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Heavy Metal Removal by Phosphate Solubilizing *Acinetobacter calcoaceticus* Isolated from Rhizosphere

Fateme Ghoreishi, Zahra Etemadifar*

Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran, P.O.Box: 81746-73441

*Correspondence should be addressed to Zahra Etemadifar, Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran, P.O.Box: 81746-73441; Tel: +983137932367; Fax: +983137932456; Email: z.etemadifar@sci.ui.ac.ir.

ABSTRACT

Worldwide industrial activities produce large amount of metal wastes. Metals remediation by microbial activities associated with phosphorus metabolism is a promising method. The goals of this study were the removal of some heavy metals by isolated phosphate solubilizing rhizobacterium (PSR) and survey the effect of total secreted materials including extracellular phosphatase on this phenomenon. PSR strain was isolated by Pikovskaya (PVK) medium and identified by 16S rDNA sequencing. Its phosphatase enzyme was assayed by colorimetric method. Metal resistance of the isolate was measured by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The metal removal was detected by atomic absorption spectroscopy. The culture supernatant was analyzed for metal removal by secreted phosphatase enzyme with or without tri-calcium phosphate (TCP), as its substrate. The isolated rhizobacterium was identified as *Acinetobacter calcoaceticus* that produced acid phosphatase enzyme. The MIC₅₀ were obtained 2.5 mM Pb(II), 2 mM Ni(II), 0.2 mM Cr(II) and <1 mM Cd(II); and the MBCs were >100 mM Ni(II), 40 mM Cd(II), 20 mM Pb(II), and 1 mM Cr(II). The removal of metals by the cells were obtained 29.45% Cr(II), 25.74% Pb(II), 18.85% Cd(II) and 11.43% Ni(II). It was not seen any nickel removal by microbial secreted products in PVK medium without TCP, whereas removal was obtained in PVK complimented by TCP 28.3%; lead removal was almost the same in both media (99%) and was not related to TCP.

Key words: Rhizobacterium, Bioremediation, Lead, Cadmium, Phosphatase enzyme.

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1. INTRODUCTION

Worldwide industrial activities produce large amount of organic and inorganic wastes that result in soil and groundwater contamination. Metals are the most concern due to their mobility and long-term persistence in environment. Abiotic remediation methods are too expensive, but metals bioremediation by microbial activities associated with phosphorus metabolism is a promising method (1). Cadmium is a toxic heavy metal poisoning risks to human health that has any physiological function within the human body and can lead to kidney, bone, and pulmonary damages. This metal is widely used in industrial processes, e.g.: as a stabilizer in PVC products, a neutron absorber in nuclear power plants, as an anticorrosive agent, as a color pigment, in the fabrication of nickel-cadmium batteries and in phosphate fertilizers (2). During the last century, lead emission to the air is mainly provided by petrol. Children are the most

susceptible to lead due to high gastrointestinal uptake and permeable blood-brain barrier. Lead in blood is bound to erythrocytes and accumulated in the skeleton by half-life about 1 month and 20-30 years respectively. The symptoms of lead poisoning are headache, irritability, abdominal pain and symptoms related to the nervous system, e.g.: sleeplessness and restlessness (3). Epidemiological investigations showed reliable data indicating the relation between nickel exposure and lung cancer risk. Nickel can cause chromosome damage both in vitro and in vivo and is found to induce DNA damage. The mechanism of nickel carcinogenesis by immunological studies is suggested the suppressive effect of nickel on Natural Killer cells activity and interferon (4). Hexavalent chromium is the greatest occupational and environmental health concern. Inhaling of some forms of Cr(VI) can cause symptoms such as a runny nose, sneezing, itching, nosebleeds, ulcers, and holes in the nasal septum and

dermal exposures may cause skin ulcers or allergic reactions (5). Recently it has been shown that microorganisms which hydrolyze organophosphate compounds and liberate orthophosphate represent a unique method to phosphate bioremediation of heavy metals (1). One of the enzymatically mediated methods for removal of Uranium is adding Glycerol-2-phosphate to Uranium contaminated water, and then treatment with *Citrobacter* spp. contained phosphatase activity. This enzyme liberates phosphate from Glycerol-2-phosphate and makes Uranium-phosphate sediment at the cell surface (6). Phosphatase enzymes are classified to acid and alkaline phosphatases that non-specific acid phosphatases (NSAPs) have three classes, A, B and C. ⁷ In addition to phosphorus liberation; other roles of NSAPs are regulation of cellular metabolism, involvement in signal-transduction and bacterial virulence (1). *Acinetobacter* is Gram-negative bacterium that is isolated from soil and water as free-living saprophyte (7). Van Groenestijn (8) in 1988 detected polyphosphatase activity in *Acinetobacter* spp. which was able to degrade native polyphosphate and was strongly stimulated by 300 to 400 mM NH₄Cl. *A. haemolyticus* was isolated from heavy metal contaminated wastewater by Zakaria et al (9) that can remediate Cr(VI) contamination in water system, but the mechanism of the removal was not studied. Moreover a strain of phosphate-releasing *A. johnsonii* was isolated from a wastewater treatment plant that was used to remove La³⁺ via precipitation of cell-bound LaPO₄ (10). The goals of this study were the removal of heavy metals (including: lead, nickel, cadmium and chromium ions) by isolated PSR, *A. calcoaceticus*, and the effect of its total secreted products include extracellular phosphatase on metal bioremediation.

