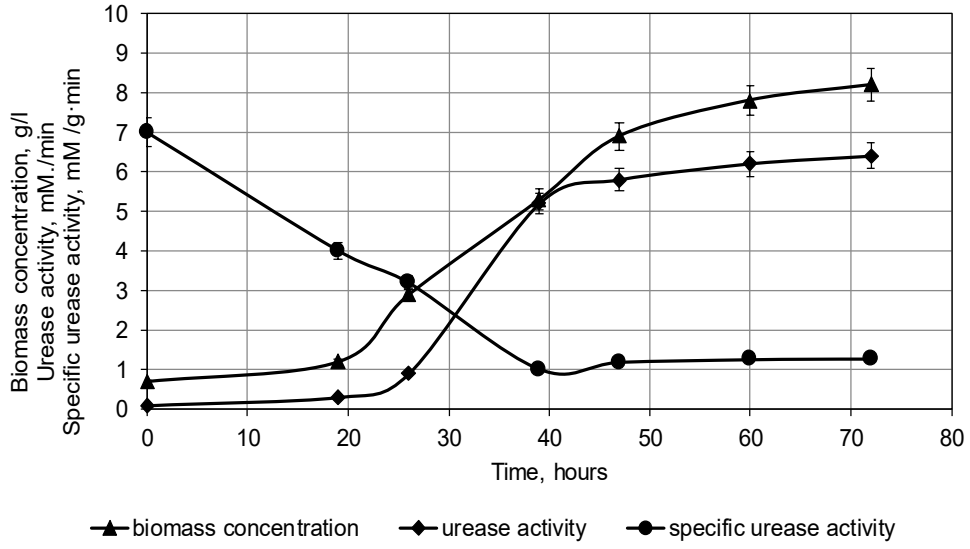
Figure 5. SEM images of cells of the strain of acid-tolerant urease producing bacteria *Staphylococcus saprophyticus* AU1.

When periodically cultivated under aeration conditions, strain UK5 showed a maximum growth rate of 0.11 h^{-1}, a maximum biomass accumulation of 8.2 g/l of dry biomass after 3 days of cultivation, and the maximum urease activity 6.4 mM hydrolyzed urea/min (Fig. 6a), while the maximum growth rate of strain AU1 was 0.15 h^{-1}, the maximum biomass accumulation was 6.9 g/l dry biomass, and the maximum urease activity was 8.1 mM hydrolyzed urea/min (Fig. 6b). Thus, the acid-tolerant strain AU1 had a maximum biomass accumulation by 19% lower, a maximum growth rate by 36.3% higher, and a maximum urease activity of the culture liquid by 26.5% higher than the same indicators for the UK5 strain.

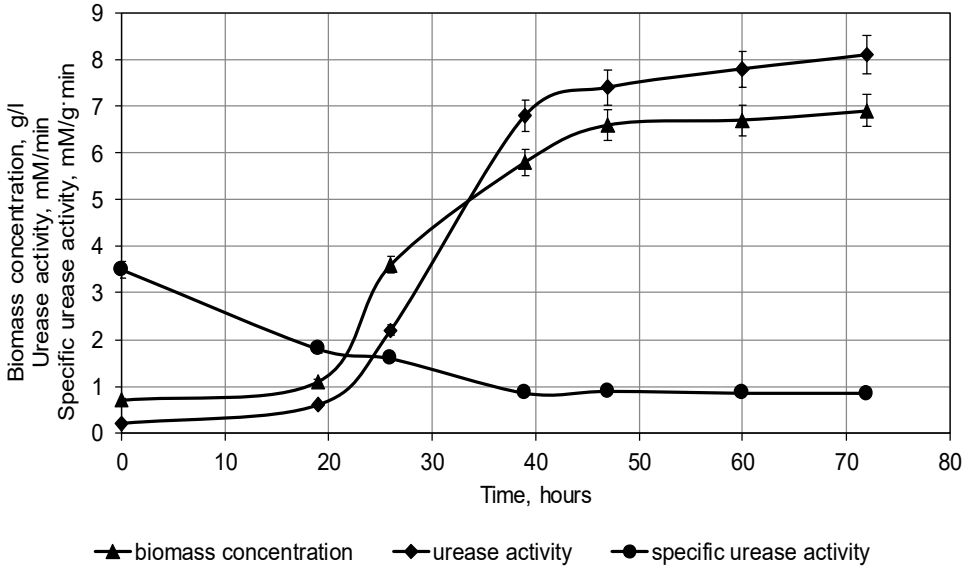
The urease activity values obtained for the acid-tolerant strain AU1 are also close to the urease activity of other strains used for biocementation, for example, *Sporosarcina paste*urii ATCC 11859, 13.3 mM hydrolyzed urea/min (Whiffin, 2004), and a strain of *Bacillus* sp., isolated from the soil in Australia, 3 mM hydrolyzed urea/min (Al-Thawadi and Cord-Ruwisch, 2012). The same urease activities were used in the biocementation of sand using pure urease enzyme: urease activity ranged from 2.5 mM/min to 10.5 mM/min (Almajed et al., 2019) and 5.9 мМ/хв (Jiang et al., 2016).

