ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 147-153
doi: 10.25083/rbl/24.1/147.153

Received for publication: August 25, 2017
Accepted: February 11, 2018

Original paper

Biosequestration of heavy metals by microbially induced calcite precipitation of ureolytic bacteria

EMAN ZAKARIA GOMAA[1]

[1]Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Cairo, Egypt

Abstract

Microbial induced calcite precipitation (MICP) using urease producing bacteria has gained great importance of research in recent years. In this study, the efficiency of ureolytic, heavy metals resistant bacteria to biomineralize toxic heavy metals has been investigated. Twenty two bacterial strains were isolated from calcareous soil samples collected from Egypt and screened for urease and calcite production as well as the tolerance to heavy metals toxicity. Micrococcus sp. NCTC -1716 was selected for subsequent studies as it was the most potent strain. Resistance of Micrococcus to increasingly concentrations of zinc, cadmium, lead and iron varied according to the metal. The capacity of Micrococcus sp. to remove heavy metals ranged from 60.66 % for Cd2+ to 97.20 % for Pb2+after 48 h of incubation. Metal sequestration via calcite precipitation showed that heavy metals are removed according to the following sequence: Pb2+, Fe2+, Zn2+, and Cd2+. Scanning electron microscope was used to examine the morphology of calcite crystals and biomineralization products, while their structure was emphasized by X-ray diffraction (XRD) analysis. The study confirmed that MICP sequesters soluble heavy metals into biominerals and proved as a promising strategy for bioremediation process.

Keywords : Bioremediation, Heavy metals, SEM, Urease, XRD

To cite this article: Gomaa EZ. Biosequestration of heavy metals by microbially induced calcite precipitation of ureolytic bacteria. *Rom Biotechnol Lett*. 2019; 24(1): 147-153. DOI: 10.25083/rbl/24.1/147.153

*Corresponding author: EMAN ZAKARIA GOMAA, Faculty of Education, Department of Biological and Geological Sciences, Ain Shams University, Cairo, Egypt
E-mail: emann7778@gmail.com

Introduction

In Nature, most heavy metals caused no toxicity as they persist at low concentrations. However, human activities such as combustion of fossil fuels, industrial progress, burning of wastes, use of agricultural fertilizers and pesticides give rise to release of non-biodegradable redundant amounts of heavy metals (H. GUO & al. [1]). Heavy metal contaminants pose a major threat to human health creating many diseases such as cancer, mental retardation and growth abnormalities. Therefore, it is necessary to search for an effective eco-friendly technique to dispose of such contaminants.

Traditional treatment mechanisms such as adsorption, filtration, evaporation, chemical precipitation, oxidation/reduction and electrolysis ion exchange used for remediation process are uneconomical, ineffective as well as the safe disposal issue of wastes (O. L. KANG & al. [2]). Many biological techniques have been used to get rid of such heavy metals from contaminated sites like bioaccumulation, bioleaching, biosorbents, biocoagulation and phytoremediation (S. CHOUDHARY& P. SAR [3]). Unfortunately, these methods are also not effective because they are expensive, take long time and result in the production of immobilized or adsorbed heavy metals back to the environment. Moreover, it cannot be applied in severe and dry climate of arid area (V. ACHAL & al. [4]).

Biomineralization based on microbially induced calcite precipitation (MICP) process provides a promising technique to remediate toxic metals from contaminated soils (C.H. KANG & al. [5]). Heavy metals are bound with calcite, which is responsible for heavy metals sequestration and ultimately resulted in significantly reduction of their levels in the environment. Provided such situation, heavy metals are removed by an effectively, economically and eco-friendly manner (C.H. KANG & al. [6]).

This study aimed at screening a number of bacterial strains isolated from calcareous soil samples collected from Egypt for urease production and calcite precipitation. The study was extended to investigate how toxicity of a range of heavy metals (zinc, cadmium, lead and iron) on growth of the most potent strain, then demonstrate the variations in heavy metals removal performance of these metals via bioprecipitation with calcite. Characterization of biomineralization products was done by scanning electron microscopy (SEM) coupled X-ray Diffraction (XRD) analysis.

