



Production of nanocalcite crystal by a urease producing halophilic strain of *Staphylococcus saprophyticus* and analysis of its properties by XRD and SEM

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Abstract

Cementation of salt-containing soils can be achieved by salt-tolerant or halophilic calcite precipitation bacteria. Therefore, the isolation of calcite-producing bacteria in the presence of salt is the first step in the microbial cementation of saline soils. Urease producing bacteria can cause calcite nano-crystals to precipitate by producing urease in the presence of urea and calcium. The purpose of this study was to isolate urease producing halophilic bacteria in order to make calcite precipitate in saline soil. The calcite and the properties of the strains were further analyzed by X-ray diffraction (XRD) and scanning electron microscope equipped with an energy dispersive X-ray detector. In this study, a total of 110 halophilic strains were isolated, from which 58 isolates proved to have the ability of urease production. Four strains were identified to produce nano-calcite using urease activity in the precipitation medium. The XRD studies showed that the size of these particles was in the range of 40–60 nm. Strain H3 revealed that calcite is mostly produced in the precipitation medium containing 5% salt in comparison with other strains. This strain also produced calcite precipitates in the precipitation medium containing 15% salt. Phylogenetic analysis indicated that these isolates are about 99–100% similar to *Staphylococcus saprophyticus*.

Keywords Nanocalcite · Urease · Halophilic bacteria · *Staphylococcus saprophyticus* · XRD

Introduction

A variety of microbial metabolic pathways including photosynthesis, ammonification, denitrification, urea hydrolysis and sulfate reduction can lead to the precipitation of carbonates in different natural environments (Hammes et al. 2003). The potential of microorganisms to synthesize calcium carbonate is called microbially induced calcite precipitation (MICP), which has many uses in civil engineering and environmental science. Urea hydrolyzing bacteria can make calcium carbonate precipitate due to bacterial hydrolysis of urea in the presence of calcium ions. Microbial precipitation of calcium carbonate results in the production of different polymorphs such as calcite, aragonite, vaterite and two hydrated crystalline components. Among these, calcite is the most common and stable bacterial calcium carbonate

polymorph (Rodriguez Navarro et al. 2012). The production of these polymorphs of calcium carbonate depends both on their growing environments and bacterial strains.

MICP has emerged as a novel technique and successful alternative for remediation and restoration of different building materials in recent years (Dhami et al. 2012, 2016). From the biological point of view, the MICP process is regulated mainly by four key factors: (1) concentration of calcium ion; (2) concentration of dissolved inorganic carbon; (3) pH; and (4) availability of nucleation sites (Hammes and Verstraete 2002). Several genera of halophilic bacteria such as *Halomonas*, *Deleya*, *Flavobacterium*, *Acinetobacter* and *Salinivibrio* and others have been reported to make carbonates precipitate in natural marine habitats (Ferrer et al. 1988a, b; Rivadeneyra et al. 1993, 2006). To date, a lot of researchers have studied carbonate precipitation in the saline aquatic environments, freshwater environments, and saline soils (Baskar et al. 2005; Novitsky 1981; Rivadeneyra et al. 2004).

In the MICP process, the hydrolysis of urea by urease can increase the pH of the medium during the MICP process. The carbon dioxide produced by urease activity or

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respiration is quickly transformed into bicarbonate (HCO_3^-) in the presence of water. This bicarbonate reacts with the hydroxyl ions to form carbonate (CO_3^{2-}) ions, and in the presence of calcium ions, the calcite is precipitated (Castanier et al. 2000). As, urease producing bacteria used for bio-cementation are active in the presence of high salt concentrations, halophilic or halotolerant, and alkaliphilic urease producing bacteria are thus preferred choices for bio-cementation (Mortensen and DeJong 2011).

Halophilic microorganisms use different strategies to overcome the stress of high salt concentrations. Halophilic archaea maintain their cytoplasmic osmotic balance by accumulating high concentrations of inorganic ions in the cytoplasm. In contrast, halophilic or halotolerant bacteria adapt themselves by accumulating high concentrations of various organic compatible solutes. These mechanisms are called “salt in cytoplasm strategy” and “salt out cytoplasm strategy”, respectively (Galinski and Trüper 1994). Some researchers such as Rivadeneyra showed that both Na^+ and Cl^- ions exert a weak or no influence on the microbial precipitation of carbonates (Rivadeneyra et al. 1991). *Deleya halophila* is a moderately halophilic microorganism, whose salt requirements and its ability to precipitate carbonates make it a useful tool in carbonate precipitation studies. Studies on this bacterium have shown that high salinity has a negative effect on calcite production (Ferrer et al. 1988b). Also, the crystals that are formed are calcite and vaterite, and the ratio of calcite to vaterite is dependent on the amount of salt and type of medium (Rivadeneyra et al. 1991). Further, aragonite is not detected even at high magnesium ion concentration in the medium (Ferrer et al. 1988b).

