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Urease purification from indigenous soil bacteria and accumulation of $CaCO_3$ crystals

Abstract. Recently, microbiologically induced calcite precipitation has been evaluated as an effective and eco friendly alternative to a repair technique in plugging of microcracks and pores in concrete for building trade. Enzymatic activity of urease synthesized by microorganisms has been induced for precipitation of $CaCO_3$ crystals. Urease activity and urease-induced calcium carbonate mineralization of indigenous soil bacteria (*Bacillus* spp.) isolated from Kilis, Turkey were studied. Total and specific urease activity of crude 19B intracellular enzyme sample were calculated as 0.67±0.003 EU/mL/min and 0.63±0.011 µmol/min/mg, respectively. Three protein bands (60.48, 23.27 and 20.17 kDa) indicating subunits of urease were detected by SDS-PAGE. Microbial calcium carbonate precipitation was analyzed by Scanning Electron Microscopy/Energy Dispersive X-Ray Analysis. Rhombohedral calcite crystals were clearly observed in SEM images. Element composition of precipitate was revealed to mostly comprise of calcium, carbon and oxygen. Results indicated that 19B strain may be evaluated for engineering applications such as remediation of concrete cracks and enhancing compressive strength of cement.

Key words: urease, *Bacillus* spp., microbial calcification, scanning electron microscopy, energy dispersive X-ray analysis.

Introduction

Urease (EC 3.5.1.5) obtained from jack bean (*Canavalia ensiformis*) is identified as the first nickel dependent metalloenzyme [1; 2]. It catalyzes the hydrolysis of urea to ammonia and carbamate and is the first enzyme to be crystallized. Carbamate formed as a result of ureolytic activity spontaneously decomposes carbonic acid. Urease was revealed to be a globulin protein with an isoelectric point of five by James Sumner in 1926 [3].

Urease is synthezised in several higher plants, yeast and microorganisms. Both plants and microorganisms use urease to supply ammonia as a nitrogen source [4; 5]. Prevailing number of eukaryotic ureases are represented by a single subunit including 840 amino acid residues. However, bacterial ureases are composed of three subunits, with e.g. 101, 106 and 567 amino acid residues identified for *Klebsiella aerogenes* urease. Exceptionally, *Helicobacter pylori* urease consists of two subunits, containing 238 and 569 acid residues [6].

Urease is an analytical tool widely used to determine urea content in blood, urine, alcoholic beverages, spring water and environmental wastewaters [7].

Ureases may be used as anticancer agents, antigen with strong stimulating ability in vaccines and in removal of urea from artificial kidney dialyzates [3; 7]. Ureases from *Staphylococcus*, *Lactobacillus*, and *Klebsiella aerogenes* are responsible of nitrogen metabolism in rumen sheep [3].

Recently, hydrolysis production of bacterial urease has been proposed as an effective alternative to cement [8]. Calcium carbonate deposits known as biocement, are calcite precipitation accumulated due to microbial urease activity [9]. Ammonia released after microbial ureolytic activity in surroundings leads to the accumulation of insoluble $CaCO_3$ by increasing pH. This is used for remediation of concrete cracks and thus increased compressive strength [10].

Consequently, the present study was focused on partial isolation of urease from indigenous soil bacterium, *Bacillus* spp., and accumulation of $CaCO_3$ crystals. Morphological properties of the latter were analyzed by scanning electron microscopy (SEM).

Materials and methods

All chemical materials in this study were supplied by Merck KGaA, Germany.

Isolation of urease producing Bacillus spp. Bacillus spp. were isolated from soil sample collected at Kilis, Turkey (36°43'48.6"N, 37°06'09.6"E). Following suspension by sterile water (1:9), soil sample was incubated at 65ºC for 30 min for stimulation of bacterial spores. 100 µL of this culture was inoculated using serial dilution technique on nutrient agar and incubated at 37°C for 24 h. Single colony was selected and maintained on nutrient agar slant for further identification and test of urease activity. Bacteria were identified by morphological (Gram staining, formation of spores and cell morphology) and standard microbiological procedures (catalase and urease test systems). To screen urease production by *Bacillus* strains, Christensen's agar medium was used, containing 20 g of urea, 1.0 g of peptone, 2.0 g of KH_2PO_4, 1.0 g of glucose, 5.0 g of NaCl, 15.0 g of agar, 0.012 g of phenol red indicator, pH 6.8±7.0. Strains were transferred to Christensen's urea agar, and incubated at 37°C for 48 h. According to the change of the medium color to pink from yellow with increase of pH, urease producing *Bacillus* strains were identified.

