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BACTERIAL Cr (VI) REDUCTION AND ITS IMPACT IN BIOREMEDIATION

Ali Pramono[(1)], MMA Retno Rosariastuti [(1,2)], Ngadiman [(1,3)]Irfan D. Prijambada[(1,4)]

[(1)]Indonesian Agricultural Environment Research Institute (IAERI), Email: ali_pramono@yahoo.com
[(2)]University of Negeri Sebelas Maret, Surakarta
[(3)] University of Gadjah Mada, Yogyakarta

ABSTRACT

Chromium is hazardous pollutant for ecosystem caused chromium especially in hexavalent form is very toxic, has high solubility and mobility, teratogenicity, mutagenicity and carcinogenicity to living system related with its oxiding power. Remediation of soil contaminated of heavy metals was important caused soil as medium for food producing. Conventional methods for heavy metals remediation consist of physical and chemical process but these applications were costly and less effective. One of the remediation technologies is the using living organisms such as microorganisms, because they have ability to reduce Cr(VI) into non toxic form, Cr(III). The aims of this research were to evaluate the reduction activity of rhizobacterial isolate and to identify the isolate which take a role in reducing chromium absorption by plant. The results showed that Isolate 39 was able to grow on LB medium containing 200 ppm Cr(VI). Isolate 39 reduced Cr(VI) up to 15 ppm concentration level in minimal medium. Isolate 39 has ability to reduce Cr(VI) both at growing cells and resting cells conditions up to 100% and 51% within 18 hours, respectively. Isolate 39 increased the phytostabilization ability of chromium by Zea mays at 30 days after seeding 3.8 times compared than control. Based on physiological characteristics and partial sequencing of 16S rRNA gene, Isolate 39 was identified as Agrobacterium sp.

Key words **:** *Agrobacterium sp, hexavalent chromium, reduction, Zea mays*

ABSTRAK

Reduksi Cr(VI) oleh Bakteri dan Manfaatnya dalam Bioremediasi. *Kromium, terutama dalam bentuk heksavalen sangat berbahaya di lingkungan karena sifatnya sangat toksik, mempunyai kelarutan dan mobilitas yang tinggi dan bersifat teratogenik, mutagenik dan karsinogenik terhadap makhluk hidup. Remediasi tanah tercemar logam berat sangat penting dilakukan karena tanah sebagai media penghasil pangan. Metode konvensional untuk remediasi logam berat meliputi proses fisika dan kimia tetapi cara-cara tersebut memerlukan biaya mahal dan kurang efektif. Salah satu teknologi remediasi adalah dengan menggunakan mikroorganisme, karena kemampuannya dalam mereduksi Cr(VI) menjadi bentuk yang kurang/tidak toksik, Cr(III). Tujuan penelitian ini adalah untuk mengetahui kemampuan reduksi isolat bakteri dan mengidentifikasi bakteri yang berpengaruh dalam penyerapan logam berat oleh tanaman. Hasil penelitian menunjukkan bahwa Isolat 39 mampu tumbuh dalam medium LB yang mengandung Cr(VI) sebesar 200 ppm dan dapat mereduksi Cr(VI) dalam medium minimal dengan kadar 15 ppm. Isolat 39 mampu mereduksi Cr(VI) baik pada kondisi sel tumbuh dan istirahat masing-masing hingga 100% dan 51% dalam waktu 18 jam. Isolat 39 meningkatkan kemampuan fitostabilisasi logam berat kromium pada tanaman jagung umur 30 hari setelah tanam sebesar 3,8 dibandingkan kontrol. Berdasarkan sifat morfologi dan hasil sekuensing gen 16S rRNA, Isolat 39 diidentifikasi sebagai Agrobacterium sp.*

