Isolation and Identification of Plant Growth Promoting and Chromium Uptake Enhancing Bacteria from Soil Contaminated by Leather Tanning Industrial Waste

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Abstract: Hexavalent chromium is considered as a priority pollutant. Phytoremediation has been widely pursued for the cleanup of heavy metal from contaminated area. The success of phytoremediation is depending on two factors: metal accumulating capability and biomass production of the plants. This paper reports on the isolation and characterization of rhizobacteria having ability to promote plant growth and increase its chromium uptake. Thirty nine of bacterial isolates were obtained from the rhizosphere of wild plants (*Sida* sp., *Sida acuta*, *Sida rhombifolia*, *Eupatorium* sp., *Acelypha* sp, *Acelypha indica*, *Amaranthus caudatus*, *Borreria* sp., *Leucas lavandulifolia*, *Eleusine indica*, *Pennisetum purpurium*, *Imperata cylindrical*, and *Vigna sinensis*) grow well on soil contaminated by leather tanning industrial waste. Three isolates, namely I26, I30, and I37, have an ability to enhance biomass production of maize (*Zea mays*) by 2.3, 2.6, and 4.0 times higher compare to the uninoculated one, respectively. The isolates also have an ability to increase chromium uptake by the maize from 7 to 14 times. All of the isolates increase the accumulation of Cr in the maize root. The 16S rDNA gene sequence of the isolates relates them to *Agrobacterium tumefaciens*.

Keywords: Maize, chromium, uptake enhancing, rhizobacteria.

INTRODUCTION

Environmental pollution by hexavalent chromium, Cr(VI), is a major public health concern [1]. It is highly soluble and mobile in water and displays toxic, mutagenic, and carcinogenic effects to living systems, including microorganisms, at low concentrations [2]. Therefore, it is considered as a priority pollutant. Major sources of Cr(VI) pollution include effluents from leather tanning, chromium electroplating, wood preservation, alloy preparation and nuclear wastes due to its use as a corrosion inhibitor in nuclear power plants [3, 4].

Many approaches have been developed and assessed to cope with the soil pollution. Current available soil clean-up technologies are often high-priced and soil disturbing so that the soil itself can rarely be utilized after the treatment. Soft and low-cost technologies, such as those afforded in ecologically engineered systems, need to be developed [5]. Plants have a natural propensity to take up metals. Plant-based environmental remediation technology has been widely pursued in recent years as an *in situ*, cost-effective potential strategy for the cleanup of trace metals from contaminated sites [6]. Phytoremediation could be the cheapest and simplest option among the available soil clean-up strategies [7].

Phytoremediation efficiency is determined by two key factors: metal accumulating capacity and biomass production of the plants. Plants with high metal accumulating capacity have been demonstrated to be potentially useful in soil cleanup, as they can take up significant amounts of metal from contaminated soils [8], but their low annual biomass production tends to limit their phytoremediation ability. The other possible alternative is the use of plants which have no high metal accumulating capacity [9], but have either high biomass or fast-growing ability that can be easily cultivated using established agronomic practices [10-12]. Extensive work has been done on the heavy metal uptake capacity of high biomass crop plants, such as Indian mustard (*Brassica juncea*), sunflower (*Helianthus annuus*), and maize (*Zea mays*) [13-15]. Clearly, if these affecting factors can be optimized, phytoremediation could be accelerated.

One approach is to add chemical agents to the soil that increase metal uptake by high biomass producing plants. In some cases, the *in situ* application of such

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chelators may pose the potential risk of groundwater pollution. Several lines of evidence suggest that soil microorganisms possess mechanisms capable of altering environmental mobility of metal contaminants with subsequent effects on the potential for root uptake and accumulation in plants [16]. This study was launched to identify maize growth promoting and chromium uptake enhancing bacteria isolated from the rhizosphere of wild plants grow well on soil contaminated by leather tanning industrial waste.

