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Determination of Biocalcification Properties of Bacillus Species Isolated from Soil

Fatma Öztürk Küp*[1], Fatma Eroğlu[2],

*[1] Erciyes Üniversitesi Fen Fakültesi Biyoloji Bölümü, KAYSERİ
[2] Erciyes Üniversitesi Fen Bilimleri Enstitüsü, KAYSERİ

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Abstract: This study aimed to determine the isolation of *Bacillus* species including urease activity from soil, morphological and molecular identification and biocalcification features. Total 11 bacteria belonging to *Bacillus* species were isolated from soil samples. Identification of the isolates was carried out with 16S rRNA sequencing analysis. Urease activity of the isolates was carried out according to the phenol-hypochlorite method. $CaCO_3$ accumulation was determined and liquid media. $CaCO_3$ crystals were analyzed in the scanning electron microscope (SEM). Urease enzyme activities of the isolates were compared to with control group *Sporosarcina pasteurii*. According to the results, F10 (*Bacillus mycoides*) was determined as the species having the highest activity with specific urease activity 8.50±0.00 U/mg. The bacterium having the lowest specific activity was determined F14 (*Bacillus thuringiensis*) with specific urease activity 2.75±0.04 U/mg value. In a liquid medium supplemented with urea and calcium chloride, the maximum $CaCO_3$ accumulation was determined to be 0.53 g in *S. pasteurii.* After the control strain, the maximum $CaCO_3$ accumulation was measured as 0.20g, 0.20 g and 0.18 g in F9, F13 and F8 isolates, respectively. According to the results of SEM examinations, it was determined that the isolates carried out calcite accumulation.

Topraktan İzole Edilen Bacillus Türlerinin Biyokalsifikasyon Özelliklerinin Belirlenmesi

Anahtar Kelimeler
Biyokalsifikasyon
Bacillus,
16S rRNA,
Üreaz aktivitesi

Öz: Bu çalışma, üreaz aktivitesine sahip *Bacillus* türlerinin topraktan izolasyonunu, morfolojik ve moleküler tanımlamalarını ve biyokalsifikasyon özelliklerini belirlemeyi amaçlamıştır. Toprak örneklerinden *Bacillus* türüne ait toplam 11 bakteri izole edilmiştir. İzolatların tanımlanması, 16S rRNA dizileme analizi ile gerçekleştirilmiştir. İzolatların üreaz aktiviteleri fenol-hipoklorit yöntemine göre gerçekleştirilmiştir. Sıvı ortamda $CaCO_3$ birikimi belirlenmiştir. $CaCO_3$ kristalleri, taramalı elektron mikroskobunda (SEM) analiz edilmiştir. İzolatların üreaz enzim aktiviteleri kontrol grubu *Sporosarcina pasteurii* ile karşılaştırılmıştır. Sonuçlara göre F10 (*Bacillus mycoides*) spesifik üreaz aktivitesi 8.50±0.00 U/mg ile en yüksek aktiviteye sahip tür olarak belirlendi. En düşük spesifik aktiviteye sahip bakteri, 2,75±0,04 U/mg spesifik üreaz aktivitesi ile F14 (*Bacillus thuringiensis*) olarak belirlendi. Üre ve kalsiyum klorür katkılı sıvı ortamda *S. pasteurii*'de maksimum $CaCO_3$ birikimi 0,53 g olarak belirlendi. Kontrol suşundan sonra maksimum $CaCO_3$ birikimi F9, F13 ve F8 izolatlarında sırasıyla 0.20g, 0.20g ve 0.18g olarak ölçülmüştür. SEM incelemelerinin sonucuna göre izolatların kalsit birikimini gerçekleştirdiği belirlenmiştir.

*Corresponding Author, email: fozturk@erciyes.edu.tr

1. Introduction

Biocalcification is a process known as lime accumulation, which is realized by organisms with urease activity, either in the soil or on inside [1]. It is formed by the precipitation of carbonate, which accumulates during the metabolic processes of microorganisms, with calcium in the form of calcium carbonate and a large number of soil microorganisms contribute to this event [2]. Some bacteria and fungi may cause the extracellular precipitation of calcium carbonate through a series of processes including photosynthesis, ammonia, denitrification, sulfate reduction and anaerobic sulfide oxidation [3].