Kata kunci: Agrobacterium sp, kromium heksavalen, reduksi, Zea may

INTRODUCTION

Chromium is a naturally occurring element in rocks, animals, plants, soil and volcanic dust and gases. Chromium predominantly occurs in the environment in one of two valence states: trivalent chromium (Cr III), which occurs naturally and is an essential dietary nutrient needed for to normal glucose, protein and fat metabolism, and hexavalent chromium (Cr VI), which, along with the less common metallic chromium (Cr 0), is most often produced by industrial processes (ATSDR, 1998). Ferrochrome production industries including are refining, chemical and refractory processing, cement-producing plants, automobile brake lining and catalytic converters for automobiles, leather tanneries and chrome pigments are mostly linked to the atmospheric burden of chromium (USEPA, 1998). There is sufficient evidence that hexavalent chromium compounds are carcinogenetic. These compounds include: calcium chromate, zinc chromate, strontium chromate and lead chromate. Occupational exposure to these hexavalent chromium compounds in chromate production, chromate pigment production and chromium plating industries have been linked to the cancer of the lung, nose and nasal sinuses (Boffetta, 1993).

Chromium ion is most carcinogenic in the form of CrO_2^{-4}, which enters the body cell by sulfate uptake pathway and is ultimately reduced to Cr(III) through a Cr (IV)-glutathione intermediate species. The hexavalent latter complex then binds with the DNA to produce a kinetically inert and potentially damaging lesion and can cause abnormal phenotype due to the formation of ROS (Bertini *et al*., 1998).

Chromium is hazardous pollutant for ecosystem caused chromium especially in hexavalent form is very toxic, has high solubility and mobility, teratogenicity, mutagenicity and carcinogenicity to living system related with its oxiding power (Cervantes *et al*., 2001). Remediation of soil contaminated of heavy metals was important caused soil as medium for food producing. Conventional methods for heavy metals remediation consist of physical and chemical process but these applications were costly and less effective. However, these methods consume high amounts of energy and large quantities of chemical reagents which are not economically feasible. Furthermore, the resultant metal-containing chemical sludge can be a potential source of metal pollution. On the other hand, biological methods such as microbiological detoxification of polluted water are economical, safe and sustainable (Shakoori *et al*., 2000). Bioremediation use microorganisms or another biological system to degrade or transform the pollutant in the controlled condition and, phytoremediation that using plant to remediate. Therefore, improvement of the interaction between plants and beneficial rhizosphere microbes would be an important component of bioremediation technology in agriculture (Glick, 2003).

A number of chromium resistant bacteria, i.e., *Bacillus* sp., *Leucobacter* sp., *Exiguobacterium* sp., *Pseudomonas* sp. (Desai *et al*., 2008), and *Brucella* sp. (Thacker *et al*., 2006), *Clostridium* sp (Nguema and Luo, 2012), *Cellulomonas* sp (Field *et al*., 2013), have been reported to reduce Cr(VI). Bacterial reduction of Cr(VI) can be considered as a mechanism of resistance to Cr(VI) (Ohtake *et al*., 1997). Cr(VI) resistance mechanisms include periplasmic biosorption, intracellular bioaccumulation and biotransformation either via direct enzymatic reaction or indirectly with metabolites (Camargo, 2005).

Bacteria living around the plant root *(rhizobacteria*) have mechanisms that resulting changes of metals ion availability in the soil to become easy or could not absorbed by plant. In this context, microbial reclamation has been a promising aspect using Cr-resistant bacteria to detoxify Cr (VI) in the rhizosphere environment. Cr-resistant rhizobacteria have been isolated from

metals contaminated soil in Desa Sambirembe, Kalasan, Sleman, Yogyakarta. Several of rhizobacteria enhanced Cr absorption in the plant tissue (*called phytoextraction*), while the others decreased Cr absorption (*phytostabilization*) (Rini, 2009), but their mechanisms are still unknown. Cr(VI) resistant strains having such detoxifying ability has raised high hope for cost effective and eco-friendly measures for sustainable agriculture in soil tract contaminated with Cr.

The aims of this research were to evaluate the reduction activity of rizhobacterial isolate and to identify these rhizobacteria which take a role in reducing chromium absorption by plant.

