Calcite dissolution by *Bacillus subtilis* SSRC102: An *in vitro* study for the reclamation of calcareous saline-sodic soils

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Dissolution of calcite by microorganisms to supply Ca²⁺ to replace Na⁺ in soil exchange sites is an important trait to reduce salinity and sodicity. An attempt was made to isolate and screen calcite dissolving bacteria for reclamation of calcareous saline-sodic soils and also to promote better crop growth. While screening the isolates for calcite solubilization index (0.37 to 2.62) and titratable acidity (0.04 to 0.25 g.l⁻¹), the isolate SSRC102 possessing higher dissolution was identified as *Bacillus subtilis*. Acetic and gluconic acid produced by *B. subtilis* SSRC102 in the presence of CaCO₃ recorded 20% of calcite dissolution with release of sufficient Ca²⁺ ions. Further, FT-IR spectra confirmed reduction of native calcite (69.1 to 62.5) suggesting their dissolution. Siderophore and extracellular polysaccharide productions might also aid in calcite dissolution and plant growth promotion as evidenced by indole acetic acid production, P and Zn solubilization.

**Keywords**: Calcite dissolution, *Bacillus* sp., calcareous saline-sodic soils, plant-soil health

**Introduction**

Soil degradation due to salinization and sodification are the major concerns of irrigated agriculture in arid and semi-arid regions of the world¹,² which are caused by low rainfall, low quality of irrigation water and high evaporations. Consequently, it decreases water uptake by plants, emergence of seedlings, penetration of root and causes imbalance in plant nutrient supply that ultimately affects their growth³. Saline-sodic soils contain high concentrations of undesirable salts of Na⁺ and carbonates which are toxic to plants by increasing the solute suction, reducing the availability of soil water to plants and also reduce the soil quality and productivity thus creating greater yield reduction in many crops⁴. Saline-sodic soils possess an EC of > 4 dS.m⁻¹, ESP of > 15% and pH of < 8.5⁵ and commonly calcareous in nature which contains calcite within the soil profile as poorly soluble form. The application of chemical amendments such as gypsum, elemental sulfur²,⁶ as well as phytoremediation using kallar grass, cotton⁷,⁸ has been used to alleviate the salinity and sodicity problems. However, some limitations encountered with these methods are high cost of chemicals, selection of suitable plants, time consumption⁹. Hence, microbial mediated calcite dissolution is recently gaining attention.

Most of the calcite dissolution (CD) research has been performed in detail without microorganisms¹⁰-¹². Microbe mediated calcite dissolution¹³-¹⁶ can be achieved by various mechanisms such as acidification by the production of organic acids¹⁷-¹⁹, inorganic acids²⁰, chelating substances²¹,²² and extracellular polysaccharide (EPS)²³. Among them, organic acids production seems to be predominant and effective way for calcite dissolution²⁴,²⁵. The present investigation was aimed at the isolation, screening and characterization of calcite dissolving bacteria (CDB) and understands the mechanism of dissolution.

**Materials and methods**

**Soil sampling and enrichment**

Calcareous saline-sodic soils were collected from three different areas (SSRA, SSRB and SSRC) of Ramnad district (Altitude of 2 m, 9.3 °N latitude and 78.8 °E longitude) in Tamil Nadu (India) based on their pH and EC levels. The collected initial soils were named as SSRAI, SSRBI and SSRCI and used as such for isolation. Enrichment was also made with 1% CaCO₃ to 100g of each soil and incubated for
15 days and those enriched soils were designated as SSRAE, SSRBE and SSRCCE, respectively. All the samples were stored at 4 °C until further analyses. The pH and EC were estimated from both the initial and enriched soils which showed initial values of 8.3, 8.2, 8.5 and 12.3, 6.0, 5.3 dS.m⁻¹ for SSRA, SSRB and SSRC, respectively.

**Isolation and Identification of calcite dissolving bacteria**

The CDB were isolated from both initial and enriched soils by serial dilution and plating technique using Devenze-Bruni (DB) agar medium. The positive isolates were selected based on clear zone formation around the colony and their solubilization index (SI) of the individual isolates. Total genomic DNA from the isolates was extracted and purified. The 16S rRNA gene sequence was amplified using universal primers, 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGGTACGACTT 3') in a thermo cycler (BioRad, USA) and amplified PCR products were sequenced (Bioserve Biotechnologies (I) Pvt. Ltd., Hyderabad, India). The sequences were aligned and compared with available sequences of bacterial lineage in the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST (Basic Local Alignment Search Tool). A phylogenetic tree was constructed using MEGA 6 program and their grouping of sequence was based on confidence values obtained by bootstrap analysis of 1000 replicates.