The purpose of this study was to isolate a potent bacterium with calcite precipitation ability in the presence of high salt concentration. To that end, this study investigated the effect of salinity on the type of crystal formation by using isolated bacteria in the precipitation media. The effect of salt amount on the type of crystalline calcium carbonate was studied by X-ray diffraction (XRD).

Materials and methods

Isolation of halophilic bacteria

About 5 g of soil sample or 5 ml of water sample were suspended in 45 ml of sterilized physiology serum and then mixed by shaking on a shaker for several hours at 120 rpm. The flasks were shaken thoroughly and the suspension was let clarified by settling. Fifty microliters of diluted samples were spread on solid tryptic soy broth (TSB) (Biolife, Italy) and Luria–Bertani media. Plates were incubated at 30 °C for 5 days. In order to isolate halophilic bacteria, salt was

added to TSB medium at different concentrations (up to 20%) (Carpa et al. 2014; Vahed et al. 2011).

Isolation of urease producing bacteria

The bacteria isolated in the previous step were selected for urease testing. For this purpose, each of the purified isolates was cultured on a solid medium containing 30 g/l TSB, 0.012 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.024 g/l $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g/l phenol red, 20 g/l urea, and 15 g/l agar (pH 6.8–7.3). In addition, due to the fact that halophilic or halotolerant bacteria need salt, 0–20 percent NaCl was added to the medium. Urease producing isolates brought about the alkalization of the culture medium and thus changed the color to pink by releasing ammonium due to the urease activity (Stabnikov et al. 2013).

Isolation of calcite-producing bacteria

Purified bacteria were cultured on precipitation medium at 30 °C for 2 weeks. Colonies were screened based on the observation of calcite precipitation by a light microscope. Precipitation medium consisted of 20 g/l urea, 2.12 g/l NaHCO_3 , 10 g/l NH_4Cl , 3 g/l nutrient broth (Merck, Germany), 25 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0–200 g/l NaCl, and 15 g/l agar. The pH of medium was adjusted to 8 with the addition of 1 N NaOH (Chahal et al. 2011).

Specific urea degradation rate

Each isolate was cultured overnight on a urea agar medium containing 5% salt and incubated at 30 °C. A loop of the active bacterium was transferred to the broth medium containing urea and 5% salt, and then 1% of it was inoculated into the culture medium when OD_{600} nm reached 0.6–0.8. Culture medium was incubated at 30 °C on a shaking incubator at 120 rpm, and every 8 h optical density (OD) and total ammonium nitrogen of each isolate were measured using a spectrophotometer at 600 nm and Nessler method (Thompson and Morrison 1951), respectively. Specific urea degradation (SUD) rate was defined as the ratio of ammonium production (AP) per unit of bacterial growth in a given time (t), which is obtained by the following formula (Dick et al. 2006):

$$\text{SUD} = \frac{\text{AP}(\text{mmol ammonium} \times \text{l}^{-1})}{[\text{OD}_{\text{cells}}] \times t (\text{h})}$$

Analysis of nanocrystals

The colonies that produced nanocrystals were cultured on precipitation medium and then were collected and dried at

Fig. 1 Morphology of monocrystal and polycrystal produced by isolates H1 (a), H3 (b), H4 (c) and H5 (d) strains cultured on precipitating medium containing 5% salt at $\times 10$ magnification. Crystals are visible outside and inside colonies