MATERIALS AND METHODS

Samples Collection

Samples were collected on April 2007 from the village of Sambirembe, Kalasan, Sleman, Yogyakarta, Indonesia (07°44'03.3"S, 110°26'38.2"E, 212 m above the sea level), where illegal dumping of leather tanning industrial waste occurred in 2001. Wild plants which were growing well on the contaminated land were carefully excavated. The wild plants were identified and classified by binomial method according to their morphological characters [17]. The results of the identification are shown in Table **1**.

Isolation of Rhizospheric Bacteria

Soil extract was prepared by mixing one kg of soil with one liter of tap water. Five mg of $CaCO_3$ was added, and then sterilized in an autoclave 120°C for 30 min. Soil particles were allowed to settle, and then filtered with double filter paper. Soil extract media were prepared by adding one g glucose, 0.5 g K_2HPO_4 to 100 mL soil extract, and 900 mL distilled water were then added. Agar (15 $g.L^{-1}$) was added to prepare soil extract agar medium. Rhizospheric soils were taken by

Table 1: Plants growing well on soil contaminated with leather tanning industrial waste, chromium content in the rhizosphere and tissue of the plant, bacterial number in the rhizosphere, arbitrarily selected bacteria from the rhizosphere, and isolates grow well in soil extract medium within 2 d of incubation

Plant	Cr conc. (ppm)		Bacterial number (CFU g^{-1} dry soil)	Arbitrarily selected isolates	Isolates grow well within 2 d (Isolat no.)
	in rhizosphere	in plant tissue			
Sida sp.	1.593	99.949	20.5×10^6	9	5 (1,2,3,4 & 5)
Sida acuta	1.329	84.084	8.0×10^6	7	1 (18)
Sida rhombifolia	1.560	13.256	4.9×10^6	7	2 (23 & 24)
Eupatorium sp.	4.634	127.711	8.6×10^6	9	2 (6 & 7)
Eupatorium sp.	0.789	19.237	15.7×10^6	8	2 (37 & 39)
Acalypha sp	7.245	40.500	12.8×10^6	5	4 (19,20,21 & 22)
Acalypha indica L.	0.910	159.660	11.5×10^6	10	10 (8,9,10,11,12,13,14,15,16 & 17)
Amaranthus caudatus	1.516	7.395	3.8×10^6	7	1 (25)
Borreria sp.	10.876	9.025	20.0×10^6	9	6 (26,27,28,29,30 & 31)
Leucas lavandulifolia	0.646	1.935	51.0×10^6	9	1 (32)
Eleusine indica	14.358	69.695	6.9×10^6	7	2 (33 & 34)
Pennisetum purpurium	1.340	9.470	11.2×10^6	6	0
Imperata cylindrical	0.326	0.194	13.9×10^6	8	1 (38)
Vigna sinensis	1.692	40.720	11.2×10^6	7	2 (35 & 36)

gentle shaking the roots of the wild plants. The soil samples were serially diluted in sterilized distilled water, and 0.1-mL samples were spread over the surface of duplicate plates of soil extract agar which had been dried for two days at room temperature. The inoculated plates were incubated for seven days at 37^{o}C. Colonies growing on the media were counted. Some colonies were selected arbitrarily according to their morphological characteristics. The selected colonies were cultured in soil extract broth for two days at 37^{o}C. Isolates having ability to grow in soil extract broth were sub-cultured several times to obtain pure cultures. Stock cultures were made in Luria Bertani (peptone 10 g.L^{-1}, yeast extract 5 g.L^{-1}, and NaC1 5 g.L^{-1}) containing 50% (w/v) glycerol and stored at –80°C.