MATERIALS AND METHODS

1. Materials

Rhizobacteria named Isolate 39 derived from Rosariastuti (*Personal Communication*). Three growth media were used in this study (in 1 l of distilled deionized water, DDI): Luria Bertani (LB) broth [10 g of peptone (Oxoid), 5 g of yeast extract (Merck) and 5 g of NaCl (Riedel-de-Haen)], LB agar [10 g of peptone, 5 g of yeast extract, 5 g of NaCl and 15 g of agar (Oxoid)] and Minimal Medium broth [3.3 g $(NH_4)_2SO_4$ (Merck), 8.7 g K_2HPO_4 (Merck), 6.8 g KH_2PO_4 (Merck), 1.23 g $MgSO_4$ (Merck) and 10 g Glucose (Rajkumar *et al*., 2005)].

2. Strains and growth condition

Isolate 39 was inoculated in LB broth containing 10 g peptone, 5 g NaCl, 5 g yeast extract in 1 l distilled water. Cultured this isolate was kept in a room temperature prior to experiment. In order to obtain the highest activity of Cr(VI) reduction, bacterial cell with log phase was used. The culture procedure was described in briefly: a single colony was inoculated into LB medium mentioned above and incubated at 30^{o}C by shaking 12 h with a speed of 125 rpm under aerobic conditions to get bacterial cell with log phase.

3. Tolerance of rhizobacterial isolates to Cr (VI)

The growth of the bacterial colonies on LB agar plates was observed after 24 h of incubation at 37^{o}C. Isolated colonies on the agar slant were streaked on LB agar medium plate containing Cr (VI) at various concentration (6, 10, 15, 20, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, and 600 ppm) using the stock chromium solution. Stock chromium solutions (1,000 ppm) were prepared as follows; 2.829 g $K_2Cr_2O_7$ (294.18 g mol^{-1}, BDH-GPRTM). The MIC is defined as the lowest concentration of Cr (VI) at which a single colony-derived streak could not grow. For bacterial isolate 20 mL LB broth was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 200 uL of the freshly prepared inoculum. These cultures were incubated at room temperature in a shaker at 125 rpm for 48 hours. Growth curves of bacterial isolate were determined with 6, 10 and 15 ppm of Cr (VI) and without chromium (Control). Absorbance was taken at 600 nm wavelength. Growth was plotted graphically.

4. Cr (VI) reduction by rhizobacterial isolates

Sterile minimal medium were aseptically inoculated with 5% of inoculums and incubated aerobically with shaking at 125 rpm. After 48 hours, the cultures spiked with Cr (VI) to a final concentration of 0, 6, 10 and 15 ppm. Cultures were terminated at 120 hours. Each treatment was replicated three times.

5. Cr (VI) reduction by growing cells

Cells at late log phase were inoculated on Minimal Medium, incubated for 48 hours at room temperature by shaking with a speed of 125 rpm and then amended with 15 ppm of Cr (VI). The cultures were incubated at the same condition as before. Media without cells was served as control. During incubation,

suspension in each sample was taken at regular interval and Cr (VI) concentration was measured. Each treatment was replicated three times.

6. Cr (VI) reduction by resting cells

Cells at late log phase were harvested by centrifugation (6,000 g) for 10 min at 4°C, washed twice with 10 mM Tris–HCl (pH 7.0) buffer and re-suspended in the same buffer solution. The cell suspension and supernatant were spiked with chromate at 15 ppm. Equivalent volumes cell suspension and supernatant fluid were assayed. Buffer solutions without cells were served as control. The rest of the procedure was the same as mentioned above for Cr (VI) reduction by growing cells.

7. Analytical methods

The pH of the solutions is determined with making use of digital pH meter (Hanna Instruments, Italy). The growth of the bacteria was monitored by the optical density at 600 nm. Hexavalent chromium was determined spectrophotometrically at 540 nm in the supernatant of the cultures after reaction with S-diphenylcarbazide in acid solution. Hexavalent chromium was determined by the diphenylcarbazide method (Thacker *et al*., 2006): 0.4 ml of solution (0.25 % (w/v) of diphenylcarbazide in acetone) was added to 0.4 ml of sample. 0.33 ml of 6 M H_2SO_4 was used to acidify the samples and adjust the final volume to 10 ml with distilled water. After 10 min of incubation at room temperature, the absorbance at 540 nm was determined by spectroscopy (Cary 50 Varian).