Materials and Methods

Isolation of urease producing bacteria

Six calcareous soil samples collected from north of the western desert and Borg El-Arab of Egypt were used for isolation of urease producing bacteria. For enrichment technique, 5 g of soil sample were inoculated into 50 ml nutrient broth amended with 20 g/l filter-sterilized urea using 0.22-mm filter (Millipore, USA) and incubated at 35oC for 72 h under shaking conditions (V. ACHAL & al. [7]). Urease producing bacteria were isolated from enriched samples using serial dilution technique followed by culturing on Christensen's Urea Agar Base (UAB) (R.M. ATLAS [8]). Colonies that showed a pinkish color change around them were marked as urease producers and selected for subsequent experiments.

Measurement of urease activity and calcite production

Urease broth medium containing (g/l): urea, 20; NaHCO3, 2.12; NH4Cl, 10, nutrient broth, 3; CaCl2.2H2O , 25, pH 7 was inoculating with overnight grown seed culture and incubated under shaking condition at 37°C for 72 h. Ammonia released from urea hydrolysis was measured as an indicator for urease activity according to the phenol-hypochlorite assay method (K. R. NATARAJAN [9]). The culture filtrate (250 µl) obtaining after centrifugation of bacterial culture was added to a mixture of 1 ml of 2.5 ml of urea (0.1 M) and 0.1 M potassium phosphate buffer (pH 8.0) and incubated at 37°C for 5 min. Phenol Nitroprusside and alkaline hypochlorite, 1 ml each were added and incubated again at 37°C for 25 min. Optical density was measured at 760 nm. Ammonium chloride (100 µg/ml) was used as standard. The experiment was done in triplicate and the average absorbance was recorded. One unit of urease is defined as the amount of enzyme that catalyzed the hydrolysis of 1 µM urea per minute under the assay conditions.

The quantity of calcium carbonate (calcite) precipitated was measured using the same medium.

The precipitate collected after centrifugation was weighed after drying at 50°C to constant weight. Bacterial growth was determined by counting the colony forming units at different time intervals and the results were expressed as log CFU/ ml. A pH meter (pH Pen Jenco 610) was used for measuring the final pH of the culture. All experiments were conducted in triplicate and the mean values were considered.

Molecular strain identification

The most efficient bacterial strain was identified using 16S rRNA gene sequencing (V. ACHAL & X. PAN [10]). The gene sequencing was done by Macrogen (South Korea). Sequence analysis was performed at the National Center for Biotechnology Information (NCBI) database, USA.

Metal toxicity experiments

For metal toxicity experiments, nutrient broth media supplemented with different concentrations (0–10 mM) of heavy metal salts (zinc chloride, cadmium sulphate, lead nitrate, and iron sulphate) were inoculated with 2% overnight grown culture (6.5 Log CFU/ ml). Each metal concentration was tested in triplicate and incubated at 35oC for 48 h. Bacterial growth was measured to examine the viability of cells in the presence of these metals (C.E. RUGGIERO & al. [11]). The lowest concentration of each heavy metal that caused no visible growth was considered as the MIC (minimum inhibitory concentration) of that metal.

Metal bioprecipitation

Metal bioprecipitation tests were performed using urease broth media supplemented with ½ MIC of each heavy metal salt. The flasks were inoculated with 2% overnight grown seed culture and incubated at 35oC for 48 h under shaking conditions. An inductively coupled plasma atomic emission spectrometer (Perkin Elemer USA, Model 2400) was used to measure the initial and final concentrations of each heavy metal and the percentage of each heavy metal removal was calculated. Furthermore, the mass of precipitation was calculated after drying to constant weight. All experiments were performed in triplicate and treatments of urease broth media without any heavy metals added and those without bacterial inoculation were used as controls.

SEM and XRD analyses

The precipitates created during the bioremediation process for each heavy metals as well as calcite precipitated were further analyzed. After completely drying and grounded into powder form, samples were gold coated with a sputter coating Emitech K575. Morphological shape and size of the crystals were examined under scanning electron microscope (JEOL, JSM 5200).