Urease purification from Bacillus strains. For preparation of bacterial inoculum, strains were grown overnight on Luria-Bertani broth at 37°C. Subsequently, 5 mL of inoculum was transferred into urea broth consisting of 1.0 g of peptone, 2.0 g of KH_2PO_4, 1.0 g of glucose, 5.0 g of NaCl and 100 mM/L urea. Cultures were incubated at 37ºC, 180 rpm for 24 h. Cells were harvested by centrifugation at 5500 rpm for 20 min after incubation. Following removal of supernatant, pellet was lyzed by suspending with 0.2 mg/mL lysozyme prepared in 0.05 M phosphate buffer (pH 7). Suspension was sonicated on ice for 30 s pulses within 5 min total period. Acquired crude enzyme was stored at -20ºC for further analyses (of enzymatic activity, total protein concentration and protein molecular weight).

Urease activity and total protein content. Amount of released ammonia was measured by phenol-hypochlorite reaction slightly modified in our lab [11]. Crude enzyme (50 µL) was added to 1.95 mL of substrate solution contaning 6.6 mg/mL of urea prepared in 0.05 M phosphate buffer (pH 7). This mixture was incubated at 37ºC for 5 min. Then, 500 µL of phenol-nitroprusside solution (0.1 g/mL phenol and 0.5 mg/mL sodium nitroprusside solutions prepared in distilled water) and alkaline sodium hypochlorite solution (5% NaOH and 26 mL/L NaOCl) at equal volume were added to this mixture. For monitoring of color development this mixture was incubated at 55ºC for 5 min in water bath. Subsequently to blue color formation, absorbance was measured at 630 nm. Calibration curve was plotted using ammonium sulfate standards (0-0.5 µmol/mL). One unit (U) of urease activity was calculated as amount of enzyme required for hydrolisation of 1 µmol of urea at 37ºC. Total protein concentration of crude enzyme was detected by Lowry assay [12].

SDS-PAGE analysis. Molecular weight of urease was determined according to Laemmli [13]. Optiprotein marker G252 (Applied Biological Materials ABM Inc., Canada) was used as a reference marker. Protein bands were monitored by staining with Coomassie Brilliant Blue R-250.

Morphological analysis of $CaCO_3$ minerals. 100 mL of overnight culture and 10% filtered urea were added to 500 mL of distilled water sample. Culture was incubated for one week at 180 rpm 37ºC and centrifuged at 5500 rpm for 20 min. $CaCO_3$ deposit (pellet) was dried on air at room temperature, in-house. After that, $CaCO_3$ crystals were observed by SEM [9]. Surface of the sample covering-stubs was coated by golden particles (Quorum Q150R Sputter Coater), viewed by SEM (FEI Quanta FEG 650) at 10 kV accelerating voltage.

Results and discusssion

Isolation and identification ureolytic Bacillus strains. Twenty aerobic, motile, Gram-positive, spore-forming, rod-shaped and catalase-positive bacterial strains were observed. Four of them were determined to be producing urease based on the medium color change to pink from yellow (Figure 1).

These isolates were entitled as 2B, 17B, 19B and XB. Similar findings related to urease activity in agar plate could be found in literature [14-17].

Intracellular urease activity. Intracellular urease activity in terms of unit (EU/mL/min) and total protein contents of crude enzyme samples are showed in Table 1.

The highest urease activity was calculated as 0.67±0.003 EU/mL/min in enzyme purified from 19B strain. The lowest activity was determined in 2B enzyme sample (0.38±0.000 EU/mL/min). As parallel to total urease activity, the lowest protein concentration was also recorded in 2B sample with 0.86±0.066 mg/mL. For specific activity measurement of enzyme purity in sample, the highest value was detected as 0.63±0.011 U/mg in 19B enzyme extract. 19B *Bacillus* isolate was revealed to show more urease activity (0.67±0.003 U/mL) and specific activity (0.63±0.011 µmol/min/mg) as compared to other isolates.