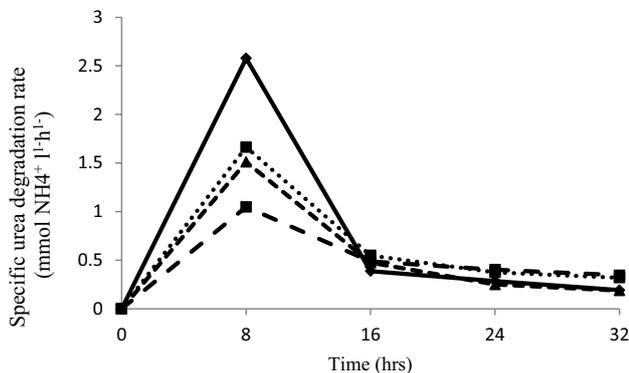
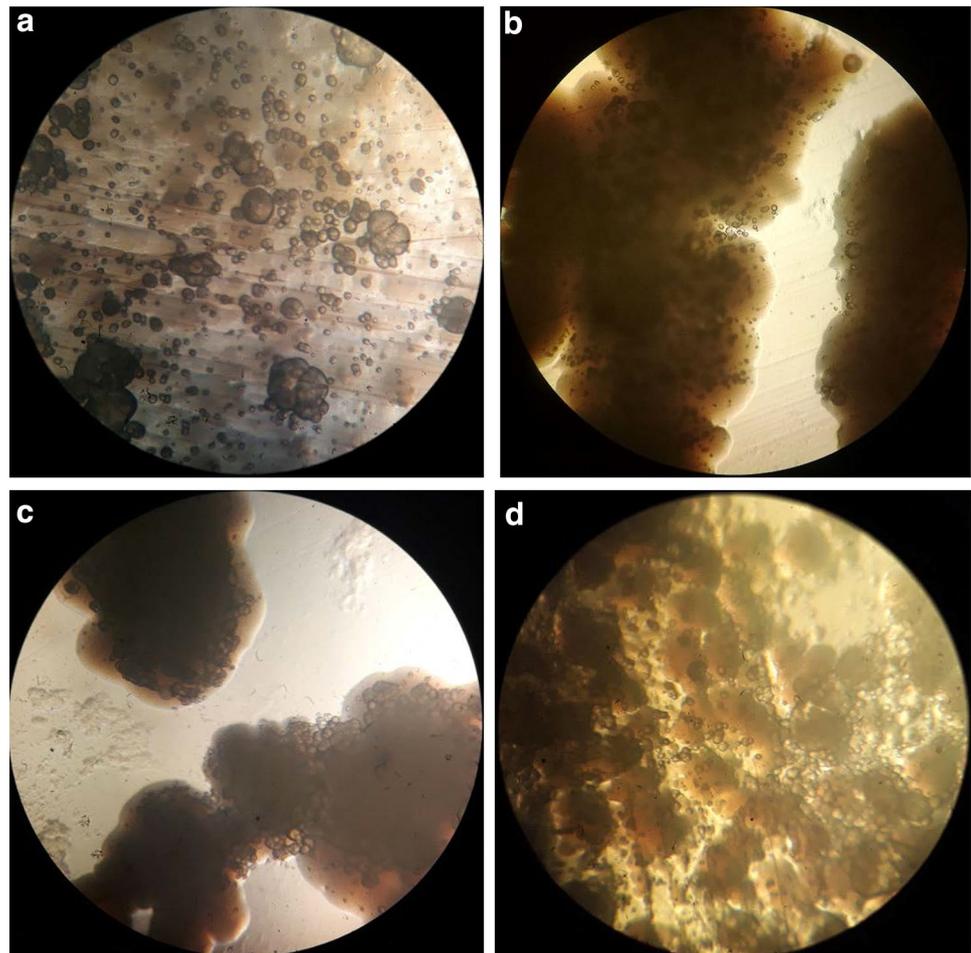


Fig. 2 The SUD rate for H1 (dashed square), H3 (dotted square), H4 (dashed triangle) and H5 (dashed diamond)

37 °C for XRD analysis. XRD measurements were done by a one-circle $\mu = \mu$ diffractometer (XRD, D8 ADVANCE, Germany). The work condition of XRD was $\text{CuK}\alpha$ radiation via a rotating anode at 40 kV and 40 mA. The data were collected at a step of $0.05^\circ 2^{-1}$ s and range of scattering angles (2θ) from 5° to 80° . The crystalline phases were

identified using the ICDD database. Crystallite particle size was determined by XRD using Scherrer formula (Yena et al. 2007). Dried samples were ground into fine powder using agate mortar and then coated with carbon, and these samples were used for size characteristics of nanocrystals by scanning electron microscope (SEM) (MIRA3 TESCAN, Czech Republic). The elementary composition of the nanocrystals was determined qualitatively by energy dispersive X-ray spectrometry (EDS) with an In Beam-SE detector.

Identification of calcifying bacterial isolates

Nanocalcite producing isolates were identified using morphological, biochemical and molecular analysis. Biochemical tests were performed according to Bergey's manual of systematic bacteriology (Brenner et al. 2005). Molecular phylogenies were determined by sequencing of 16S rRNA using universal primers 27F-YM (Frank et al. 2008) and 1492R-Y (Gökçekus et al. 2011). Bacterial DNA was extracted using boiling method. The 1.5 ml of bacterial culture was centrifuged for 10 min at $6000\times g$ and the pellet was suspended in biology grade water and centrifuged at