X-Ray Diffraction (XRD) analysis was used to investigate the crystal structure of calcite and metal precipitates. The dried samples were spread uniformly onto glass plate and employed with X-ray diffractometer (INEL X-ray diffractometer). The samples were identified by comparing the X-ray profile of the samples with standards of International Center Diffraction Databases (ICDD) (C.H. KANG & al. [6]).

Statistical analysis

The results were presented as mean± standard deviation of triplicate experiments. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of $p<0.05$.

Results and Discussions

In the present study, 22 morphologically different bacterial strains were isolated from calcareous soil samples. These natural environments are regarded as potential candidate for isolation of ureolytic bacteria capable of yielding urease enzyme stable under harsh conditions such as high temperature and alkaline environments. As a primary test, the isolates were screened qualitatively for urease production using UAB medium. Based on the intensity of pink color observed on urea agar media, ten efficient bacterial strains were selected. Ammonia released from urea hydrolysis act to raise the pH of the culture to over 9.0 causing a color change of the indicator dye, phenol red, from yellow to pink (M.B. BURBANK & al. [12]). Further, the selected strains were quantatively evaluated for the rate of urease production and calcite precipitation.

Metal toxicity is an important factor to be considered for remediation process as it is directly related to the survival and activity of bacteria in contaminated sites (Y.H. LI & al. [13]). So, the strains were tested for their heavy metals resistance. Among the selected strains, WD-2, WD-5, WD-9, BA-3 and BA-7 were the highest urease producers and showed

significant heavy metals resistance efficiency as well. The results presented in Fig. (1) show that strain WD-9 exhibited the highest urease activity (6.50 U/ml), followed by WD-5 (4.14 U/ml), BA-3 (3.36 U/ml), BA-7 (2.20 U/ml) and WD-2 (1.98 U/ml). In addition, the highest calcite production was recorded for WD-9 (10.0 mg/ml), followed by BA-3 (9.32 mg/ml), WD-5 (8.22 mg/ml), BA-7 (7.40 mg/ml), and WD-2 (5.11 mg/ml).

Strain WD-9 was selected as the most effective isolate for urease activity and calcite precipitation. Furthermore, it showed the highest metal resistance against all metal tested (Zn2+, Cd2+, Pb2+ and Fe2+) compared to other strains. Strain WD-9 was identified using 16S rRNA gene sequence analysis. The sequence alignment for the comparison of 1,500 bp by BLASTN software showed a high homology of 98% to Micrococcus sp. NCTC -1716.

Type of bacteria, substrate concentration, pH, temperature and salinity are among the factors that control the ureolytic reaction rate (N. DHAMI & al. [14]). The urease production profile of Micrococcus sp. was recorded over 120 h. As shown in Fig. 2, maximum growth (9 log CFU/ ml) and urease activity (6.60 U/ml) were recorded at 72 h, afterwards a gentle reduction was observed, probably due to reduction of urea concentration in the medium. In the beginning of the incubation time, pH of the media was 7.0, and it raised to its maximum level (9.60) at 96 h. Shifting pH toward alkalinity correlated with increasing the density of calcite precipitate, reached its highest level (10.80 mg/ml) at 120 h. Under alkaline conditions, there is a considerable increase in carbonate production which binds calcium ions resulting in the deposition of calcite. This mechanism plays a vital role in biomineralization process (C.X. QIAN & al. [15]).

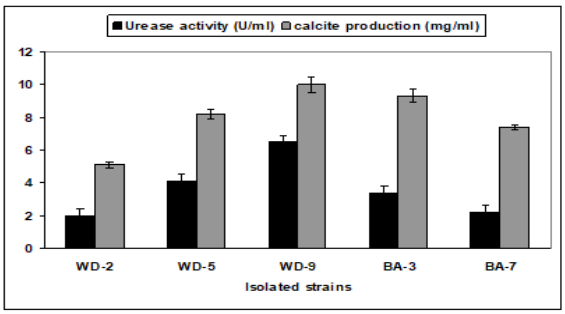

Figure 1. Urease activity and calcite production by isolated bacterial strains. Bars represent standard deviations.