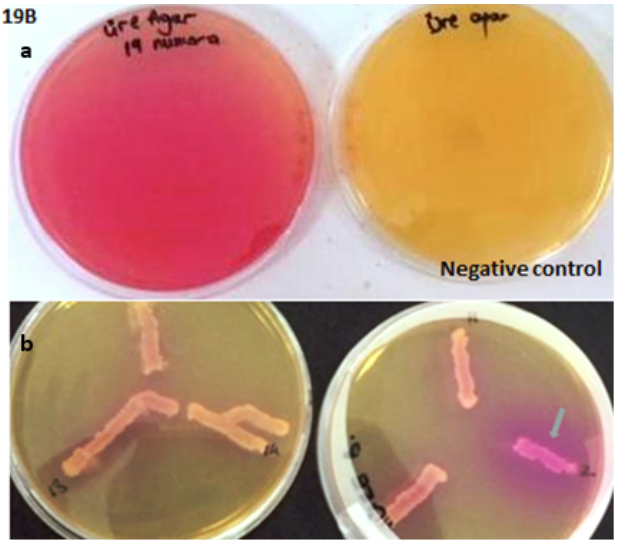


Figure 1 – Screening of urease-producing *Bacillus* spp. Note: (a) pink color indicating urease-producing, (b) yellow color indicating non-urease activity

Similarly, Tepe et al. (2019) intracellular urease purified using *Bacillus amyloliquefaciens* U17 isolated from calcareous soil of Denizli (Turkey) and specific enzyme activity found as 0.615±0.092 µmol/min/mg [18]. In other study, different bacterial strains were isolated from soil samples collected on the farm field to test urease activity of isolates. The value of urease activity varied within 0.072 and 0.88 U/mL [14]. Similar observations were reported by Kim et al. (2015). For *B. subtilis* having functional urease, detectable value of urease specific activity equaled to 0.113±0.006 U/mg protein [19]. Enzyme activity findings (0.55 U/mg) of urease extracted from bacterial sources reported by Mohammed et al. (2014) [20] were similar to our results.

Urease characterization. As can be seen from the Figure 2, the crude enzyme samples have many protein bands with different molecular weights. Protein bands belonging to urease in 19B sample were determined in comparison with bands having same molecular weight in other crude extracts.

Table 1 – Total and specific urease activities of cell-free enzyme samples

	Urease activity (EU/mL/min)	Protein amount (mg/mL)	Specific activity (µmol/min/mg)
2B	0.38±0.000	0.86±0.066	0.44±0.033
17B	0.65±0.003	1.30±0.022	0.50±0.006
19B	0.67±0.003	1.07±0.001	0.63±0.011
XB	0.58±0.014	1.08±0.004	0.53±0.011

Consequently, three protein bands (60.48, 23.27 and 20.17 kDa) indicating the subunits of urease were calculated. These findings of molecular weight exhibited similarity with previous study: 70, 60 and 55 kDa for *B. sphaericus* MTCC 5100 [21]; 66, 45, 29 and 15 kDa for *Proteus mirabilis* [20]; 11, 13 and 61 kDa for *B. pasteurii* [22].

Mineralogical results. Microbial cells adhere to different substrates for providing nutrition in natural environments. The cells metabolize nutrients to acquire energy resulting in minerals precipitated as by-product. The accumulation of calcium carbonate precipitates based on bacterial activities improves soil quality [15]. This accumulation is known as microbial induced calcite precipitation (MICP). As a result of biomineralization by ureolytic bacteria, $CaCO_3$ forms such as calcite, aragonite, vaterite and hydrated crystalline are obtained [18].

Morphological analysis of minerals for 19B strain having the highest urease activity was tested. Calcium carbonate precipitation potential of 19B strain was analyzed by SEM/EDX. Results are presented on Figure 3.