**Titratable acidity (TA)**

Fifteen selected isolates were estimated for TA by inoculating them onto DB liquid medium and incubated for 24 h at 30 °C under shaking condition at 120 rpm. After incubation, one ml of the cell free culture supernatant was titrated against 10 mM NaOH in the presence of phenolphthalein indicator until the appearance of pink colour.

**Calcite dissolution**

*B. subtilis* SSRCI02 that had highest TA was selected for further studies on calcite dissolution. The quantification of calcite dissolution was done using cell free culture supernatant of *B. subtilis* SSRCI02 grown in DB medium and was subjected to the analysis of calcium, carbonates, bicarbonates, pH and TA analysis at periodical intervals till 5 days of incubation.

The organic acid profile of 24 h old *B. subtilis* SSRCI02 grown in the presence of CaCO₃ was measured by injecting 30 μl of 0.2 μm filtered cell free supernatant in High Performance Liquid Chromatography (HPLC) with a UV detector at 210 nm. The organic separation was carried out on Cosmosil packed column (Nacalai Tesque, Japan) using 10.8% Acetonitrile in 0.0035 M H₂SO₄ as mobile phase with 0.6 ml.min⁻¹ flow rate. The data integration and analysis was done using Autochrom software. HPLC grade organic acids (No. 47264 from Sigma Aldrich, USA) were used as standards. Further, FT-IR spectra of the same sample analyzed in JASCO FT-IR 6800 fitted with diamond enabled Attenuated Total Reflectance (ATR) sample holder and DLaTgs detector with a wavelength range of 400-4000 cm⁻¹. Spectral measurements were done in triplicates and 64 scans were recorded for all samples at a 4 cm⁻¹ resolution.

**Siderophore, EPS production and biofilm formation**

The culture supernatant of *B. subtilis* SSRCI02 was used for determination of per cent siderophore units, EPS production and biofilm formation.

**Plant growth promoting activity of *B. subtilis* SSRCI02**

Cell free culture supernatant of 48 h old *B. subtilis* SSRCI02 (0.5 ml) grown in DB medium was quantified for IAA. The phosphorous and zinc solubilization by *B. subtilis* SSRCI02 was also determined by point inoculation onto Sperber’s hydroxyl apatite and Bunt and Rovira medium, respectively. Antagonistic effect of *B. subtilis* SSRCI02 was tested against two phytopathogens viz., *Rhizoctonia solani* and *Macrophomina phaseolina* by employing dual culture technique and their per cent inhibition was determined.

**Statistical analysis**

All the data were subjected to statistical analysis with soft-ware, Microsoft Excel for Windows 2007 add-in with XLSTAT version 2010.5.05 (XLSTAT, 2010). Statistically significant differences were analyzed using analysis of variance (ANOVA) at 5% significance level.

**Results**

**Soil sampling and enrichment**

The soil samples collected from three different regions of Ramnad showed an increase in both pH and EC due to enrichment. The pH and EC of the enriched soils were 8.47, 8.39, 8.65 and 12.6, 6.2, 5.5 dS.m⁻¹ for SSRA, SSRB and SSRC, respectively. Overall, the pH and EC was increased from 1.7 to 2.3% and 2.7 to 3.5% respectively. The soil microbial populations of enriched soils were also increased due to CaCO₃ enrichment (data not given).
**Isolation, screening and identification**

A clear halo zone around the colony was considered as screening criteria for selection of positive CDB isolates (Fig. 1). The CDB population was significantly influenced by CaCO$_3$ enrichment in the soil as observed with an increased population of 7.42, 7.39 and 7.16 from 7.18, 6.95 and 7.14 log$_{10}$cfu.g$^{-1}$ soils in SSRA, SSRB and SSRC samples, respectively (Table 1).

A total of thirty eight isolates (17 from initial and 21 from enriched soils) were obtained from all the soil samples. These isolates were further subjected to plate screening for calcite dissolution based on SI which ranged from 0.37 to 2.62 (Fig. 2). The highest SI was observed in SSRCE25 with 2.62 followed by SSRAI21 and SSRCI03 (2.50). Of the 38 isolates, the 15 selected isolates had the highest calcite SI of more than 1.5 and were identified based on 16S rRNA gene sequence. Except one isolate, SSRCE29, identified as *Staphylococcus arlettae*, all others were close to the genus, *Bacillus*. The identity, per cent similarity and submitted Genbank Accession number are presented in table 2.