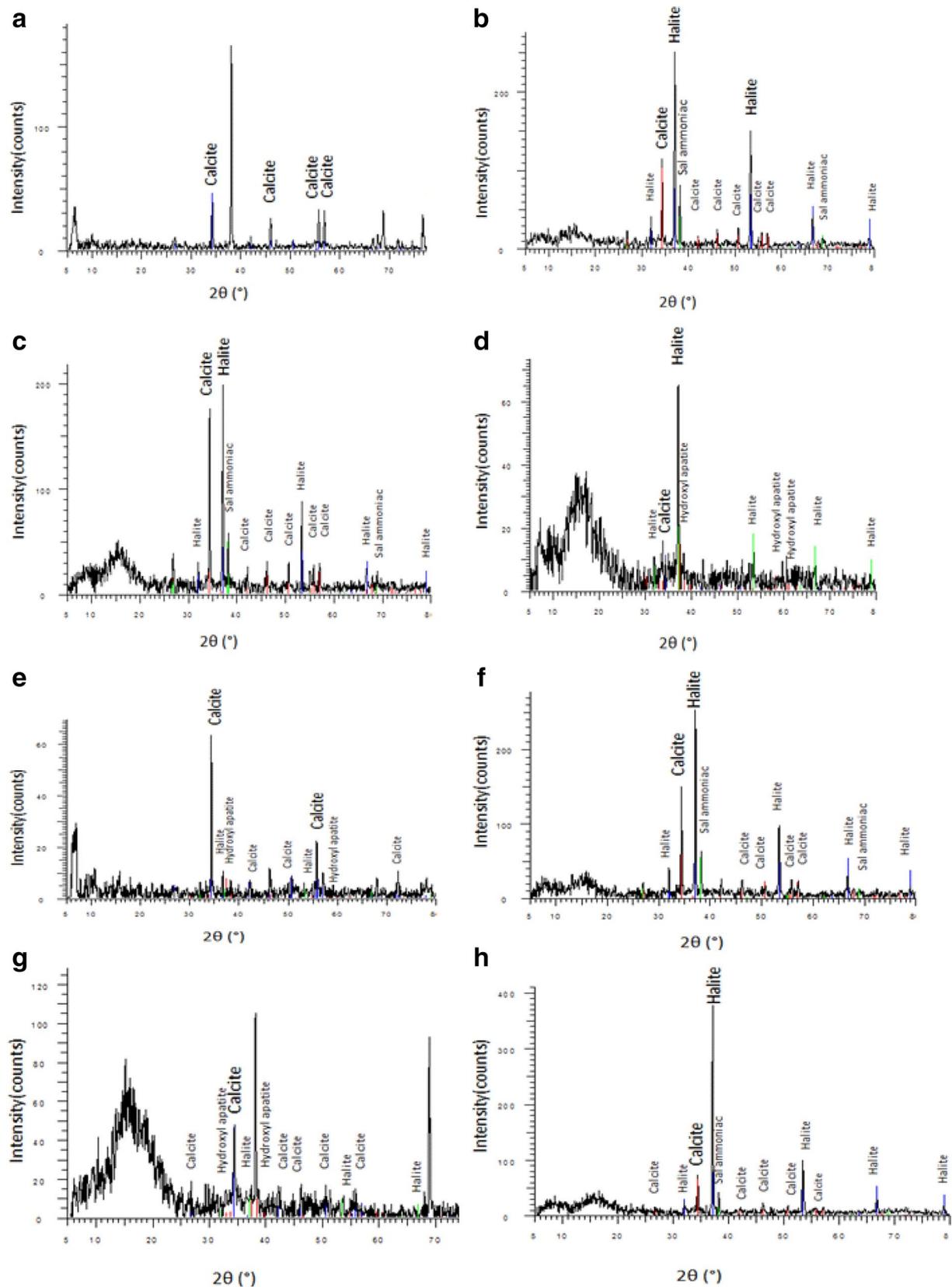


Fig. 3 XRD patterns of precipitates collected from isolates 1, 3, 4, and 5, cultured on precipitating medium with different NaCl concentrations: **a** H1, without added NaCl, **b** H1, 5% NaCl, **c** H3, 5% NaCl,

d H3, 15% NaCl, **e** H4, without salt, **f** H4, 5% NaCl, **g** H5, without added NaCl, **h** H5, 5% NaCl

6000×g for 10 min. The cells were washed three times using this water, subjected to two freeze–thaw cycles, suspended in 1 ml of molecular biology grade water, boiled at 100 °C in a water bath for 15 min, and centrifuged at 6000×g for 5 min (Yang et al. 2008). The supernatant was used directly for PCR or stored at –20 °C.

Amplification reactions were conducted in 25 µl volume containing 1 µl of each primer (10 µmol/µl), 0.5 µl of dNTPs (10 mM), 2.5 µl of 10× PCR buffer, 0.75 µl of MgCl₂ (50 Mm), 18 µl of PCR H₂O, template DNA extracted from 1 µl of bacterium, and 0.25 µl of Taq polymerase (5 U/µl) (Sina Colon, Iran). The PCR was performed in a Thermal Cycler (T100, Bio-Rad, USA) using appropriate programs optimized for these primers. After denaturation of DNA through heating for 4 min at 94 °C, the PCR program was run for 35 cycles; each cycle consisted of these steps: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min (Gökçekus et al. 2011). This was followed by a final elongation step for 5 min at 72 °C. After amplification, purified PCR product was sequenced in both directions using an automated sequencer by Macrogen (Seoul, Korea). Sequences were edited by Finch TV version 1.4.0. (URL: <http://www.geospiza.com/finchtv.html>) and assembled with BioEdit version 7.1.3.0 (URL: <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). All the assembled sequences were aligned with the published sequence using BLASTN program, and sequences were submitted to NCBI database. Phylogenetic tree of bacterial 16S rRNA was studied based on Bayesian inference (MrBayes) (Huelsenbeck and Ronquist 2001). For Bayesian phylogenetic inference, we used MrBayes v3.2.6 (MCMC ngen 6 million generations, samplefreq 1000, nchains 4 and the first 25% of the trees sampled as burn-in) (Ronquist and Huelsenbeck 2003). The analyses were stopped when the standard deviation of split frequencies was well below 0.01. Trees were visualized using TreeGraph2 (Stöver and Müller 2010).

