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ISOLATION, SCREENING AND IDENTIFICATION OF LEAD AND CADMIUM RESISTANT SULFUR OXIDIZING BACTERIA

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Sulfur oxidizing bacteria (SOB) oxidize elemental sulfur (S°) or reduce sulfur compounds to generate sulfuric acid which reduces the pH of media. In this study, lead and cadmium tolerant SOB were isolated by using thiosulphate medium from samples collected from different ecologies and screened on account of pH reduction, sulfate ions production, phosphorus solubilization index and phosphorus bioleaching tests. Results indicated that sulfate ions production (800 mg/L by *Bacillus* sp. strain SS-16) and phosphorus solubilization (713 mg/L by *Bacillus* sp. strain SS-16) from rock phosphate were significantly increased as compared to control due to pH reduction (net decrease of 4.52 points). The tolerance of isolated SOB by minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) was evaluated at different concentrations of lead and cadmium and *Bacillus* sp. strain SS-16 showed its ability to tolerate lead and cadmium up to 1000 and 180 mg/L, respectively. Organic acids production ability of three efficient isolated SOB (SS-4, SS-8 and *Bacillus* sp. SS-16) was evaluated under normal and stressed (lead and cadmium polluted) conditions. Results indicated that short chain organic acids amount increased under Pb and Cd stressed conditions as compared to normal medium. The isolated SOB were further studied for their morphological and biochemical properties. The most efficient bacterium belonged to sewage sludge ecology and was identified as *Bacillus* sp. SS-16.

Keywords: Sulfur oxidizing bacteria, phosphorous solubilization, MIC, MBC, heavy metal resistant bacteria, organic acid profiling

INTRODUCTION

Heavy metal pollution of soil has become global environmental problem due to intensively increasing industrialization and agricultural activities (Mcgrath *et al*., 1995). In Pakistan, there is an unchecked disposal of raw sewage from domestic and industrial areas on cropped soils. Concentrations of heavy metals (Pb, Cd and Cr) in vegetable fields irrigated with untreated sewage are advancing towards phytotoxic levels (Hussain, 2000; Qadir *et al*., 2000). Accumulation of heavy metals in crop plants through soil-root interface is a critical hazard (Sharma and Agrawal, 2006; Yoon *et al*., 2006; Maimon *et al*., 2009). Thus, under this scenario, there is a need to reclaim soil contaminated with heavy metals.

Phytoremediation, an emerging green technology is receiving much more attention for rehabilitation of soils polluted with heavy metals because it is non-intrusive and inexpensive means of remediating contaminated soils (Alkorta and Garbisu, 2001; Prasad and Freitas, 2003; Wei *et al*., 2004; Ali *et al*., 2013). Phytoremediation can take place in many ways, but phytoextraction is an in-situ promising environment friendly technique for the reclamation of polluted soil (Nascimento and Xing, 2006). But, the success of phytoextraction is hindered by less solubility of heavy metals in soil solution and low bioavailability to plants (Turgut *et al*., 2004). The use of elemental sulfur in soil has been suggested to decrease pH and increase solubility and bioavailability of heavy metals in soil (Kayser *et al*., 2000).

In Pakistan, the soils are mostly alkaline (pH>7) and calcareous ($CaCO_3$>3%) in nature (Sharif *et al*., 2000; Jafar *et al*., 2012) with 15% soils are poor in sulfur (<10 mg/kg SO_4^{-2}), and 30% (11-30 mg/kg SO_4^{-2}), 33% (31-99 mg/kg SO_4^{-2}) and 22% (mg/kg SO_4^{-2}) soils fall in acceptable, adequate and high range, respectively (NFDC, 1992; Sutaria, 2016). Sulfur is the most important plant nutrient after nitrogen, phosphorus and potassium (Jamal *et al*., 2010) and is a natural acidifying element. But due to greater fixation, low availability of plant nutrients is an immense issue. Plant roots take sulfur mainly in the form of sulfate (SO_4^{-2}). Sulfur oxidizing bacteria (SOB) oxidize elemental sulfur into sulfate in alkaline calcareous soils and produce sulfuric acid which decreases the soil pH and increases the solubility and plant availability of nutrients (Hitsuda *et al.,* 2005; Abdou, 2006; El-Tarabily *et al.,* 2006) as well as heavy metals and thus improve the condition of alkali and heavy metal polluted soils.

Although, majority of the reported SOB are associated with genera *Thiobacillus, Thiothrix, Thiomicrospira, Achromatium* and *Desulfuromonas*, but heterotrophic bacteria

also oxidize reduced sulfur compounds (Das *et al*., 1996). Most agricultural soils are deficient in *Thiobacilli* (Lawrence and Germida, 1991). Soils inoculated with sulfur oxidizing bacteria speed up the natural biological sulfur oxidation leading towards increased plant yield (Amal *et al*., 2014). Keeping this in view, the present study was conducted for isolation and screening of lead and cadmium resistant SOB.

