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BIOEXTRACTION OF IRON THROUGH *Bacillus* sp. SP10 FROM MINERAL OXIDE AND ITS APPLICATION AS PLANT MICRONUTRIENT

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ABSTRACT

Iron is an essential micronutrient for all living organisms because of its critical role in metabolic processes. In plants it plays a key role in the synthesis of chlorophyll, some vital enzymatic and metabolic processese. However, most of the iron in nature is present as precipitates or in insoluble forms. Therefore, biological mechanism has been employed to mineralize Fe(III) oxides into Fe(II) through microbial action to increase the availability of soluble iron. An iron reducing bacterial strain was isolated based on its tolerant limits and iron mineralizing ability. The strain *Bacillus* sp. SP10 effectively mineralized iron in synthetic medium possessing 1% starch and pH 7. In a 25 day's column study, the soluble Fe(II) as micronutrient showed a gradual increase in each seepage collected from different treatment columns. Similarly, the plants grown on soil obtained from treatment D (i.e. **1% starch + 1% *Bacillus* sp. SP10 inoculum + 0.25% anthraquinone-2,6-disulphonate) column displayed good growth and maximum shoot length, mainly due to the increased accessibility of iron through microbial mineralization. The mineralization of Fe in soil was established through SEM and EDX analysis.**

Keywords: *Bacillus* sp., column study, ferric hydroxide, iron mineralization

INTRODUCTION

Soil is a basic environmental component that covers most land and provides a structural and nutritional support to the plants that help in the development and survival of human being. At the same time, soil is the most endangered part of our ecosystem which is prone to be contamination. Heavy metal contamination in soil is one of the major problems that causes toxic effects on human health (Verma and Kuila, 2019) and hampers plant growth. Some metals like nickel, copper, iron and arsenic are beneficial to human and plants but still its occurrence in high concentration is toxic (Valko *et al*., 2016). Human activities such as mining of ores, accumulation of pesticides, gas extraction and industrialization affect the natural ecosystem and require renovation techniques (Ahuti, 2015; Mgbemene *et al*., 2016). Exploitation of metal-based wastes through bioremediation is one of such renovation technique which uses indigenous microbes or microbes from different site (Santos *et al*., 2011). The use of indigenous microbes eliminates the need of monitoring (Asha and Sandeep, 2013).

The mechanisms of biological management include biomineralization, bio-transformation, bio-accumulation, biosorption, metal-microbe interactions and bioleaching (Verma and Kuila, 2019). These mechanisms use microorganisms to convert toxic heavy metals into non-toxic forms or convert insoluble heavy metals to soluble forms (Verma and Kuila, 2019). For the growth and development, various microbes utilize heavy metals as per their needs as essential micronutrient. For example, anaerobic bacteria use Fe^{2+} whereas most of the other bacteria utilize Fe^{3+} as a nutritional substrate

(Ahemad, 2014). A few microbes derive their energy by oxidizing some organic compounds with Fe(III) or Mn(IV) as electron acceptor (Lovley and Philips, 1988). Microbes are utilized according to their ability and mode of action towards heavy metals. Some microbes are used to mineralize organic pollutants into CO_2, H_2O or metabolic intermediates as end-products (Verma and Kuila, 2019). Many microbes significantly modify metal solubility so reduce their metal state (Lovley *et al*., 1991). Some heavy metals cannot be destroyed completely; instead organic metal complex are transformed into less toxic, water soluble and precipitated forms (Garbisu and Alkorta, 2001).

Iron is one of the essential micronutrient for all life forms (Rickard, 2012). Organisms develop different mechanisms to acquire this iron. After oxygen, silicon and aluminium, iron is considered to be the 4th most abundant element (Yi *et al*., 2012) and the second most available metal after aluminium (Pierre *et al*., 2002) It is an important micronutrient for plant. Although the availability of iron is abundant in soils, but due to its low solubility the plants suffer from iron deficiency which results in low yield and inferior quality of crops (Ma and Ling, 2009). Not only plants, but humans also suffer from iron deficiency. Inadequate iron in human causes anaemia and may interrupt in certain metabolic activities like oxygen transport, DNA synthesis, electron transport, etc. This scenario can be overcome by intake of iron-fortified foods (Man *et al*., 2021).