Results

Isolation of calcite precipitating bacteria

A total of 110 halophilic strains were isolated, from which 58 isolates had the ability of urease production. The microscopic studies of colonies showed that only four isolates were able to produce crystals on precipitation medium. Strains were isolated from sediments of Kish Island (H1), water of Kish Island (H3, H4) (Kish Island, Iran) and tap water from Ahvaz (H5) (Ahvaz, Iran). All the four isolates produced crystals on precipitation medium. As seen in Fig. 1, these isolates produced different types of calcite morphology.

Specific urea degradation rate

According to SUD rate, it was shown that strain H5 has the maximum urease activity. This strain produces ammonium 2.55 mmol/l in an hour. The SUD rate of some strains is illustrated in Fig. 2. H3 produces 1.66 mmol of ammonium per liter in an hour.

XRD analysis

On the basis of Fig. 3, strain H1, in precipitation medium without additional salt, deposited only calcite (Fig. 3a), but in precipitation medium with 5% salt (Fig. 3b), in addition to calcite, halite and sal ammoniac were also produced. No crystals were formed in precipitation medium containing 10% salt by this strain.

Strain H3 showed the most amount of calcite production in precipitation medium containing 5% salt in comparison with other strains. In addition to calcite, halite and sal ammoniac were detected in the dried colony powder of this bacterium (Fig. 3c). This strain was also able to produce calcite precipitates in precipitation medium containing 15% salt (Fig. 3d). The amount of calcite in the medium containing 5% NaCl was more than in the other medium with 15% NaCl.

Studies on strain H4 showed that this strain could also produce calcite precipitates in a precipitation medium with or without 5% salt (Fig. 3e, f). In precipitation medium with 10% salt, only halite and hydroxyapatite were produced, and calcite was not detected (not shown here). Strains H1, H4 and H5 produced more calcites in a medium containing 5% NaCl compared to a salt free medium. In strain H5,

Table 1 A summary of the types of crystal produced by strains H1, H3, H4 and H5 examined in the media with different salt concentrations

Strain	Salt concentrations			
	0%	5%	10%	15%
H1	Calcite	Calcite Halite Sal ammoniac	–	ND
H3	Clacite	Calcite Halite Salammoniac	Hydroxylapatite Calcite Halite	Hydroxylapatite Calcite Halite
H4	Hydroxylapatite Calcite Halite	Calcite Halite Salammoniac	Hydroxylapatite Halite	ND
H5	Hydroxylapatite Calcite Halite	Calcite Halite Salammoniac	–	ND

–: no crystal produced, ND: not determined

XRD analysis showed calcite and halite in the precipitation media with or without salt. In this strain, hydroxyapatite was produced in the precipitation medium without salt, and sal ammoniac was produced in the precipitation medium of 5% salt. But in the precipitation medium with 10% salt, no crystals were produced by strain H5 (not shown here). Table 1 summarizes the types of crystals produced by the strains examined in different salt concentrations.

SEM and EDX analysis

Figure 4 shows the image of the SEM of the crystals formed by isolate H1. SEM showed that calcite nanocrystals produced by strain H1 are homogeneous in shape and size. According to SEM analysis, it seems that calcite nanoparticles have been integrated by the bacteria trapped inside them. The XRD studies showed that the size of these

