INTRODUCTION

Iron (Fe) is the fourth most abundant element in the earth’s crust [34] and it has been recognized as a physiological requirement for all types of life. It has a central role in some essential biochemical reactions like electron transport during respiration and photosynthesis [27]. It is involved in the synthesis of chlorophylls, maintenance of chloroplast structure and its function. The change of iron valence from Fe(III) to Fe(II) is an essential characteristic for cellular redox reactions [27]. Despite the high abundance of iron in many soils, its bioavailability is restricted to soils with aerobic and neutral pH environments. Iron is predominantly found in the ferric form of Fe bearing minerals with limited solubility in aerobic conditions, thus cannot provide sufficient available iron for plants [22, 28, 29]. Iron deficiency damages chlorophyll biosynthesis and affects chloroplast development in plants [9]. Therefore, iron availability is directly involved in plant productivity. Chlorosis due to the lack of iron availability in calcareous soils with high pH is a major agricultural problem that results in reduced crop yields in almost 30% of cultivated soils worldwide [9]. Plants exhibit two distinct strategies to acquire iron. In strategy I in nongraminaceous plants [11, 27], iron solubility increases by secretion of protons that induces Fe(III)–chelate reductase [27]. The generated Fe(II) is then transported into the plant by the Fe-regulated transporter [9, 27]. Strategy II is based on the excretion of low molecular mass secondary amino acids known as ‘phytosiderophores’ which chelates scanty soluble iron [6]. Siderophores also are produced by a wide range of soil microorganisms [5] and hence they improve iron supply for plants growth [27]. Furthermore, in anaerobic conditions and in the absence of other terminal electron acceptors, some bacterial species such as Geobacter sp and Shewanella sp can couple oxidation of organic matter to ferric iron reduction, via a biological process termed dissimilatory metal reduction (DMR) [23, 33]. A variety of Fe(III)—reducing organisms have been isolated and described from a broad diversity of environments [19, 32]. These bacteria have been observed at up to 10^5 cells per gram of agricultural soils [12]. In calcareous soils, iron predominant form is strongly insoluble at neutral and basic pHs and bacterial strains with high iron reduction capacity could amend Fe(II) availability for plants [27]. Dong et al. [7], Vodyanitskii [29] and

Isolation and Identification of Ferric Reducing Bacteria and Evaluation of their Roles in Iron Availability in Two Calcareous Soils

N. Ghorbanzadeh, A. Lakzian, G. H. Haghnia, and A. R. Karimi

Agricultural College, Ferdowsi University of Mashhad, Iran., Department of Soil Science
e-mail: nasrin_gh908@yahoo.com

Received October 18, 2013

Abstract—Iron is an essential element for all organisms which plays a crucial role in important biochemical processes such as respiration and photosynthesis. Iron deficiency seems to be an important problem in many calcareous soils. Biological dissimilatory Fe(III) reduction increases iron availability through reduction of Fe(III) to Fe(II). The aim of this study was to isolate, identify and evaluate some bacterial isolates for their abilities to reduce Fe(III) in two calcareous soils. Three bacterial isolates were selected and identified from paddy soils by using 16S rRNA amplification and then inoculated to sterilized and non-sterilized calcareous soils in the presence and absence of glucose. The results showed that all isolates belonged to Bacillus genus and were capable of reducing Fe(III) to Fe(II) in vitro condition. The amount of Fe(III) reduction in sterilized calcareous soils was significantly higher when inoculated with PS23 isolate and Shewanella putrefaciens (S. putrefaciens) (as positive control) compared to PS16 and PS11 isolates. No significant difference was observed between PS11 and PS16 isolates in the presence of indigenous microbial community. The results also revealed that glucose had a significant effect on Fe(III) reduction in the examined calcareous soil samples. The amount of Fe(III) reduction increased two-fold when soil samples were treated with glucose and inoculated by S. putrefaciens and PS23 in non-sterilized soils.

Keywords: Bioreduction, Bacillus sp, Ferric iron, Paddy soils, Shewanella putrefaciens

DOI: 10.1134/S1064229314120059
and syringe and then sealed with thick butyl rubber
lamine-reducible ferric iron (Fe³⁺–HR) were mea-
surements were then centrifuged at 700 rpm for 10 min and
the Fe(III) reducing bacterial community. Suspen-
sions used to prepare dilution series. Nutrient
agar plates were inoculated by 100
supernatants used to prepare dilution series. Nutrient
and temperature of the sampling sites are 1186 mm
15 cm of two different paddy Inceptisol soils in Guilan
province, northern Iran. Mean annual precipitation
in two calcareous soils collected from Khorasan prov-
ince, eastern Iran. Mean annual precipitation and
in a 2% agarose gel containing 0.5
72°C for 2 min. The final extension was carried out at
72°C for 5 min. The reaction products were separated
and visualized under UV light.

Sokolova [24] have reported the reduction of struc-
tural ferric iron in soil components such as clay miner-
als and oxyhydroxides, but there are few studies
regarding soil indigenous bacteria with iron reduction
ability. Valencia-Cantero et al. [27] reported that
rhizospheric bacterial populations could reduce hydroxylamine-reducible Fe(III) present in alkaline
soils. The current study was primarily designed to iso-
late and identify some iron reducing bacteria to eval-
uate the Fe(III) reduction ability of selected isolates in
two iron deficient calcareous soils.