MATERIALS AND METHODS

***Sample collection and growth medium for isolation of SOB*:** Samples were collected from different ecologies like sewage sludge (SS), sulfur contaminated soil (SCS), sewage water (SW), industrial waste sludge (IWS), tannery effluent (TE), cow dung (CD) and normal soil (NS) and isolation of SOB was performed by using dilution plate technique on thiosulfate medium ($Na_2S_2O_3$, 5.0 g; K_2HPO_4, 0.1 g; $NaHCO_3$, 0.2 g; NH_4Cl, 0.1 g dissolved in 1.0 L distilled water) using bromo cresol purple as an indicator (Beijerinck, 1904). The pH of the medium was adjusted to 8.0 before sterilization. One gram or one milliliter of collected samples was added to 20 mL of the sterilized medium poured in test tubes. Then the tubes were incubated at 30°C for 14 days. Change in color from purple to yellow denoted the growth of SOB in the test tubes. Sewage sludge sample produced bright yellow color that indicated the presence of efficient SOB. Then 100 µL of bacterial culture was poured on thiosulfate agar plates containing bromo cresol purple. After 3 days, growth was appeared and 23 individual colonies were picked and streaked on thiosulfate agar plates containing bromo cresol purple.

Screening of Efficient Isolates:

***Color change*:** Among 23 colonies, 11 isolates were selected as effective SOB on the basis of color change on thiosulfate agar plates from purple to yellow and further analyzed for different parameters.

***The pH reduction test*:** One milliliter fresh culture of selected isolates prepared in thiosulphate medium (pH 8.0) was inoculated in flasks containing 20 mL thiosulphate medium with bromo cresol purple (pH 8.0) and incubated at 30°C for 16 days. Screening of isolates was performed on the account of their capability to reduce the pH of medium. The pH of the samples was determined through pH meter (Kent Eil 7015, England). The experiment was conducted with three replications.

***Sulfate ions production test*:** Sulfate ions produced during the growth of SOB in thiosulfate medium were determined by adding 1:1 barium chloride solution (10% w/v) in bacterial culture supernatant (Cha *et al*., 1999). White turbidity was appeared due to formation of barium sulfate. That turbidity was measured at 450 nm by spectrophotometer (ANA-720W, Tokyo Photo-electric Company Limited, Japan). The values obtained were compared with standard sulfate curve that was constructed by dissolving K_2SO_4 in distilled water (Kolmert *et al*., 2000). The experiment was performed with three replications.

***Phosphorus solubilization index (PSI)*:** For the determination of PSI, fresh culture of selected isolates (0.1 mL) was placed on thiosulphate tricalcium phosphate (TCP) agar (0.5%) plates and incubated for 8 days at 30°C. The experiment was performed with three replications. Phosphorous solubilization zones were formed on agar plates and PSI was quantified by using the following formula (Edi-Premono *et al*., 1996).

$$\text{PSI} = \frac{\text{Colony diameter} + \text{Holozone diameter}}{\text{Colony diameter}}$$

***Quantitative phosphorus solubilization*:** Phosphorous solubilzation efficiency of selected SOB was checked by rock phosphate bioleaching test. The experiment was performed with three replications. Each conical flask contained 100 mL thiosulphate medium (pH 8.0) along with 0.5% rock phosphate. After autoclaving the flasks were inoculated with 1.0 mL broth culture of each selected isolate in 3 flasks, and 3 flasks were kept as un-inoculated control. The flasks were incubated (100 rev min^{-1}) at 30°C for 16 d. After 8 and 16 days of incubation, aliquot samples (5 mL) were drawn and centrifuged. The supernatants were analyzed for P solubilization. The amount of soluble P was calculated through molybdenum blue method (Watanabe and Olsen, 1965).

***Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC)*:** MIC of selected SOB isolates for Pb and Cd was determined. The colonies were picked from formerly grown bacteria and streaked on the freshly LB agar medium supplemented with different concentrations of Pb. The same procedure was repeated with isolated SOB under various concentrations of Cd. The concentration of metals at which the number of colonies reduced as compared to control plate, was considered as MIC. After determination of MIC, MBC was determined. The concentration of metals at which no colony grew or the number of colonies was reduced by 99.9% as compared with the control plates, was considered as MBC (Zeng *et al*., 2009; Kafilzadeh *et al*., 2010; Shamim and Rehman, 2012). Lead nitrate [$Pb(NO_3)_2$] and cadmium nitrate [$Cd(NO_3)_2$] salts were used as a source of Pb and Cd, respectively. The experiment was performed with three replications.

***Analysis of organic acids produced by SOB*:** Organic acids production ability of three efficient isolated SOB, selected on the basis of above conducted tests, was determined under normal, lead (600 mg Pb L^{-1}) and cadmium (80 mg Cd L^{-1}) stressed medium following the method of Li *et al.* (2009). Levels of Pb and Cd applied to medium were selected through MIC and MBC. The experiment was conducted with three replications. Organic acids were determined by HPLC (Shimadzu, Japan) with LC-10 AT, UV-visible and SPD-10 AV, after running the samples along with standards for organic compounds.

***Morphological and biochemical characterization of SOB*:** Colony and cell morphology of selected isolates was studied by the method of Bergey and Boone (2009). Gram staining was done by the method of Vincent (1970). Presence of catalase and oxidase in the selected SOB was identified following the methods described by MacFaddin (1980) and Steel (1961), respectively. Siderophores production was measured following the method of Schwn and Neilands (1987). Selected isolates were analyzed for their ability to utilize S°. Isolates were inoculated in sterilized Starkey medium amended with 10 g S° (Starkey, 1935) and incubated at room temperature for 15 days. Growth and elemental sulfur utilization were assessed by pH reduction of medium. Temperature tolerance test for selected isolates was performed. Isolates were inoculated in sterilized thiosulfate medium and incubated at different temperatures (20, 28, 38 and 45°C) for 15 days and growth was observed (Priyanka *et al*., 2014).