Changes in urease activity of *Staphylococcus saprophyticus* AU1 cells depending on the pH of the environment

The dependence of the activity of acid urease of *Staphylococcus saprophyticus* AU1 on the pH of the medium was determined in the range from 4.0 to 8.0 (Fig. 7).



a



b

Figure 6. Change in biomass concentration, g dry biomass/l (▲), urease activity, mM/min (■) and specific urease activity (●) during batch cultivation of *Bacillus* sp. UK5 (a) and *Staphylococcus saprophyticus* AU1 (b).

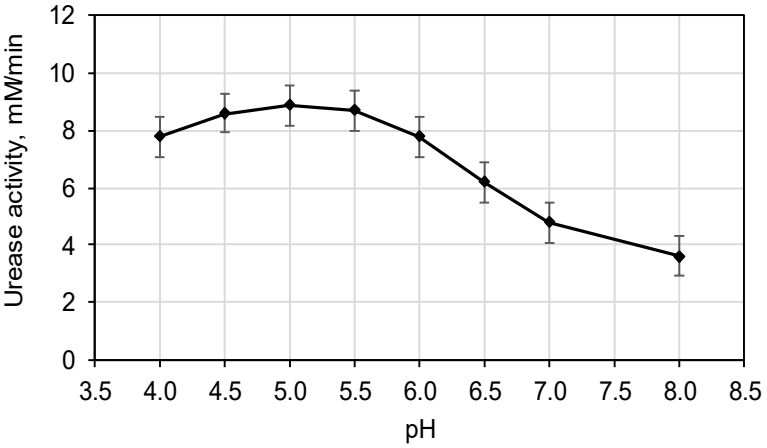


Figure 7. Urease activity of *Staphylococcus saprophyticus* AU1 depending on the pH of the medium.

The highest urease activity was observed in the pH range from 4.5 to 5.5, gradually decreasing with pH increasing. The optimal pH value for the action of acidic ureases, according to the literature, was 4.5 for urease, the producer of which was the strain *Enterobacter* sp. R-SYB082 (Yang et al., 2010); 4.2 for urease produced by the strain *Arthrobacter mobilis* SAM 0752 (Miyagawa et al., 1999). Commercially available Nagapshin urease, produced by Nagase Chemtex Corporation (Kyoto, Japan) and used in (Gowthaman et al., 2021), was active in the pH range from 4.0 to 8.0 with an optimum for action at pH 5.0.

Changes in urease activity during inactivation of *Staphylococcus saprophyticus* AU1 cells

The use of inactivated bacterial cells for biocementation has been proposed to maintain environmental biosafety. As has been shown in the work (Stabnikov et al., 2016), inactivated bacterial cells of *Yaniella* sp. VS8, which retained urease activity, can be used for bioclogging of soil. The use of pure urease enzyme in large-scale biocementation is expensive. In addition, the use of bacterial cells has advantages such as the creation of calcium carbonate crystallization centers in the processed materials due to the adhesion of cells on their surface. When comparing the effectiveness of such methods for inactivating cells of bacteria *Yaniella* sp., based on rupture of the cell membrane, such as treating a cell suspension with ultrasound; incubation for 1 hour in ethanol solutions and incubation in sodium dodecyl sulfate solution with a final concentration of 0.5% (w/v), it was shown that (1) sonication (750 watts; 20 megahertz; amplitude 50%) for 30 s, 60 s, and 300 s did not inactivate cells; (2) incubation of bacterial cells in 25% (v/v), 50% (v/v), and 70% (v/v) ethanol solutions significantly reduced the concentration of living cells from $5.2 \cdot 10^8$ CFU/ml to $4.9 \cdot 10^2$ CFU/ml, while (3) treatment with a 0.5% dodecyl sulfate solution for 120 minutes ensured complete inactivation of cells, while maintaining urease activity.

