Production of Bioagent for Calcium-Based Biocement

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Abstract Biocements and biogrouts are developing extensively as new materials alternative to cement and toxic chemical grouts. The most popular type of biocement is a mixture of urease-producing bacteria, urea and calcium salt. Thus, development of biotechnology to produce biomass of urease-active bacteria for large-scale biocementation is an important biotechnological task. Two strains of urease-producing bacteria, *Yaniella* sp. VS8 and *Bacillus* sp. VS1 that synthesized inducible and constitutive urease, respectively, were used in the present study. It was shown that low cost biomass of urease-active bacteria can be produced from the hydrolyzed excessive activated sludge of municipal wastewater treatment plant. The biomass of *Yaniella* sp. VS8 grown in this medium diminished the hydraulic conductivity of sand from 4.8×10^{-4} m/s to $5\cdot10^{-8}$ m/s after several biotreatments with solution of 1.5 M urea and 0.75M $CaCl_2$.

Keywords *Bacillus* sp., *Yaniella* sp., Activated sludge, Biocement, Biogrout.

INTRODUCTION

New area of Biotechnology, Construction Biotechnology, is developing in two directions: 1) biotechnological production of construction materials, and 2) biotechnology of construction processes [1]. Biocements and biogrouts are developing extensively as new construction materials alternative to cement and chemical grouts. Most popular type of biocement and biogrout produce calcium carbonate minerals due to activity of urease-producing bacteria (UPB) in the presence of urea and calcium ions [1-9]. The most popular type of biocementation and biogrouting is based on microbially-induced calcium carbonate precipitation (MICCP). It is a sequence of the following steps: 1) adhesion of cells of urease-producing bacteria on the soil particle/rock surface; 2) creation a microgradient of carbonate/bicarbonate concentration and the pH in the site of cell attachment due to hydrolysis of urea by urease; and 3) formation of calcium carbonate crystals that are attached to the soil particle or rock surface [1, 8].

$$(NH_2)_2CO + 2\,H_2O + CaCl_2 \xrightarrow{UPB} CaCO_3 \downarrow + 2\,NH_4Cl$$

Biomass of urease-producing bacteria is one of the major components of the biocement. Therefore, the important task for the development of biocementation biotechnology is the method to obtain UPB biomass for large-scale applications using cheap raw materials.

The medium ATCC 1376 NH4-YE, which contains yeast extract, 20 g; $(NH_4)_2SO_4$, 10 g; 0.13 M Tris buffer (pH 9.0), 1L, is recommended for cultivation of urease-producing bacteria *Sporosarcina pasteurii* (former *Bacillus pasteurii*) and is most often used for laboratory studies of biocementation. This medium was used for cultivation of *Sporosarcina pasteurii* ATCC 11859 and *Bacillus pasteurii* ATCC 6453 [10-12]. Sometimes, Tris buffer is replaced with distilled water, but the pH of the medium is adjusted to 9.0 [9]. Very often ammonium sulfate is replaced with urea, 20 g/L, and addition of urea to medium for cultivation of UPB is usually recommended. Liquid medium is sterilized by autoclaving for 15 – 20 min at 121°C, meanwhile solution of urea has to be sterilized by filtration through 0.2 μm Millipore filter to avoid thermal decay. The strain of *Sporosarcina pasteurii* DSMZ 33 was grown in Nutrient Broth supplemented with 2 % (w/v) urea [13]. Tryptic Soy Broth with urea, 20 g/L, was recommended for cultivation of *Sporosarcina pasteurii* DZM 33 by Collection of Microorganisms and Cell Cultures, Braunschweig, Germany [14, 15]. Yeast extract with urea was used for cultivation of *Bacillus sphaericus* LMG 225 57 [5, 16]. Nutrient Broth supplemented with 2% urea was used for cultivation of *Sporosarcina pasteurii* NCIMB 8841 [17]. Sometimes, medium contains both ammonium sulfate and urea [18, 19].

In some studies, extra source of carbon was added to medium to enhance the conditions for cultivation of UPB. For example, 100 mM sodium acetate was added to the medium containing yeast extract, urea, and ammonium sulfate for cultivation of enrichment culture of UPB [20], and calcium acetate, 26 g/L, was added to the medium containing Nutrient Broth, 3 g/L,

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ammonium chloride, 10 g/L, sodium bicarbonate, 2.1 g/L, and urea, 10 g/L, for cultivation of urease-producing bacteria *Sporosarcina pasteurii* and *Bacillus sphaericus* [21].