***Molecular identification of selected heavy metal tolerant SOB*:** The selected SOB isolate was identified by amplifying, sequencing and analyzing its 16S rRNA gene sequence. For this purpose, crude DNA of the selected isolate Bacillus sp. SS-16 was separated from the cell culture using proteinase K treatment (Cheneby *et al.*, 2004). The universal primers were used for PCR amplification (forward primer: 50'-AGA GTT TGA TCH TGG CTC AG-30' and reverse primer: 50'-TAC GGH TAC CTT GTT ACG ACT T-30'). The PCR reaction was performed by using 2.5 µL crude DNA as a template (Hussain *et al.*, 2011). The size of the amplified 16S rRNA was checked by segregating on 1% agarose gel along with GeneRuler 1kb DNA (Fermentas). The 16S rRNA PCR product was refined using a PCR Purification Kit (Favorgen, Taiwan) and sequenced by Macrogen (Seoul, Korea). 16S rRNA of Bacillus sp. SS-16 was matched with the known nucleotide sequences using BlastN (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree was developed through multiple alignments using Mega 6 Software.

The data were analyzed using analysis of variance technique by Statistix 8.1 statistical package (Statistix, USA). Mean values were compared using Tukey's (HSD) test at $p < 0.05$ (Steel *et al.*, 1997). Means and standard errors were calculated with MS Excel, 2016.

RESULTS

All the collected samples from different ecologies i.e. sewage sludge (SS), sulfur contaminated soil (SCS), sewage water (SW), industrial waste sludge (IWS), tannery effluent (TE), cow dung (CD) and normal soil (NS), were tested for the presence of sulfur oxidizing bacteria. It is revealed from the data that different ecologies contain different number of SOB. However, highest population was observed in sewage sludge (Fig. 1). For further isolation sewage sludge was used and eleven strains were selected as SOB on the basis of color change.

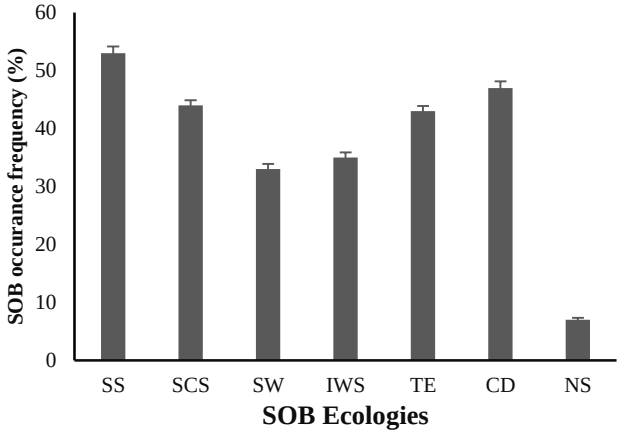

Figure 1. Frequency of sulfur oxidizing bacteria in the sampling ecologies indicating highest number of SOB in sewage sludge (average of three replicates). SS (sewage sludge), SCS (sulfur contaminated soil), SW (sewage water), IWS (industrial waste sludge), TE (tannery effluent), CD (cow dung), NS (normal soil).

Figure 2 shows the effect of 11 selected SOB isolates on pH reduction of thiosulfate medium during 16 days of incubation. Data reveals that maximum reduction (net decrease of 4.52 points) in pH was recorded in case of *Bacillus* sp. SS-16 (Fig. 3) after 16 days of inoculation followed by SS-8 with 3.72 pH (net decrease of 4.28 points). While the minimum decrease was observed in isolate SS-21 giving a pH value of 5.37 (net decrease of 2.63 points). Four SOB isolates SS-4 (3.86), SS-7 (4.10), SS-2 (4.18) and SS-13 (4.25) showed pH value between 3.86 and 4.25 (net decrease in pH of 3.75 to 4.14 points). After eight days of inoculation, *Bacillus* sp. isolate SS-16 gave maximum significant reduction in pH (3.78) of thiosulfate medium with net decrease in pH of 4.22 points followed by the SS-8 with 4.12 pH (net decrease in pH of 3.88). pH of 6 SOB SS-1 (5.10), SS-2 (4.55), SS-4 (4.25), SS-7 (4.49), SS-11 (4.90), SS-13 (4.67) and SS-17 (4.78) ranged from 4.25 to 5.10 with net decrease in pH ranging from 3.10 to 3.75 points. Isolates SS-3 (7.21) and SS-21 (7.49) gave minimum net reduction in pH that ranged from 0.51 to 0.79 points.

The results revealed that nine isolates out of eleven produced free sulfate contents at 8^{th} day of incubation while at 16^{th} day of incubation, all SOB isolates produced the free sulfate contents (Fig. 4). *Bacillus* sp. isolate SS-16 produced highest amount (801 mg/L) of sulfate ions at 16^{th} day of incubation while same isolate produced the sulfate ions of 480 mg/L at 8^{th} day of incubation. Isolates SS-4 and SS-8 produced 410 and 436 mg/L sulfates ions at 8^{th} day of incubation,

respectively, while these isolates produced 745 and 775 mg/L at 16th day of incubation, respectively. Sulfate ions production due to SS-1, SS-2, SS-7, SS-11, SS-13 and SS-17 ranged from 259 to 385 mg/L at 8th day of incubation. While, sulfate ions produced due to these isolates ranged from 527 to 712 mg/L at 16th day of incubation. Rest of the strains produced sulfate ions with non-detectable range at 8th day of incubation.