Effect of Rhizobacterial Inoculation on the Growth and Chromium Uptake of Maize

Peat moss (C 36.11 %, N 0.97 %, P 0.03 %, K 0.02 %, Ca 2.32 %, Mg 0.19 %, S 0.30 %, and pH 6) was chosen as the carrier for the microbial inoculums. Bacterial cells were grown overnight in 5-mL Luria Bertani Broth. The culture (approx. 10^{9} cells.mL^{-1}) was mixed with 10 g of passed through a 2-mm sieve, and then sterilized peat moss. Sand was used for the chromium uptake experiment. The sand was washed by tap water, air-dried, passed through a 2-mm sieve, and sterilized. Five kg of sterilized sand was placed into one pot. Each pot was amended with a tableted compound fertilizer (PT Saraswati Anugerah Makmur, Surabaya, Indonesia) containing 322.2 mg N, 78.1 mg P, and 178.2 mg K, 102.3 mg Ca, 27.2 mg Mg, and 35.8 mg S. Peat moss-based bacterial inoculums (10.0 g) were added to each pot for the inoculation treatment while the uninoculated control treatment received similar amounts of autoclaved carrier-based inoculums in order to minimize any possible variation in soil properties. Before seeding, *Z. mays* grains were surface-sterilized by shaking in 70% ethanol for 5 min, followed by shaking in 2% sodium hypochlorite for 30 min, and then rinsed with sterile water for five times 5-min each time. Three urface-sterilized grains were sown in the sand at the place of the bacterial inoculums. Five sample pots were periodically weighed and water was added to bring back to their initial weights to maintain moisture at water holding capacity. Experiment pots were watered with the average volume of water added to the sample pots throughout the development period of the maize grown. At seven days after seeding, the plants were thinned to one plant per pot. Chromium was added 14 d after seeding in the form of $K_2Cr_2O_7$ to reach a final concentration of 6 ppm Cr(VI) in soil. Plant biomass was harvested 30 d after Cr(VI) exposure. The roots and shoots were separated and dried in oven at 75°C to constant weights. Powder of the root and shoot materials were dissolved in dilute acid mixture (HClO4:HNO3; 1:1 v/v) and their Cr content was determined using atomic absorption spectroscopy. Absorbed Cr in plant parts were calculated from plant dry weights and their Cr content.

Identification of Rhizobacterial Isolates by Phenotypic and Genotypic Characterizations

Rhizobacterial isolates that increase chromium uptake and accumulation by maize were selected for identification.

a. Phenotypic Characterization

Phenotypic characterization such as cell morphology/cells type, Gram stain reaction, motility, aerobicity, ability to grow at 40°C, catalase test, oxidase test, urea hydrolysis, gelatin hydrolysis, indole test, growth on Hofer's alkaline broth, 3-ketolactose production, growth on NaCl 2 % and acid production from glucose were carried out according to *Bergey's Manual of Systematic Bacteriology* [18].

b. Genotypic Characterization

Genotypic characterization was done by 16S rDNA gene sequencing. Genomic DNA of bacterial isolates was extracted from 5-mL bacterial cultures grown overnight following a previously described method [19]. Bacterial pellets were suspended in 410 µL of TE buffer (10 mM Tris-HCl pH8 and 1 mM EDTA pH8). A volume (50 µL) of 60 mg.mL^{-1} fresh lysozyme (Sigma, Milwaukee, WI) solution was added to the cell suspension and incubated for 30 min at 37 °C with occasional mixing. Subsequently, 30 µL of 10% SDS (sodium dodecyl sulfate) and 3 µL of 20 mg.mL^{-1} proteinase K were added, mixed and the incubation was continued for another 30. Afterward, 100 µL NaCl 5 M and 100 µL CTAB (N-cetyl-N,N,N,-trimethylammonium bromide) 10 % were added, gently mixed, and incubated at 65 ºC for 10 min. The mixture was extracted with 600 µL ice cold chloroform by gently mixing, centrifuged at 12,000 rpm for 5 min, and the aqueous phase was taken carefully and removed into new sterile microcentrifuge tube. DNA present in the aqueous phase was precipitated with addition of 0.6 vol. ice cold isopropanol and incubation for one hour on ice. The precipitate was washed with ice cold 70% ethanol. After dried up, the precipitate was resuspended in 50 µL of TE buffer.