2. MATERIALS AND METHODS

2.1. Materials

All materials were prepared from Merck and Sigma Companies. For isolation and screening of phosphate solubilizing bacteria, PVK agar medium was used which is containing (g l⁻¹): Glucose 10, MgSO₄.7H₂O 0.1, KCl 0.2, FeSO₄.7H₂O 0.002, Ca₃(PO₄)₅ 5, (NH₄)₂SO₄ 0.5, and yeast extract 0.5. Loria Bertani (LB) agar containing (% w/v): yeast extract 0.5, NaCl 1, tryptone 1, pH=7 by 0.1 M NaOH was used for preparation of bacterial culture and molecular identification. For DNA extraction cetyl trimethyl ammonium bromide (CTAB) /NaCl solution containing: CTAB powder 10 g, and 80 ml of NaCl 0.7 M was prepared. TE buffer 10X containing: 10 ml of Tris-HCl 1 molar pH=8, 0.5 ml of EDTA 0.5 M pH=8, and 88 ml deionized water was prepared. Other solutions for DNA extraction include: SDS 10%, Proteinase K 20 mg/ml, NaCl 5 M, Chloroform/Isoamyl alcohol mixture 24/1, Isopropanol, NaCl 3 M, ethanol 70%. Potassium hydroxide (KOH) 3% solution and oxidase disk were used for catalase and oxidase tests, respectively. The concentration of 400 mM stock solutions of each metal salt (NiCl₂, PbNO₃, CdCl₂, K₂Cr₂O₇) were prepared in distilled water.

PCR reaction components, which were purchased from Fermentas co., include: PCR buffer, dNTPs, MgCl₂, Taq DNA polymerase. Universal Primer pair, 27F and 1492R were constructed by Takapouzist and used for 16S rDNA sequencing. Solutions used for phosphatase assay include: p-nitrophenyl phosphate (pNPP) 7.6 mM, acetate buffer 50 mM pH=5.5, concentrated NaOH, and Tris (pH 10) 100 mM.

2.2. Apparatus

The instruments used in this microbiological study were include: Incubator (Memmert; Germany), Autoclave (Iran; Tolid), Shaker (Bottmingen, Switzerland), Spectrophotometer (Eppendorf Biophotometer, Germany), Microtiter-plate (cell culture model), 0.45 µm millipore filter (Orange scientific; Spain), ELISA reader (Stat-fax), and Centrifuge (Eppendorf, Germany). Thermal cycler (Eppendorf 5331, Germany), Gel documenter (Biometra; Germany), and Power supply (Nogen Pars; Iran) were used for molecular identification. Analytical instruments include: Atomic absorption spectroscopy machine (Analyst 300 model machine, PerKinElmer; USA) for heavy metal analysis, and FTIR machine (JASCO model 6300; Japan) for infrared spectroscopy.

2.3. Organism

The soil adhering to the roots of *Brassica oleracea* was separated by gentle tapping, using sterilized forceps and stored in 4°C. After sifting, samples of 0.1 gr soil was suspended in 5 ml of 0.9% sterilized NaCl solution and after shaking for 30 min in 150 rpm, 0.1 ml of each serial dilution (1:10⁻⁵) was spread on Pikovskaya's (PVK) agar containing tri-calcium phosphate (TCP) as the insoluble phosphate substrate, incubated for 3 days at 30°C. Colonies with clear halo were marked positive for phosphate solubilizing (11).

2.4. Biochemical and molecular identification of bacteria

PSR isolate was cultured on Loria Bertani (LB) agar and was characterized for Gram staining and biochemical analysis. The isolated PSR was also tested for catalase (12) and oxidase (13). The genomic DNA of the isolated PSR was extracted by CTAB method (14). The identification of PSR was done based on 16S rDNA sequencing, using two general primers 27F and 1492R in Polymerase Chain Reaction (PCR) by Thermal cycler Eppendorf 5331. The total PCR reaction mixture was 50 µl comprising 2.5 µl PCR buffer, 0.5 µl dNTPs, 1.2 µl MgCl₂, 0.2 µl Taq polymerase, 1 µl from each primer and 3 µl genomic DNA. The thermocycling conditions involved an initial denaturation at 95°C for 5min, followed by 30 cycles of 94°C for 30sec, 64°C for 45sec, and 72°C for 70sec and final extension at 72°C for 5min (15). The 16S rDNA partial sequence was analyzed using the BLASTn (<http://www.ncbi.nlm.nih.gov>) search algorithm and aligned to the nearest neighbors.

2.5. Phylogenetic tree

In order to studying the ancestor of strain, phylogenetic tree was drawn via Mega6 software according to the 16srDNA sequencing, by neighbor-joining method.

2.6. Growth curve of the isolated PSR strain

In order to check the time of logarithmic growth of the PSR strain in liquid medium (LB), growth curve was drawn. The amount of 1 ml culture suspension with an optical density at 600nm (OD₆₀₀) of 0.08 to 0.13 was added to 99 ml of sterile medium and incubated at 30°C with 180 rpm shaking for 48 hours. The optical density was read at 600 nm every two hours. The growth curve obtained by Excel 2013 software (16).