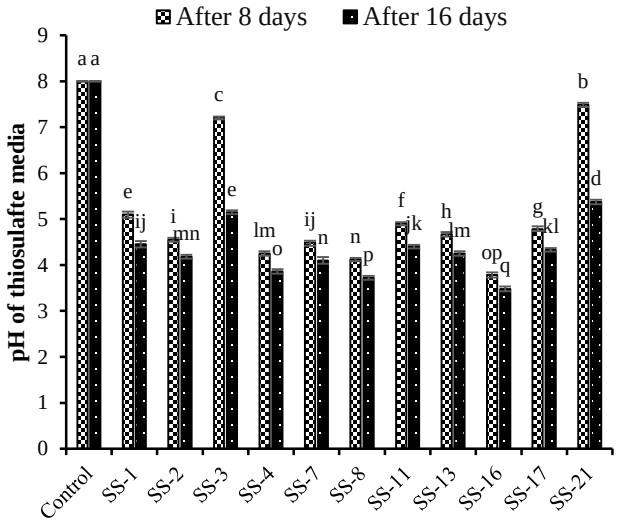

Figure 2. pH reduction in thiosulfate medium due to inoculation of SOB at 8th and 16th day of inoculation (average of three replicates).

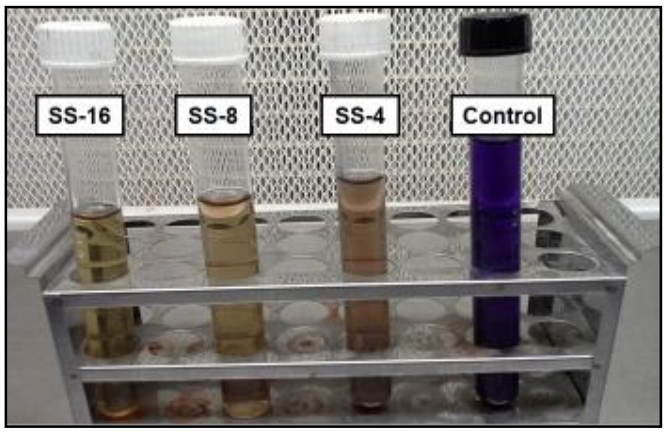

Figure 3. pH reduction test.

All eleven selected isolates were examined for phosphorus solubilization index (Fig. 5). According to data highest PSI (7.13) was observed in case of *Bacillus* sp. isolate SS-16 followed by isolate SS-8 with 6.56 PSI, while minimum PSI (1.2) was noted in case of isolate SS-21. PSI observed due to SS-1, SS-2, SS-3, SS-4, SS-7, SS-11, SS-13 and SS-17 was 1.64, 4.59, 1.39, 5.26, 5.27, 2.31, 3.82 and 3.24, respectively. No holozone was noticed in thiosulfate agar plates where no inoculation was done (control).

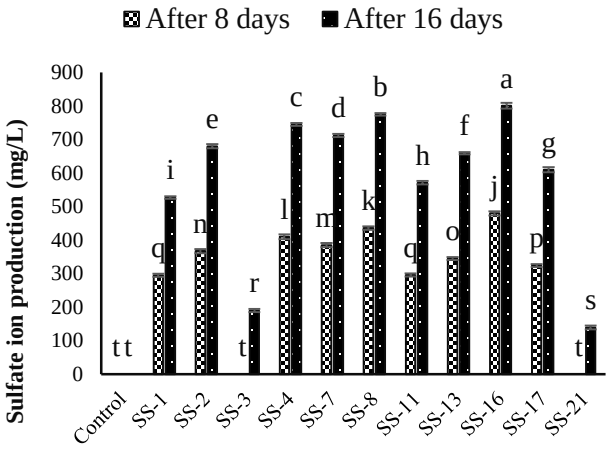

Figure 4. Sulfate ions production in thiosulfate medium due to inoculation of SOB at 8th and 16th day of inoculation (average of three replicates).

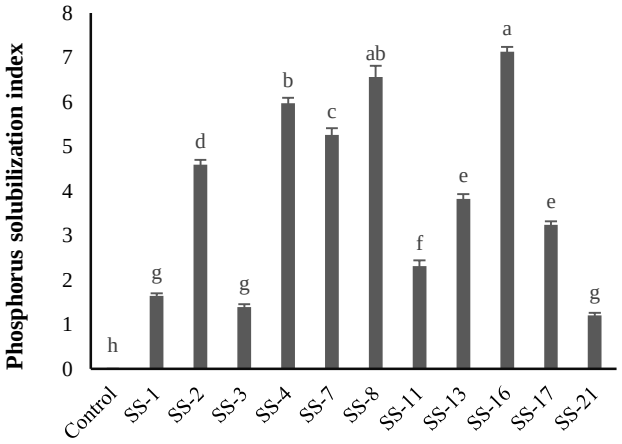

Figure 5. Phosphorus solubilization index of selected SOB (average of three replicates).