Urease is a nickel-containing enzyme; therefore some authors used media with an addition of nickel chloride. Addition of 10 μm Ni^{2+} ions to the yeast extract medium increased urease activity, however, higher concentrations of nickel led to its significant dropping [22]. The similar results were obtained for recombinant strain of *Esherichia coli*, containing a plasmid that encodes *Bacillus pasteurii* urease [23]. Addition of nickel in concentration of 5 – 100 μM enhanced microbial calcite precipitation. Optimal concentration of Ni^{2+} was 5 μM, however concentration of Ni^{2+} above 1000 μM totally suppressed calcium precipitation. Addition of 0.1 mM $NiCl_2$ to the medium with yeast extract, 20 g/L, and 0.17 M ammonium sulfate, was used for cultivation of *Bacillus shaericus* MCP-11 [10]. Another medium for cultivation of UPB contained 10 μM of nickel ions [24].

The pH of medium for cultivation of UPB is usually adjusted to 9.0 – 9.5 [19, 25, 26], but sometimes medium has lower pH, for example 8.6 [18]. However, all media described above are too expensive for the large scale construction application of biocementation.

One of the ways to reduce the cost of biocementation is the use of low cost medium for cultivation of UPB. It was shown that replacement of yeast extract with cheap soybean meal in the medium, that was used for cultivation of *Sporosarcina pasteurii*, increased urease activity in comparison with ATCC 1376 NH4-YE medium [11]. The industrial effluent of the dairy industry, lactose mother liquor (LML), was proposed as a growth medium for cultivation of UPB *Sporosarcina pasteurii* [26]. Urease activity of culture liquid and concentration of UPB biomass grown in LML – urea media (10% LML, 5 g NaCl, 2 % urea, and 25 mM $CaCl_2$) were almost the same that were obtained on YE-medium (1 g yeast extract, 5 g NaCl, 2% urea, and 25 mM $CaCl_2$).

It is known that hydrolyzed activated sludge can be used for cultivation of different microorganisms [28, 29]. There was attempt to use activated sludge, produced on wastewater treatment plant, for growth of urease-producing bacteria *Sporosarcina pasteurii* ATCC 11859 [26]. Sludge was treated with 0.5M NaOH for 20 min with following neutralization to pH 8.0 with H_2SO_4. However, this hydrolysate did not sustain growth of UPB. So, until now, the majority of researchers use expensive microbiological media with addition of urea after its cold sterilization by filtration. This technique is not suitable for large scale cultivation of UPB in case of their practical, large-scale use for biogrouting and biocementation.

The aim of the present study was to find a low cost medium to produce biomass of UPB suitable for industrial-scale biocementation/bioclogging.

MATERIALS AND METHODS

Microorganisms and their Identification

Two strains of urease-producing bacteria were used in this study. One of them was the strain *Bacillus* sp.VS1. This strain was used in our previous research for bioclogging and biocementation of sand [3, 14, 15, 30].

New strain of UPB was isolated from enrichment culture of urease-producing bacteria using sand after several weeks biocementation of sand in $1m^3$ tank. Ten-fold dilutions of enrichment culture grown in liquid TSB medium (composition is shown below) were used to grow colonies at 30°C for 6 days in Petri dishes with Tryptic Soya Broth (Difco Laboratories, Detroit, Michigan, USA with urea solidified by addition of 20 g Bacto Agar (Difco)/L with phenol red. Phenol red was used as a pH indicator: its color is yellow at the pH 6.8, but gradually changes to red/bright pink color at the pH above 8.2. Colony on Petri dish with higher urease activity was isolated for further study.

The morphology of the bacterial cells was observed by scanning electron microscopy (SEM, Zeiss EV050, UK). Gram-staining was performed using Gram-stain kit (Difco Laboratories, Detroit, MI, USA). The nearly full-length 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) with forward primer 27F and reverse primer Universal 1492R [25]. Purified PCR products were sequenced using the ABI PRISM 3730xlDNAsequencer and the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit. Primers 27F, 530F, 926F, 519R, 907R, and 1492R were adopted to sequence both strands of the 16S rRNA gene. The sequences were finally assembled to produce the full-length sequence and the full-length sequence was compared with all other sequences available in the NCBI Genbank database using BLAST (http://blast.ncbi.nlm.nih.gov).

Media and Conditions Used for UPB Cultivation

TSB medium for microbial cultivation had the following composition: Tryptic Soya Broth (Difco Laboratories, Detroit, Michigan, USA), 30 g; NaCl, 20 g, $NiCl_2 \cdot 6H_20$, 24 mg, distilled water 1L, pH 8.2. For the aseptic cultivation, TSB medium was sterilized at 121°C for 15 min. Stock solution of urea, 100 g/L, was sterilized by filtration through Millipore filter with diameter 0.2 μm to avoid urea loss due to thermal treatment, and added to sterile medium, 200 mL to 800 mL of medium. Batch cultivation of urease-producing bacteria (URB) was under shaking conditions at 200 rpm at temperature of 25°C.