In prebiotic times, soluble ferrous iron concentrations were largely present but later due to the irreversible oxidation of ferrous iron and ferric iron precipitation in the form of oxides and hydroxides, the availability of iron became low (Pierre *et al*., 2002). Reduction or mineralization of iron is a key process in iron uptake. Reduction of Fe(III) oxide is largely controlled by microbial processes. Microbes reduce Fe(III) to Fe(II) and derive energy from this respiration to support its growth (Vargas *et al*., 1998). The solubility of iron is extremely low in calcareous soils, which worldwide cover about third of arable land (Romheld and Nikolic, 2006). Tt is necessary to increase the availability of iron for plants which, in turn, helps in improving yields and quality that consequently increases human nutrition and health (Nikolic, 2018). The present study was aimed to explore mineralization of iron as micronutrient from synthetic oxide and assess its effect on plant growth. Moreover, the optimum conditions under which maximum mineralization of Fe occur was explored.

MATERIALS AND METHODS

Sample collection and isolation of bacterial strains

The soil samples were collected at 10 cm depth from different areas pertaining to the Salem Steel Plant (11.6600°N, 78.0276°E) and Dalmia Magnesite Corporation (11.7193°N, 78.1056°E) situated in Salem district of Tamil Nadu, India. The samples were transferred to the bioremediation laboratory of Periyar University using sterile polythene bags. The pour plate technique was employed to enumerate and isolate bacterial colonies from the collected samples using nutrient agar medium. The plates were incubated at 35^{o}C for 24 h. Individual colonies were enumerated and isolated based on their morphological characteristics. The isolates were labelled and tentatively identified by Gram's staining, spore production, morpho-cultural characteristics and important biochemical tests (Buchanan and Gibbons, 1974).

Primary screening of Fe(III) mineralizing bacterial isolates

To assess the tolerance limit of isolates the nutrient agar medium was enriched with variable concentrations of Fe(III) (100 - 1000 ppm), prepared by using analytical reagent grade $FeCl_3$. The test isolates were spotted onto the nutrient agar plate and the isolates were observed for their growth and survival after 24 h incubation at 35^{o}C (Kanwal *et al*., 2004).

Hydrolysis of various carbon sources

Different carbon sources *viz*., starch, cellulose and glucose at 1% level were assessed for their hydrolysis by the efficient bacterial strains selected after primary screening to observe their tolerance

limits in higher concentrations of Fe(III) and its effective transformation. The tests performed included starch hydrolysis, cellulose hydrolysis and triple sugar ion fermentation using appropriate medium and standard methods as per Chakraborty *et al*. (2011) and Saryono *et al*. (2019).

Preparation of synthetic Fe(III) oxide

Equal volumes of 0.4 M ferric chloride and 1 M sodium hydroxide was thoroughly mixed and left as such for a few hours. Then the collected precipitate was washed with sterile distilled water, air-dried and powdered for further studies (Bonneville *et al*., 2004). A deep reddish-brown precipitate was obtained after the reaction of aqueous ferric chloride with aqueous sodium hydroxide. The precipitate in solution was amorphous Fe(III) oxide which was purely insoluble in water. This Fe(III) oxide was used as raw material and insoluble mineral.

Secondary screening for Fe-mineralizing bacteria

The five most efficient bacterial strains obtained from primary screening of Fe (III) and hydrolysis of carbon sources were chosen for secondary screening for mineralization of Fe(III) oxide. The bacterial inoculum at 1% was inoculated into 100 mL mineral salt medium (MSM) containing (L^{-1}) 1.17 g NaCl, 0.30 g KCl, 0.15 g HN_4Cl, 0.41 g $MgCl_2.6H_2O$, 0.11 g $CaCl_2$, 0.20 g KH_2PO_4, 0.07 g Na_2SO_4, 2 g $NaHCO_3$ and pH 8 ± 0.2. The MSM was also amended with 1% starch as carbon source and 0.25 g synthetic Fe(III) oxide and incubated for 24 h at 35^{o}C. Then 10 mL sample was taken and centrifuged at 4000 rpm for 10 min. The supernatant collected was estimated for Fe(II) content by 1,10-phenanthroline method using UV-Vis spectrophotometer (Model: Cyberlab UV100) at 510 nm (Sivakami *et al*., 2012). After every 24 h, the same method was followed for a period of 7 days and the results were plotted on a graph using the values estimated from the standard graph.