2.7. Heavy metals resistance

Microtiter-plate was used to measure the bacterial resistance in nickel, lead, chromium and cadmium. A 400 mM stock of each metal salt (NiCl₂, PbNO₃, CdCl₂, K₂Cr₂O₇) was prepared and filtrated by 0.45 µm millipore filter. The wells contained 100µl of sterilized LB broth plus 50µl heavy metals at concentrations ranges from 0.5 to 100mM for Ni, 0.1 to 100mM for Cr, and 1 to 100mM for Pb and Cd. Each well was inoculated with a 50µl of overnight PSR culture with an optical density at 600nm (OD_{600nm}) of 0.08 to 0.13. After shaking for 1 h in 100 rpm, they were incubated at 30°C for 48 h (17). Growth was monitored at OD_{600nm} using a microtiter plate spectrophotometer. Each experiment was performed in triplicate and negative control (without bacterial cells) was prepared for each metal. Then minimal inhibitory concentration (MIC₅₀) for each metal salt was measured. In order to measure the minimal bactericidal concentration (MBC) 10 µl of wells that had no sensible growth was spread on the LB agar medium and incubated at 30°C for 72h. The lowest concentration of each metal was required to kill the bacterial cells is MBC (18).

2.8. Preparation of supernatants

Briefly, supernatants from 3 day's PSR cultures (explained above) were obtained by centrifugation at 6000 rpm for 20 min (19).

2.9. Phosphatase enzyme assay

Phosphatase activity was measured as the ability of the supernatant to release p-nitrophenol (pNP) from p-nitrophenyl phosphate (pNPP) as the substrate. For acid phosphatase assaying, bacterial supernatant was incubated with 7.6 mM pNPP in 50 mM acetate buffer (pH 5.5) at 37°C. After the appropriate incubation at 37°C, because pNP is colorless at acidic pH, concentrated NaOH was added to the reactions, and the production of pNP was monitored at 410 nm. For determining the alkaline phosphatase activity, the pNPP hydrolysis reactions were performed in 100 mM Tris (pH 10) (20).

2.10. Inductive or potentially phosphatase enzyme production

Two media were prepared: PVK in dissolved phosphate limited condition and PVK plus 2 g/l K₂HPO₄ in rich dissolved phosphate state. The isolated PSR was cultured in both media and incubated at 30°C for 72h with 180 rpm shaking. Phosphatase activity in both media was assayed as explained at previous section.

2.11. Metal removal by the isolated PSR

Cells of 72h cultured PSR in PVK broth were separated by centrifugation at 11000 ×g for 20 min and washed twice by 0.9% sterilized NaCl solution. Mixture contained PSR cells with an optical density at 600nm (OD_{600nm}) of 0.7, 50 mM glucose, 100 mM Tris-HCl buffer and 2 mM of each salt metal (pH=7) prepared and incubated at 30°C for 10h with 170 rpm shaking. The amount of remained metals ions in the culture supernatant was measured by atomic absorption spectrometry using an Analyst 300 model machine (PerKinElmer-USA) after the samples were digested with concentrated nitric acid (21, 22). The culture media including each metal separately without bacterial cells also was analyzed as controls.

2.12. Metal removal by extracellular products of the isolated PSR

PVK broth medium was inoculated by isolated PSR strain and incubated at 30 °C for 3 days. Then it was centrifuged at 6000 rpm for 20 min. The supernatants include crude secreted materials incubated for 10h at 30°C with 170rpm shaking after adding two different metal solutions, one with TCP as phosphatase substrate and other without it. The remained metal in each culture supernatant was measured by atomic absorption spectrometry using an Analyst 300 model machine (PerKinElmer-USA) after the samples were digested by concentrated nitric acid (20, 21). Negative control, without bacterial supernatant also was analyzed as blank sample.

2.13. Statistical analysis

All statistical analysis was prepared by IBM SPSS Statistic ver. 20.0 for Windows. The One-Way ANOVA test was used to survey the significance of the results.

3. RESULTS AND DISCUSSION

3.1. Bacterial identification

The isolated PSR strain was a Gram-negative, non-motile bacterium with coccobacilli morphology, and showed catalase positive, oxidase negative and non-reductive nitrate characteristics. The result of 16S rRNA gene amplification for molecular identification of the isolated strain is shown in Figure 1. The 1500 bp DNA band represents the PCR produced by 27F and 1492R general primers.

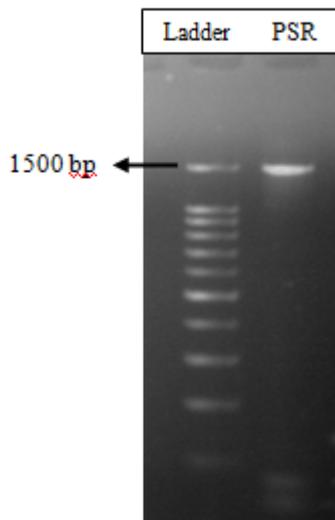


Figure 1. Gel electrophoresis of 16S rRNA gene amplification of SSC2 strain by Universal primers (27F and 1492R)

The edited sequence of the PCR products is shown in Figure 2 (https://www.ncbi.nlm.nih.gov/nucleotide/KU685396). The result of the BLAST search of the 16S rRNA gene

sequences indicated that the isolate was closely related to *A. calcoaceticus* with 99% similarity (Table 1). This bacterium is part of the normal human intestinal flora and also is a soil bacterium.