Determination of Biomass Concentration

Accumulated bacterial biomass was measured either as the content of total suspended solids or by counting colony-forming units (CFU) on Petri dishes. Dry weight of bacterial biomass was determined after centrifugation and drying at 105°C to a constant weight. Ten-fold dilutions of enrichment or pure cultures were used to enumerate colonies at 30°C after 5 days growth of bacteria in Petri dishes filled aseptically with sterile Tryptic Soya Broth (Difco Laboratories, Detroit, Michigan, USA) solidified by addition of 20 g Bacto Agar (Difco)/L.

Measurement of Urease Activity

Urease activity (UA) was measured as the amount of ammonium produced from 1M solution of urea per minute [3]. Amount of ammonium produced from urea was determined using the electric conductivity meter showing linear correlation (R^2 = 0.9997) between the molar concentrations of NH_4^+ (Y) and the changes of electric conductivity of solutions (ΔX) in mS/cm for 5 min.

Experimental Set Up

To check is urease produced by the studied urease-producing bacterial strains *Yaniella* sp VS8 and *Bacillus* sp. VS1 either a constitutive or an inducible enzyme, batch cultivation of bacteria was conducted in the YE-medium of the following composition, g/L: yeast extract, 20; NaCl, 20; $NiCl_2$, 0.02, phenol red, 0.01, distilled water to 1L This medium, 100 mL, was placed in 300 mL flasks. Sterilization of the YE-medium was at 121°C for 15 min. Nothing was added to the flask 1; 20 mL of stock solution of urea with concentration of urea 100 g/L, sterilized by filtration through Millipore filter with diameter 0.2 μm, was added into sterile medium in the flask 2; NH_4Cl, 20 g/L was added into the flask 3 before thermal sterilization. Inoculum, 10 mL per 100 mL of medium, was added into each flask. Cultivation was conducted on the shaker at 200 rpm at the temperature of 25°C. Urease activity was determined for 5 min contact of bacterial cells with 1M solution of urea.

To determine influence of urea concentration on urease activity of *Yaniella* sp. VS8, batch cultivation was conducted using sterile YE-medium with the initial pH adjusted with 1M NaOH to 7.2. Concentrations of urea were 5, 10, and 20 g/L in the flasks 1, 2 and 3, respectively. Inoculum, which was culture liquid of *Yaniella* sp. VS8 stored at 4°C, was added in quantity 5% (v/v).

To compare several media for UPB growth and urease synthesis, cultivation was conducted for 48 h (for *Bacillus* sp. VS1) or 64 h (for *Yaniella* sp. VS8) at the temperature of 25°C on the shaker at 200 rpm in the 300 mL flasks with 100 mL of media of the following basic composition: NaCl, 20 g/L; $NiCl_2$, 24 mg/L; urea, 20 g/L. Different sources of carbon and energy were added to this basic medium, g/L: (1) TSB, 30; (2) TSB, 30; yeast extract (YE), 10; (3) YE, 20; (4) YE, 40; (5) YE, 40; acetate, 10.

Activated sludge from municipal wastewater treatment plant (MWWTP) was tested as possible source of nutrients for UPB. Sludge was allowed to settle down, then supernatant was removed, and the concentrated sludge was used for the medium preparation. Content of total suspended solids (TSS) in this concentrated sludge was 5.7 g TSS/L. Acid hydrolysis of sludge using 98 % H_2SO_4 was conducted. Concentrated sulfuric acid, 3 mL, was added per 100 mL of sludge. Acid hydrolysis of activated sludge was conducted at 80°C for 1 h. The hydrolysate of activated sludge (HAS) has been neutralized to the pH value of 8.0 with 5M NaOH, and was supplemented with NaCl, 20 g/L. The medium was not transparent and had a lot of suspended flocks of biomass. This medium, 100 mL, was placed in each of three 300 mL conic flasks. Nothing was added to the flask 1. Solution of $NiCl_2$, 24 mg/L, was added to the flasks 2 and 3. Glucose, 3 g/L, was added to the flask 3. No urea was added into the medium for cultivation of *Bacillus* sp. VS1. Sterile solution of urea, 20 g/L, was added into the medium for cultivation of *Yaniella* sp. VS8. The flasks were placed on the shaker, and bacteria cultivation was conducted at 200 rpm and the temperature of 25°C.

The experiments were made in triplicate to check the repeatability and quantify the changes of bacterial cultivation parameters statistically.