Effect of starch and pH on Fe mineralization

The most efficient bacterial strain exhibiting maximum potential to mineralize ferric (Fe^{3+}) into ferrous (Fe^{2+}) was selected. The strain was subjected to Fe mineralization study in 100 mL MSM supplemented with 0.25 g Fe(III) oxide and various concentrations of soluble starch (0.25, 0.5, 0.75, 1.0 and 1.25%). The inoculated medium was incubated under shaking condition at 35^{o}C. The samples were drawn aseptically after every 24 h and pellets separated by centrifugation at 4000 rpm for 10 min. From the cell free supernatant, the soluble Fe(II) was determined as micronutrient by 1,10-phenanthroline method (Harry Pyenson and Tracy, 1945). The study was conducted under constant shaking conditions for 7 days. Another study was carried out in MSM with variable pH levels (pH 5, 6, 7, 8 and 9) on Fe mineralization.

Biomineralization of iron in soil through column approach

The redox transformation of iron from contaminated soil was examined using four glass column setups (Plate 1). The columns used were glass of 35 cm length and 4 cm width. Prior to use, each column was sterilized 4 times by using absolute alcohol. One column was filled with garden soil without ferric oxide (as control treatment). The other three columns were filled with soil amended with synthetic Fe(III) oxide as un-mineralized element (Ayyasamy and Lee, 2009). Each column was tightly closed with holed caps at the top. The tube from the top of the column was inserted into collection vessel and the tube from the bottom was connected to the reservoir containing the respective synthetic medium supplemented with starch as a sole carbon source (Plate 1). The medium was drawn into the column using a set of peristaltic pump (Ravel, RH-P100S-100-2H). The column treatments comprised of A) MSM broth + sterile deionized water without carbon

Plate 1: Mineralization of iron through column approach

source as control, B) MSM broth + 1% starch + 1% inoculum (*Bacillus* sp. SP10), C) MSM broth + 1% starch + 1% inoculum + 0.25% humic acid, and D) MSM broth + 1% starch + 1% inoculum + 0.25% anthraquinone-2,6-disulphonate. The 200 mL medium prepared, as per treatment, was pumped into their respective columns. The samples were collected from the collection vessel of each column after each 24 h and the mineralization of iron estimated by using standard method (Harry Pyenson and Tracy, 1945). This study was carried out for a period of 25 days. The structure and presence of soluble Fe in most effective column was tested through scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) analysis.

Phytotoxicity assay of microbial mineralized soil in different columns

Fertile garden soil and microbially-mineralized soil obtained from different treated columns were mixed in variable ratios (0:1, 1:1, 1:2, 1:3 and 1:0). The mixed soils were taken in separate sterile cups. Fertile garden soil alone was maintained as control. Viable healthy seeds of green gram (*Vigna radiata*) were surface sterilized with 0.1% $HgCl_2$ solution for 2 min and washed with sterile distilled water thoroughly (Sun *et al*., 2020). Five seeds were sown in each cup and allowed to germinate. The moisture content was maintained by irrigating with tap water as and when required. The seed germination and growth parameters were assessed after every 24 h for 10 days. The average shoot length of plants in each cup was measured. After day 10, the plants were uprooted and washed thoroughly with distilled water and length of shoot and root measured.

Statistical analysis: All the above experiments were conducted in completely randomized design and each treatment was replicated three times. The data generated was analysed for mean, standard error and critical difference at 5% level of significance.

RESULTS AND DISCUSSION

Bacterial strains and screening

Based on the morphological and cultural characteristics 29 bacterial strains were isolated which fell under four major genera namely *Alcaligenes*, *Bacillus*, *Lactobacillus*, and Enterobacteriaceae group. *Bacillus* was the predominant group (Fig. 1). Among the 29 bacterial strains tested, only 11 showed survival up to 1000 ppm Fe(III) [Table 1]. The strains isolated were tested for their tolerance against Fe^{3+} In nutrient agar medium. Sivakami *et al*. (2012) also isolated similar bacterial genera from iron contaminated soil. Further from the tolerance study, hydrolysis of carbon source and screening of Fe(III) oxide mineralizing bacteria were carried out. In this study, only one bacterium namely *Bacillus* sp. strain SP10 showed potential efficiency in Fe(III) mineralization.