The inactivating effect of dodecyl sulfate was confirmed by scanning electron microscopy, which showed the destruction of treated bacterial cells of urease-producing

bacteria. Based on these data, it was proposed to use sodium dodecyl sulfate to inactivate *Staphylococcus saprophyticus* AU1 cells; its inhibitory effect on bacteria is based on the dissolution of the cell membrane (Hansen et al., 2011). The cell suspension with pH 5.0 was added with calculated amount of sodium dodecyl sulfate and kept at a room temperature. Data on keeping a bacterial suspension of *Staphylococcus saprophyticus* in a 0.5% sodium dodecyl sulfate are given in Table 1.

Table 1

Effect of treatment time of *Staphylococcus saprophyticus* AU1 cells in 0.5% sodium dodecyl sulfate on their urease activity and survival

Time of incubation, min	Urease activity, mM hydrolyzed urea/min during incubation in a solution of 1M urea, min *		CFU/ml**
	5	30	
0	7.7	4.8	$1.2 \cdot 10^8$
10	4.2	7.7	$2.9 \cdot 10^2$
30	3.8	6.9	$0.7 \cdot 10^1$
60	3.4	5.9	6.0
90	3.3	5.7	0

* the measurement accuracy was less than 10%.

** the measurement accuracy was less than 20%.

Incubation of bacterial suspension of *Staphylococcus saprophyticus* AU1 in a 0.5% sodium dodecyl sulfate significantly reduced the concentration of living cells, and exposure for 40 minutes allowed their complete inactivation. Urease activity decreased, but remained quite high – the percentage of losses during processing for 40 minutes was 26% of the original (Table 1). Complete inactivation of cells of *Yaniella* sp.VS8, when treated with 0.5% sodium dodecyl sulfate solution, was observed after 120 minutes of exposure (Stabnikov et al., 2016).

According to the results, urease activity of the treated cells increased with the time of incubation in a 1M urea solution, which indicates the constitutive nature of urease synthesis in *Staphylococcus saprophyticus*, which becomes more accessible when the cell ruptures. This is also evidenced by data on changes in urease activity during incubation in a 1 M urea solution (Table 2).

Table 2

Urease activity (UA) for different times of incubation of bacterial cells in 1 M urea solution

Cells of *S. saprophyticus* AU1	UA*, mM hydrolyzed urea/min during contact with 1M urea solution, min					
	5	30	60	90	120	150
Live untreated	7.7	4.8	3.9	3.6	3.0	2.1
Inactivated after incubation in 0.5% sodium dodecyl sulfate solution for 90 minutes	3.3	5.7	6.9	5.7	3.9	2.3

* The measurement accuracy was less than 10%.

Thus, the most suitable method for inactivating bacterial cells while maintaining their urease activity is incubation of a bacterial suspension in a 0.5% sodium dodecyl sulfate for 90 minutes, which will ensure biosafety when using *Staphylococcus saprophyticus* AU1 cells for biocementation.

Staphylococcus saprophyticus AU1 bacteria were grown under sterile conditions in a liquid medium prepared from Tryptic Soy Broth under the shaking at 200 rpm at room temperature. Cells were inactivated by adding 0.5% (w/v) dodecyl sulfate and holding for 90 min. The treated cells were separated by centrifugation at 5000 rpm for 10 minutes, resuspended in 2% (w/v) NaCl solution and used for biocementation of sieved river sand.