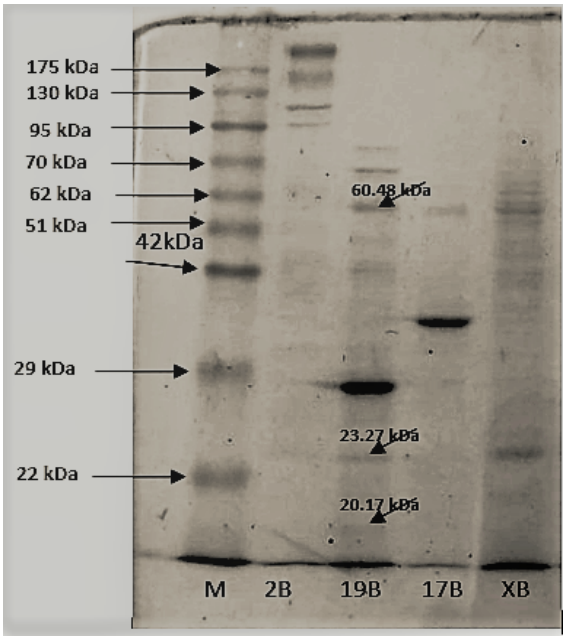
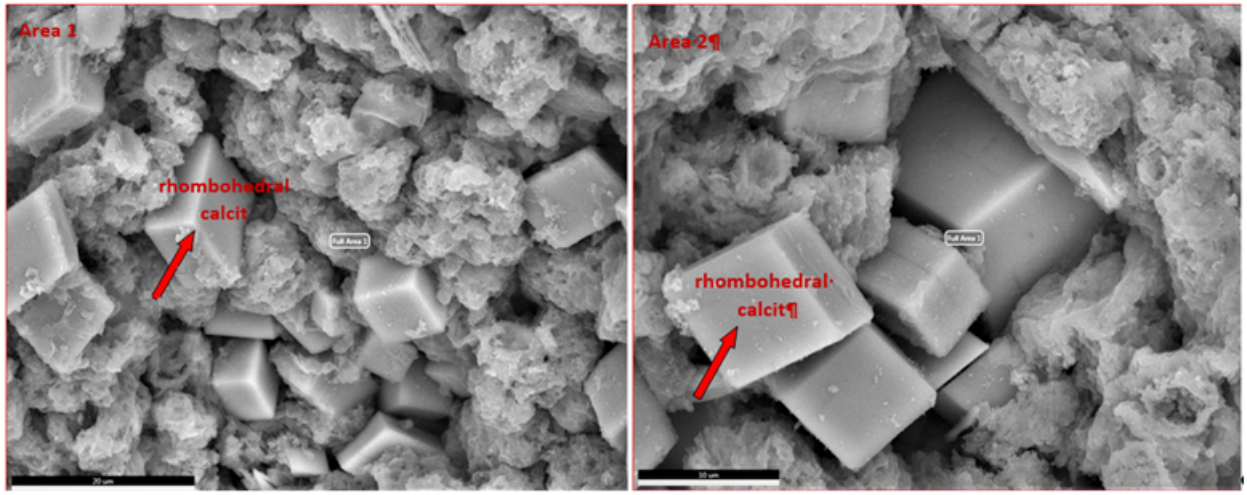
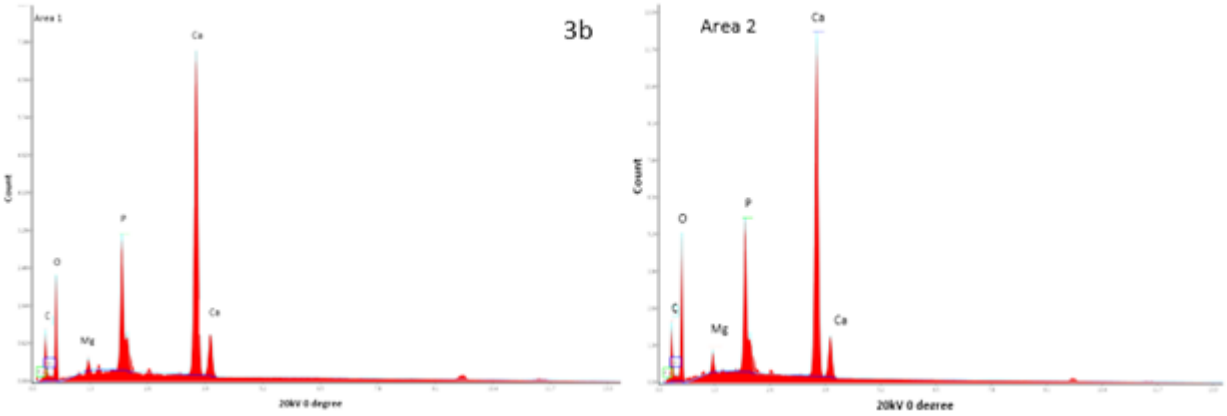


Figure 2 – Molecular weight analysis of ureases by SDS-PAGE using a 12% sperating gel. Note: M – marker DNA.

According to our results, calcites with a rhombohedral crystalline structure are clearly observed (Figure 3a). The EDX peaks (Figure 3b) and quantitative analysis (Table 2) belonging to rhombohedral crystalline show elemental composition of the precipitate.