Species of the *Bacillus* group can precipitate calcite on their cell constituents and in their micro-environment by conversion of urea into ammonia and carbon dioxide. The bacterial degradation of urea locally increases the pH and promotes the microbial deposition of carbon dioxide as calcium carbonate in a calcium-rich environment. An endospore-forming soil microorganism, *Bacillus pasteurii*, has been used as the urease producer. *Bacillus pasteurii* produces intracellular urease that constitutes close to 1% of the cell dry weight [4]. It has been reported in studies that *Bacillus sphaericus* and *Bacillus lentus* have biocatalytic properties other than *Bacillus pasteurii*. There is great value for the restoration of the calcareous monuments that have been destroyed by biochemical production of calcite crystals by bacteria with calcifying properties and for the improvement of openness and cracks [5].

Despite the importance of these bacteria in the repair of cracks and slits, very few bacteria have been studied in this regard. In this study, *Bacillus* species isolated from soil were characterized according to morphological and molecular methods and tried to determine urease activities and biocatalytic properties.

2. Material and Method

2.1. Sample collection, bacteria isolation and identification.

Soil samples were collected from the commercial bags and placed in radiation-sterilized polypropylene bottles. After sweeping the top surface of the soil, a soil sample of about 2 to 5 cm from the surface was taken from the surface with a sterile spatula. The samples were stored at +4 °C until used.

To enrich the soil samples for urease-producing bacteria, 1 g of soil was inoculated into 50 ml of nutrient broth containing 2% urea, and incubated at 130 rpm for 120 hours at 37 °C (pH 8.0). Serial dilutions of 10^{-1} to 10^{-7} were prepared from the samples, 0.1 ml of seeds were added to the nutrient agar (NA) medium at 10^{-3} dilution, and incubation was allowed at 37 °C for one night. At the end of the incubation, the growing colonies were sown on urease agar medium, which is the urease selective medium, to control urease activity [6]. Colonies with different morphological structures growing on the urease agar medium were selected and gram stained. Gram positive rod-shaped bacteria were selected as the test material. *Sporosarcina pasteurii* NCIM 2477 procured from the National Collection of Industrial Microorganisms (NCIM) were used in screening studies to select the maximum urease producer.

Chromosomal DNA was extracted from overnight grown bacterial cells by alkaline lysis [7]. The 16S rRNA gene region was amplified by PCR using 5'-AGAGTTTGATCCTGGCTCAG-3 'forward and 5'-AAGGAGGTGATCCAGCCGCA-3' reverse primers. The following program was used in PCR: 95°C for 5 minutes for the initial denaturation of the template DNA followed by 35 cycles of 94°C denaturation for 15 seconds, 53°C annealing for 15 seconds and 72°C extension for 1.5 minutes, followed by a 5-minute final extension at 72°C and a 4°C hold. Sequence analysis of amplified PCR products was made with service acquisition. The obtained 16SrRNA gene sequence was compared with those from GenBank using the BLASTN program. A phylogenetic tree was constructed by the neighbor-joining method using MEGA5.2 software.

2.2. Urease activity and $CaCO_3$ accumulation in liquid medium

The urease activities of the bacteria were performed according to the phenol-hypochlorite method. According to this method, a mixture of 1 ml 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 ml urea (0.1 M) was added to 250 µl bacterial culture. The mixture was incubated for 5 min at 37 °C and 1 µl of phenol nitropuricide and alkaline hypochlorite were added at the end of the incubation and then incubated at 37 °C for 25 min. At the end of the incubation, samples were measured by spectrophotometer at a wavelength of 626 nm one unit of urease is defined as the amount of enzyme hydrolyzing 1 µmol urea/min. Ammonium chloride (50 to 100 µM) is used as the standard [8].

Nutrient broth supplemented with 2% urea and calcium chloride (NB-U/Ca) was used to measure $CaCO_3$ accumulation in the liquid medium [6]. 30 ml of NB-U/Ca were inoculated with 2% bacterial culture and allowed to incubate at 30 ˚C for 7 days at 130 rpm. Accumulated $CaCO_3$ was filtered with Whatman filter paper and dried at 60 °C for 3 hours.

The weight of the accumulated $CaCO_3$ is calculated according to the following equation.

$$Wc = Wfc – Wf \quad (1)$$

where (Wfc) is the weight of filter paper containing precipitant; and (Wf) is the weight of empty filter paper.