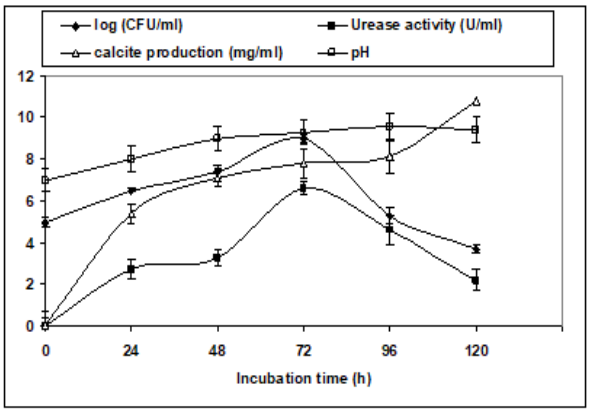

Figure 2. Time course of growth, urease activity, calcite production and final pH of Micrococcus sp. cultivated in urease broth medium. Values are averages from three independent experiments. Bars represent standard deviations.

Effect of heavy metals on bacterial growth

From the presented results, Micrococcus sp. possess high ureolytic and calcite deposition efficiency, be non-pathogenic and an alkalophilic strain that could grow under harsh conditions as well. This strain can be regarded as a possible candidate for bioremediation processes.

However, heavy metal toxicity affects the microbial growth and efficiency of MICP. Therefore, it is a prerequisite to select isolates exhibit ureolytic capability and heavy metal tolerance to upgrade the efficiency of bioremediation process (C.H. KANG & al. [16]).

Results illustrated in Fig. 3 showed that cadmium exhibited the highest toxicity on Micrococcus sp. with a minimum inhibitory concentration of 2 mM, whilst zinc and iron were similar, with an MIC of 3.5 mM. Resistance of the strain to lead was the highest, with growth unhindered below 5 mM. As a result, MIC values of Micrococcus sp. were superior to that reported for other strains. It is worth noting that survival of the strain at high metal concentrations cultivated in urea-amended medium suggested that the metal removal capacity offers a defense mechanism against metal toxicity.

Results showed that the least toxicity of lead to the cells. Lead has been recognized as one of the most hazardous heavy metals among environmental

pollutants. Electric battery manufacturing, mining activities as well as products including lead cause irregular supply of lead to the environment (D. HUANG & al. [17]).

Metal bioprecipitation

Biomineralization is defined as the extracellular production of minerals by a microorganism which has various potential applications in bioengineering field (L.C. VERONICA & al. [18]). Sequestration of inorganic pollutants, including heavy metals, is one of these applications that play an essential role in bioremediation process (S.H. RAUT & al. [19]). Microorganisms have to be enzymatically active to interact with the pollutants and convert them into harmless products. Calcite deposition capacity of urolytic bacteria results in mineralization of soluble heavy metal ions and their ultimate conversion to carbonates. Hence, production of calcium carbonate as a stable mineral phase displays permanent sequestration of contamination (A. LOPEZ-MORENO & al. [20]).

The ability of Micrococcus sp. to remove heavy metals via precipitation process catalyzed by urease hydrolysis is investigated. Upon cultivation of Micrococcus sp. in urease broth medium supplemented with ½ MIC of each heavy metal, it was found that Pb2+ recorded the highest removal rate (97.20%) while the lowest removal efficiency was recorded for Cd2+ (60.66 %) after 48 h (Fig.4). These results indicate that biosequestration process is superior to biosorption one which achieved only 36.07 % removal of lead by Micrococcus luteus DE2008 (Z.M. PUYEN & al. [21]).

Results presented in fig (4) showed that the highest precipitated mass was recorded for Pb 2+ (18.00 mg/ml), followed by Fe2+ (16.20 mg/ml), Zn2+ (14.98 mg/ml) and Cd2+ (11.73 mg/ml).