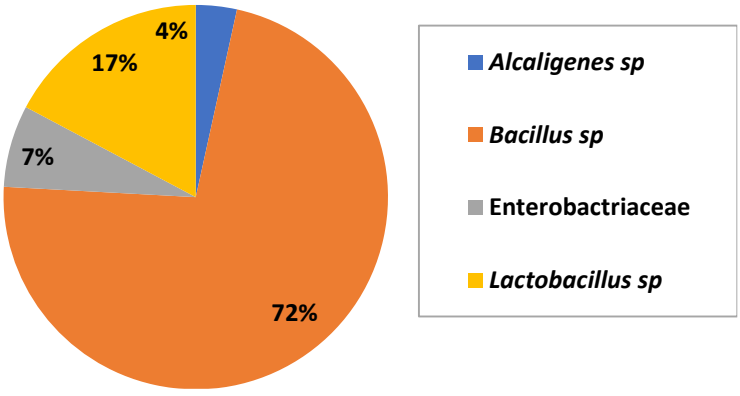

Fig. 1: The bacterial genera (%) isolated from metal rich sites

Hydrolysis of carbon sources

Among the various hydrolysis tests performed, maximum bacterial strains were able to use starch as their sole carbon source while only a few bacterial strains could ferment glucose and no strains were able to utilize cellulose (Table 1). *Bacillus* sp. strain SP10 showed maximum zone of hydrolysis (31 mm) in starch hydrolysis test. Hence, starch was selected as a suitable carbon source for further studies.

Table 1: Hydrolysis of carbon source by bacterial strains selected after primary screening

S. No.	Bacterial strains	Starch hydrolysis (mm)	Cellulose hydrolysis (mm)	Triple sugar iron (TSI) test			
				Slant pH	Medium bud reaction	Gas production	H_2S production
1	SP4	24	-	Alkaline	Acid	-	-
2	SP5	13	-	Alkaline	Alkaline	-	-
3	SP6	14	-	Acid	Acid	+	-
4	SP7	-	-	Acid	Acid	-	+
5	SP8	13	-	Alkaline	Alkaline	-	-
6	SP10	31	-	Alkaline	Acid	-	-
7	SP11	2	-	Alkaline	Alkaline	-	-
8	SP13	23	-	Alkaline	Alkaline	-	-
9	SP19	-	-	Alkaline	Acid	-	-
10	SP23	2	-	Alkaline	Alkaline	-	-
11	SP25	-	-	Alkaline	Acid	-	-

- indicate negative; + = positive

It has previously been reported that *Bacillus* sp. utilize glucose as their carbon source (Santos and Martins, 2003).

Mineralization of Fe(III) oxide

In secondary screening the bacterial strains that showed $\geq$ 20 mm diameter zone of clearance in starch hydrolysis test were selected for testing their ability to mineralize ferric oxide. Based on this parameter five *Bacillus* sp. strains namely SP4, SP10, SP11, SP13 and SP23 were selected. Among these strains, *Bacillus* sp. SP10 showed maximum conversion of ferric to ferrous in a period of 7 days (Fig. 2) and was selected as potential bacterial strain for further studies. All the tested bacterial strains showed resistance to iron (1000 mg L^{-1}) as well as hydrolysed carbon sources.

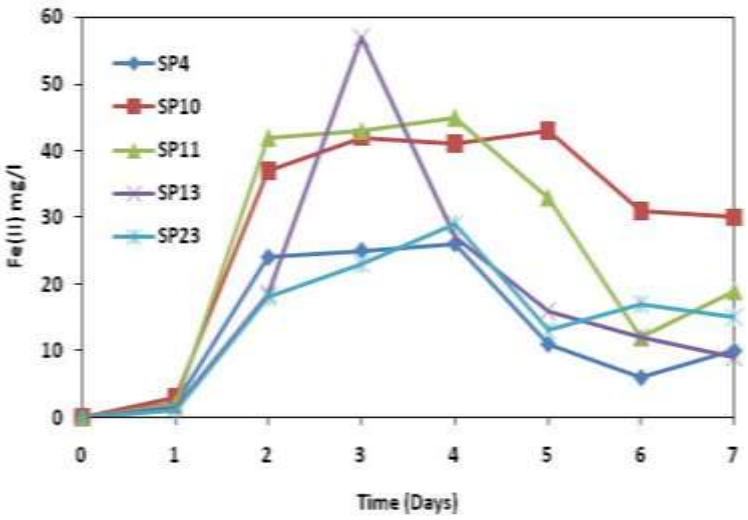

Fig. 2: Secondary screening of in selected *Bacillus* sp. strains for Fe mineralizing bacteria