Preparing a bone meal solution

Bone meal was used as a source of calcium. As shown in Gowthaman et al. (2021) calcium and phosphorus are present in bone meal as hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. Hydrochloric acid dissolution of bone meal (bone hydroxyapatite) was carried out according to the equation:

$$Ca_{10}(PO_4)_6(OH)_2 + 8\ HCl \longrightarrow 10\ Ca^{2+} + 6\ HPO_4^{2-} + 8\ Cl^- + 2\ H_2O$$

pH and calcium concentration were measured during dissolution Ca^{2+} (Fig. 8).

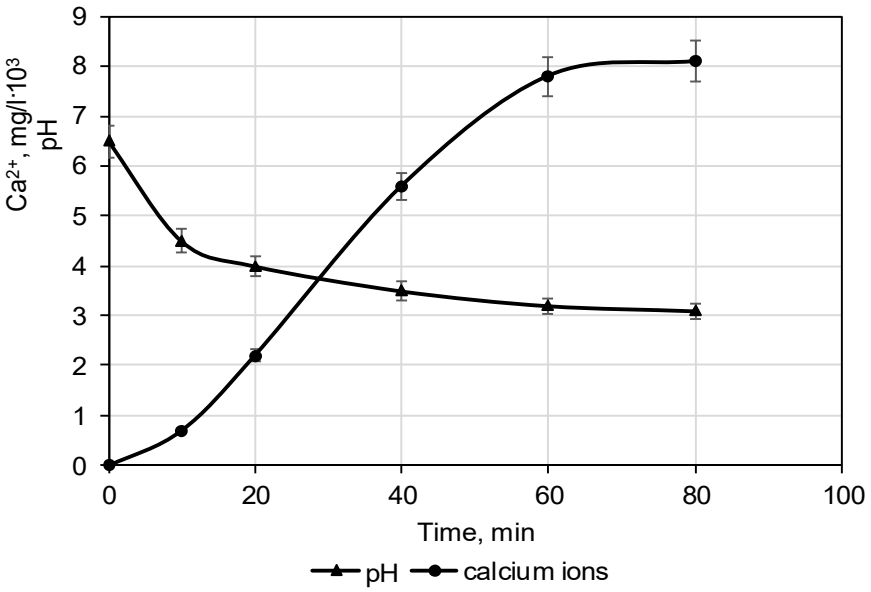


Figure 8. Changes in pH and calcium concentration when bone meal is dissolved in hydrochloric acid.

The final pH value after 80 min of bone meal dissolution was 3.5, and the concentration of calcium ions was 7800 mg/l. The remaining bone meal was filtered off and the resulting solution was used in further studies. The indicators of the filtered solution of Ukrainian bone meal hydrolysate were similar to those obtained in the study of Japanese researchers: 8800 mg/l of calcium ions and a pH value of 3.4 (Gowthaman et al., 2021).

Biocementation of sand using hydrolyzed bone meal as a source of calcium

The possibility of using bone meal as a source of calcium for biocementation was tested using inactivated cells of an acid-tolerant urease producer. Bone meal is one of the excellent and inexpensive sources of calcium and inorganic phosphate and can be used as a cementing material instead of calcium chloride. To use bone meal as a component for the formation of biomement, it was pre-dissolved in dilute hydrochloric acid. Bone meal dissolved in acid along with urea and a source of acidic urease was introduced into sand for biocementation. The components for biocementation were: (a) bone meal hydrolysate, (b) a suspension of inactivated cells of acid urease bacterial producer, and (c) urea. Sand was biocemented at the ratio of calcium and urea ion concentrations according to Table 3.

Table 3

Variants for sand biocementation

Sample	Concentration of Ca^{2+}, g/l	Concentration of urea, g/l	Mole ratio
1	7.8	1.8	0.25
2	7.8	3.9	0.50
3	7.8	5.7	0.75

For biocementation, sifted river sand was used. Particle size distribution and average size were measured using a Bettersizer S3 Plus particle size analyzer (Bettersize Instruments, Dandong, China) (Fig. 9).