(a)



(b)

Figure 3 – SEM image of $CaCO_3$ minerals.
Note: (a) – SEM image of rhombohedral calcite produced by 19B urease activity; (b) – element analysis of precipitate.

Table 2 – EDX quantitative analysis of rhombohedral calcites

Element	Area 1		Area 2	
	Weight%	Atomic %	Weight%	Atomic %
C	10.14	17.73	10.13	17.19
O	42.52	55.81	46.12	58.77
Mg	0.94	0.81	1.42	1.19
P	8.69	5.89	8.8	5.79
Ca	37.71	19.76	33.54	17.06
Total	100.01	100.00	100.01	100.00

Composition of calcite observed to mostly consist of calcium, carbon and oxygen elements. This is indicated that rhombohedral crystalline formed as a result of microbial calcium carbonate precipitation. In EDX analysis, % weight of Ca produced by B19 was detected as 33.54-37.71 %.

Similar calcite morphologies to our results was showed by using *Sporosarcina pasteurii* strain ATCC 11859 [10]. Tao and Whenkun (2012) also indicated to the calcites or calcites/vaterite crystal forms of calcium carbonate precipitation induced by *Bacillus pasteurii* [23]. In other study, it was noted that B*acillus* strain with high urase activity showed high microbial calcification (calcite shape) [24]. Anitha et al. (2018) detected needle-like crystals of biocement produced by *Bacillus cereus* KLUVAA [5]. The calcium carbonate mineralization property of *Paenibacillus favisporus* U3 were revealed and rhombohedral vaterite and layered calcite crystals verified by mineralogical analyses by Tepe et. al. (2019) [18]. Present study and previous works indicated that different bacterial genus may produce different crystal structures, which may vary from strain to strain.

Conclusion

In this study, the $CaCO_3$ mineralization and urease activitiy properties of *Bacillus* strains, indigenous soil bacterium, were investigated. Four urease-producing *Bacillus* strains from Kilis soil were purified. Total and specific urease activity of crude 19B intracellular enzyme sample were calculated as 0.67±0.003 EU/mL/min and 0.63±0.011 μmol/min/mg, respectively. Urease was determined to be a trimeric enzyme of three 60.48, 23.27 and 20.17 kDa molecular weight subunits by SDS-PAGE. The rhombohedral crystalline calcite accumulation of B19 strain having high urease activity was revealed by SEM/EDX analysis. Our results emphasized that 19B strain may be evaluated for geological and engineering applications, such as remediation of concrete cracks and enhancing compressive strength of cement. The hardness and durability of biocement obtained by B19 bacterial suspension transferring to sand samples requires further research.

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References

1 Balasubramanian A., Ponnuraj K. (2010) Crystal structure of the first plant urease from Jack Bean: 83 years of journey from its first crystal to molecular structure. J. Mol. Biol., vol. 400, pp. 274-283. https://doi.org/10.1016/j.jmb.2010.05.009.

2 Dixon N.E., Gazzola C., Blakeley R.L., Zerner B. (1975) Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J. Am. Chem. Soc., vol. 97, pp. 4131-4133. https://doi.org/10.1021/ja00847a045.

3 Sujoy B., Aparna A. (2013) Enzymology, immobilization and applications of urease enzyme. Int. Res. J. Biologic. Sci., vol. 2, no. 6, pp. 51-56.

4 Mobley H.L.T., Hausinger R.P. (1989) Microbial ureases: significance, regulation, and molecular characterization. Microbiol Rev., vol. 53, pp. 85-108. https://doi.org/10.1128/MMBR.53.1.85-108.1989.

5 John A., Isaacson H. R., Bryant M.P. (1974) Isolation and characteristics of a ureolytic strain of Selenomonas ruminantium. J. Dairy Sci. vol. 57, pp. 1003-1014. https://doi.org/10.3168/jds.S0022-0302(74)85001-0.

6 Hausinger R.P., Karplus P.A. (2001) Handbook of metalloproteins. John Wiley Sons, Ltd., Chichester. P. 965.

7 Akkas T. (2016) Novel methods to prepare cross-linked enzyme aggregates (CLEA). challenging immobilization models – urease and pepsin. Dissertation, University of Sabanci, 95 p.

8 Krishnapriyaa S., Venkatesh Babub D.L., Prince Arulraj G. (2015) Isolation and identification of bacteria to improve the strength of concrete. Microbiologic. Res., vol. 174, pp. 48- 55. https://doi.org/10.1016/j.micres.2015.03.009.