GTCGAGCGGAGTGATGGTGCTTGCACTATCACTTAGCGGGACGGGTGAGTAATGCTTAGGAATCTGCC
TATTAGTGGGGGACAACATTTGAAAAGGAATGCTAATACCGCATACTGCTACGGGAGAAAGCAGGGGAT
CTTCGGACCTTGCCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGG
CGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG
GCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGTGGACGT
TACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGTAATACAGAGGGTGCAAGCGTTAAT
CGGATTTACTGGGCGTAAAGCGCGCTAGGCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTT
GGGAATTGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAA
ATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTGCG
AAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGTCTACTAGCCGTTG
GGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACT
AAAAC TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAATTCGATGCAACGCGAA
GAACCTTACCTGGGCCTTGACATAGTAAGAACTTCCAGAGATGGATTGGTGCCTTCGGGAACCTTACATA
CAGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGGTAGTCCCGCACGAGCGCAACCCTTT
TCCTTATTTGCCAGCGAGTAATGTCGGAACCTTAAGGAATACTGCCAGTTGACAAACCTG

Figure 2. *Acinetobacter calcoaceticus* strain SCC2 16S ribosomal RNA gene, partial sequence, KU685396

Table 1. Results of the 16S rDNA sequence BLAST at NCBI

Accession	Identity	E value	Query cover	Total score	Max score	Description
NR 117619.1	99%	0.0	99%	1911	1911	<i>Acinetobacter calcoaceticus</i> strain ATCC 23055 16S ribosomal RNA gene, complete sequence
NR 113343.1	99%	0.0	99%	1911	1911	<i>Acinetobacter calcoaceticus</i> strain JCM 6842 16S ribosomal RNA gene, partial sequence
NR 114958.1	99%	0.0	99%	1911	1911	<i>Acinetobacter calcoaceticus</i> strain ATCC 23055 16S ribosomal RNA gene, partial sequence
NR 114922.1	99%	0.0	99%	1911	1911	<i>Acinetobacter calcoaceticus</i> strain CIP 81.8 16S ribosomal RNA gene, complete sequence

NR 114921.1	99%	0.0	99%	1911	1911	Acinetobacter calcoaceticus strain LMG 1046 16S ribosomal RNA gene, complete sequence
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According to the phylogenetic tree (Figure 3), the nearest strain to the isolated PSR strain SCC2 sequence is *A. calcoaceticus*, which confirmed molecular detection. The

identified sequence was deposited in NCBI GenBank by accession no. KU685396.