The column study using synthetic soil containing ferric oxide revealed that the iron mineralization from its insoluble form to soluble form was maximum when the soil was supplemented with 1% carbon source, 1% *Bacillus* sp. inocumlum and 0.25% ADQS or humic acid. This is in agreement with the reports that the dissolution of iron is relatively higher in media supplemented with ADQS or humic acid as compared to the medium without supplementation (Ayyasamy *et al*., 2009; Ayyasamy and Lee, 2009; Ayyasamy and Lee, 2012). The presence of Fe in mineralized sample was tested with SEM and EDX. The image with aero mark showed Fe gathering from the synthetic oxide and the peak obtained through SEM and EDX confirmed that the presence of Fe in sample after mineralization in soil. EDX measurement revealed that the samples obtained from the synthetic oxide contained O and other trace elements. As depicted in the image, the Fe peak obtained through EDX analysis confirms that the presence of Fe in the sample after mineralization.

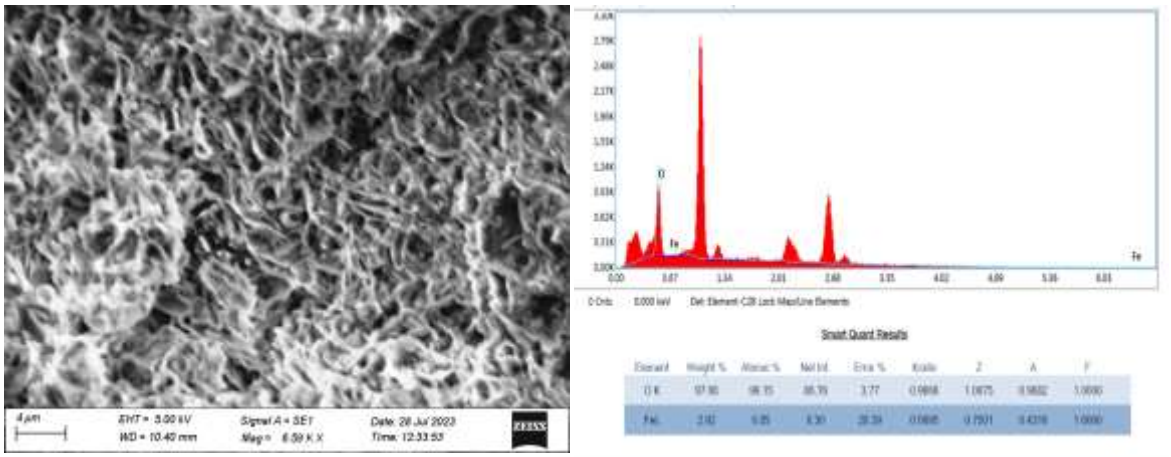

Plate 2: SEM and EDX analysis of Fe obtained from treatment column D; SEM image showing the surface structure of mineralized Fe, (left side); EDX analysis showing the presence of Fe in the treated sample (right side

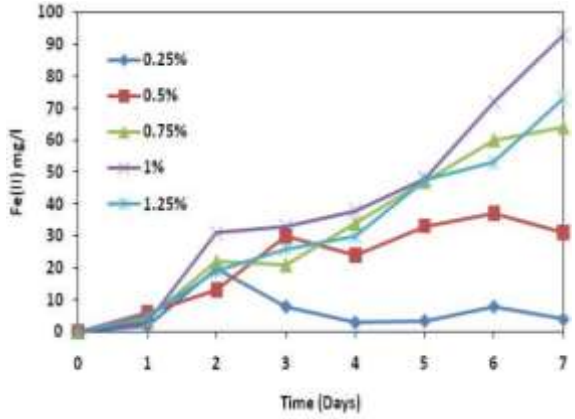

Fig. 3: Effect of various concentrations of starch on Fe mineralization by *Bacillus* sp. SP10