2.3. SEM and EDX analysis of the isolates.

The calcite accumulation of the bacteria was determined by scanning electron microscopy, calcium amount EDX analysis. Samples were completely dried at room temperature, and then examined at accelerating voltages ranging from 30 to 35 kV by an SEM (Zeiss EVO50). Samples were gold coated with a sputter coating Emitech K575 prior to examination.

3. Results and Discussion

In our study, a bacterium isolated from soil was grown by adding urea to the enriched media and 11 strains with urease activity were identified by 16S rRNA sequence analysis (Table1).

Table 1. Names of *Bacillus* spp. isolated from soil and mapped species and similarities in Genbank

Isolates no	Species name	Genbank species match	Similarity rate (%)
F4	*Bacillus thurigiensis*	*B. thuringiensis* ATCC 10792	99
F6	*Bacillus subtilis*	*B. subtilis* JCM 1465	99
F7	*Bacillus thurigiensis*	*B. thuringiensis* ATCC 10792	82
F8	*Bacillus macroides*	*B. macroides* LMG 18474	96
F9	*Bacillus macroides*	*B. macroides* LMG 18474	99
F10	*Bacillus mycoides*	*B. mycoides* NBRC 101228	98
F11	*Bacillus thurigiensis*	*B. thuringiensis* ATCC 10792	99
F12	*Bacillus macroides*	*B. macroides LMG* 18474	98
F13	*Bacillus macroides*	*B. macroides LMG* 18474	98
F14	*Bacillus thurigiensis*	*B. thuringiensis* Bt407	96
F15	*Bacillus mycoides*	*B. mycoides NBRC* 101228	96

The sequences of the 16S rRNA gene region of the isolates were compared with those of the gene bank, and a phylogenetic tree was constructed according to the maximum similarity index with the program MEGA5.2 (Figure 1). *Clostridium frigoris* species was used as the outer group.