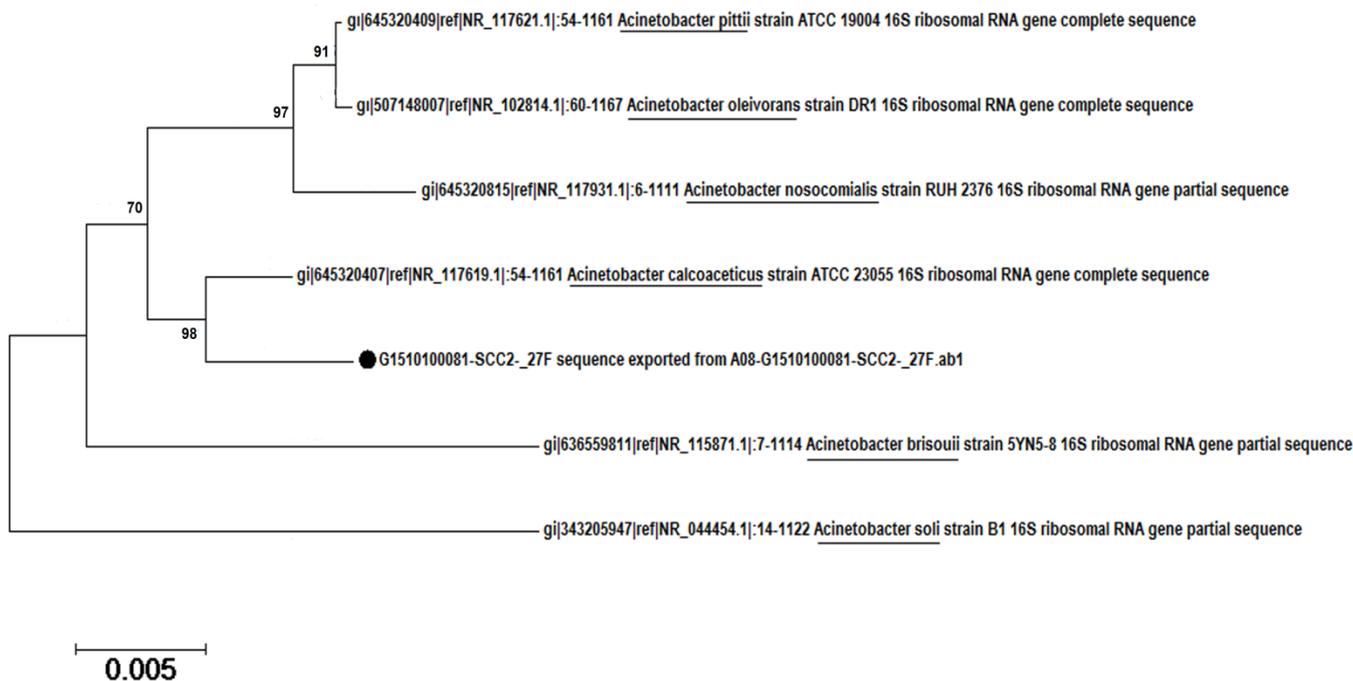


Figure 3. Phylogenetic tree of isolated strain based on 16S rDNA sequence draw with Mega 6 software; Isolated strain in this study is shown with solid circle (SCC2)

3.2. Growth curve of the isolated strain SCC2

Growth curve of the PSR was shown in Figure 4. Logarithmic growth of the PSR strain SCC2 prolonged

within 10-50 hours, generation times. The LB medium without any inoculation was used as blank medium with no increase in optical density during the incubation time.

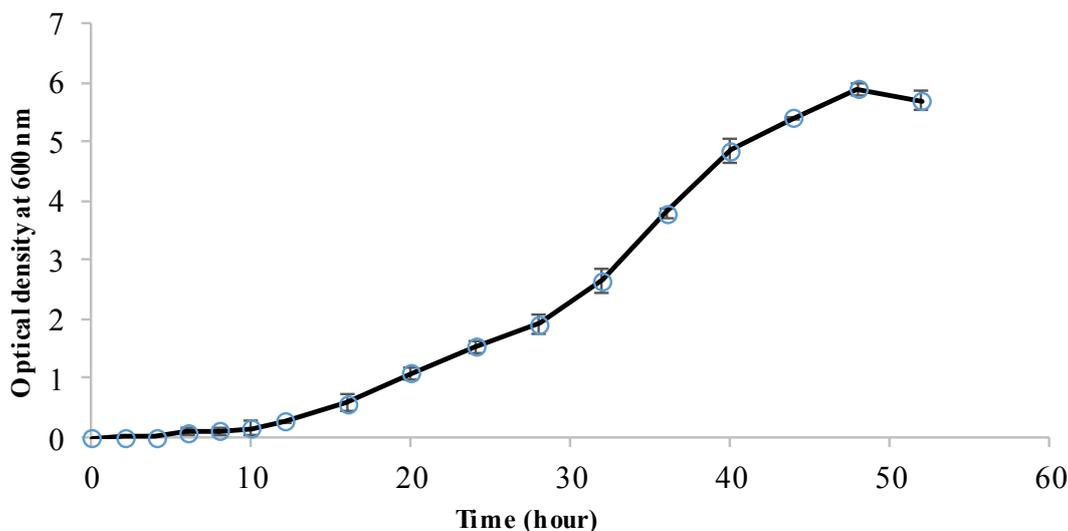


Figure 4. Growth curve of isolated *Acinetobacter calcoaceticus* SCC2 in 50 ml LB broth, the flask with a capacity of 250 ml incubated at 30 ° C with 170 rpm shaking

3.3. MIC and MBC of metals for SCC2 strain

The minimal inhibitory concentration (MIC) of heavy

metals ions in LB broth medium inoculated by the cell suspension at logarithmic phase were 2 mM Ni(II) (Figure 5a), 0.2 mM Cr(II) (Figure 5b), 2.5 mM Pb(II) (Figure 5c), and <1 mM Cd(II) (Figure 5d). The order of the toxicity of

the metals to strain *A. calcoaceticus* was found to be Cr(II) > Cd(II) > Ni(II) > Pb(II). MICs of nickel, chromium, lead and cadmium for PSR strain are shown in Table 2.

Table 2. MIC and MBC of metals for isolated PSR

Metal	MIC50 (mM)	MBC50 (mM)
Nickel	2	>100
Chromium	0.2	1
Lead	2.5	20
Cadmium	<1	40