**MATERIALS AND METHODS**

**Soil sampling.** Soil samples were collected from 0–
15 cm of two different paddy Inceptisol soils in Guilan
province, northern Iran. Mean annual precipitation
and temperature of the sampling sites are 1186 mm
and 17.5°C respectively. Paddy soil samples were used
just for isolation of Fe(III) reducing bacteria. The
Fe(III) reduction ability of bacterial isolates was tested
in two calcareous soils collected from Khorasan prov-
ince, eastern Iran. Mean annual precipitation and
temperature of the sampling sites are 259.7 mm and
13.7°C respectively. Table 1 shows some chemical and
physical properties of the two calcareous soils.
DTPA–extractable iron (Fe–DTPA) and Hydroxy-
lamine-reducible ferric iron (Fe³⁺–HR) were mea-
sured in two calcareous soils by the methods of [14]
and [16] respectively.

**Bacterial isolation.** Soil suspensions (1 : 10) (W/V)
in sterile water was prepared and shaken in incubator-
shaker at 120 rpm in dark at 25°C for a week to activate
the Fe(III) reducing bacterial community. Suspens-
sions were then centrifuged at 700 rpm for 10 min and
supernatants used to prepare dilution series. Nutrient
agar plates were inoculated by 100 µL from some dilu-
tions (10⁻³ to 10⁻⁹) and incubated in dark at 25°C for
a week. Fifty different bacterial colonies were excised
randomly based on their morphology and purified and
then stored at –4°C.

**Screening procedure.** Five mL of nutrient broth
supplied with 20 mM HEPES, 0.1 mM Ferrozine and
400 µM Ferric citrate. Nutrient broth poured to the
oven dried serum bottles which previously urged with
N₂ for 5 min (to remove O₂) by using a sterile needle
and syringe and then sealed with thick butyl rubber
stoppers. The bottles were inoculated with 100 µL of
each bacterial isolates suspension (10⁸ cells mL⁻¹) and
incubated for 3 days at 30°C in darkness. Fe(III)
reduction was measured spectrophotometrically and
the absorbance determined at wavelength of 562 nm.

**DNA extraction and PCR amplification.** DNA of
three selected bacterial isolates with high Fe(III)
reduction ability was extracted using a simple and
rapid method for DNA preparation as described by
Chen and Kuo [3] 16S rRNA gene of each bacterial
isolate was amplified using two fd1 (5’-AGAGTT
TGATCTTGCTGTC-3’) and rd1 (5’-AAGGAG
GTGATCCAGGC-3’) primers as described previ-
ously [2, 3]. The standard 25-µL PCR reaction mix-
tones contained 100 mM dNTPs, 10 mM MgCl₂,
15 mM each primer, 0.2U Taq DNA polymerase and
1 ng template bacterial DNA. Negative controls con-
tained all components for the PCR except the tem-
plate DNA. The reactions were run on a DNA thermal
cycler for a 35–cycle amplification series. After initial
denaturation of the reaction mixture at 95°C for
3 min, each cycle included denaturation at 95°C for
1 min, annealing at 63°C for 1 min, and extension at
72°C for 2 min. The final extension was carried out at
72°C for 5 min. The reaction products were separated
in a 2% agarose gel containing 0.5 µg of ethidium bro-
mide per mL and visualized under UV light.

**Sequencing and phylogenetic analyses.** Selected rep-
resentative PCR amplicons were subjected to cloning
and sequencing by Takapoozis Company
(http://www.takapouzist.com). Amplified sequences
were deposited in the NCBI Gen Bank and compared
with sequences from data base (www.ncbi.nlm.nih.gov/
blast) using the blast algorithm.

**Fe (III) reducing bacteria selection.** *S. putrefaciens*
is a well-characterized, facultative anaerobic bacte-
rium with a demonstrated ability to couple the oxida-
tion of organic matters under anoxic conditions [7].
This bacterium was provided from Microbial Bank of
Iran (Central Collection of Industrial Fungi and Bacte-
teria, Iran- Karaj). *S. putrefaciens* was routinely cul-
tured aerobically in tryptic soy broth (30 g L⁻¹) from
the stock culture, which was kept in 40% glycerol at
–80°C. PS23, PS16 and PS11 isolated from paddy
soils were selected for Fe(III) reduction experiment.

**Bacterial culture.** *S. putrefaciens* and three *Bacillus*
isolates was grown aerobically in tryptic soy broth

<table>
<thead>
<tr>
<th>Soil characteristics</th>
<th>FC*</th>
<th>Silt</th>
<th>Clay</th>
<th>Sand</th>
<th>CCE</th>
<th>C-org</th>
<th>pH₅</th>
<th>Fe-DTPA, mg/Kg</th>
<th>Fe³⁺–HR**, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil A</td>
<td>14</td>
<td>40</td>
<td>17</td>
<td>42</td>
<td>16</td>
<td>0.77</td>
<td>7.64</td>
<td>1.7</td>
<td>27.11</td>
</tr>
<tr>
<td>Soil B</td>
<td>11.2</td>
<td>32</td>
<td>16</td>
<td>52</td>
<td>16</td>
<td>0.25</td>
<td>7.62</td>
<td>2.7</td>
<td>22.63</td>
</tr>
</tbody>
</table>

* — Field Capacity.
** — Hydroxylamine Reducible Fe(III).