These metals can substitute for calcium as they are similar to it in atomic radius and form comparable metal carbonate structures (H.H. TENG & L. ZHAO [22]). However, on existing surfaces of calcite, rapid sorption of metal can form an extra outer layer, preventing further sorption and limiting dissolution of the mineral (Y. DU & al. [23]).

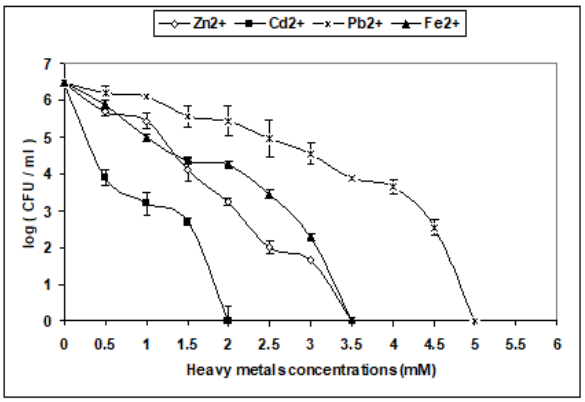

Figure 3. Metal toxicity experiments of zinc chloride, cadmium sulphate, lead nitrate, and iron sulphate on Micrococcus sp. cultivated in nutrient broth media supplemented with different heavy metal concentrations. Values are averages from three independent experiments. Bars represent standard deviations.

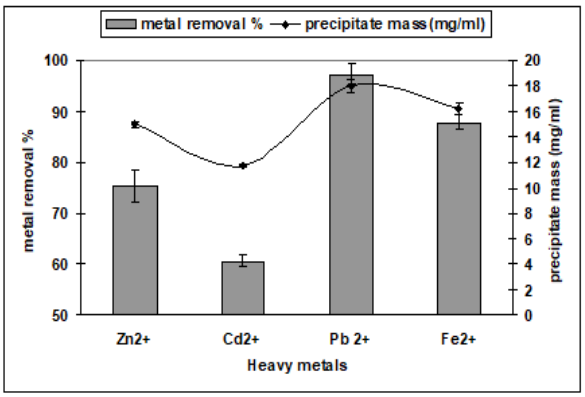

Figure 4. The removal rates and precipitated mass of individual heavy metals by Micrococcus sp. after 48 h of incubation. Values are averages from three independent experiments. Bars represent standard deviations.

SEM and XRD analyses

Many factors affecting the morphology and structure of metal carbonates precipitated during bacterial ureolysis such as bacterial strain, rate of urea hydrolysis, type of metal and activities of other enzymes such as carbonic anhydrase (N.K. DHAMI & al. [24]). In the present study, the biomineralization products as well as calcium carbonate crystals precipitated by Micrococcus sp. were visualized by SEM. Figure 5(A-E) showed that heavy metal crystals exhibited different morphological form, spherical, cuboid, rhombohedral, rod or irregular shape and their size is in the range of 10–50 µm.

To further confirm the role of MICP in heavy metals bioremediation process, mineral constituents of the precipitated samples were analyzed by X-ray diffraction (XRD) analysis. Fig 6A confirmed that the white precipitate produced by Micrococcus sp. is calcite. XRD peaks of Fig. 6 (B-E) showed that the metals are co-precipitated with CaCO3 in bioremediation experiment as ZnCO3, CdCO3, PbCO3 and FeCO3, respectively which showed evidence of transformations of metals into geochemically stable calcite species. Furthermore, this analysis indicated the presence of calcite crystals as principally mineral of all the carbonates precipitated.