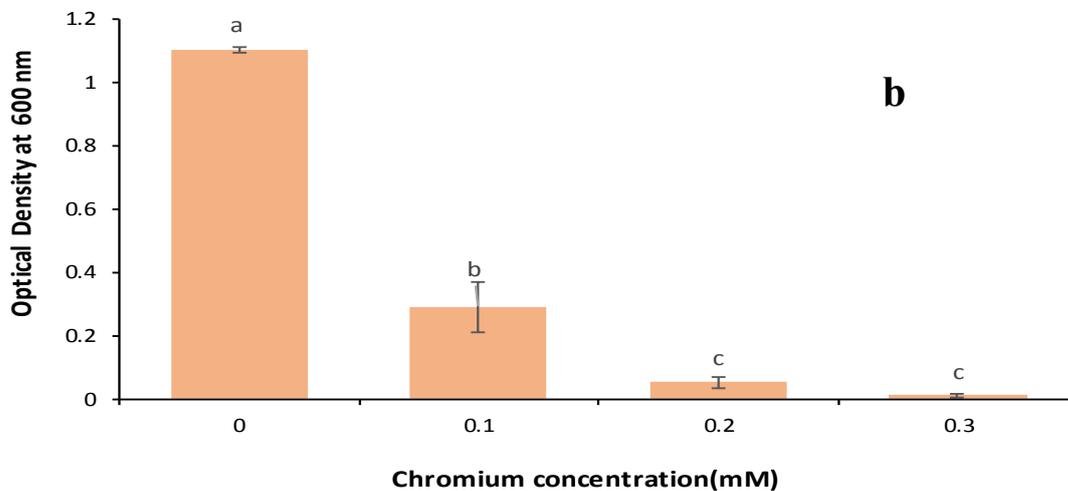
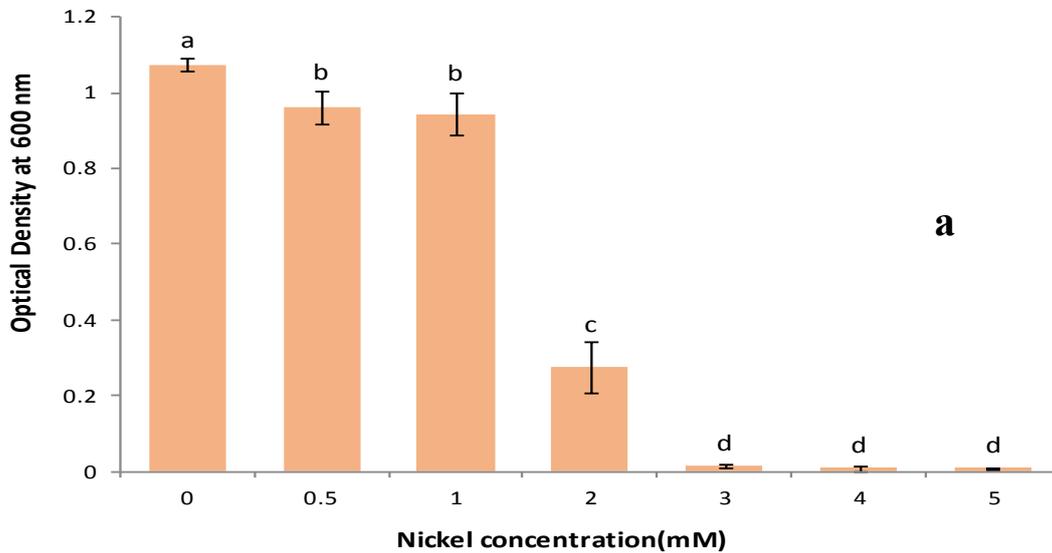
MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration

The minimal bactericidal concentration (MBC) of the metals in the liquid LB medium containing heavy metal ions is shown in Table 2. The order of the toxicity of the metals to *A. calcoaceticus* strain SCC2 was found to be Cr(II) > Pb(II) > Cd(II) > Ni(II).

production

Phosphatase assay in two media with or without soluble phosphate (K_2HPO_4) source was obtained the same. These results indicated that enzyme production by SCC2 strain was non-inducible and this bacterium produced phosphatase in the presence or absence of soluble phosphate.