Quantitative analysis of phosphorus solubilization after inoculation (Fig. 6) revealed that *Bacillus* sp. isolate SS-16 dissolved 431 and 713 mg/L at 8th and 16th day of inoculation, respectively, followed by SS-8 that solubilized 391 and 681 mg/L phosphorus at 8th and 16th day of inoculation, respectively. Minimum phosphorus solubilization of 41 and 169 mg/L was noted in case of SS-21 at 8th and 16th day of inoculation, respectively. Phosphorus solubilization due to rest of the isolates ranged from 63 to 375 and 220 to 646 mg/L at 8th and 16th day of inoculation, respectively. No dissolution was detected in case where no inoculation was done (control). Figures 7 and 8 show the resistance of selected eleven isolates to different concentrations of Pb and Cd in terms of MIC and MBC. Tolerance test indicated that isolates SS-1 and *Bacillus* sp. strain SS-16 showed highest resistance to Pb (1000 mg/L

MIC) each followed by SS-7 which survived under 900 mg/L Pb. Similarly, under cadmium stress, these isolates SS-1 and *Bacillus* sp. strain SS-16 showed the maximum MIC (180 mg/L Cd), followed by SS-7 with 140 mg/L Cd MIC. Minimum tolerance in term of MIC for Pb (300 mg/L Pb) and Cd (40 mg/L Cd) was recorded in case of SS-11. Rest of the strains showed different response to the different levels of Pb and Cd.

For MBC test, isolates SS-1 and *Bacillus* sp. strain SS-16 showed highest resistance to Pb (1200 mg/L MBC) each followed by SS-7 with 1000 mg/L Pb. Similarly, under cadmium stress, these isolates SS-1 and *Bacillus* sp. strain SS-16 showed the maximum MBC (200 mg/L Cd) each followed by SS-21 with 180 mg/L Cd MBC. Minimum tolerance in term of MBC for Pb (400 mg/L Pb) and Cd (60 mg/L Cd) was recorded in case of SS-11. Rest of the strains showed different response to the different levels of Pb and Cd.

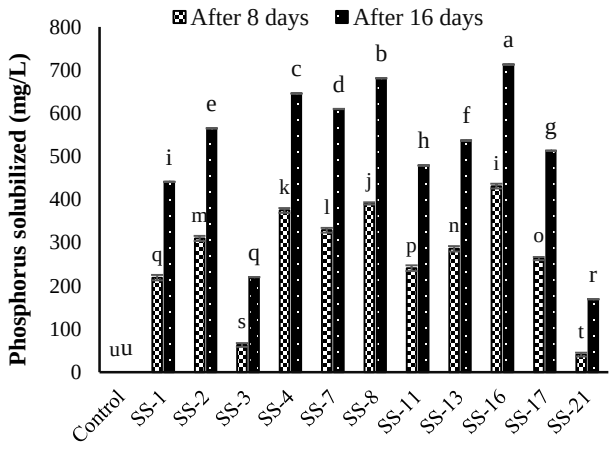

Figure 6. Quantification of P solubilization through bioleaching test (average of three replicates).

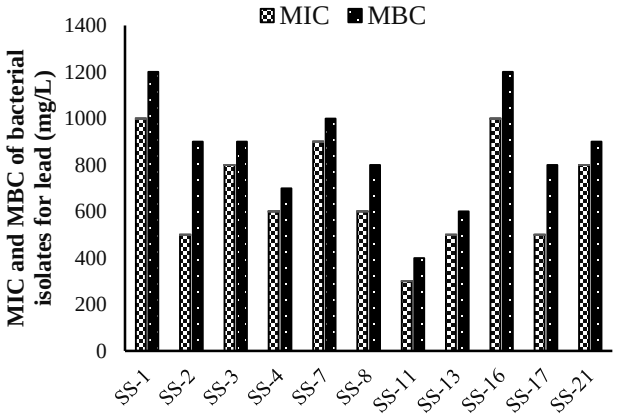

Figure 7. Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of Pb for SOB isolates (average of three replicates).

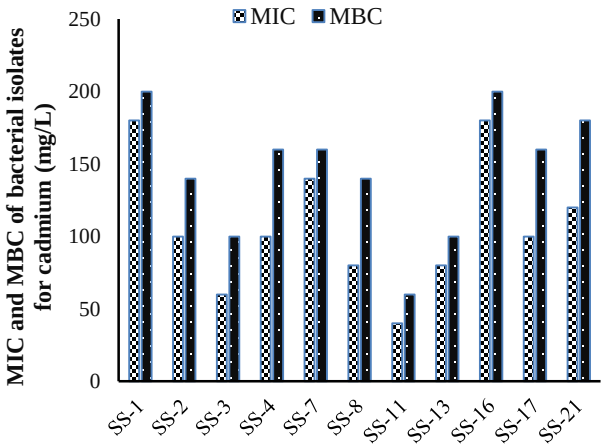

Figure 8. Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of Cd for SOB isolates (average of three replicates).

Different organic acids were determined in the culture of strains SS-4, SS-8 and *Bacillus* sp. strain SS-16 under normal and stressed (Pb and Cd) conditions (Table 1). Under normal conditions, major organic acid in the profile of SS-4 was tartaric acid (362 µg/mL) and the lowest was pyruvic acid (1.03 µg/mL). Fumaric acid was the second most abundant organic acid with 342 µg/mL amount in the profile. Under Pb stress, pyruvic acid increased from 1.03 to 1766 µg/mL. Similarly, tartaric acid increased from 362 to 1613 µg/mL. Other acids decreased approximately 100 µg/mL in Pb stress compared to normal conditions.