RESULTS AND DISCUSSION

Characteristics of Urease-Producing Bacteria Used in this Study

The strain *Bacillus* sp. VS1 (GenBank accession number of nucleotide sequence of 16S rRNA JF896459) isolated from sand on the medium with urea [3, 14, 15] was used in his study. It is similar to the strain *Bacillus* sp. CPB 2 (GenBank accession number of nucleotide sequence of 16S rRNA AF548874) (identity was 99%) isolated for ureolytic microbial calcium carbonate precipitation in Ghent University, Belgium [21]. BLAST results suggested that the closest relative of the strain *Bacillus* sp. CPB 2 is *Bacillus pasteurii* belonging to Risk group 1 according to The Technical Rules for Biological Agents (TRBA). The strain *Bacillus* sp. VS1 is aerobic, spore-forming, Gram-positive rods with the size 0.5 μm in width and 2 μm in length (Figure **1a**). Colonies are circular, entire, and opaque (2 - 4 mm in diameter).

The strain VS8 (GenBank accession number of nucleotide sequence of 16S rRNA KT 182991) was selected from enrichment culture of urease-producing bacteria. Isolated strain was similar to *Enteractinococcus* sp. YIM 101632 (identity was 98 %) and Y*aniella* sp. YIM 100590 (identity was 96 %). The strain Y*aniella* sp. YIM 100590 was considered representing a novel species of a new genus within the family *Micrococcaceae*, for which the name *Enteractinococcus coprophilus* gen. nov., sp. nov. was proposed [32]. This strain was alkalophilic (was able to grow at pH 7.0–11.0 with the optimal pH 8.0), and halotolerant (tolerated up to 11% (w/v) NaCl). The slightly halophilic (growth occurred with 0-20% (w/v) NaCl), and facultatively alkaliphilic (growth occurs at pH 6.0–10.5) actinobacterium strain *Yaniella soli* sp. nov. was isolated from the forest soil in China [33]. An orange bacterial strain *Yaniella fodinae* sp. nov. was isolated from a soil sample of a coal mine [18]. Some strains from the genus *Yaniella* are halotolerant [33, 34]. We did not find any reference in literature that representative of this genus caused any infection. The bacteria of the genus Y*aniella* belong to Risk group 1. Strain VS8 is Gram-positive cocci, non-sporulating, halotolerant, alkaliphilic, aerobic bacterium. Cells have average diameter about 1 μm, non-motile (Figure **1b**). Colonies are circular (0.3–1.0 mm in diameter), usually white, but become orange growing on TSA with content of NaCl 5%. Strain was able to grow between the pH values of 6.5 and 11.0.

Is the Addition of Urea to the Media Necessary for Urease Activity?

Usually, urease-producing bacteria are cultivated in the medium containing urea [5, 14-19]. Separate sterilization of urea and addition it to the medium complicates production of a large quantity of urease-producing bacterial biomass for large-scale biocementation.

Theoretically, to ensure synthesis of urease, urea should be added to the medium in the case when urease-producing bacteria synthesize an inducible enzyme, and it is possible to cultivate UPB in the medium without urea in case when synthesized urease is a constitutive enzyme. Testing what type of urease is produced by *Bacillus* sp. VS1 or *Yaniella* sp. VS8 was

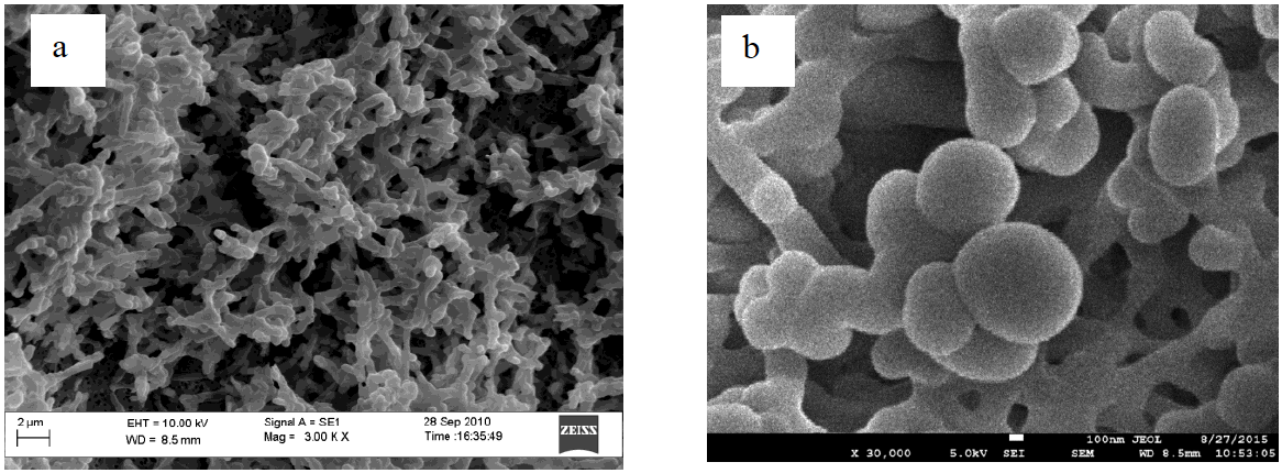

Figure 1: SEM image of the cells *Bacillus* sp. VS1 (**a**) and *Yaniella* sp. VS8 (**b**) on the membrane filter.