Universal bacterial 16S rDNA primers, 20F (5'-GAGTTTGATCCTGGCTCAG'-3, the *Escherichia coli* numbering system, [20]) and 1500R (5'-GTTACCTTGTTACGACTT'-3) (Invitrogen, Carlsbad, CA), were used to amplify 16S rRNA gene fragments with the isolated bacterial genomic DNA as a template following the protocol of Weisburg *et al*. [21] with modification in using GoTaq Green Master Mix (Promega, Madison, WI). PCR was carried out in a GeneAmp@ PCR system 9700 (Applied Biosystems, Foster City, CA). The following conditions were used for DNA amplification: Initial denaturation at 94°C for three min, followed with 30 cycles consisting of one min at 94°C, one min at 50°C, and one min 30 s at 72°C, plus an additional final extension step of five min at 72°C. Accuracy of the PCR products (about 1500 bp in size) were confirmed by electrophoresis through a 0.8% horizontal agarose gel containing 0.5 $\mu g.mL^{-1}$ ethidium bromide. Gels were examined under u.v. light and photographed [22]. PCR products were purified using Agarose Gel DNA Extraction Kit™ (Roche Applied Science, Indianapolis, IN).

Sequencing of the 16S rDNA was carried out on an ABI PRISM 3100-*Avant*™ Genetic Analyzer (Applied Biosystems, Foster, CA) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). In addition to 20F and 1500 R primers, the following four primers were also used for the sequencing of the 16S rDNA; i.e. 520F (5'-CAGCAGCCGCGGTAATAC-3'), 520R (5'-GTATTAC CGCGGCTGCTG-3'), 920F (5'-AAACTCAAATGAA TTGACGG-3') and 920R (5'-CCGTCAATTCATTTG AGTTT-3').

Multiple alignments of the sequences (ca. 715 bases) determined were performed with a program GENETYX WIN Ver. 3 (Software Development, Tokyo, Japan). A search of GenBank with BLAST program (www.ncbi.nih.nlm.gov/Blast.cgi) [23] was used to identify named bacterial species. Clustal X2.0 (ftp://ftp.ebi.ac.uk/pub/software/clustalw2) was used to construct the phylogenetic tree and then drawn using NJ (neighbor-joining) plot (http://pbil.univlyon1.fr/ software/njplot.html).

RESULTS AND DISCUSSION

Isolation of Bacteria from the Rhizosphere of Wild Plants Growing Well on the Contaminated Soil

At a site where illegal dumping of leather tanning industrial waste was occurred in 2001, several wild plants, as listed in Table **1**, were observed to grow well. There were 14 plants species belong to 10 genera and eight families of which three species each belonged to Malvaceae and Poaceae, two species each belonged to Asteraceae and Euphorbiaceae, and one species each belonged to Amaranthaceae, Rubiaceae, Fabaceae and Labiateae. Chromium content in the rhizosphere and tissue of the plants were then analyzed and shown in Table **1**. The results showed that none of the wild plant species accumulated chromium at a level above 1000 ppm. Therefore, none of them could be classified as a hyperaccumulator [24]. Among the wild plants that grow well in the contaminated soil, *Acalypha indica* was found to have the highest bioconcentration factor, followed by two species of *Sida*.

Chehregani and Malayeri [25] have reported that *Euphorbia cheiradenia*, *Scariola orientals*, *Centaurea virgata*, *Gundelia tournefortii* and *Eleagnum angustifolia* found to grow in the mining area could accumulate heavy metals in different organs. *Acalypha australis* have been reported to grow at contaminated sites in Korea and accumulate chromium in shoot at a level of 265 ppm [26]. *Sida sp* and *Sida acuta* have been reported to grow at several dumping sites at Ghana [27] and Colombia [28].