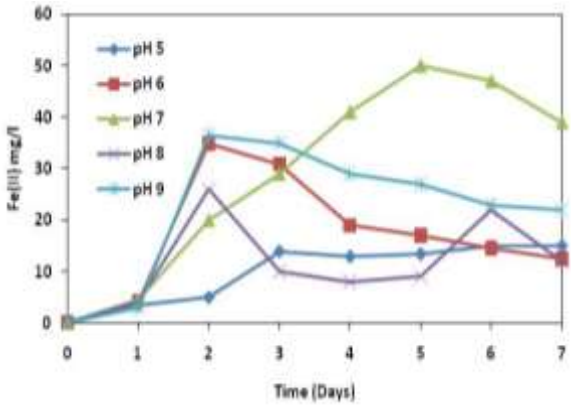

Fig. 4. Effect of various pH on Fe mineralization by *Bacillus* sp. SP10

Among the various concentrations of starch tested, 1% showed maximum mineralization of Fe (93 mg L^{-1}) and 0.25% concentration showed least mineralization ((3 mg L^{-1})) [Fig. 3]. In another study, maximum mineralization of iron occurred at pH 7 (50 mg L^{-1}) while the mineralization activity under other pH conditions were remarkably low (Fig. 4).

Biomineralization of iron in synthetic soil using column approach

Four treatment columns (A, B, C and D) were used to assess the biomineralization of Fe(III) oxide in synthetic soils under various conditions. The 1% starch + 1% *Bacillus* sp. SP10 treated columns (B, C and D), irrespective of the presence of humic acid or anthraquinone-2,6-disulphonate, showed significant increase in the mineralization of ferric to ferrous iron. Control treatment (A) showed low concentrations of ferrous iron which may be attributed to the absence of iron solubilizing bacteria in the synthetic medium and soil (Fig. 5). Under optimum conditions, the *Bacillus* sp. strain SP10 showed better Fe mineralization in the soil supplemented with insoluble Fe(III) oxide.

SEM and EDX analysis of mineralized Fe

Since treatment D (1% starch + 1% *Bacillus* sp. SP10 + 0.25% anthra-quinone-2,6-disulphonate) in column study showed significantly higher mineralization of Fe, therefore, the structure and presence of soluble Fe obtained from this treatment column by the action of *Bacillus* sp. strain SP10 were tested through SEM and EDX analysis. Plate 2 shows the surface structure of mineralized Fe analysed through

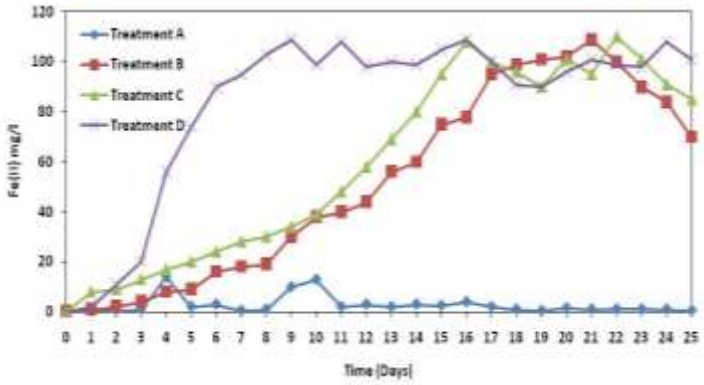

Fig. 5: Mineralization of synthetic iron oxide in soil through column approach

SEM analysis. The Fe peak obtained in EDX depicted the presence of Fe as micronutrients in the sample after mineralization.

The soils from various columns were tested for phytotoxicity. The results revealed that the plants in inoculum + starch treated soils had better growth in comparison to the plants raised in control soil. This revealed that *Bacillus* sp. strain SP31converted iron to its soluble form and improved its uptake. Reports indicate that iron-based materials do not show phytotoxicity within the test dose range (Sun *et al*., 2020).

Phytotoxicity assay of microbial mineralized soil

The seeds of green gram were sown in soils in different columns that were mixed in various ratios of fertile: treated soil (0:1, 1:1, 1:2, 1:3 and 1:0). (Fig. 6). The results that plant length in treatment B, C, and D was higher in comparison to the control (treatment A). This might be due to the absence of mineralization in treatment A soil. Whereas in treatment B, C and D soils the ferric iron was mineralized