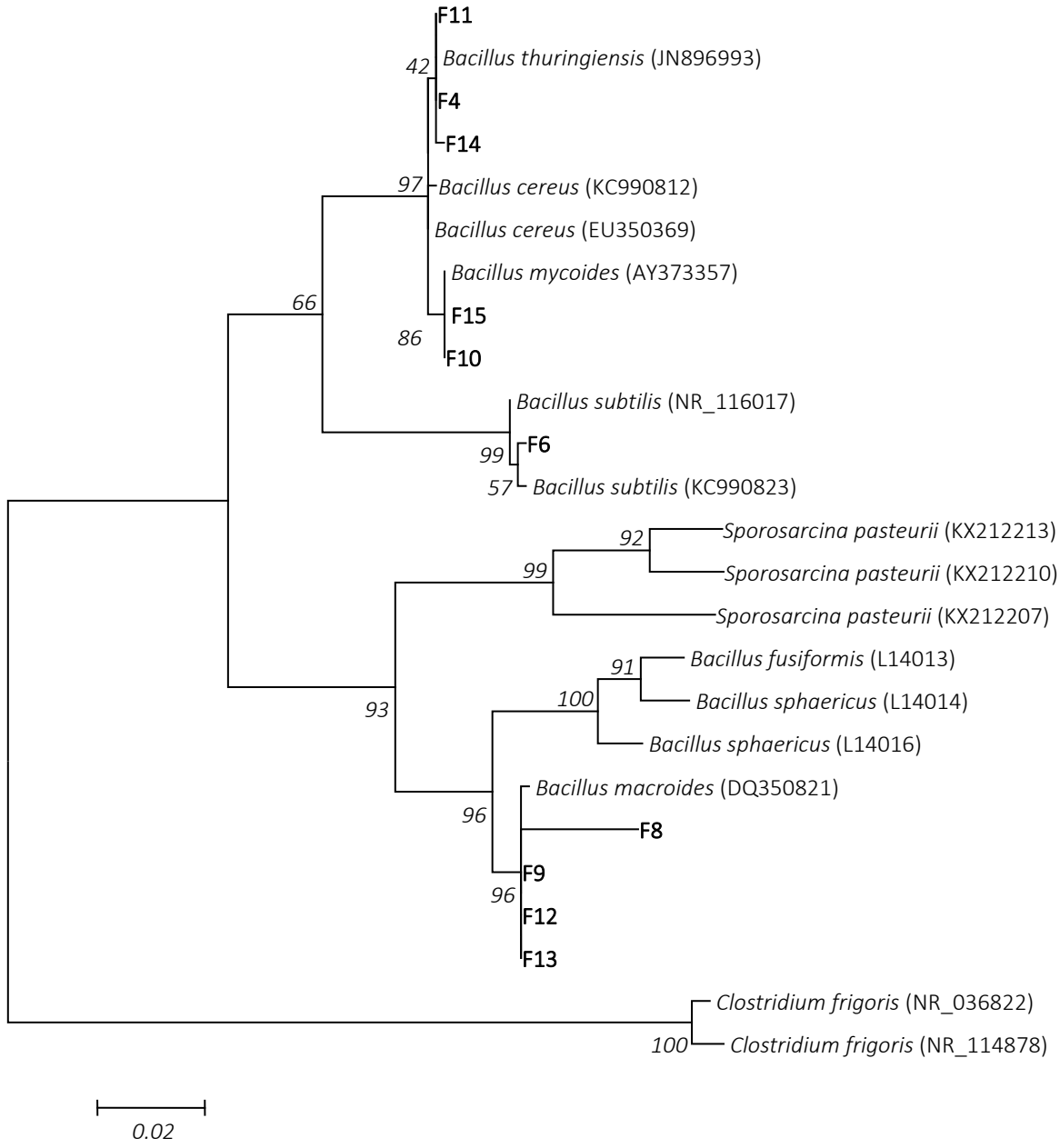

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences of the isolated strains as compared to known bacterial species. The NCBI accession numbers for known strains from the NCBI database are listed in parenthesis. Numbers at the nodes indicate the bootstrap values on neighbor joining analysis.

A change in coloration following incubation on urea broth was recorded as an urease positive reaction. When organisms utilize urea, ammonia is formed and this in turn makes media alkaline which then produces red pink colour. This increase in pH causes the indicator to change from yellow to deep pink and is a positive test for urea hydrolysis [9]. Isolates in broth showed a rapid and intense pink color development indicating its urea hydrolysis ability by urease enzyme. As a result of the urease activity analysis, it was determined that the control strain *Sporosarcina pasteurii* had the highest activity with 11.64 U/mg. After the control strain the species with the highest activity among the species belonging to the genus Bacillus was identified as F10 (*B. mycoides*) isolate at 8.43 U/mg. Specific activities of the other isolates of F9, F6, F12 and F4 were 8.16, 7.53, 7.55 and 6.42 U/mg, respectively. The specific activity of the F8, F11, F13 and F15 bacteria was found to be lower than the other bacteria. When compared to the other bacteria, it was determined that the bacterium with the lowest specific activity was F14 isolate at 2.66 U/mg (Table2). Since specific activity is dependent on the purity of the protein, pH, temperature and other conditions of the assay [10].

Table 2. Specific urease enzyme activity, $CaCO_3$ accumulation in liquid medium and Ca amounts according to EDX assay of ısolates and *S. pasteurii*

Isolates	Specific urease activity (U/mg)	$CaCO_3$ accumulation in liquid medium	Ca amount (in weight %)
F4 (*B. thuringiensis*)	6,59±0,08	0.03	31,10
F6 (*B. subtilis*)	7,96±0,03	0.04	39,25
F7 (*B. thuringiensis*)	7,26±0,16	0.04	38,77
F8 (*B. macroides*)	4,43±0,01	0.18	28,12
F9 (*B. macroides*)	8,21±0,00	0.20	22,43
F10 (*B. mycoides*)	8,50±0,00	0.09	33,03
F11 (*B. thuringiensis*)	4,41±0,00	0.07	30,93
F12 (*B. macroides*)	7,57±0,01	0.07	30,93
F13 (*B. macroides*)	8,28±0,01	0.20	22,38
F14 (*B. thuringiensis*)	2,75±0,04	0.06	34,01
F15 (*B. mycoides*)	4,77±0,03	0.12	27,44
Sporosarcina pasteurii	11,64±0,03	0.53	48,52

In liquid medium supplemented with urea and calcium chloride, the maximum $CaCO_3$ accumulation was determined to be 0.53 g in control strain *S. pasteurii.* After the control strain, the maximum $CaCO_3$ accumulation was measured as 0.20 g, 0.20 g and 0.18 g in F9, F13 and F8 isolates, respectively. $CaCO_3$ accumulations of F15, F10, F12, F11, F14, F6 and F7 isolates were determined as 0.12 g, 0.09 g, 0.07 g, 0.07 g, 0.06 g, 0.04g, 0.04 g, respectively. The minimum $CaCO_3$ accumulation was determined to be 0.03 g in the F4 isolate (Figure2, Table2).