8. Chromium Absorption in Maize Plant

The effect of bacterial isolated treatments for bioremediation on maize (*Zea mays*) plant was studied in small pots containing 5 kg of sand soil under natural condition. After 7 days from the date of seeding, the plants inoculated with Isolate 39 culture and treated with chromium at 6 ppm of Cr (VI) concentration after 7 days. A similar type of experiment was settled for the control (uninoculated) in parallel. Biomass plant were harvested at 30 days after Cr (VI) exposure. The roots and shoots were separated for determining chromium contents. The uptake of chromium in the plant roots and shoots were determined by digestion and measuring chromium content by flame atomic absorption spectrophotometer (Banks *et al*., 2006). Root and shoot samples were extracted for total Cr using perchloric acid digestion method. Exactly 0.5 g of oven dried and ground plant materials was placed in a 125 ml digestion tube, mixed with 4 ml of $HNO_3/HClO_4$, (1:1, v/v) and allowed to stand overnight. The samples were then placed in a digestion block and heated until the digested liquid was clear. The contents in the digestion tube were diluted to 10 ml with deionized water and filtered (filter paper) into 125 ml Erlenmeyer flasks. The filtrates were analyzed by flame atomic absorption spectrophotometer.

9. Identification of bacterial isolated by 16S rRNA gene sequencing

Bacterial genomic DNA was extracted following a previously described method (Ausubel *et al*., 2002). Aseptically transfer 1.5 ml of the overnight culture of bacterial isolate into a sterile 1.5 ml microcentrifuge tube. Centrifuge at top speed (12,000 rpm) for 5 minute in microcentrifuge at room temperature. Aspirate and discard spent medium and add another 1.5 ml of culture to pellet. Centrifuge, discard spent medium, and repeat the procedure with another 1.5 ml of culture. At the last aspiration, leave the pellet as dry as possible but be sure not to aspirate the cells. Resuspend the cell pellet in 410 µl buffer TE solution (0.1 mM Tris-HCl pH8 and EDTA pH8). Add 50 µl of fresh

lysozyme solution with 60 mg/ml concentration, vortex and incubated at 37 ºC for 30 minutes with homogenized each 10 minutes. Add 30 µl SDS 10 % and 10 µl Proteinase K with 20 mg/ml concentration and homogenized, then incubated at 37 ºC for 30 minutes with homogenized each 10 minutes. Add 100 µl NaCl 5 M and 100 µl CTAB 10 % and slowly homogenized, incubated at 65 ºC for 10-15 minutes. Add 600 µl ice cold cloroform, mix 100 times, centrifuged at 12.000 rpm for 5-10 minutes. Three layers will be formed, the upper layer taken carefully and removed into new sterile microcentrifuge tube and add 500 µl ice cold isopropanol, homogenized and incubated for 1 hour in ice storage, and again centrifuged at 12.000 rpm for 5 minutes. Discharged the formed supernatant and added 500 µl ice cold ethanol 70 % to the pellet, incubated for 1 hour in ice storage, and again centrifuged at 12.000 rpm for 5 minutes. After centrifuged, decanted and dried the pellet by letting it stand for about 20 minutes to discharge remain ethanol. Resuspend the pellet in 50 µl of TE buffer.

Polymerase Chain Reaction (PCR) was used to isolation and amplification of 16S rRNA gene from genome DNA bacterial isolates. The genomic DNA was used as template in (10 µl) PCR reactions consisting of mix 5 µl Go Taq Green™, 2 µl distilled water, 1 µl of 10F primer and 1 µl of 1541R primer with 50 pmol/µl concentration. Amplification cycles (30 cycles in total) consisted of initial denaturation step at 94 ºC for 4 minutes, followed by a 2 minutes denaturation step at 94 ºC, a 1 minute annealing step at 50 ºC, and a 5 minutes elongation step at 72 ºC and a final extension step at 72 ºC. PCR product stored at 4 ºC. Electrophoresis of PCR product used agarose gel 0.8%. If the result of electrophoresis formed clear lane of each well with 1500 bp size, it is noted that gene was readily to be sequenced. The base sequencing of the PCR product was done at Ibaraki University, Japan. The sequence data were aligned and analyzed to identify the bacterium and its closest neighbors using BLAST program (www.ncbi.nih.nlm.gov/Blast.cgi) (Altschul *et al*., 1997) at NCBI database. The phylogenetic tree was constructed using aligned sequences by the neighbor joining using Clustal X and NJ Plot programs.