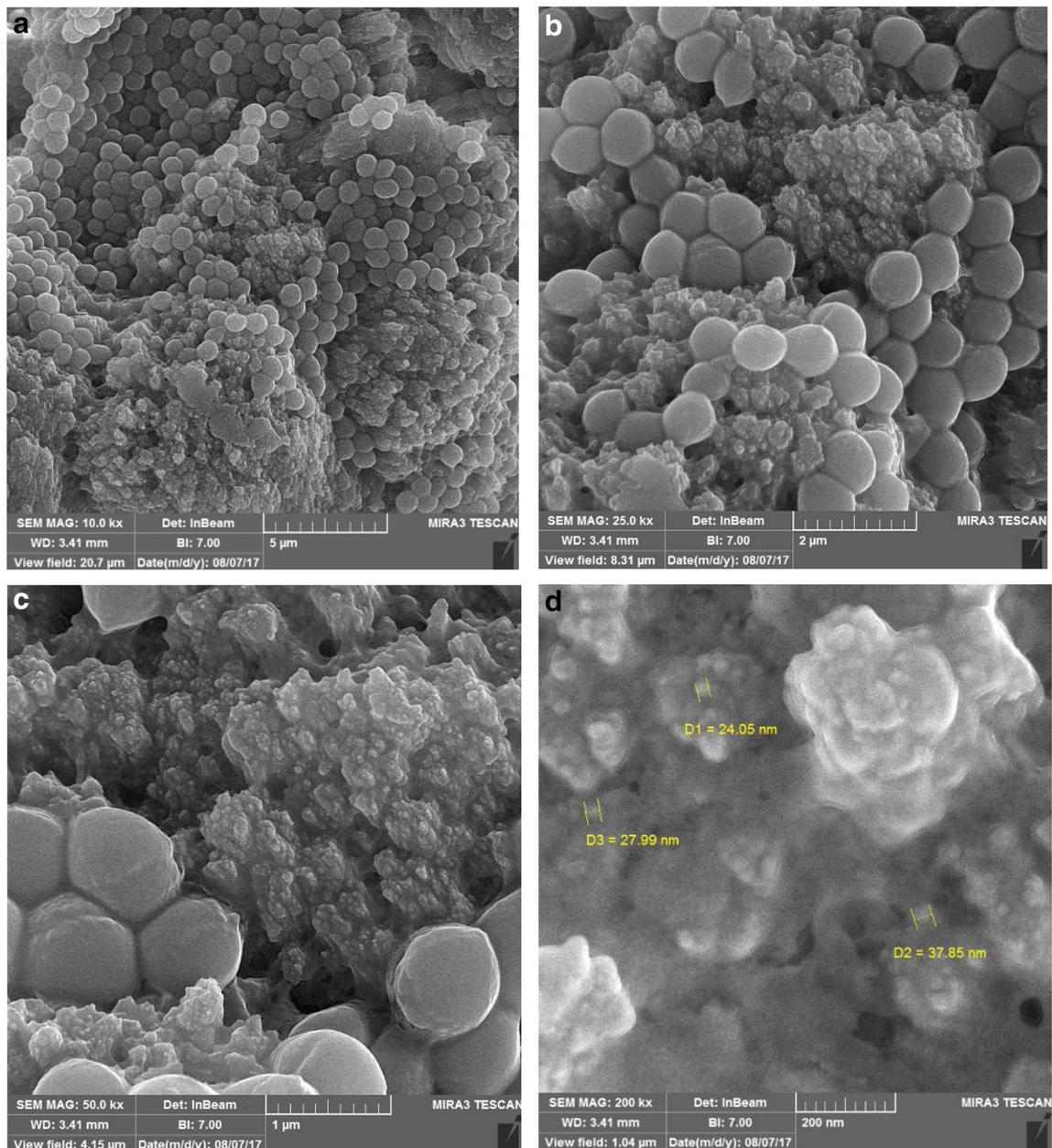


Fig. 4 SEM and EDX images of aggregation of nanocrystals produced by strain H1 on bacterial surfaces in precipitating medium with different degrees of magnification. The scale bar is 5, 2, 1 micron and 200 nm, respectively

particles was in the range of 40–60 nm. Figure 5 shows energy dispersive X-ray (EDX) spectrum of precipitates of H1, which proves the presence of its constituent elements of calcite.

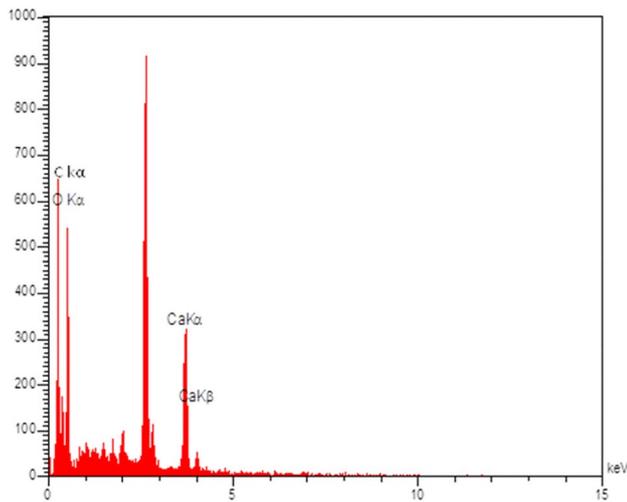


Fig. 5 SEM–EDX analyses of three areas from a calcinated precipitate produced by strain H1. The scale bar is 5, 2, 1 micron and 200 nm, respectively

Bacteria identification

These strains were identified by biochemical and molecular analysis. The results of the biochemical tests of the isolates are presented in Table 2. All the strains can use maltose, sucrose, fructose and glucose as their carbon and energy sources. These isolates can grow in the presence of 15% of salt and therefore can tolerate and grow in the highly salty soils. The 16S rRNA gene sequence of strains 1, 3, 4 and 5 showed to be similar, respectively, by 99.85%, 99.92%, 99.92%, and 99.92% to *Staphylococcus saprophyticus*. Their 16S rRNA sequences were deposited in GenBank under the accession numbers of MG655151, MG655152, MG655153 and MG655154, respectively. Figure 6 shows phylogenetic tree of isolates according to MrBayes. The amplified PCR product was approximately 1500 bp in length, and the alignment length was 1391 characters (including indels). H5 and H3 strains, respectively, were placed in separate clads of I and IV with 0.926 the posterior probability, while H1 and H4 strains were branched together in one cluster.