**Titratable acidity**

The titratable acidity of selected isolates ranged from 0.04 to 0.25 g.l$^{-1}$ (Fig. 3) with the highest TA in SSRCI02 (0.25 g.l$^{-1}$) followed by SSRBE29 (0.23 g.l$^{-1}$) and the least TA was found in SSRAE42 and SSRCE29 (0.04 g.l$^{-1}$) and is on par with each other. The isolate SSRCI02 identified as *B. subtilis* with highest TA was selected for further studies.

**Calcite dissolution**

The calcite dissolution behavior of *B. subtilis* SSRCI02 showed an initial linear decrease in pH (8.02) till 4$^{th}$ day (5.55), later increased to 6.36 on 5$^{th}$ day. In contrast, TA showed an increasing trend until 4$^{th}$ day (0.73 to 1.99 g.l$^{-1}$) and decreased on 5$^{th}$ day (1.23 g.l$^{-1}$). The calcium carbonate content of the medium was slowly decreased from 5.0 to 4.03 g.l$^{-1}$ with simultaneous increase in calcium and bicarbonate contents in the spent medium. The Ca$^{2+}$ content increased linearly and reached maximum at 5$^{th}$ day with 4.03 g.l$^{-1}$. Whereas, the maximum bicarbonate content was observed at 4$^{th}$ day (0.71 g.l$^{-1}$) and decreased later. From this experiment, 20% of calcite dissolution by *B. subtilis* SSRCI02 was recorded (Fig. 4).

The isolate *B. subtilis* SSRCI02 produced organic acids such as gluconic acid, lactic acid, acetic acid, fumaric acid, propionic acid and phytic acid.

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**Table 1 — Effect of CaCO$_3$ on CDB population (log$_{10}$cfu.g$^{-1}$ soil) in calcareous saline-sodic soils.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Initial</th>
<th>Enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSRA</td>
<td>7.18$^{\pm 0.03}$</td>
<td>7.42$^{\pm 0.02}$</td>
</tr>
<tr>
<td>SSRB</td>
<td>6.95$^{\pm 0.05}$</td>
<td>7.39$^{\pm 0.04}$</td>
</tr>
<tr>
<td>SSRC</td>
<td>7.14$^{\pm 0.09}$</td>
<td>7.16$^{\pm 0.01}$</td>
</tr>
</tbody>
</table>

Values are mean $\pm$ SE (n=3) and within each row, values followed by same letters are not significantly different from each other according to DMRT (p≤0.05).
Among them, the predominant organic acid was acetic acid (931.6 µg.ml⁻¹) followed by gluconic acid (896.1 µg.ml⁻¹). Other acids were at lower levels; propionic acid (28.3 µg.ml⁻¹) and lactic acid (10.4 µg.ml⁻¹). In addition, minimal amount of phytic acid (10 µg.ml⁻¹) and fumaric acid (0.27 µg.ml⁻¹) were also recorded when B. subtilis SSRCI02 was supplemented with CaCO₃ (Fig. 5). On the other hand, the medium without CaCO₃ did not produce acetic acid, gluconic acid, propionic acid and lactic acid whereas minimal amount of formic acid (75.2 µg.ml⁻¹) and fumaric acid (0.09 µg.ml⁻¹) were observed (data not given).

The culture supernatant of B. subtilis SSRCI02 analyzed by ATR-FT-IR, the spectral data showed the changes in vibration and alteration of structure with reduced intensity (69.1 to 62.5%) of CaCO₃ at 1636 cm⁻¹. Further, the presence of additional new peaks at wave number of 1038 and 1301 cm⁻¹ with strong C-O and medium OH groups was observed in treated sample which were not present in the sample un-amended with CaCO₃ (Table 3 and Fig. 6).

Siderophore, EPS and biofilm formation

The siderophore and EPS production of B. subtilis SSRCI02 was higher in CaCO₃ amended medium with 93.1% and 13.1 µg.ml⁻¹, respectively compared to