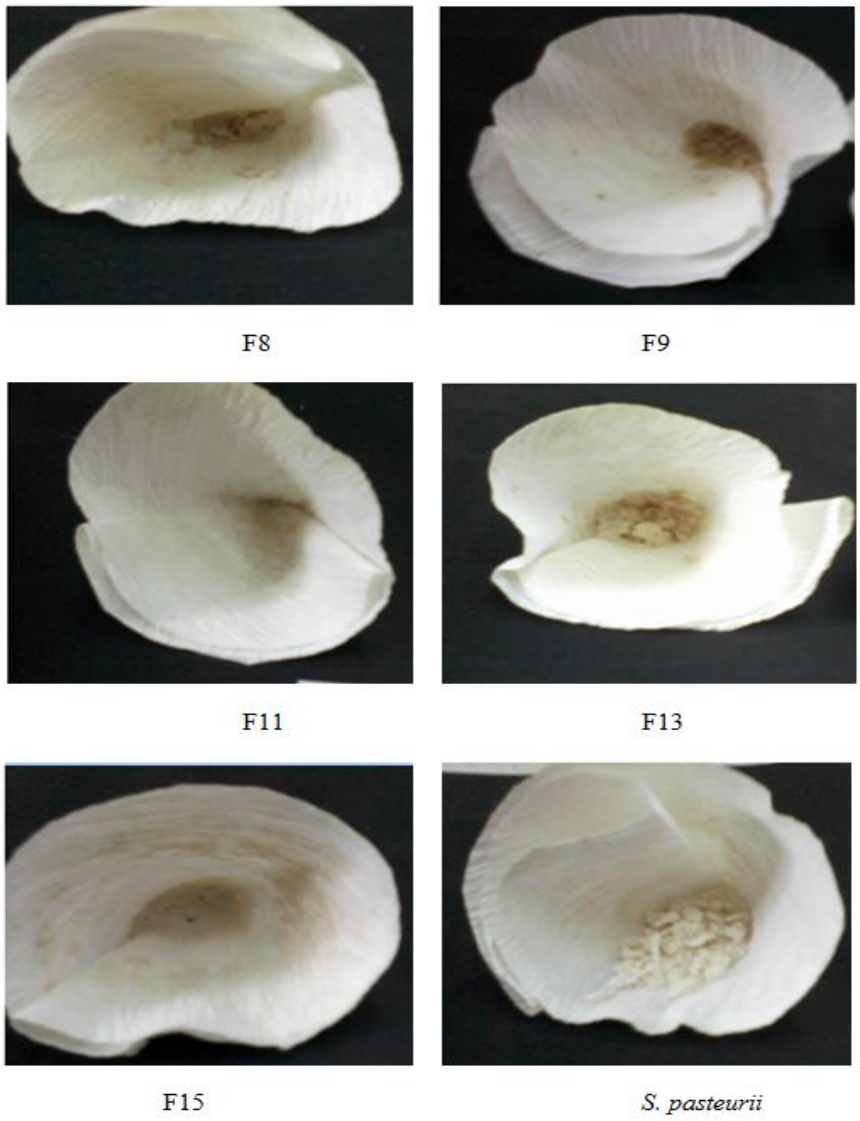

Figure 2. $CaCO_3$ accumulation formed by *S. pasterurii* and some isolates after 7 days of incubation in NB-U/Ca liquid medium.

The formation of $CaCO_3$ precipitant is due to the hydrolysis of urea which results in the production of ammonia and carbonate. Ammonia release act to raise the pH of the medium, while the condition favors calcium carbonate precipitation. Carbonate binds calcium ions present in the medium resulting in the formation of calcium carbonate crystals which are deposited in broth media. [11-14]. Urease activity and calcium carbonate accumulation in liquid media were also shown to have biocatalytic properties in the isolates with enzyme activity in general.