RESULTS AND DISCUSSION

1. Tolerance of rhizobacterial isolates to Cr (VI)

The Isolate 39 showed maximum tolerance against Cr (VI) at a concentration of 200 ppm on LB agar plates. The growth of the isolates was slightly inhibited by Cr (VI) more than 15 ppm (Figure 1). The isolates exhibited a significantly lower Cr (VI) tolerance in minimal medium than in LB agar. These results indicate that Cr (VI) reduction ability of the bacteria used was not related to their resistance to Cr (VI) on agar medium. Generally, nutrient-rich media with yeast extract and tryptone have been used for determining the Cr (VI) toxicity and its reduction by bacteria. In such media, the real toxicity of Cr(VI) could be masked or underestimated due to complexation of Cr(VI) with organic components (Desai *et al*., 2008; Caravelli *et al*., 2008). Whereas, minimal medium supplemented with glucose at a low level (1%), used in our study, eliminated/ minimized the possible complexation of Cr (VI) with media components and allowed the assessment of the toxicity of Cr (VI) to bacteria more accurately. Furthermore, these results suggest that the actual resistance of the bacteria to a pollutant should be determined preferably in liquid media.

To minimizing complexation the Cr (VI) by medium component and probably evaluate Cr VI) toxicity to bacteria more truthfully, we used minimal medium supplemented with glucose at low level (1%). The result of this experiment showed that isolate culture in minimal medium Cr(VI) at the start of growth could not grow at 6 ppm Cr VI) exposure. Therefore,

Isolate 39 was grown previously for 48 hours to reach the end of exponential phase, then to be spiked Cr (VI) and again incubated to know its effect to cell growth (Figure 1).

Visually, medium without Cr (VI) (control) still show yellowish colour at the end of incubation, while the isolates cultured medium exposure Cr (VI) whitish whereas colour intensity increase as strong as chromium level. Even we did not measure Cr (VI) remain in the culture, the changes liquid colour and raising the amount of cell after 3 days incubation indicated that Cr (VI) have been reduced. Cr (VI) reduction to less toxic Cr (III) is base mechanism for bacterial resistance to Cr (VI) (Cervantes *et al.*, 2001).

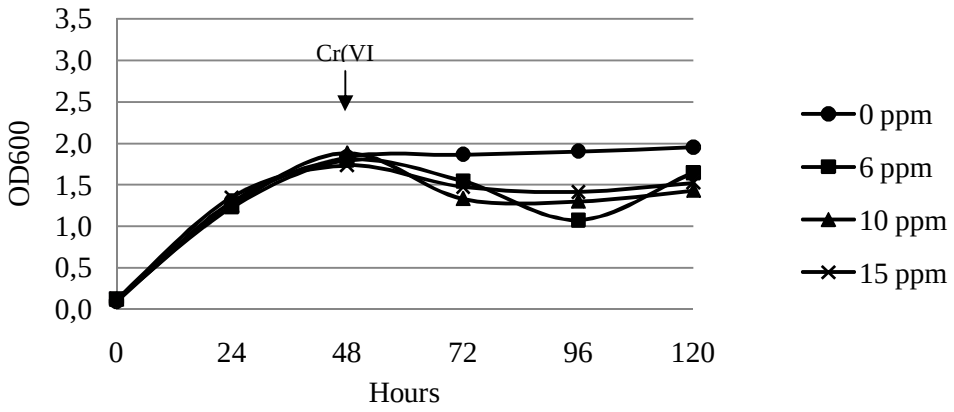

Figure 1
The growth of Isolate 39 on minimal medium at various concentrations of Cr (VI)