Good growth of plants indicate that the chromium exist on a state and valence that are neither available nor harmful for the plants. Availability and toxicity of chromium is affected by microbial activity around the rhizosphere [29]. Therefore, isolation of bacteria existing in the rhizosphere of plants that grow well on chromium contaminated area is interesting. The isolated rhizobacteria can be used to assist phytoremediation of soil contaminated by chromium. The rhizospheric soils of the plants were used as a source for bacterial isolate. Bacterial number in the rhizosphere, arbitrarily selected bacteria from the rhizosphere, and isolates grow well in soil extract medium within two days were shown in Table **1**. The bacterial numbers in the rhizosphere of plants that grow well on chromium contaminated area were varied from 10^6 to 10^7, indicating that the bacteria grow well on chromium contaminated soil. Thirty nine rhizobacteria were then arbitrarily selected and isolated. The rhizosphere of *Acalypha indica* contribute 10 isolates, followed by *Borreria* sp. (six isolates), *Sida* sp. (five isolates), *Acalypha* sp. (four isolates), two isolates each from the rhizosphere of *Eleusine indica*, *Sida rhombifolia* and *Vigna sinensis,* two isolates each from the rhizosphere of two species of *Eupatorium* sp., and one isolate each from the rhizosphere of *Sida*

acuta, *Amaranthus caudatus*, *Leucas lavandulifolia*, and *Imperata cylindrical*. No isolate was selected from the rhizosphere of *Pennisetum purpurium.*

The Effect of Rhizobacterial Isolates Inoculation on the Growth and Chromium Uptake by Maize

Chromium toxicity on plant was indicated by inhibition of roots and shoots growth and that inhibition affected the dry weight of biomass. The 39 rhizobacterial isolates were evaluated for their ability in assisting chromium uptake by maize. The collected data were dry weights of the roots, shoots, and total biomass; as well as Cr content in the roots, shoots, and total biomass (Table **2**).

Table **2** shows the effect of 39 rhizobacterial isolates inoculations on plants grow and their uptake of chromium. Inoculation by 11 isolates was shown to increase root growth for more than five times compared to the than the uninoculated one. Inoculation with isolate I27 and I30 can even increase the dry weight of roots by 10 times higher, i.e. 0.76 g for the uninoculated plants to become 7.97 g and 7.62 g for plants inoculated with isolate I27 and I30, respectively. On the other hand, inoculation by seven isolates was shown to increase shoot growth for more than two times higher than the uninoculated one. Inoculation with isolate I37 can even increase the dry weight of shoots by 3 times, i.e. 3.06 g for the uninoculated plants to become 9.42 g for plants inoculated with isolate I37. Moreover, inoculation of maize with isolates I37 resulted in an increase of plant dry weight four times, i.e. from 3.82 g for the uninoculted plants to become 15.47 g.

In addition to increasing the growth of maize, inoculation by some isolates were able to increase the concentration of chromium in plant tissues of maize. Inoculation by isolate I30 was shown to increase chromium concentration in roots, from 531.2 ppm for the uninoculated plants to become 1241.5 ppm for the inoculated one. In addition, inoculation by isolates I27, I20, and I32 was shown to increase chromium concentration in shoot from 93.9 ppm to become 165.3 ppm, 172.2 ppm, and 214.4 ppm, respectively. Overall, the effect of rhizobacterial isolates inoculations on the concentration of chromium in the plant as a whole shows that inoculation by isolate I26, I27, I29, I30, I32, and I37 can increase chromium concentration in plant from 180.9 ppm for the uninoculated plants to become 583.0 ppm, 428,4 ppm, 452.1 ppm, 953.4 ppm, 488.6 ppm, and 363.7 ppm, respectively.

The increase of plant biomass and the concentration of cadmium in it have meant an increasing amount of cadmium which is uptaken by the plant. Inoculation by isolates I26, I30, and I37 was shown to increase chromium uptake and its accumulation in roots for more than 10 times, i.e. from 403.7 μg in the uninoculated plants to become 5021.8 μg, 9460.2 μg, and 5396.0 μg, respectively. In addition, inoculation by isolates I12, I20, and I32 was shown to increase chromium uptake and accumulation in shoot from 287.3 μg to become 752.5 μg, 862.7 μg, and 626.0 μg, respectively. Overall, the effect of rhizobacterial isolates inoculations on the uptake and accumulation of chromium in the plant as a whole shows that inoculation by isolate I26, I30, and I37 can give the highest increase of chromium uptake by plant from 691.0 μg for the uninoculated plants to become 5078.4 μg, 9543.4 μg, and 5626.8 μg, respectively. Nouri *et al*. [30] suggest that plants having an ability to uptake and accumulate heavy metal can be used for phytoremediation. Selection of the isolates was then based on the effect of their inoculation on chromium uptake and accumulation by the maize. Isolate I26, I30, and I37 which give the highest increase of chromium uptake and accumulation by plant were selected accordingly.