Calcium carbonate deposits of the isolates were also detected by scanning electron microscopy. According to SEM images, the isolates were found to carry out calcium carbonate accumulation (Figure3).

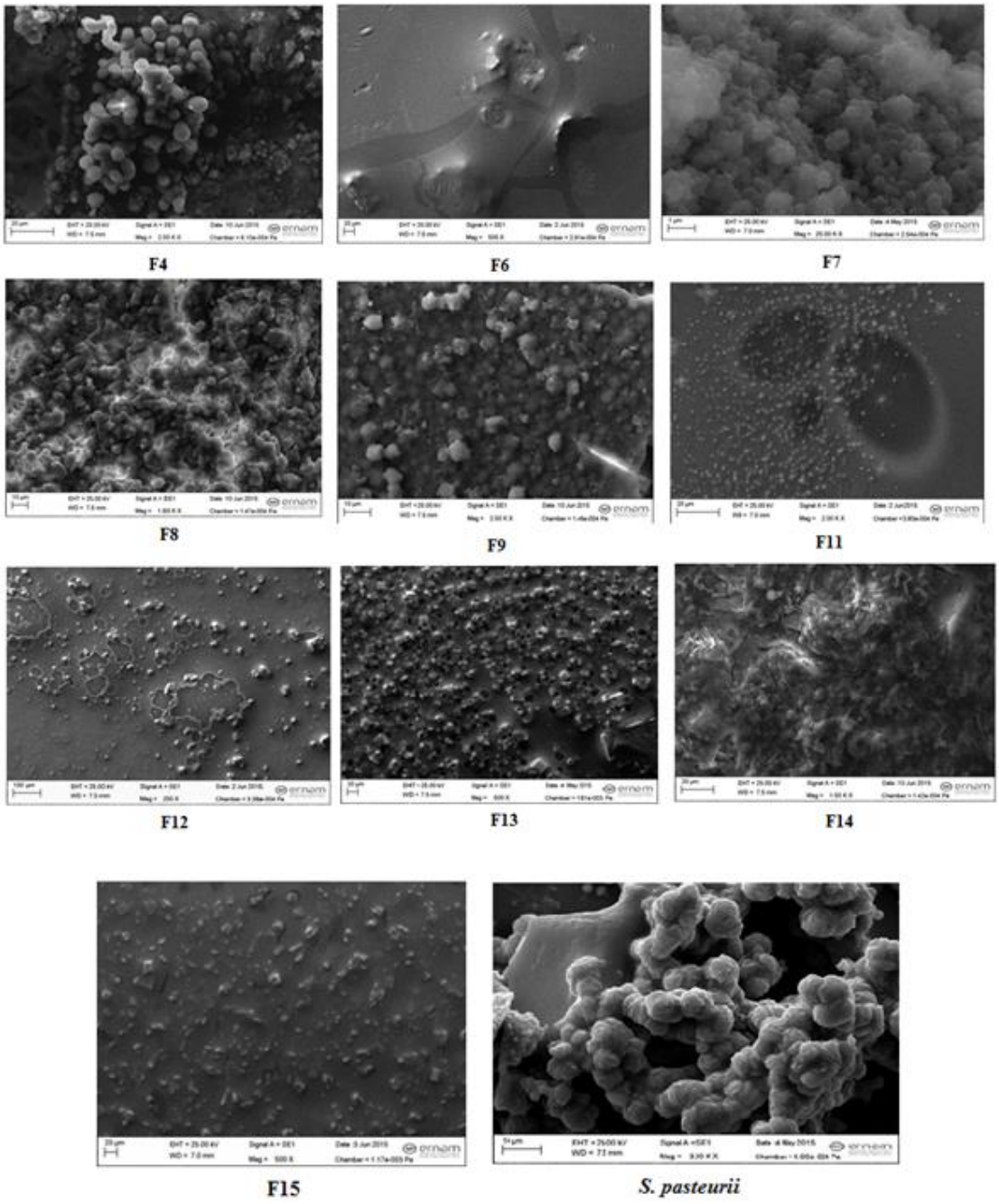

Figure 3. SEM micrograph of microbiologically induced calcite precipitation of the isolates and *S. pasteurii.*