3.4. Inductive or potentially phosphatase enzyme



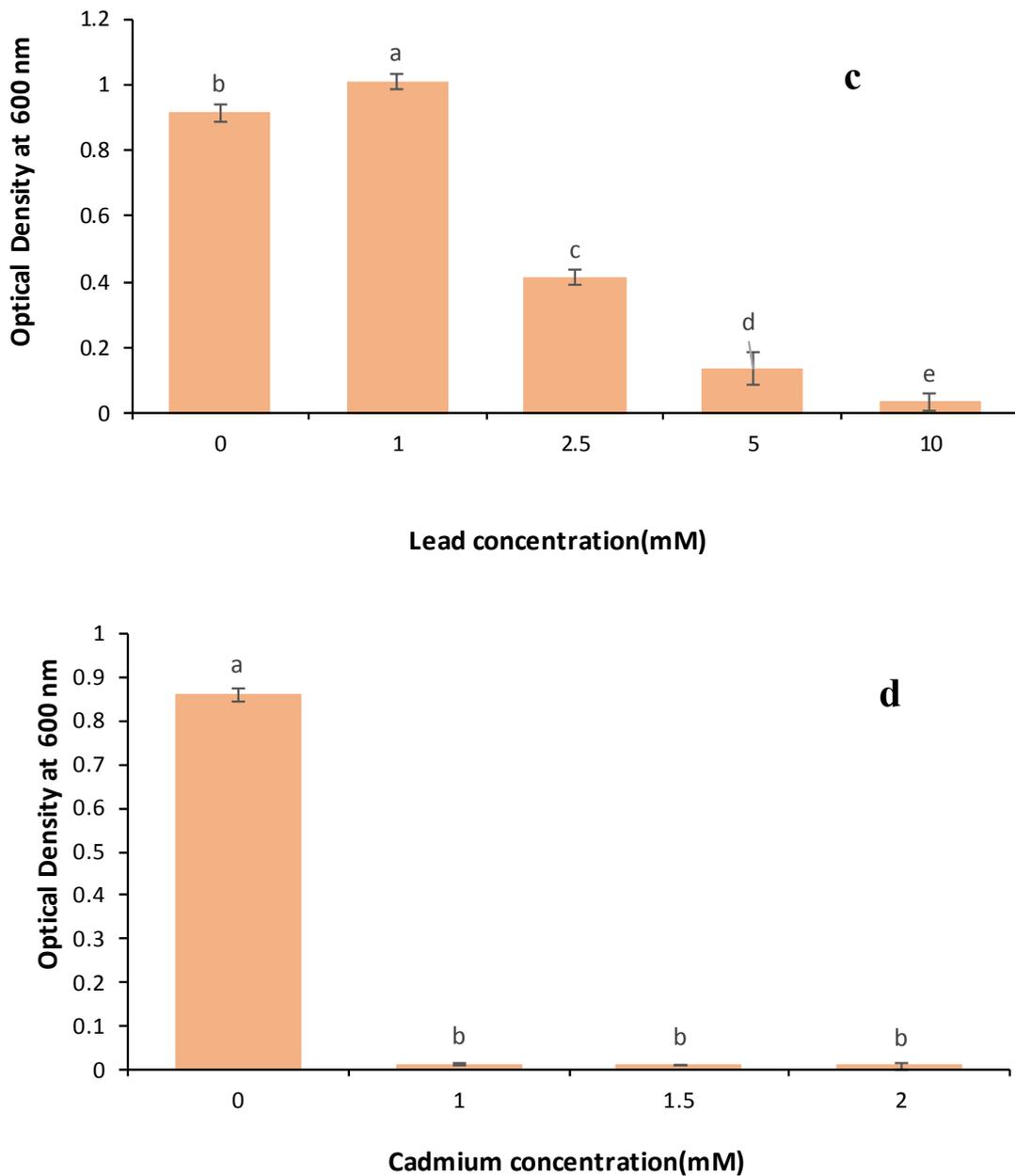


Figure 5. Resistance of *Acinetobacter calcoaceticus* SCC2 to heavy metals, in LB broth medium incubated at 30°C for 48 hours. (a) NiCl₂, (b) K₂Cr₂O₇, (c) PbNO₃, (d) CdCl₂. All values are mean of three replicates. Error bars represent ± standard deviation (SD). Bars with different letters show significantly (p < 0.05) different (Duncan's test)

3.5. Metal Removal by Cells of the isolate

According to the Figure 6, the heavy metal removal percentage by the cells of *A. calcoaceticus* SCC2 was 29.45% (Cr(II)), 25.74% (Pb(II)), 18.85% (Cd(II)), and 11.43% (Ni(II)). The order of the removal percentage by

the cells was Cr(II) > Pb(II) > Cd(II) > Ni(II). Although the resistance of isolated *Acinetobacter* to chromium salt was lower and nickel salt was higher than other metals, but the removal percentage was reverse so that it was 29.45% for chromium and just 11.43% for nickel.

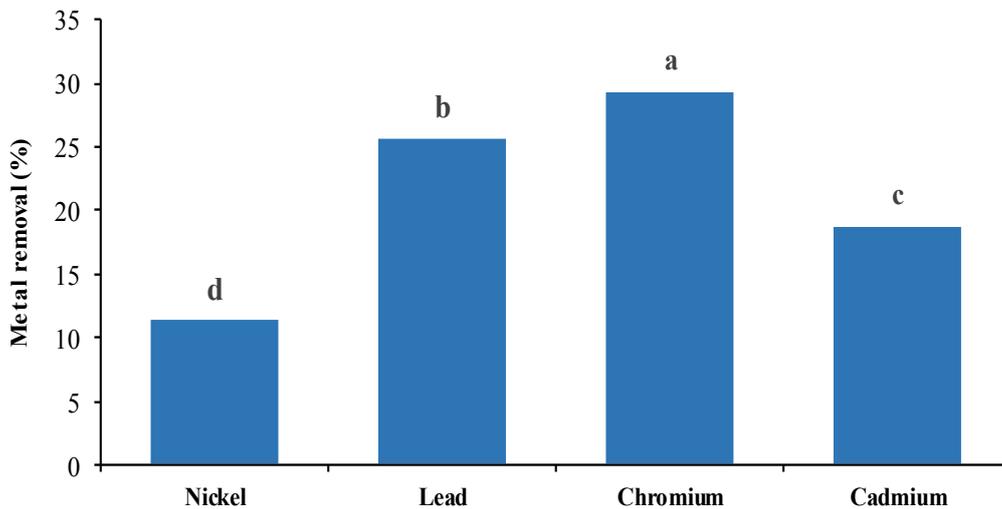


Figure 6. Metals removal by *Acinetobacter calcoaceticus* SCC2 cells ($OD_{600nm} = 0.7$), during 10h, at 30°C and 170 rpm shaking. Dissimilar letters indicate a significant difference between variables

3.6. Metal Removal by extracellular secreted products

As shown in Figure 7, the removal of nickel by crude extracellular secreted products was 0% in PVK medium without TCP and 28.3% in PVK broth medium

complimented by TCP as phosphatase substrate (higher than removal by the cells of the isolated strain). However, lead salt removal in both media was almost the same.