9 Anitha V., Abinaya K., Prakash S., Seshagiri Rao, A., et al. (2018) Bacillus cereus KLUVAA mediated biocement production using hard water and urea. Chem. Biochem. Eng. Q., vol. 32, no. 2, pp. 257-266. https://doi.org/10.15255/CABEQ.2017.1096.

10 Okwadha G.D.O., Li J. (2010) Optimum conditions for microbial carbonate precipitation. Chemosphere, vol. 81, pp. 1143-1148. https://doi.org/10.1016/j.chemosphere.2010.09.066.

11 Azarno T. (2013) Optimization of urease production for the production of microbial biocement. Dissertation, University of Ege, 69 p.

12 Lowry O.H., Rosebrough N.J., Farra N.J., Randall R.J. (1951) Protein measurements with the

Folin phenol reagent. J. Biol. Chem., vol. 193, pp. 265-275.

13 Laemmli U.K. (1970). Cleavage of the structural proteins during assembly of the head of bacteriophage T4. Nature, vol. 227, pp. 680-685. https://doi.org/10.1038/227680a0.

14 Agarwal A., Banerje S., Dev K. (2015) Comparative characterization of urease secreting bacterial and fungal isolates from soil sample of farm fields. Int. J. Adv. Sci. Tech. Res., vol. 5, no. 4, pp. 138-150.

15 Bibi S., Oualha M., Ashfaq M.Y., Sulemimanb M.T. (2018) Isolation, differentiation and biodiversity of ureolytic bacteria of Qatari soil and their potential in microbially induced calcite precipitation (MICP) for soil stabilization. Royal Soc. Chem., vol. 8, pp. 5854-5863. https://doi.org/10.1039/C7RA12758H.

16 Cinar C. (2015) Isolation and characterization of Bacillus thuringiensis from olive tree related habitats. Dissertation, University of Izmir Institute Technology. 127 p.

17 Phanga I.R.K., Chanb Y.S., Wonga K.S., Lau S.Y. (2018) Isolation and characterization of urease-producing bacteria from tropical peat. Biocatal. Agric. Biotechnol., vol. 13, pp. 168-175. https://doi.org/10.1016/j.bcab.2017.12.006.

18 Tepe M., Arslan S., Koralay T., Mercan Dogan N. (2019) Precipitation and characterization of CaCO3 of Bacillus amyloliquefaciens U17 strain producing urease and carbonic anhydrase. Turkish J. Biol., vol. 43, pp. 198-208. https://doi.org/10.3906/biy-1901-56.

19 Kim J.K., Mulrooney S.B., Hausinge R.P. (2005) Biosynthesis of active Bacillus subtilis urease in the absence of known urease accessory proteins. J. Bacteriol., vol. 187, no. 20, pp. 7150-7154. https://doi.org/10.1128/JB.187.20.7150-7154.2005.

20 Mohammed S.O., Ahmed E.S., Hafez E.E., Khalid A., et al. (2014) Characterization and purification of urease enzyme from new Proteus mirabilis strain. J. Adv. Sci. Res., vol. 5, no. 4, pp. 8-11.

21 Kumar Singh A., Singh M., Verma N. (2017) Extraction, purification, kinetic characterization and immobilization of urease from Bacillus sphaericus MTCC 5100. Biocatal. Agric. Biotechnol., vol. 12, pp. 341-347. https://doi.org/10.1016/j.bcab.2017.10.020.

22 Follmer C., Real-Guerra R., Wasserman G.E., Olivera-Severo D., et al. (2004) Jackbean, soybean and Bacillus pasteurii ureases. Eur. J. Biochem., vol. 271, pp. 1357-1363. https://doi.org/10.1111/j.1432-1033.2004.04046.x.

23 Tao D., Wenkun Z. (2012) Optimization of calcium carbonate precipitation for Bacillus pasteurii. Appl. Mech. Mater., vol. 178-181, pp. 676-679. https://doi.org/10.4028/www.scientific.net/AMM.178-181.676.

24 Varenyum A., Mukherjeel A., Reddy M.S. (2010) Characterization of two urease-producing and calcifying Bacillus spp. isolated from cement. J. Microbiol. Biotechnol., vol. 20, no. 11, pp. 1571-1576. https://doi.org/10.4014/jmb.1006.06032.