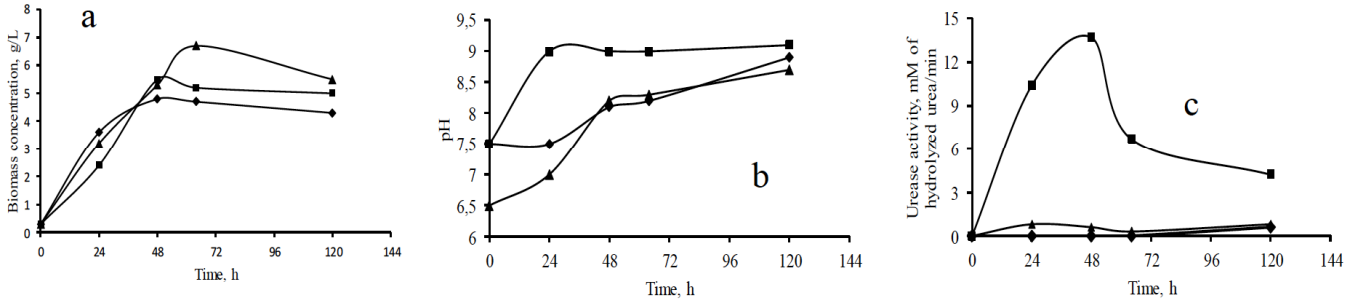

Figure 2: Changes of growth parameters of *Yaniella* sp. VS8 in media YE (⬥); YE+urea (■); YE+NH_4Cl (▲): biomass concentration, g of dry biomass/L (**a**); pH (**b**); urease activity, mM of hydrolyzed urea/min (**c**).

conducted. Parameters of *Yaniella* sp. VS8 growth in the media without urea or containing urea are shown in Figure **2**.

It was previously established that the strain *Yaniella* sp. VS8 does not grow at a pH lower than 6.5. So, the initial pH in all media was adjusted to 8.3. The pH decreased after 24 hours of cultivation to 7.5 in the medium with yeast extract and to 7.0 in the medium with yeast extract added with ammonium chloride. The pH was adjusted with 1M NaOH in these media to 8.3. The stable pH 9.0 was maintained in the medium with yeast extract added with urea. Maximum urease activity, 13.7 mM hydrolyzed urea/min, was observed in the medium added with urea after 64 hours of cultivation. However, in the media without urea, practically there was no urease activity at all. Thus, urease of the strain *Yaniella* sp. VS8 was inducible enzyme.

Other results have been obtained for *Bacillus* sp. VS1 grown in the media without or with urea (Figure **3**).

Bacillus sp. VS1 can grow at a slightly acid pH, so there was no need to adjust the initial pH of the media. The pH of YE medium was 6.5. Culture liquid of *Bacillus* sp. VS1 possessed the highest urease activity in the yeast extract medium with ammonium chloride (up to 20.3 mM hydrolyzed urea/min after 48 hours of cultivation). It was 19.2 mM hydrolyzed urea/min for the yeast extract medium without any addition of nitrogen source, and 10.7 mM hydrolyzed urea/min for the yeast extract medium added with urea. Thus, urease of *Bacillus* sp. VS1 is a constitutive enzyme. However, it was shown for *Yaniella* sp. VS8 that cells grown in the medium without urea, and, as a consequence, without urease activity, demonstrated fast increase of urease activity being transformed into the medium containing urea. For example, urease activity was observed when urea, 20 g/L, was added to the medium with yeast extract after 48 hours of growth. The pH of medium after next 24 hours was 8.9, urease activity was 4.5 mM hydrolyzed urea/min. Moreover, increase of urease activity was observed in the samples of bacterial cells without urease activity after their contact with urea solution (Table **1**).

Thus, the cells of *Yaniella* sp. VS8, grown in the medium without urea, being submerged in the solution of urea, synthesized an inducible urease enzyme, and the level of their urease activity reached very fast the level of urease activity of cells grown in the medium with urea. So, to obtain real value of urease activity of culture liquid it is necessary to make measurement in very short time, for example, 5 min. If cells are in contact with urea solution for a relatively long time, the values will be quite different. If bacterial culture grows in the medium with high enough concentration of urea, the value of urease activity become lower with the