Inoculations of rhizobacteria have been reported to stimulate the growth of *Brassica juncea* and protect the plant from metal toxicity [31-33]. Luo *et al*. [34] also reported that plant-growth-promoting *Bacillus* sp. increase biomass production as well as manganese and cadmium uptake by sweet sorghum. The rhizobacteria are reported to influence the growth, yield, and nutrient uptake of plant growing in a contaminated soil by an array of mechanisms. They may help plant by increasing supply of nutrients, such as phosphorus, sulphur, iron and copper, and producing plant hormones but they may also lessen toxic effects of heavy metals on the plants through the secretion of acids, proteins, and other chemicals that serve as an effective metal sequestering [35]. Prijambada and Proklamasiningsih [36] reported that organic acids may lessen the toxic effect of aluminum on soybean.

Identification of Rhizobacterial Isolates by Phenotypic and Genotypic Characterizations

The results of phenotypic characterization were shown in Table **3**. The four isolates show identical characteristics which are in agreement with the characteristics of bacteria belonged to the phylum of

Table 2: The effect of rhizobacterial isolates inoculation on root, shoot and plant dry weight; Cr contents in the root, the shoot, and the plant; their phytoextraction and phytostabilization coefficients

Inoc. with isolates	Root d.w. (g)		$[Cr]_{root}$ (ppm)		Shoot d.w. (g)		$[Cr]_{shoot}$ (ppm)		Plant d.w. (g)		$[Cr]_{plant}$ (ppm)	
None	0.76	cd	531.2	bcdefg	3.06	defgh	93.9	bcde	3.82	def	189.2	defg
1	1.54	cd	473.4	cdefgh	3.99	cdefgh	91.0	bcde	5.53	bcdef	188.8	defg
2	1.02	cd	312.8	efgh	4.03	cdefgh	40.3	de	5.04	cdef	90.2	fg
3	1.03	cd	460.3	def	2.99	defgh	32.6	de	4.02	def	113.8	efg
4	1.47	cd	309.9	bcdef	4.77	bcdefgh	73.9	bcde	6.23	bcdef	130.6	efg
5	0.31	d	367.3	f	1.25	h	144.9	abcd	1.55	f	190.2	defg
6	0.83	cd	393.5	def	3.20	defgh	24.4	de	4.03	def	98.5	fg
7	0.59	d	256.3	def	3.03	defgh	88.7	bcde	3.61	def	115.6	efg
8	0.30	d	47.8	f	1.26	h	53.0	bcde	1.56	f	71.0	g
9	1.03	cd	225.5	cdef	4.34	bcdefgh	49.5	bcde	5.37	cdef	83.9	g
10	0.97	cd	333.6	def	3.82	cdefgh	25.1	de	4.79	cdef	88.1	g
11	1.26	cd	385.0	bcdef	4.98	bcdefgh	26.8	de	6.24	bcdef	106.7	fg
12	1.50	cd	254.8	bcdef	6.63	abcde	113.5	abcde	8.13	bcde	138.8	defg
13	0.37	d	320.4	ef	2.10	efgh	28.6	de	2.47	ef	71.9	g
14	0.98	cd	197.7	bcdef	6.22	abcdef	25.9	de	7.20	bcdef	53.8	g
15	1.07	cd	159.8	cdef	4.25	bcdefgh	50.9	bcde	5.31	cdef	74.6	g