In the acid profile of SS-8, production of different acids varies in normal and stressed conditions. Highest amount of tartaric acid was observed under normal conditions while this acid increased under Pb and Cd stressed conditions up to 1750 and 1638 µg/mL, respectively. Pyruvic acid also increased under Pb and Cd stressed conditions. In the profile of *Bacillus* sp. strain SS-16, major organic acid was tartaric (826 µg/mL) while the lowest was oxalic acid (2.15 µg/mL) under normal conditions. Under Pb stress, pyruvic acid was highest (1570 µg/mL) while the second major acid in the profile of *Bacillus* sp. strain SS-16 was tartaric acid (1424 µg/mL) and lowest was succinic acid (7.23 µg/mL). Under Cd stress, highest amount of tartaric acid was 1798 µg/mL. Pyruvic acid also increased as compared to unstressed condition. Citric and malonic acids also increased under Pb stress as compared to unstressed condition.

The biochemical characterization of the isolated SOB was performed (Table 2). It is evident from the results that SOB strains SS-2, SS-3, SS-7, SS-8, SS-13, *Bacillus* sp. strain SS-16 and SS-21 were Gram positive while the other four strains SS-1, SS-4, SS-11 and SS-17 were Gram negative. Amongst 11 SOB strains 6 strains SS-2, SS-4, SS-7, SS-8, SS-13 and *Bacillus* sp. strain SS-16 utilized elemental sulfur. SS-2, SS-

Table 1. Organic acid profile from the cell culture of SS-4, SS-8 and SS-16 under normal, lead and cadmium stressed conditions (average of three replicates).

Treatment	Organic acids (µg/mL)	SS-4	SS-8	SS-16
Normal	Pyruvic acid	1.03 ± 0.01	514.96 ± 2.96	560.32 ± 2.46
	Tartaric acid	362.34 ± 4.14	733.97 ± 4.22	826.51 ± 6.24
	Citric acid	48.77 ± 0.56	67.02 ± 0.39	64.38 ± 0.49
	Oxalic acid	2.97 ± 0.03	1.03 ± 0.01	2.15 ± 0.02
	Malic acid	135.39 ± 1.55	251.39 ± 1.44	249.30 ± 1.88
	Methyl malonic acid	176.28 ± 2.02	1.13 ± 0.01	2.18 ± 0.02
	Malonic acid	178.81 ± 2.04	11.42 ± 0.07	11.69 ± 0.09
	Fumeric acid	342.10 ± 3.91	285.34 ± 1.64	262.63 ± 1.98
	Succinic acid	105.73 ± 1.21	3.26 ± 0.02	4.18 ± 0.03
Lead (Pb) Stress	Pyruvic acid	1766.13 ± 7.75	1070.87 ± 4.70	1570.43 ± 6.89
	Tartaric acid	1400.85 ± 10.6	1750.18 ± 13.2	1412.25 ± 10.7
	Citric acid	189.26 ± 1.43	4.18 ± 0.03	261.04 ± 1.97
	Oxalic acid	2.04 ± 0.02	3.84 ± 0.03	13.32 ± 0.10
	Malic acid	228.28 ± 1.72	27.81 ± 0.21	270.50 ± 2.04
	Methyl malonic acid	1.05 ± 0.01	5.20 ± 0.04	8.24 ± 0.06
	Malonic acid	3.26 ± 0.02	2.97 ± 0.02	293.78 ± 2.22
	Fumeric acid	268.78 ± 2.03	8.03 ± 0.06	293.85 ± 2.22
	Succinic acid	39.95 ± 0.30	8.24 ± 0.06	7.23 ± 0.05
Cadmium (Cd) Stress	Pyruvic acid	824.05 ± 3.61	911.35 ± 4.00	824.05 ± 3.61
	Tartaric acid	1638.16 ± 12.4	1798.99 ± 13.6	1638.16 ± 12.4
	Citric acid	21.42 ± 0.16	12.38 ± 0.09	21.42 ± 0.16
	Oxalic acid	9.15 ± 0.07	7.23 ± 0.05	9.15 ± 0.07
	Malic acid	22.53 ± 0.17	347.13 ± 2.62	22.53 ± 0.17
	Methyl malonic acid	12.20 ± 0.09	8.24 ± 0.06	12.20 ± 0.09
	Malonic acid	210.06 ± 1.59	18.27 ± 0.14	210.06 ± 1.59
	Fumeric acid	1180.79 ± 8.91	309.10 ± 2.33	1180.79 ± 8.91
	Succinic acid	747.88 ± 5.64	4.18 ± 0.03	747.88 ± 5.64

Means are written with standard error (mean ± SE)

Table 2. Morphological and biochemical characterization of SOB.