Table 2 Biochemical test results of isolates

Characteristics	<i>S. saprophyticus</i> (ATCC15305)	Strain H1	Strain H3	Strain H4	Strain H5
Pigment	d ^a	+	+	+	+
Catalase	+	+	+	+	+
Coagulase	–	–	–	–	–
Oxidase	–	–	–	–	–
Urease	+	+	+	+	+
Hemolysis	–	–	–	–	–
Deoxyribonuclease (DNase agar)	–	–	–	–	–
Novobiocin resistance	+	+	+	+	+
Growth in the presence of 10 and 15% NaCl					
10%	+	+	+	+	+
15%	d	+	+	+	+
Aerobic acid production from					
Maltose	+	+	+	+	+
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	+
Lactose	d	+	–	+	–
Glucose	+	+	+	+	+
Starch	–	–	–	–	–
Xylose	–	–	–	–	–
Galactose	–	–	–	–	–
Mannose	–	–	–	–	–

^a11–89% strain positive

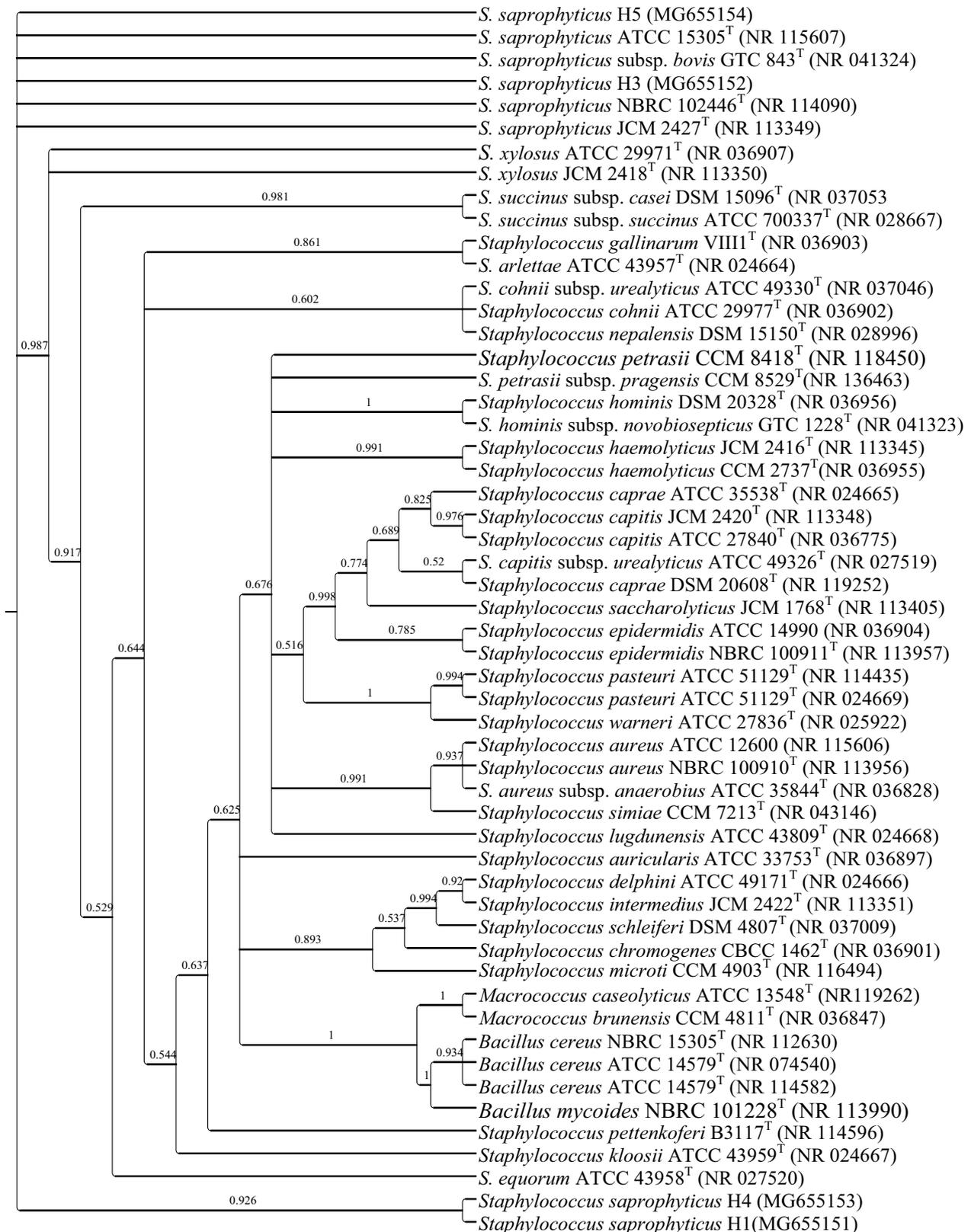


Fig. 6 Phylogenetic tree of isolated bacterial 16S rRNA studied based on Bayesian inference (MrBayes). *S.*: *Staphylococcus* (Ronquist and Huelsenbeck 2003)

Discussion

Many studies have suggested that urease-producing bacteria can produce calcite through urease enzyme activity. Our data showed that urease activity was present in four isolates when tested in the urease activity assay media. Studies of Hammes et al. (2003) and Wei et al. (2015) showed a good relationship between urease activity and calcite production. Four isolates of *S. saprophyticus*, *Sporosarcina globispora*, *Bacillus lentus*, and *Sporosarcina* sp. with a high level of urease activity were isolated from calcareous soils and sands. *S. saprophyticus* had a higher SUD rate than other strains, but there was not a strong relationship between the calcite precipitation and urease activity (Kim and Youn 2016). Similarly, we did not observe a strong relationship between urease activity and calcite production in these strains. H5 strain had a good urease activity in comparison with other strains. However, H3 strain produced more calcites than other strains. The main crystal produced by *Deleya halophila* in the presence of salt, which is a moderately halophilic microorganism, was aragonite (Rivadeneira et al. 1996). In the present study, isolates H1, H3, H4 and H5 produced calcite in the precipitation medium with or without salt. In contrast to the above research, there was not any aragonite produced by these strains as determined by XRD analysis of crystals. Additionally, in the presence of strains such as H4 and H5, more calcites were produced in the precipitation medium containing 5% NaCl in comparison with salt-free medium. In the precipitation medium containing 5% salt, calcite is mostly produced by isolate H3 in comparison with other strains. Unlike other strains, this strain also produced calcite precipitates when the salt concentration reached 10%. In addition, this strain produced calcite in the 15% salt containing precipitation medium. Lots of research have shown that an increase in salinity decreases calcite precipitation. In the medium of halophilic bacteria *Exiguobacterium mexicanum*, calcium carbonate precipitation decreased significantly by 10% salt stress compared with 3.6–7.5% salt stress (Bansal et al. 2016). All strains in this study produced calcite in salt free precipitation medium, but by increasing the salt concentration the amount of deposited calcite decreased and then disappeared in some strains (e.g. H1, H4, and H5).