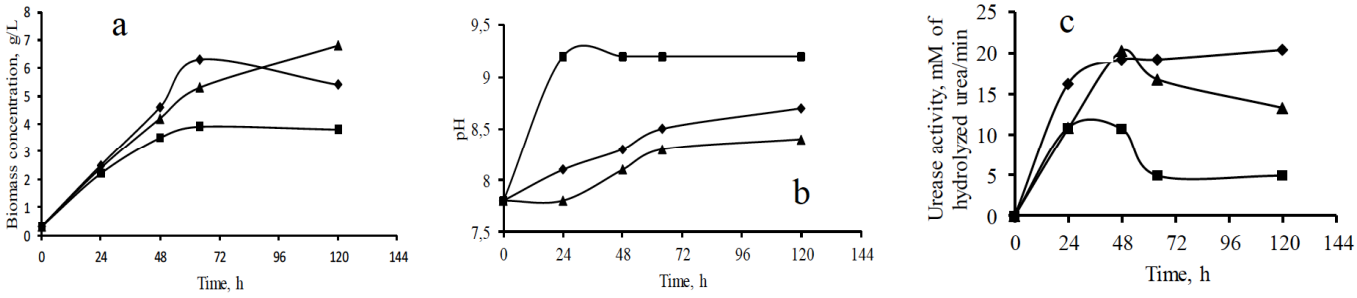

Figure 3: Changes of growth parameters of *Bacillus* sp. VS1 in media YE (◆); YE+urea (■); YE+NH_4Cl (▲): biomass concentration, g of dry biomass/L (**a**); pH (**b**); urease activity, mM of hydrolyzed urea/min (**c**).

Table 1: Average Urease Activity (UA) Determined for *Yaniella* sp. VS8 or *Bacillus* sp. VS1 Grown in Different Media

Duration of cell contact with 1M urea solution, min	Average UA, mM of hydrolized urea/min, for different time intervals					
	Yaniella sp. VS8			*Bacillus* sp. VS1		
	YE	YE+urea	YE+NH_4Cl	YE	YE+urea	YE+NH_4Cl
5	0	12.8	0	14.2	4.9	13.8
30	0.3	4.7	0.2	11.1	3.7	6.6
270	0.8	3.1	1.7	7.2	3.6	6.3
360	0.9	3.1	2. 9	6.3	3.5	6.3
1000	1.3	1.9	1.7	1.5	1.6	1.8

increase of time of bacterial cells contact with solution containing urea. Meanwhile, in case when bacterial culture grows in the medium with a low concentration of urea or at the absence of urea, the value of urease activity becomes higher with the increase of time of bacterial cells contact with the solution containing urea. Urease activities of bacterial culture *Yaniella* sp. VS8 grown in the media with the content of urea 5, 10, or 20 g/L are shown in Table **2**.

Determination of urease activity on the third day of cultivation showed the following values, mM hydrolyzed urea/min: 1.3, 2.4, and 8.4 (contact of cells with 1M urea was 5 min); 1.7, 2.1, and 6.8 (contact of cells with 1M urea was 30 min), and 2.0, 2.2, and 3.0 (contact of cells with 1M urea was 220 min) for the media with the content of urea 5, 10, and 20 g/L, respectively.

It must be taken into account that time needed to determine urease activity depends on the medium composition. For example, according to data shown in Table **3**, urease activity of culture liquid of *Yaniella* sp. VS8 after 64 hours of cultivation was 8.4 mM hydrolyzed urea/min in the medium containing 20 g/L urea which is higher than 1.3 mM hydrolyzed urea/min and 2.4 mM hydrolized urea/min in the media containing 5 g/L and 10 g/L urea, respectively. However, cells of *Yaniella* sp. VS8, grown in the media with a low urea concentration being transferred to the solution of 1M urea showed after certain time significant increase of urease activity (Table **3**). After 24 hours of contact there was no difference in urease activity for culture liquids obtained in the media with different concentration of urea.

According to these data, there is no need to grow urease-producing bacteria in medium containing urea notwithstanding this enzyme is inducible or constitutive. Even if content of urea in the medium will be low or absent, addition of urea in cultural liquid resulted in

Table 2: Parameters of *Yaniella* sp. VS8 Growth in Media with Different Urea Concentrations*

Time, h	Medium pH			UA, mM of hydrolized urea/min			Biomass, g/L		
	1	2	3	1	2	3	1	2	3
24	8.4	8.8	9.0	0.4	0.3	0.7	3.2	3.4	3.2
48	8.6	8.8	9.0	0.7	2.0	2.3	3.2	3.4	3.2
64	8.6	8.8	9.0	1.3	2.4	8.4	4.2	4.7	5.6
88	8.7	8.9	9.0	2.3	2.9	4.9	4.1	4.6	5.4

*Note: concentrations of urea were 5 g/L, 10 g/L, and 20 g/L in the media 1, 2, and 3, respectively.