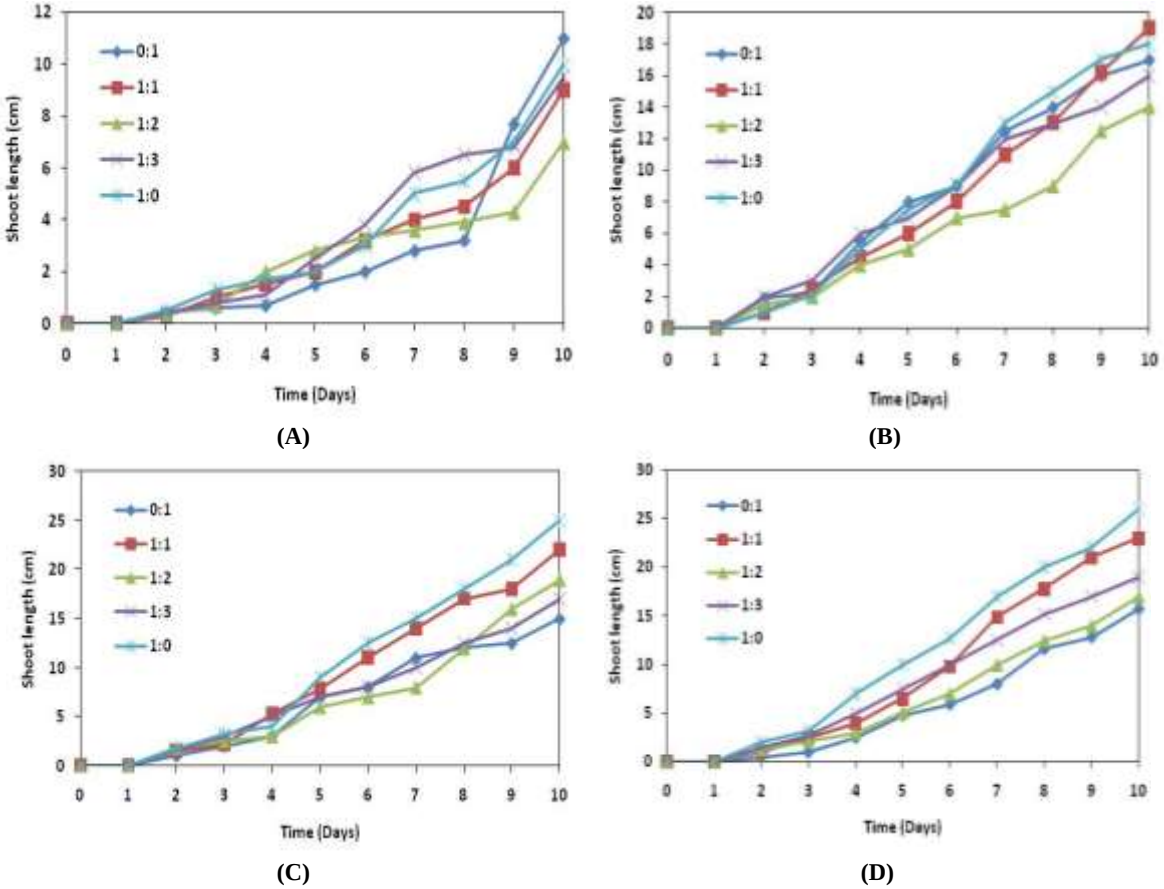

Fig. 6: Effect of treated soils obtained from various treatment columns on the growth of green gram: [A) control soil without C source supplement; B) 1% starch + 1% inoculum (*Bacillus* sp. SP10); C) 1% starch + 1% inoculum + 0.25% humic acid; and D) 1% starch + 1% inoculum + 0.25% + anthraquinone-2,6-disulphonate]

into ferrous using the potential bacterial strain (SP10). This shows that iron in its soluble form of ferrous is essential for the growth of plants. Moreover, the soils mixed in 1:1 ratio was able to produce plants that were merely close to the growth of plants in control fertile soils (1:0). The plants grown on treated soil alone (0:1) also had significant growth but shorter than control plants.

The soils obtained from the column were tested for phytotoxicity. The results show that the plants sown on treated soils with microbial strain have grown well when compared with the plants sown in soil without microbial strain. This infers that the microbial strain has converted iron in its soluble form for the uptake of plants as an essential micronutrient. It is said that iron-based materials do not show phytotoxicity within the dose range (Sun *et al*., 2020).

Conclusion: The *Bacillus* sp. strain SP10 was identified as a potential bacterium capable to mineralize iron from its insoluble form. Mineralization was maximum at pH 7.0 in the medium supplementation with 1% starch as carbon source. The column study revealed that the addition of soil organic matter such as ADQS and humic acid supported the mineralization process. The phytotoxicity assay depicted that the bio-mineralized soil procured from treated columns greatly supported the plant growth in comparison to untreated soil. It may be concluded that although iron is naturally present in soils its low solubility makes iron as a less available element in the surrounding. Hence, it is necessary to increase iron solubility for improvement in the growth and development of plants.

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