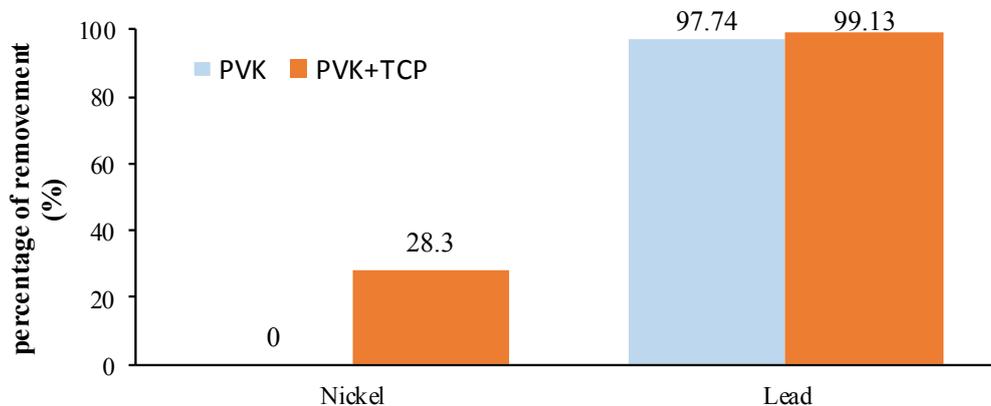


Figure 7. Removal of nickel and lead ions by extracellular secreted materials of *Acinetobacter calcoaceticus* SCC2 in PVK medium with or without tri-calcium phosphate incubated at 30°C for 10h with 170 rpm shaking

Acinetobacter is Gram-negative rod bacterium that is isolated from soil and water as free-living saprophyte (7). *A. haemolyticus* was isolated from heavy metal contaminated wastewater by Zakaria (9) that could remediate Cr(VI) contamination in water system, but the mechanism of the removal was not studied. In addition a strain of phosphate-releasing *A. johnsonii* was isolated from a wastewater treatment plant that was used to remove La^{3+} via precipitation of cell-bound $LaPO_4$ (10). In other studies, chromate (VI) removal from tannery effluent by *Acinetobacter* sp. was indicated (23), and hexavalent chromium was removed by *Acinetobacter* sp. in a chromium-contaminated activated sludge (24). Shakibaie et al. identified a silver (Ag) resistant *A. baumannii* BL54 that was isolated from clinical samples and removed silver from photographic wastewater effluent (25). Moreover, a

biofilm producing rhizobacterium *A. calcoaceticus* P23 with the ability of phenol degradation reported by Yamaga et al. (26). In this study, PSR that was isolated from the roots of *Brassica oleracea*, was identified as *A. calcoaceticus* strain SCC2. According to the phosphatase assay in two media, with or without phosphorus source, phosphatase production by this strain was potential and was not inducible by phosphate limitation. Because phosphorus is a growth limiting factor, phosphate solubilizing bacteria (PSB) with the ability to dissolve insoluble phosphate complexes, provide absorbable and utilizable forms of phosphate for plants and could be used as bio-fertilizer in sustainable agriculture (27). Low turbidity ($OD_{600}=0.7$) of *A. calcoaceticus* SCC2 cells removed approximately 30% of 2mM $K_2Cr_2O_7$ (containing 208 mg Cr(VI)), while in the study by Zakaria (9) less

quantities of Cr(VI) removed by their isolate, so that 1 gram dry weight of *A. haemolyticus* cells removed about 38% of Cr(VI) from the 100 mg/l suspension of the metal. With comparison of two mentioned studies and according to the enriched medium in Zakaria study (nutrient broth with glucose) it may be concluded that *A. calcoaceticus* SCC2 isolated in our study is more efficient than *A. haemolyticus* in the removal of chromium. High MBC of Cd(II) (40 mM) against low MIC (1 > mM), and high MIC of Pb(II) (2.5 mM) unlike low MBC (20 mM) show that the metal resistance changes in the course of the time. Higher removal percentage of chromium in contrast to its lower MIC suggested that the removal percentage of this metal is not related to its MIC. The removal agent for Ni(II) was probably the secreted phosphatase enzyme in the bacterial supernatant, since the nickel was removed just in the presence of the phosphatase substrate (TCP). Unlike nickel, the removal of lead in both medium, with or without insoluble phosphatase substrate (TCP) were similar, suggesting that the other bacterial secreted products such as polysaccharides, proteins, nucleic acids, siderophores and so on were effective in the metal removal process (28, 29). In addition, the isolated *Acinetobacter* cells in this study could remove nickel and lead from metal suspensions that show the role of cells in the intracellular metal accumulation or precipitation at the cell surface. On the other hand, biosurfactant (emulsan) production by *A. calcoaceticus* was reported previously that is applicable in metal bioremediation by this bacterium (30).

4. CONCLUSION

A. calcoaceticus is a suitable strain for heavy metal bioremediation and its phosphatase enzyme probably plays role in nickel removal. This bacterium can be a suitable candidate for bioremediation of heavy metals because of its more performance in metal removal than other strains. Role-playing of cell surface and secreted products maybe upgrade the metal removal in this strain. In addition, the rhizobacteria with the ability of metal bioremediation can be used as bio-fertilizer to promote sustainable farming along with the ability to remove heavy metals and degrade the pollutants in the rhizosphere.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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