Characteristics	Isolates										
	SS-1	SS-2	SS-3	SS-4	SS-7	SS-8	SS-11	SS-13	SS-16	SS-17	SS-21
Morphology	C	SR	C	SR	SR	SR	SR	SR	SR	SR	C
Gram reaction	-	+	+	-	+	+	-	+	+	-	+
Colony character	SIY	SRW	SIW	SRW	SRW	SRW	SRW	SRY	SRW	SRY	SIW
Catalase	-	+	-	+	-	+	-	+	+	-	-
Oxidase	+	+	-	+	+	+	+	+	+	+	-
Siderophore	-	+	-	+	-	+	-	-	+	+	-
S° utilization	-	+	-	+	+	+	-	+	+	-	-
pH reduction	+	++	+	+++	++	+++	+	++	+++	+	+
Growth at 5°C	-	+	-	+	+	+	-	+	+	-	-
Growth at 28°C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Growth at 38°C	++	+++	++	+++	+++	+++	++	+++	+++	++	++
Growth at 45°C	++	++	-	++	++	++	++	++	++	++	-

+ve sign shows the presence while -ve sign shows absence of the character, C: cocci, SR: small rod, SIY: smooth, irregular, yellow, SRW: smooth, round, white, SIW: smooth, irregular, white, SRY: smooth, round, yellow

4, SS-8, SS-13 and *Bacillus* sp. strain SS-16 were positive in catalase production while rest of the strains were negative. Except SS-3 and SS-21 isolates, all other isolates were positive in oxidase production. Similarly, SS-2, SS-4, SS-8, *Bacillus* sp. strain SS-16 and SS-17 showed siderophore production while remaining strains were negative in siderophore production.

Results of temperature tolerance test revealed that all 11

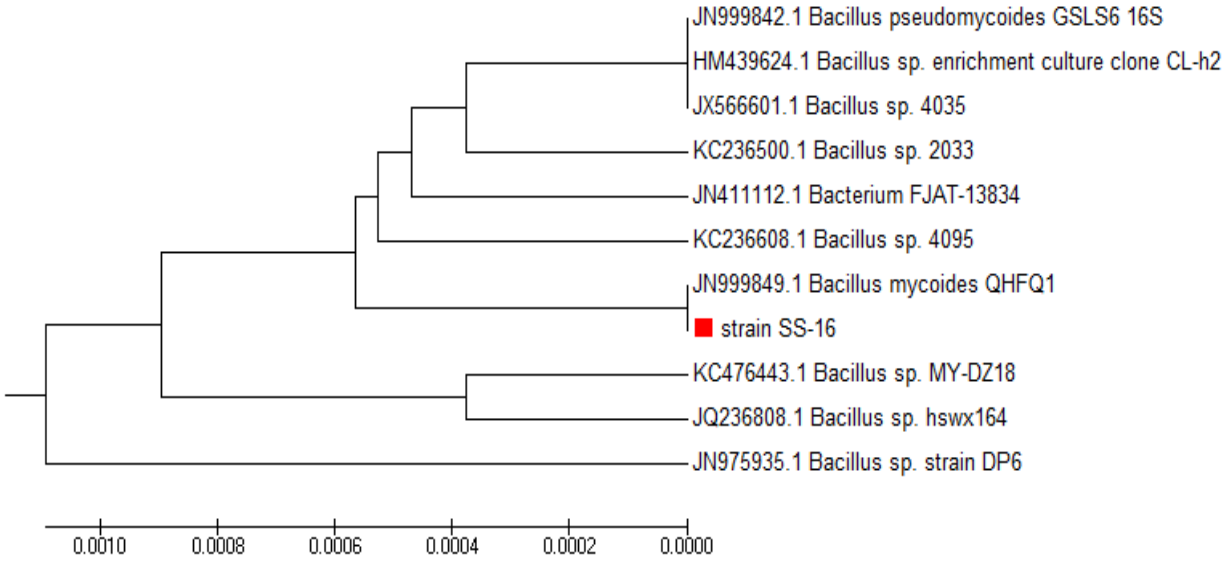

Figure 9. UPGMA phylogenetic analysis resulting from the multiple alignment of 16S rRNA gene sequence of *Bacillus* sp. with those of other bacterial strains found in Gene Bank database.

isolates were able to flourish in a wide range of temperature between 5-45°C, however the optimum being 28-38°C as shown in Table 2. On the basis of the results of pH reduction, sulfate ions production and phosphorus solubilization tests and also phosphorus solubilization index, *Bacillus* sp. strain SS-16 was selected as most efficient SOB by using principle component analysis for identification. Factor coordinates of all sulfur oxidizing isolates are presented in Table 3. Maximum coordinate value (3.175) was observed in case of *Bacillus* sp. strain SS-16. The selected most efficient SOB isolate (SS-16) was identified as *Bacillus* sp. accession no. MF170226 using 16S rRNA technique.

Table 3. Factor coordinates of sulfur oxidizing bacteriaon the basis of pH reduction, sulfate ions production, phosphorus solubilization index and phosphorus bioleaching tests.

Observation	F1	F2	F3	F4	F5
Control	-5.367	-1.175	-0.324	-0.010	-0.009
SS-1	-0.307	0.547	-0.674	-0.112	0.027
SS-2	1.283	-0.060	-0.083	0.049	0.031
SS-3	-3.174	0.574	0.606	0.050	-0.064
SS-4	2.216	-0.247	0.223	0.007	-0.040
SS-7	1.657	-0.180	0.075	0.097	0.043
SS-8	2.602	-0.314	0.336	0.009	0.006
SS-11	0.072	0.427	-0.534	0.003	-0.060
SS-13	0.903	0.108	-0.251	0.074	0.024
SS-16	**3.175**	-0.367	0.370	-0.147	-0.019
SS-17	0.522	0.225	-0.341	0.032	-0.005
SS-21	-3.583	0.462	0.597	-0.053	0.066