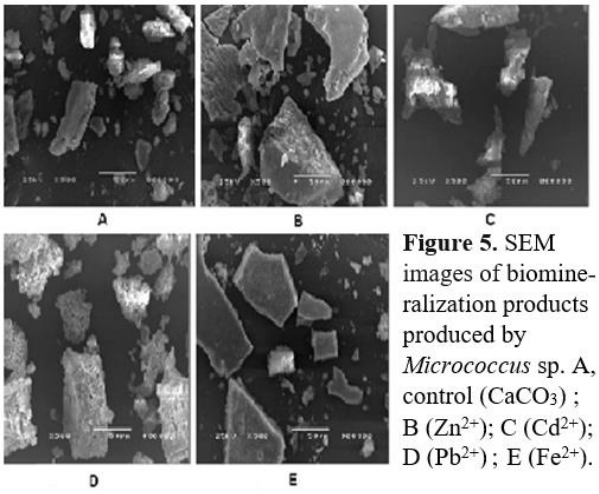

Figure 5. SEM images of biomineralization products produced by *Micrococcus* sp. A, control ($CaCO_3$) ; B (Zn^{2+}); C (Cd^{2+}); D (Pb^{2+}) ; E (Fe^{2+}).

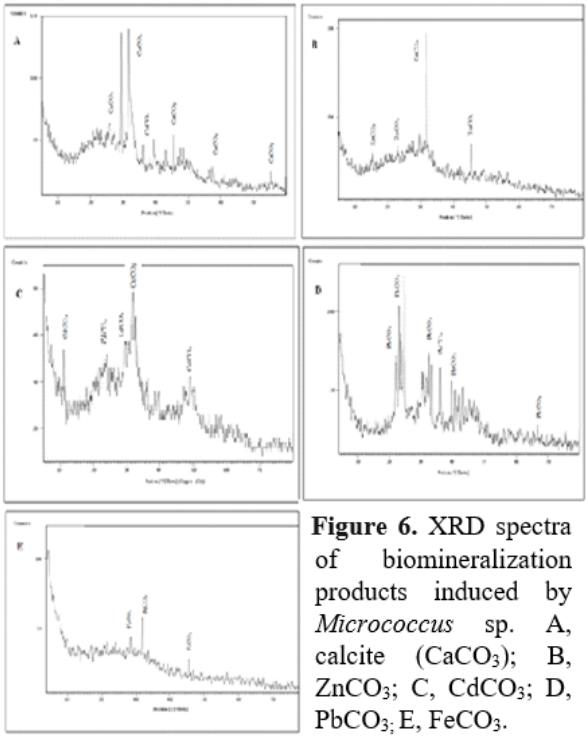

Figure 6. XRD spectra of biomineralization products induced by *Micrococcus* sp. A, calcite ($CaCO_3$); B, $ZnCO_3$; C, $CdCO_3$; D, $PbCO_3$; E, $FeCO_3$.

Conclusions

The results of the present study indicate that the biomineralization process based on ureolysis-driven calcium carbonate precipitation could be applied for soil bioremediation in a variety of environments.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

References

1. H. GUO, S. LUO, L. CHEN, X. XIAO, Q. XI, W. WEI, G. ZENG, C. LIU, Y. WAN, J. CHEN, Y. HE, Bioremediation of heavy metals by growing hyperaccumulator endophytic bacterium Bacillus sp. L14. Bioresour. Technol., 101, 8599–8605 (2010).
2. O. L. Kang, N. Ramli, M. Said, M. Ahmad, S.M. Yasir, A. Ariff, Kappaphycus alvarezii waste biomass, A potential biosorbent for chromium ions removal. J. Environ. Sci., 23 (6), 918–922 (2011).
3. S. Choudhary, P. Sar, Uranium biomineralization by a metal resistant Pseudomonas aeruginosa strain isolated from contaminated mine waste. J. Hazard. Mater., 186, 336–343 (2011).
4. V. Achal, X. Pan, D. Zhang, Remediation of copper-contaminated soil by Kocuria flava CR1 based on microbially induced calcite precipitation. Ecol. Eng., 37, 1601–1605 (2011).
5. C.H. Kang, S.J. Oh, Y.J. Shin, S.H. Han, I. H. Nam, Bioremediation of lead by ureolytic bacteria isolated from soil at abandoned metal mines in South Korea. Ecol. Eng., 74, 402–407 (2015).
6. C.H. Kang, Y.J. Shin, P. Anbu, I.H. Nam, J.S. So, Biosequestration of copper by bacteria isolated from an abandoned mine by using microbially induced calcite precipitation. J. Gen. Appl. Microbiol., 62, 206–212 (2016).