The SUD profile in Fig. 2 suggests that urease activity behavior of each strain is specific to itself and there is a specific point for optimum activity. H3 strain produces calcite precipitation in 5% salt containing medium more than in 15% salt containing and salt-free precipitation media. Ferrer et al. (1988b) showed that optimal calcium carbonate crystal formation occurs at a salt concentration of 2.5% in *Deleya*

halophila. In another study by Del Moral et al. (1987), calcite precipitation was tested with various species of moderately halophilic bacteria and it was found that these bacteria produced calcite and vaterite at 2% total salt, pure calcite at 10% salt, and mainly aragonite at 20% total salt.

The very good production of calcite by isolate 3 in the 5% salt containing medium suggested that this strain may be a good industrial strain for cementation of saline soils. The most notable result of the study is application of halophilic urease producing bacteria in cementation of saline soil. This study demonstrated there are a lot of bacteria in saline soil that can cause precipitation in biocementation with stimulation. Use of indigenous halophilic bacteria of salty soil is a new important method for cementation of salty soil.

The presence of other ions, such as magnesium, could interfere with calcite formation (Sánchez-Román et al. 2007). High concentration of magnesium ion could also result in deposition of magnesium carbonate trihydrate ($MgCO_3 \cdot 3H_2O$) besides the formation of calcite in MICP process (Cheng et al. 2014). In this study, like those conducted by Sánchez-Román et al. (2007) and Cheng et al. (2014), in addition to calcite, other crystals such as halite, sal ammoniac, and hydroxyapatite were deposited. In the present study, the XRD analysis showed the presence of calcite in culture media, and EDX analysis demonstrated that chemical composition of materials would be $CaCO_3$. In studies by Bansal, SEM analysis of the mortar specimens subjected to bacterial treatment revealed the presence of dense matrix of crystals with visible rod shaped bacterial cells in close association with crystals (Bansal et al. 2016). A similar arrangement was seen in H4 strain between nanocrystals and bacteria. The reason for this phenomenon is that bacteria act as nucleation sites in calcite precipitation.

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