16	0.89	cd	919.0	bcdef	4.81	bcdefgh	59.8	bcde	5.70	bcdef	193.9	defg
17	1.28	cd	99.2	bcdef	4.52	bcdefgh	87.2	bcde	5.80	bcdef	91.7	fg
18	2.44	cd	288.0	abcd	6.92	abcd	40.2	de	9.37	bcd	99.4	fg
19	1.42	cd	147.2	bcdef	5.95	abcdefg	25.5	de	7.37	bcdef	48.7	g
20	1.57	cd	710.2	bcdef	5.01	bcdefgh	172.2	ab	6.58	bcdef	300.9	cdefg
21	1.09	cd	904.3	cdef	4.02	cdefgh	19.0	de	5.10	cdef	207.3	defg
22	5.23	ab	260.3	bcdef	1.78	gh	40.1	de	7.01	bcdef	205.2	defg
23	6.53	a	273.7	bcde	2.27	efgh	32.8	de	8.80	bcde	200.4	defg
24	6.45	a	308.7	bcd	2.77	defgh	104.5	abcde	9.22	bcd	245.9	defg
25	6.25	a	299.0	bcdef	1.61	gh	16.3	e	7.86	bcdef	241.1	defg
26	7.09	a	708.3	bcde	1.62	gh	34.9	de	8.71	bcde	582.5	bc
27	7.97	a	491.4	abcd	1.91	fgh	165.3	abc	9.87	abcd	428.4	cde
28	7.03	a	312.3	bcde	1.40	gh	63.7	bcde	8.43	bcde	270.6	defg
29	7.52	a	524.7	bcd	1.55	gh	99.7	abcde	9.07	bcd	451.7	bcd
30	7.62	a	1241.5	abcd	2.39	defgh	34.8	de	10.01	abcd	940.4	a
31	7.45	a	337.0	abcd	2.17	efgh	32.2	de	9.62	abcd	268.3	defg
32	2.95	bcd	760.0	bcdef	2.92	defgh	214.4	a	5.87	bcdef	657.7	b
33	3.52	bc	305.4	ab	8.35	abc	56.8	bcde	11.87	ab	125.5	efg
34	1.67	cd	287.7	bcdef	4.34	bcdefgh	23.6	de	6.01	bcdef	93.0	fg
35	1.32	cd	335.3	bcdef	5.73	abcdefgh	78.0	bcde	7.04	bcdef	130.1	efg
36	1.32	cd	359.5	bcdef	5.69	abcdefgh	83.1	bcde	7.01	bcdef	133.2	efg
37	6.05	a	891.9	a	9.42	a	24.5	de	15.47	a	411.9	bcde
38	1.43	cd	394.9	bcdef	5.97	abcdefg	16.8	e	7.40	bcdef	87.9	g
39	2.45	cd	47.8	abc	8.62	ab	41.3	cde	11.07	abc	43.2	g

Each value is a mean of 3 replicates.

Table 3: Morphological and Physiological Characteristics of the Selected Isolates

Characteristics	I26	I29	I37
Cell morphology	Rod	Rod	Rod
Gram staining	-	-	-
Motility	+	+	+
Oxygen requirement	Aerob	Aerob	Aerob
Growth at 40°C	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Urea hydrolisis	+	+	+
Gelatin hydrolysis	-	-	-
Indole production	-	-	-

proteobacteria, the class of alphaproteobacteria. The morphological and physiological characteristics showed good correspondence to the characteristics of the order of Rhizobiales. In order to further specify the classification, the four isolates were further examined for characteristics that can distinguish *Agrobacterium* from *Rhizobium*. The results of the further examination showed that the four isolates were able to grow on a medium containing 2 % of NaCl, on Hofer's alkaline broth, producing 3-ketolactose, and producing acid from glucose. The characteristics were in agreement to the characteristics of the genus *Agrobacterium*.