DISCUSSION

The study demonstrated that sewage sludge and cow dung are SOB enriched ecologies due to presence of sulfur or reduced sulfur compounds. SOB rely on sulfur oxidation for their energy demand (Pokorna *et al*., 2007). Phosphorous solubilizing potential of selected SOB through bacterially produced sulfuric acid as a result of sulfur oxidation has been explained in this study. Biological sulfur oxidation is an exceptional quality of SOB for oxidation of sulfur or sulfur compounds and generation of sulfuric acid. Thus, biological sulfur oxidation results in the production of sulfuric acid. SOB showed variance in their ability to oxidize sulfur. However, the most efficient isolate (*Bacillus* sp. strain SS-16) oxidized S compounds swiftly and generated sulfate ions in massive amount and dropped pH of thiosulfate medium. In agreement to our findings, variation in S oxidation by SOB isolates has also been reported in previous studies (Hassan *et al*., 2010; Yang *et al*., 2010).

Furthermore, highly efficient SOB (*Bacillus* sp. strain SS-16 and SS-8) generated sulfuric acid quickly and started making holozones from the first day of inoculation and as a result their phosphorus solubilization index (PSI) was very high (Islam *et al*., 2007). The strains with high PSI are documented highly effective in enhancing the solubilization of phosphorus in thiosulfate medium containing rock phosphate (Hariprasad and Niranjana, 2009; Ahemad and Khan, 2010). It has been reported earlier that during tricalcium phosphate bioleaching test, bacterially produced sulfuric acid not only solubilized phosphorus from tricalcium phosphate but also reduced the pH of thiosulafte medium (Ullah *et al*., 2014). The most

efficient SOB produced huge concentration of sulfuric acid and dropped pH of medium within short period of time. Similarly, in the present study, most efficient SOB isolates (SS-8 and SS-16) decreased the pH during pH reduction test and enhanced the phosphorus solubilization. Hence, pH reduction in the thiosulfate medium signify the efficiency and potential of SOB for P solubilization (Aria *et al*., 2010; Oh *et al*., 2010; Ullah *et al*., 2013).

In the present study, efficient isolates (SS-8 and *Bacillus* sp. strain SS-16) produced highest quantity of sulfates in the sulfate ions production test. It has been reported in the previous studies that the sulfate concentration in leach solutions illustrated the fundamental potential of SOB to oxidize sulfur or sulfur compounds (Ullah *et al*., 2014). The most proficient SOB quickly oxidized sulfur compounds and generated sulfate ions that form sulfuric acid whereas less efficient SOB showed gradual sulfur oxidation ability and for that reason low level of sulfates were present in thiosulfate medium. So, SOB can be screened on account of quantity of sulfate ions present in their leach solutions (Lee *et al*., 2005; Yang *et al*., 2010).

Results of rock phosphate solubilization experiment have demonstrated that the isolates with highest sulfate ions production ability (SS-8 and *Bacillus* sp. strain SS-16) resulted in maximum solubilization of phosphorus from rock phosphate. On the other hand, isolated SOB with low sulfate ions production ability (SS-1 and SS-21) dissolved least amount of P form rock phosphate as detected in leach solutions. It revealed that the concentration of both sulfate ions as well as phosphorus increased in medium as a result of pH reduction (Bhatti and Yawar, 2010). Moreover, other selected isolates have potential to utilize elemental sulfur or thiosulphate and carbon dioxide as the only sources of energy and carbon, respectively. Moreover, they depicted great potential to generate sulfate ions intensively as a result of pH reduction of media (Kelly and Wood, 2000; Vidyalakshmi and Sridar, 2007; Babana *et al*., 2011).

Isolated SOB depicted their abilities to tolerate lead and cadmium concentrations (in terms of MIC and MBC) which might be associated with different adaptive mechanism such as change in shape and structure of the cell (Varghese, 2012; Abbas *et al.,* 2014), production of organic acid (Li *et al*., 2009) and metabolites (Haferburg *et al*., 2009). Present study demonstrated that production of organic acids increased under Pb and Cd stressed conditions as compared to unstressed condition. Similar results have been observed by other researchers (Li *et al*., 2009; Jones, 1998; Ehrlich, 1998). It has been studied that inclusion of trace components to culture media can activate the production of numerous secondary organic acids and metabolites (Sprocati *et al*., 2006; Haferburg *et al*., 2009; Li *et al*., 2009). Further, Haferburg *et al*. (2009) proposed that production of different metabolites in response to trace component stress may be involved in detoxification of trace components by chelation. Li *et al.* (2009) revealed that production of carboxylic acids from bacterial cells was activated by trace components and these acids have also been increased the mobility of different components in soil. It has also been reported that trace components in plant cells are detoxified by complexation with carboxylicacids (Salt *et al*., 1999; Kupper *et al*., 2004). Therefore, strains such as *Bacillus* sp. SS-16 that have ability to produce largest amount of organic acids in response to heavy metal stress can be used to detoxify metal contaminants in the soil and also to improve the growth of plants grown under metal stressed conditions.

***Conclusion*:** Heavy metal tolerant sulfur oxidizing bacterium (*Bacillus* sp. SS-16) has potential of pH reduction, phosphorus solubilization and also organic acids production ability under Pb and Cd stressed conditions. Finally, it would be beneficial in future to investigate the effect of *Bacillus* sp. SS-16 on plant growth promotion under calcareous as well as heavy metal polluted soil to restore soil health in an environmentally sustainable way.

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