7. V. Achal, A. Mukherjee, S. Goyal, S. Reddy, Corrosion prevention of reinforced concrete with microbial calcite precipitation. ACI. Mater. J., 2, 157–164 (2012).
8. R.M. Atlas, Handbook of Microbiological Media, 4th ed. ASM press. Taylor and Francis group, LLC, (2010).
9. K. R. Natarajan, Kinetic study of the enzyme urease from Dolichos biflorus. J. Chem. Educ., 73, 556–557 (1995).
10. V. Achal, X. Pan, Characterization of urease and carbonic anhydrase producing bacteria and their role in calcite precipitation. Curr. Microbiol., 62, 894–902 (2011).
11. C.E. Ruggiero, H. Boukhalfa, J.H. Forsythe, J.G. Lack, L.E. Hersman, M.P. Neu, Actinide and metal toxicity to prospective bioremediation bacteria. Environ. Microbiol., 7, 88–97 (2005).
12. M.B. Burbank, T.J. Weaver, B.C. Williams, R.L. Crawford, Urease activity of ureolytic bacteria isolated from six soils in which calcite was precipitated by indigenous bacteria. Geomicrobiol. J., 29, 389-395 (2012).
13. Y.H. Li, B. Xia, Q. S. Zhao, F.Q. Liu, P. Zhang, Q. J. Du, Removal of copper ions from aqueous solution by calcium alginate immobilized kaolin. J. Environ. Sci., 23(3), 404-410 (2011).
14. N. Dhami, M. Reddy, A. Mukherjee, Improvement in strength properties of ash bricks by bacterial calcite. Ecol. Engin., 39, 31-35 (2012).
15. C.X. Qian, R.X. Wang, L. Cheng, J.Y. Wang, Theory of microbial carbonate precipitation and its application in restoration of cement-based materials defects. Chinese J. Chem., 28, 847–857 (2010).
16. C.H. Kang, S.H. Han, Y.J. Shin, S.J. Oh, J.S. So, Bioremediation of Cd by microbially induced calcite precipitation. Appl. Biochem. Biotechnol., 172, 1929–1937 (2014).
17. D. Huang, G. Zeng, X. Jiang, C. Feng, H. Yu, G. Huang, H. Liu, Bioremediation of Pb-contaminated soil by incubating with Phanerochaete chrysosporium and straw. J. Hazard. Mater., 134, 268-276 (2006).
18. L.C. Veronica, B.V. Liliana, M.A. Carlos, Indigenous microorganisms as potential bioremediators for environments contaminated with heavy metals. Int. Biodet. Biodeg., 69, 28-37 (2012).
19. S.H. Raut, D.D. Sarode, S.S. Lele, Biocalcification using B. pasteurii for strengthening brick masonry civil engineering structures. World J. Microbiol. Biotechnol., 30, 191–200 (2014).
20. A. Lopez-Moreno, J.D. Sepulveda-Sanchez, E.M. Mercedes Alonso Guzman, S. Le Borgne, Calcium carbonate precipitation by heterotrophic bacteria isolated from biofilms formed on deteriorated ignimbrite stones, influence of calcium on EPS production and biofilm formation by these isolates. Biofoul., 30, 547–560 (2014).
21. Z.M. Puyen, E. Villagrasa, J. Maldonado, E. Diestra, I. Esteve, A. Sole , Biosorption of lead and copper by heavy-metal tolerant Micrococcus luteus DE2008. Bioresour. Technol., 126, 233–237 (2012).
22. H.H. Teng, L. Zhao, Surface behavior of calcite upon uptake of Cd2+ and Pb2+. Geol. J . China Univ., 18, 193–202 (2012).
23. Y. Du, F. Lian, L. Zhu, Biosorption of divalent Pb, Cd and Zn on aragonite and calcite mollusk shells. Environ. Pollut., 159, 1763–1768 (2011).
24. N.K. Dhami, A. Mukherjee, M.S. Reddy, Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. J. Microbiol. Biotechnol., 23, 707–714 (2013).