Table 3: Average Urease Activity of Culture Liquid of *Yaniella* sp. VS8 in the Media with Different Concentrations of Urea*

Time of cells contact with 1M urea solution, min	UA, mM of hydrolized urea/min		
	1	2	3
5	1.28	2.38	8.42
30	1.70	2.14	4.83
180	2.04	2.24	3.00
1200	1.41	1.65	1.65

*Note: concentrations of urea were 5 g/L, 10 g/L, and 20 g/L in the media 1, 2, and 3, respectively.

urease synthesis, and urease activity become equal to one in the medium with high enough concentration of urea. The biomass of *Yaniella* sp. VS8 grown in this medium diminished the hydraulic conductivity of sand from 4.8×10^{-4} m/s to $5\cdot10^{-8}$ m/s after several biotreatments with solution of 1.5 M urea and 0.75M $CaCl_2$.

Urease Activity of Microbial Biomass Used for Biocementation

It is under the question what source of urease is better for biocementation: a) culture liquid after cultivation of urease-producing bacteria [9, 14, 15, 35]; b) bacterial cells separated from culture liquid [30]; c) supernatant without bacterial cells [35]; d) extract from UPB biomass containing urease [36].

In our experiments, cells of *Yaniella sp.* VS8 or *Bacillu*s sp. VS1 were separated from supernatant by centrifugation of 20 mL of culture liquid and concentrated cells were resuspended in 20 mL of 2% NaCl. Urease activities of initial culture liquid, supernatant and resuspended cells are shown in Table **4**.

Supernatant after centrifugation of *Bacillus* sp. VS1 cell suspension had urease activity of 3.3 mM hydrolyzed urea/min, meanwhile supernatant of *Yaniella* sp. VS8 cell suspension had almost zero urease activity. Resuspended cells of both strains had lower urease activity (UA) than initial culture liquids (Table **4**). The decrease of UA of resuspended cell suspension in comparison with initial culture liquid was 12% for *Yaniella* sp. VS8 and 48% for *Bacillus* sp. VS1. It can be explained that urease of *Bacillus* sp. VS1 was present in the cells and in supernatant, but urease of *Yaniella* sp. VS8 was present only in the cells and absent in supernatant.

Cells were also concentrated by centrifugation and resuspended in 10 mL of NaCl (concentrated in 2 times) and in 5 mL of NaCl (concentrated in 4 times). Concentrated cell suspensions had proportionally higher urease activity (Table **4**). So, it is possible to produce bioagent with high urease activity using concentrated suspension of UPB cells.

Use of Hydrolyzed Activated Sludge for UPB Cultivation

The most often used source of carbon and energy for cultivation of urease-producing bacteria are yeast extract [5, 9, 11, 12, 16, 36, 37] and tryptic soya broth [3, 14, 15]. According to the results of batch cultivation of *Yaniella* sp. VS8 in media with YE or TSB added with urea, the best medium for growth and urease synthesis was yeast extract with concentration 20 g/L (Table **5**).

Higher concentration of yeast extract diminished concentration of accumulated bacterial biomass and urease activity of culture liquid. An addition of acetate to medium with yeast extract slightly increased biomass concentration, but significantly reduced urease activity.

Cells of *Bacillus* sp. VS1, grown in the medium with TSB, showed the highest urease activity. Addition of TSB, 30 g per 1L of distilled water, gives the medium containing in (g/L): casein peptone (pancreatic), 17.0; soya peptone (papain digest), 3.0; glucose, 2.5; sodium chloride, 5.0; dipotassium hydrogen phosphate, 2.5.

Yeast extract and TSB both rich in organic sources of nitrogen-containing proteins, peptides, amino acids, B vitamins, and trace elements. However, these media are expensive for the large scale applications. Waste activated sludge from wastewater treatment plants (WWTPs) that treat domestic wastewater contains organic matter, N, P, K, and other nutrients [28]. Hydrolyzed activated sludge was proposed for cultivation of different microorganisms: yeasts (*Candida, Hansenula,* etc.), mycelium fungi (*Spicaria,*

Table 4: Urease Activity of Culture Liquid, Supernatant, and Cells of *Bacillus* sp. VS1 and *Yaniella sp.* VS8

Object	UA, mM of hydrolyzed urea /min for the strain	
	***Bacillus* sp. VS1**	***Yaniella* sp. VS8**
Culture liquid	10.5	5.8
Supernatant	3.3	0.2
Cells resuspended in 2% NaCl	5.9	5.1
Cells concentrated in 2 times in 2% NaCl	10.8	9.4
Cells concentrated in 4 times in 2% NaCl	19.2	18.4

Table 5: Use of Different Media for Cultivation of *Yaniella* sp. VS8 and *Bacillus* sp. VS1

Medium	*Yaniella* sp. VS8		*Bacillus* sp. VS1	
	UA, mM of hydrolyzed urea /min	Biomass, g/L	UA, mM of hydrolyzed urea /min	Biomass, g/L
TSB, 30 g/L	6.6	2.9	14.1	4.5
TSB, 30 g/L + YE, 10g/L	8.7	3.2	n.d.	n.d.
YE, 20 g/L	13.7	5.6	11.2	5.0
YE, 40 g/L	11.3	4.9	11.2	7.1
YE, 40 g/L + acetate, 10g/L	5.7	5.3	12.4	6.9