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Table 2 — Identification of the CDB isolates by 16S rRNA analysis.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Isolates</th>
<th>Closely related to and their Accession No.</th>
<th>Identity</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SSRAI08</td>
<td>Bacillus safensis (NR_041794.1)</td>
<td>99%</td>
<td>KX673631</td>
</tr>
<tr>
<td>2</td>
<td>SSRAI21</td>
<td>Bacillus mojavensis (NR_118290.1)</td>
<td>99%</td>
<td>KX673632</td>
</tr>
<tr>
<td>3</td>
<td>SSRAE36</td>
<td>Bacillus methylotrophicus (NR_116240.1)</td>
<td>99%</td>
<td>KX673633</td>
</tr>
<tr>
<td>4</td>
<td>SSRAE37</td>
<td>Bacillus aryabhattai (NR_115953.1)</td>
<td>99%</td>
<td>KX673634</td>
</tr>
<tr>
<td>5</td>
<td>SSRAE40</td>
<td>Bacillus methylotrophicus (NR_116240.1)</td>
<td>99%</td>
<td>KX673635</td>
</tr>
<tr>
<td>6</td>
<td>SSRAE41</td>
<td>Bacillus tequilensis (NR_104919.1)</td>
<td>99%</td>
<td>KX673636</td>
</tr>
<tr>
<td>7</td>
<td>SSRAE42</td>
<td>Bacillus aryabhattai (NR_115953.1)</td>
<td>99%</td>
<td>KX673637</td>
</tr>
<tr>
<td>8</td>
<td>SSRBE23</td>
<td>Bacillus subtilis (NR_118486.1)</td>
<td>100%</td>
<td>KX673638</td>
</tr>
<tr>
<td>9</td>
<td>SSRBE29</td>
<td>Bacillus licheniformis (NR_118996.1)</td>
<td>98%</td>
<td>KX673639</td>
</tr>
<tr>
<td>10</td>
<td>SSRCI02</td>
<td>Bacillus subtilis (NR_112686.1)</td>
<td>100%</td>
<td>KX602660</td>
</tr>
<tr>
<td>11</td>
<td>SSRCI03</td>
<td>Bacillus pumilis (NR_074977.1)</td>
<td>100%</td>
<td>KX673640</td>
</tr>
<tr>
<td>12</td>
<td>SSRCE25</td>
<td>Bacillus tequilensis (NR_104919.1)</td>
<td>99%</td>
<td>KX673641</td>
</tr>
<tr>
<td>13</td>
<td>SSRCE28</td>
<td>Bacillus tequilensis (NR_104919.1)</td>
<td>98%</td>
<td>KX673977</td>
</tr>
<tr>
<td>14</td>
<td>SSRCE29</td>
<td>Staphylococcus arlettae (NR_024664.1)</td>
<td>98%</td>
<td>KX673978</td>
</tr>
<tr>
<td>15</td>
<td>SSRCE30</td>
<td>Bacillus amyloliquefaciens (NR_075005.1)</td>
<td>97%</td>
<td>KX673642</td>
</tr>
</tbody>
</table>
control (90.1% and 4.3 µg.ml\(^{-1}\)). Similarly, biofilm formation of the isolate was higher in medium supplemented with CaCO\(_3\) registering 0.2 OD\(_{600nm}\) than control (0.1 OD\(_{600nm}\)).

**Plant growth promoting activity**

The IAA production by \textit{B. subtilis} SSRCI02 was 16.3 µg.ml\(^{-1}\) while the phosphorous and zinc solubilization was recorded with a solubilization index of 2.67 and 1.33, respectively. The isolate also possess the ability to inhibit two phyto-pathogens such as \textit{Rhizoctonia} sp. and \textit{Macrophomina} sp. with 33.8 and 27.5% inhibition, respectively.

**Discussion**

Soil salinity and sodicity are the major constrains for agricultural productivity especially in arid and semi-arid regions due to less rainfall, faulty irrigation or poor drainage practices which leads to soil degradation\(^{5,6,43,44}\). Replacement of accumulated Na\(^+\) ions in the soil exchange sites with Ca\(^{2+}\) ions in the saline-sodic soils can alleviate this problem. Many of the saline-sodic soils were calcareous in nature; however, calcium as calcite is present in poorly soluble or precipitated form. Hence for dissolution, chemicals amendments and plant-mediated processes has been widely used. Nevertheless, there are very limited studies on microbial mediated dissolution of calcite in saline-sodic soils. In the present study, focus has been given to develop a bioinoculant for reclamation of calcareous saline-sodic soils. To accomplish the task specific enrichment were made with these soils to obtain an efficient calcite dissolving bacteria for calcareous saline-sodic soils and their probable mechanisms for calcite dissolution were assessed in this study.

The increase in soil pH, EC and microbial population observed in enriched saline-sodic soils might be due to the addition of CaCO\(_3\). Preliminary screening of these bacterial isolates for efficient calcite dissolution based on solubilization index is an important criterion used in many studies\(^{45-47}\) and the SI ranged from 0.37 to 2.62. Furthermore, in the present investigation, TA was considered as critical factor in selecting best isolates, as TA had direct relationship with the mineral solubility\(^{31,48}\). From the result, the highest TA producing \textit{B. subtilis} SSRCI02 (0.25 g.l\(^{-1}\)) was further analyzed for calcite dissolution under \textit{in vitro} condition.