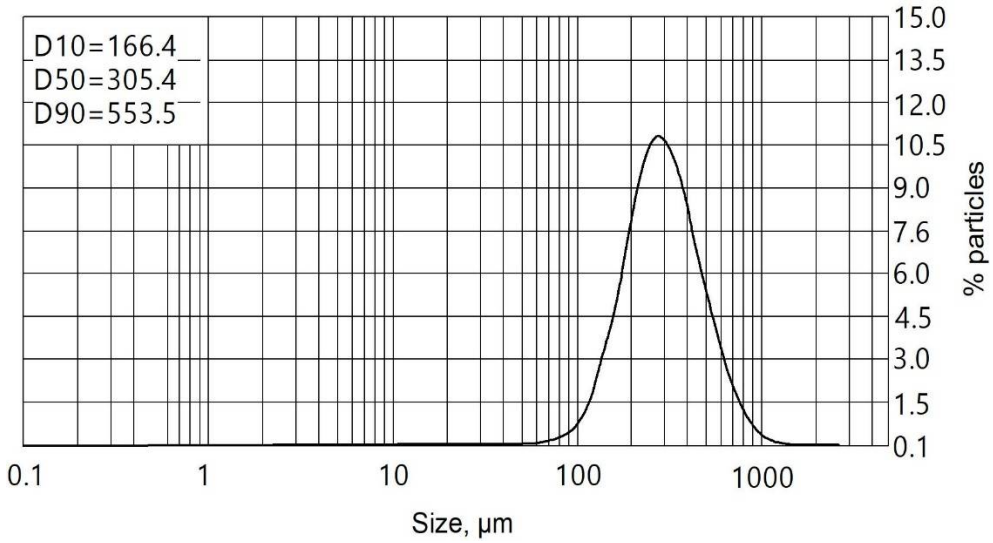


Figure 9. Size distribution of sifted sand particles.

Thus, the main sand fractions had particles ranging from 200 to 300 μm (30.31%) and 300.0 to 400 μm (23.74%). D10=166.4 μm; D50=305.4 μm; D90=553.5 μm, that is, 10% of the sand had particles less than 166.4 μm, 50% less than 330 μm and 90% less than 1600 μm.

Sand according to particle size is classified as fine – particle size from 75 to 425 μm; medium – particle size from 425 to 20500 μm, and coarse - particle size from 2000 to 4750 μm. That is, the sand that was used for biocementation in the present research was fine. For comparison, biocementation studies typically use: ASTM (American Society for Testing and Testing Materials) graded sand with an average particle size of 400 μm; standard sand with rounded particles (Societe Nouvelle du Littoral, France Ser) with an average particle size of 420 μm; standard Ottawa sand with an average size of 300 μm with particle size varying from 150 to 1180 μm.

Sand, 60 g, was placed in a column with a volume of 50 ml and an internal diameter of 30 mm. Suspension of inactivated bacterial cells (20 ml) was slowly supplied into the column. The sand with inactivated cells was kept for 2 hours for cell adsorption, and then the suspension was drained by gravity. The sand was washed with 20 ml of water. After gravity removal of water, 20 ml of biocementing solution was supplied into the column according to Table 3. Incubation was carried out for 24 hours, and the remaining solution was removed by gravity.

To increase the strength of the treated sand, a biocementing solution was injected into each column 8 times. The control was a sample in which a calcium solution was supplied, but without urea. The results of calcium accumulation showed that in the control calcium was not retained in the sand, but was washed out. Thus, only in the presence of urea with an increase in pH due to its enzymatic hydrolysis, dissolved calcium is converted into an insoluble calcium-phosphorus-containing compound, which fills the pores of the sand and holds it together. Thus, a small amount of calcium during biocementation of this type will be precipitated in the form of calcium carbonate, and the bulk will be precipitated as a calcium-phosphorus-containing compound that allows reducing the amount of urea used for biocementation, and, as a result, significantly diminishing the release of ammonium and ammonia into the environment. The change in permeability of sand is shown in Figure 10.

Sand biocemented by the proposed method does not have the same strength as with conventional biocementation due to the formation of calcium carbonate, and its water permeability has decreased only to $2 \cdot 10^{-5}$ m/s, when with biocementation it is usually consists about $1 \bullet 10^{-6}$ m/s. It is not practical to carry out a larger number of bio-treatments, but the data obtained allow the use of bio-cementation of this type to strengthen the soil to reduce its erosion, for example after an earthquake, or to combat dust erosion to reduce atmospheric pollution (Stabnikov et al., 2013b). The main advantage of this method is the possibility of a significant reduction (by 75%) emissions of ammonium and ammonia into the environment.