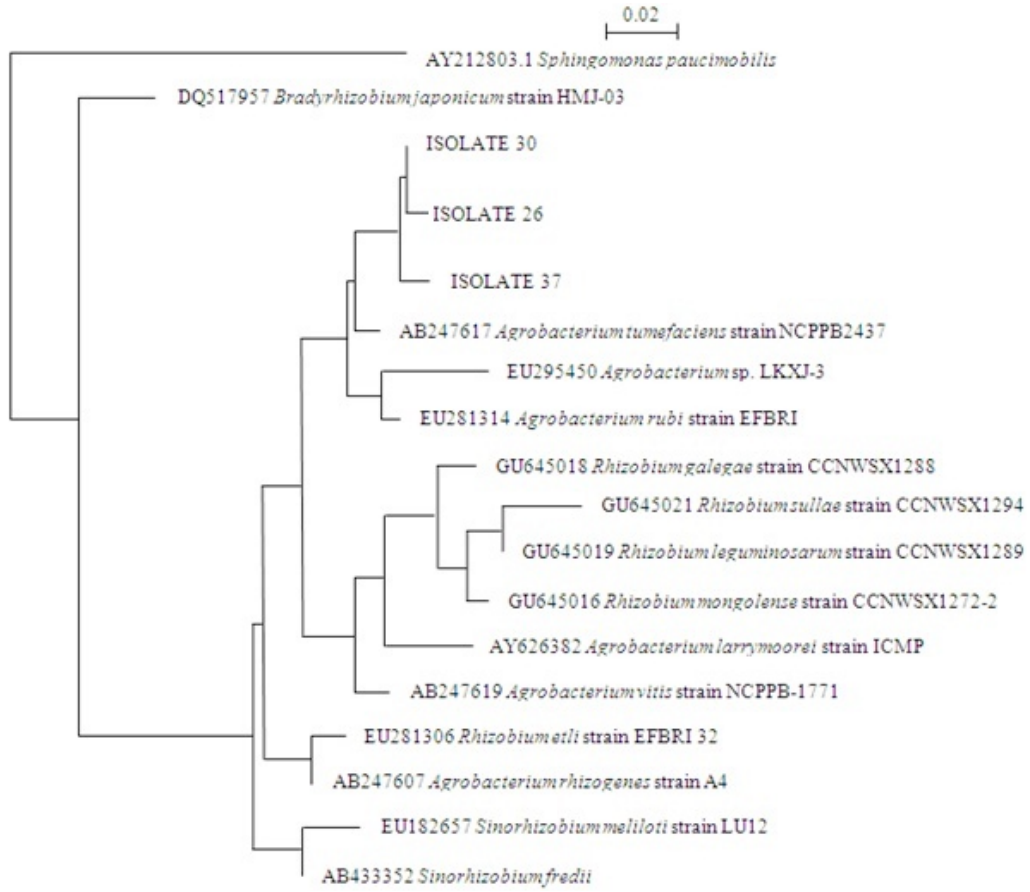

Figure 1: Phylogenetic relationship between the selected isolates (I26, I30, and I37) with published 16S rDNA sequences of 14 closely related members of Rhizobiales.
Sphingomonas paucimobilis was used as the out-group.

Genotypic characterization was then employed to confirm the result. Partial sequencing of 16S rRNA genes from isolates I26, I30, and I37 have been done and the results were compared with the sequences of 16S rRNA deposited in GenBank by BLAST search on www.ncbi.nih.nlm.gov/Blast.cgi. The 16S rRNA sequences of the isolates were highly homologous to *Agrobacterium tumefaciens,* with greater than 99% sequence identity. To confirm the position of the isolates in phylogeny, a number of representative *Agrobacterium* strains were selected for construction of a phylogenetic tree, using *Sphingomonas paucimobilis* as the out-group (Figure **1**).

Various species of bacteria like *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance the plant growth [35]. However, there have been no reports before indicating that *Agrobacterium* can improve plant growth and their ability for heavy metal uptake.

CONCLUSIONS

Thirty nine of bacterial strains were isolated from contaminated soil of the leather tanning industrial waste for their possible use in Cr(VI) bioremediation. The inoculation of rhizobacteria I26, I30, and I37 increased chromium uptake and accumulation in the maize by seven to 14 times higher than the uninoculated one, suggesting the potential use of these strains as effective agents to assist phytoremediation. Isolate I26, II30, and I37 have a close relationship with *Agrobacterium tumefaciens*.

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