Calcite dissolution by \textit{B. subtilis} SSRCI02 revealed that a reduction in pH and an increase in TA have well correlated with the calcite solubility. The solubility of minerals through TA has already been reported that the maximum solubilization occurred under strong acidic conditions\(^{49}\). The reduction in calcium carbonate content is the main phenomena for the dissolution of calcite to release the calcium ions. In the present study also, the CaCO\(_3\) content decreased with the release of calcium ions as well as bicarbonates from day one suggesting the dissolution starts at the beginning the culture growth.

The enzymatic activities such as carbonic anhydrase and phosphatase help the multicellular organisms for the attachment, penetration and dissolution of calcareous substrates\(^{40}\). In the present CD experiment, the highest phosphatase activity (93.4 U.ml\(^{-1}\)) observed on 4\(^{th}\) day of incubation with acidic pH (5.5) and TA (1.99 g.l\(^{-1}\)) elucidates the role of these enzymes on calcite dissolution. Further, increase in calcium carbonate at the end of incubation might be due to precipitation\(^{51}\). Acidolysis is the main mechanism for mineral weathering by means of organic acids production such as acetic, gluconic, formic, oxalic as well as inorganic acids such as nitric, sulphuric acid. The dissolution of calcite is regulated by the production of organic acids\(^{52}\). In the present study, the isolate \textit{B. subtilis} SSRCI02 produced different organic acids and among them; acetic acid and gluconic acid were predominant. FT-IR results confirmed the CD as the intensity of calcite reduced from 69.1 to 62.5% by \textit{B. subtilis} SSRCI02. In addition, the presence of additional peaks with strong C-O and medium OH groups might be attributed to the presence of organic acids as evidenced by HPLC chromatogram (Fig. 5).

### Table 3 — FT-IR spectrum of \textit{B. subtilis} SSRCI02.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Functional group</th>
<th>Bond</th>
<th>Intensity</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated(^{d})</td>
<td>1636</td>
<td>Alkene</td>
<td>C=C</td>
<td>Variable</td>
<td>Stretching (non conjugated C=C)</td>
</tr>
<tr>
<td>Treated(^{d})</td>
<td>1636</td>
<td>Alkene</td>
<td>C=C</td>
<td>Variable</td>
<td>Stretching (non conjugated C=C)</td>
</tr>
<tr>
<td></td>
<td>1038</td>
<td>Alcohols</td>
<td>C-O</td>
<td>Strong</td>
<td>Stretching</td>
</tr>
<tr>
<td></td>
<td>1301</td>
<td>OH</td>
<td>Medium</td>
<td>Deformation</td>
<td></td>
</tr>
</tbody>
</table>

FT-IR spectrum of treated\(^{d}\) (\textit{B. subtilis} SSRCI02) and untreated\(^{d}\) (Control) samples showed the changes in their intensity at the wavenumber 1038 and 1301. These peaks were not observed in control sample.
The production of siderophore and EPS as well as biofilm formation are considered as mechanisms for the mineral dissolution and attachment of bacteria on the mineral surface. While, the isolate B. subtilis SSRCI02 recorded higher siderophore, EPS and biofilm in calcite amended condition than the unamended control which signifies their role in calcite dissolution. This is the first preliminary findings that showed the role of siderophore in calcite dissolution.

Besides calcite dissolution, B. subtilis SSRCI02 showed a plant growth promoting activity like IAA production which helps crop growth. P and Zn solubilization helps the plant uptake nutrients from soil. Additionally, pathogen suppression ability of B. subtilis SSRCI02 might help plants to be healthy with yield enhancement. Hence, calcite dissolution, plant growth promotion, phytopathogen inhibition of B. subtilis SSRCI02 makes this as a potential bioinoculant to remediate calcareous saline-sodic soils.

Conclusion

The present study identified a calcite dissolving bacterium, B. subtilis SSRCI02 with a potential to dissolve 20% calcite by releasing sufficient Ca$^{2+}$ ions and the possible mechanisms of CD might be by secretion of organic acids, siderophore, EPS with biofilm formation. Further, plant growth promoting and antagonistic ability of B. subtilis SSRCI02 are complimentary advantages in effective reclamation of calcareous-saline-sodic soils.

Acknowledgement

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