Also, calcium amounts were analyzed by EDX. As a result of the analysis, it was determined that the highest calcium amount was in control with (in weight %) 48.52% *S. pasteurii*. The highest calcium amount after the control strain was determined in F6, F14, F10, F2, F12, F4, F11 isolates, respectively, with 39.25%, 34.01%, 33.03%, 31.95%, 31.36%, 31.10%, 30.93%. Calcium amount in other isolates was determined forF1, F8, F15, F9, F13 and F3 respectively, with 29.03%, 28.12%, 27.44%, 22.43%, 22.38 % and 19.78% (Table 2). The presence of high amounts of calcium in the bacterial isolates confirmed the presence of calcite in the form of calcium carbonate. The isolates were grown at a higher rate in the presence of oxygen and consequently induced active precipitation of calcium.

CONCLUSION

In this study, isolates of *Bacillus* species with urease activity were isolated from soil samples. The identification of the isolated bacteria was carried out according to morphological characteristics and 16S rRNA sequence analysis. The urease activities of the isolates identified at the species level were determined and the biocatalytic properties of the active

bacteria were tried to be determined. In our study, it was determined that F13 and F9 isolates, identified as *B. macroides* with biocalcification properties, formed calcite in excess in the liquid medium. Also, the F10 isolate, identified as *B. mycoides*, was found to be high in urease activity. It is recommended that further investigation to utilize these isolates in several applications should be performed.

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REFERENCES

[1] Ramachandran, S. K., Ramakrishnan, V., Bang, S. S. 2001. Remediation of concrete using microorganisms. ACI Materials Journals. 98 (2011), 3–9.

[2] Sarda, D., Choonia, H. S., Sarode, D. D., Lele, S. S. 2009. Biocalcification by *Bacillus pasteurii* urease: a novel application). Journal of Industrial Microbiology and Biotechnology. 36(2009), 1111–1115.

[3] Castanier, S., Metayer-Levrel, G. Le., Perthuisot, J. P. 1999. Ca-carbonates precipitation and limestone genesis–the microbiol geologist point of view. Sedimentary Geology. 126(1999), 9-23.

[4] Braissant, O., Verrecchia, E. P. And Aragno, M. 2002. Is the contribution of bacteria to terrestrial carbon budget greatly under estimated. Naturwissenschaften, 89(2002), 366-370.

[5] Dick, J., Windt, W. De., Graef, B. D., Saveyn, H., Meeran, P. V., Belie, N., Verstraete W. 2006. Biodeposition of a calcium carbonate layer on degraded limestone by Bacillus species. Biodegradation. 17(2006), 357- 67.

[6] Hammad, I. A., Talkhan, F. N., Zoheir, A. E. 2013. Urease activity and induction of calcium carbonate precipitation by *Sporosarcina pasteurii* NCIMB 8841. Journal of Applied Sciences Research. 9(2013), 1525-1533.

[7] Maniatis, T., Fristsh, E. F., Sambrook, J. 1989. In Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York,

[8] Giffel, M. C., Beumer, R. R., Granum, P. E., Rombouts, F. M. 1997. Isolation and characterization of Bacillus cereus from pasteurised milk in house hold refrigerators in the Netherland. International Journal of Food Microbiolgy. 34(1997), 307-318.

[9] Prescott, H., 2002. Laboratory Exercise in Microbiology, Fifth edition. The Mc GrawHill.

[10] Mobley, H., Hausinger, R. 1989. Microbial ureases: Significance, regulation, and molecular characterization. Microbiological Reviews. 53(1989), 85–108.

[11] Stocks-Fischer, S., Galinat, J.K., Bang, S.S. 1999. Microbiological precipitation of CaCO3. Soil Biology and Biochemistry. 31(1999), 1563-1571.

[12] Hammes, F., Boon, N., Villiers, J. de.,Verstraete,W., Siciliano, S.D. (2003a). Strain-Specific Ureolytic Microbial Calcium Carbonate Precipitation. Applied and Environmental Microbiology. 69(2003a), 4901-4909.

[13] Whiffin, V.S. 2004. Microbial $CaCO_3$ Precipitation for the Production of Biocement. PhD thesis. Murdoch University, Western Australia.

[14] Al-Thawadi, S.M, 2008. High Strength In-Situ Biocementation of Soil by Calcite Precipitating Locally Isolated Ureolytic Bacteria. PhD thesis. Murdoch University, Western Australia.