Thus, compared with the conventional biocementation method based on microbially initiated calcium carbonate precipitation, the use of bone meal hydrolysate as a source of calcium when using acid-tolerant urease offers significant environmental and economic advantages, namely: (a) reduction in the release of ammonium and toxic ammonia into the environment; (b) reduction in material costs, and (c) solution of bone waste disposal problem.

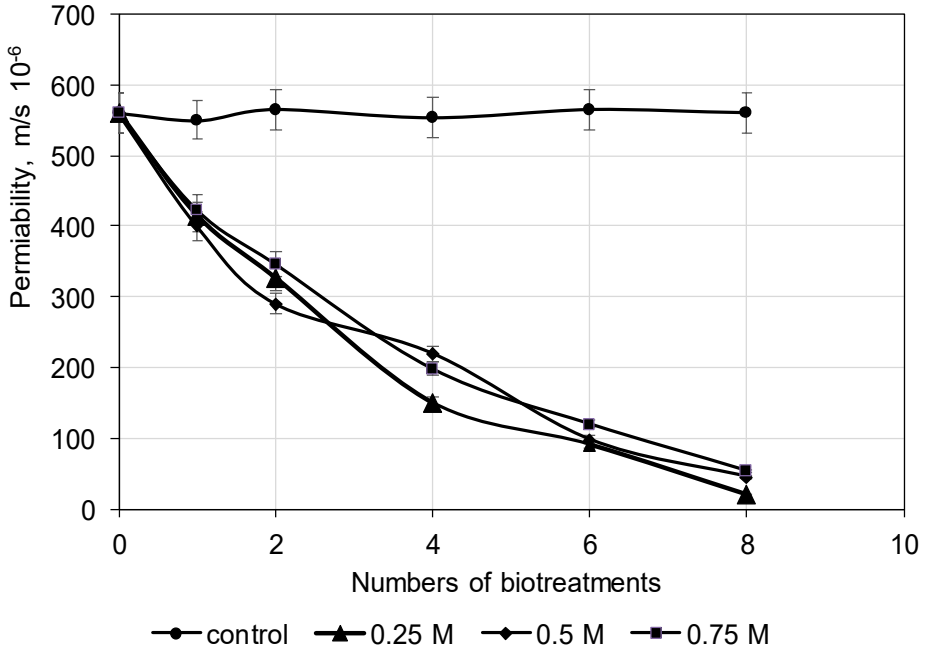


Figure 10. Changes in the water permeability of sand during biocementation using bone meal hydrolysate and a reduced amount of urea (0.25 M, 0.5 M, and 0.75 M), the hydrolysis of which occurred under the action of inactivated cells of acid urease producer.

Conclusions

1. Selection of a bacterial strain that synthesized acid urease was carried out among bacteria isolated from acidic soil. The strain with the highest urease activity was identified by rRNA gene amplification and sequencing as *Staphylococcus saprophyticus* AU1.
2. The physiological properties of the strain were studied: the maximum growth rate of strain AU1 was 0.15 h^{-1}, the maximum accumulation of biomass was 6.9 g/l of dry biomass, and the maximum urease activity was 8.1 mM hydrolyzed urea/min. The highest urease activity of the *Staphylococcus saprophyticus* AU1 strain was observed at the pH range from 4.5 to 5.5, gradually decreasing with increasing pH.
3. To ensure environmental biosafety, the use of inactivated bacterial cells that retain urease activity has been proposed for biocementation. Complete inactivation of cells was observed when cells were treated with 0.5% sodium dodecyl sulfate solution for 90 minutes.
4. Application of bone meal containing calcium in the form of hydroxyapatite allows reducing the amount of urea during the biocementation process. The water permeability of sand biocemented using, hydrolyzed bone meal and acid-tolerant urease, was $2 \cdot 10^{-5}$ m/s, which makes it possible to use biocementation of this type to strengthen the soil to reduce its liquefaction, for example after an earthquake, or to control dust erosion for reduction of atmospheric pollution.

5. The main advantage of this method is the ability to significantly reduce by 75% urea consumption and, thereby, reduce emissions of ammonium and ammonia into the environment. In addition, the problem of bone waste disposal is solved and the cost of materials for biocementation is reduced.

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