Endomyces, Trichosporon, Penicillium, etc.), Actinomycetes (*Streptomyces,* etc.) and rod-shaped bacteria (*Bacillus, Bacterium*, etc.) [29]. There was attempt to use sludge produced on wastewater treatment plant for cultivation of urease-producing bacteria *Sporosarcina pasteurii* ATCC 11859 [26]. Sludge was treated with 0.5M NaOH for 20 min with following neutralization to pH 8.0 with H_2SO_4. However, this hydrolysate did not sustain growth of urease-producing bacteria.

In our study more harsh hydrolysis conditions were applied to activated sludge, described in section Materials and Methods. Hydrolyzed activated sludge (HAS) was non-homogeneous liquid with content of TSS 5.7 g/L. Urease activity of culture liquid and biomass concentration were detected in the media every day. During cultivation of *Yaniella* sp. VS8 and *Bacillus* sp. VS1 on HAS, duration of lag-phase increased in comparison with yeast extract medium, probably due to adaptation of bacteria to initial high TSS concentration. So, results for 4 days of bacterial cultivation in the hydrolyzed activated sludge were compared with ones obtained for 3 days of bacterial cultivation in the medium with yeast extract (Table **6**).

The number of urease-producing bacteria *Yaniella* sp. VS8 grown on the hydrolyzed activated sludge was almost the same and even a little bit higher than the cells number in the medium with 4% of yeast extract. Meanwhile, urease activity was lower than in the medium with yeast extract. The reason of this phenomenon may be the inactivation of urease by activation of protease synthesis, which is needed for bacterial growth in the media containing proteins [14]. Urease activity of cultural liquid of the strain *Bacillus* sp. VS1 grown on the hydrolyzed activated sludge diminished more significantly in comparison with yeast extract medium. The possible explanation is that this strain synthesized both endocellular and extracellular urease, and inactivation of urease in supernatant is going faster. Addition of glucose, 0.3%, or salt of Ni did not change significantly either urease activity of culture liquid or bacterial cells growth yield. However, bacterial cells of the strain *Bacillus* sp. VS1, growing in hydrolyzed activated sludge or in hydrolyzed activated sludge with glucose, hydrolyzed 45% and 72% of urea, respectively, for 24 hours in the solution of 1M urea.

Therefore, cultivation of urease-producing bacteria *Yaniella* sp. VS8 and *Bacillus* sp. VS1 for large scale biocementation applications could be conducted using the cheap media based on hydrolyzed activated sludge. The strain *Bacillus* sp. VS1, which synthesized constitutive urease, grew well and showed sufficient for biocementation urease activity in the hydrolyzed activated sludge without urea addition. In case of cultivation of the strain *Yaniella* sp. VS8, which synthesized inducible urease, urea was added to the

Table 6: Use of Hydrolyzed Activated Sludge (HAS) for Cultivation of *Yaniella* sp. VS8 and *Bacillus* sp. VS1

Medium	*Yaniella* sp. VS8		*Bacillus* sp. VS1	
	UA, mM of hydrolyzed urea /min	Biomass, CFU/mL	UA, mM of hydrolyzed urea /min	Biomass, CFU/mL
YE, 40 g/L	11.3	$1.1 \cdot 10^8$	11.2	$1.8 \cdot 10^9$
HAS	7.5	$6.2 \cdot 10^8$	3.6	$1.2 \cdot 10^8$
HAS+ $NiCl_2$	7.5	$5.7 \cdot 10^8$	3.7	$1.4 \cdot 10^8$
HAS+ $NiCl_2$ + glucose	8.2	$8.1 \cdot 10^8$	4.5	$7.2 \cdot 10^8$

medium. However, taking into account our studies on influence of urea concentration on growth and development of this bacterial strain, cultivation on HAS can be conducted without addition of urea. Urease will be activated during biocementation process under supplying of biocementing solution, which consists of $CaCl_2$ and urea.

CONCLUSIONS

Urease-producing bacteria *Bacillus* sp. VS1with constitutive urease and *Yaniella* sp. VS8 with inducible urease can be cultivated on the relatively cheap medium from the hydrolyzed activated sludge of municipal wastewater treatment plant to obtain biomass for large scale geotechnical applications on bioclogging and biocementation. There is no strict requirement in addition of urea to the medium for cultivation of urease-producing bacteria. Addition of urea to culture liquid at the end of cultivation resulted in fast urease synthesis so that urease activity become equal to one in the medium with high concentration of urea.

To produce bioagent with high urease activity concentration of UPB cells should be used.

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