2. Cr (VI) reduction by rhizobacterial isolates

Cr (VI) reduction by growing cells. Isolate 39 was grown previously for 48 hours to reach the end of exponential phase, then be spiked Cr (VI) and again incubated. This experiment result indicated that cell growth of Isolate 39 was inhibited by presence Cr (VI) in the minimal medium and Cr (VI) reduction was associated with cell growth (Figure 2). Although the cell amount was decreasing after Cr (VI) exposure, but Isolate 39 could survive and grow after 12 hours. This case showed that Isolate 39 could tolerate Cr (VI) toxicity at 15 ppm level. Analysis of Cr (VI) remaining in the supernatant show that Cr (VI) has been reduced. Decreasing of Cr (VI) done by Isolate 39 cultures reach up to 0.8 ppm after 12 hours incubation from Cr (VI) initial level 15 ppm (Table 1).

The rhizobacterial isolate has been adapted in the chromium contaminated environment resulting could grow and proliferate at the toxic level. The cell growth was inhibited by Cr(VI) compound induced frame-shift mutation, base pair substitution on G-C and A-T in the bacterial cell. But, the bacteria could probably repair DNA damage resulted by Cr(VI) toxicity. The decreasing of cell amount and increasing of lag period by Cr(VI) perhaps cell need adaptation time to DNA repair during Cr(VI) exposure in the liquid medium (De Flora *et al.*, 1990; Bae *et al.*, 2000). Isolate 39 could reduce Cr(VI) after they were grown until the end of exponential phase, either with Cr(VI) induction at the start of growth or without induction (data not shown). Cr(VI) reduction need available energy in organic or inorganic form (Rahman *et al.*, 2007).

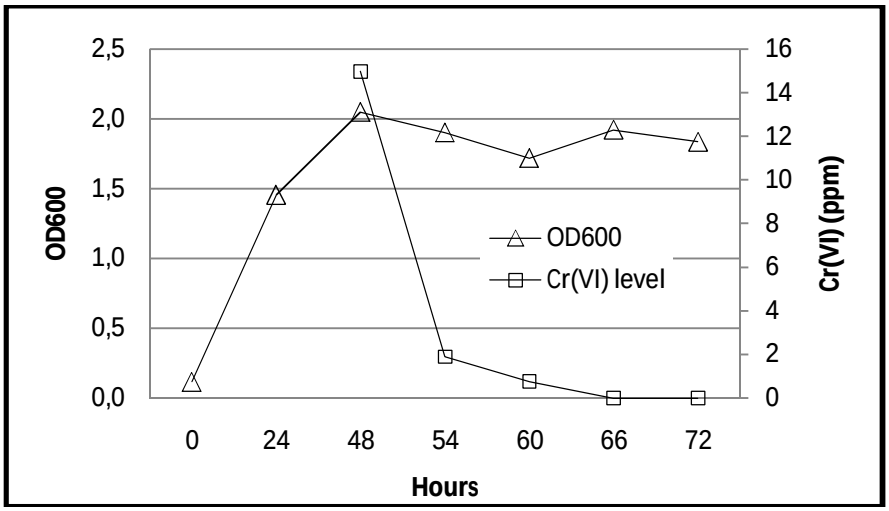

Figure 2
The cell growth of Isolate 39 and Cr(VI) reduction by growing cells treatment. OD_{600} (△), Cr (VI) level (□). Cells were exposured by Cr(VI) started 48 hours of growing.

Table 1
The Cr (VI) level at growing cells treatments of Isolate 39

Treatments	Cr(VI) level (ppm)*) Hours				
	48	54	60	66	72
Growing cell	15.0	1.9	0.8	0.0	0.0
Resting cell	15.0	8.1	7.7	7.3	7.2
Supernatant	15.0	1.0	0.8	0.0	0.0
Control	15.0	15.0	14.8	14.6	14.2

*) Average of 3 replications

Decreasing in Cr (VI) level done by Isolate 39 at the growing cells condition perhaps was caused by biosorption of bacterial cell wall, chromate reductase activity, and metabolites such as organic acids. Biosorption is the process of taking the metals by microorganism passively (no power). Metals ion binding by biosorption on organisms is using capsule, extracellular polymer and functional groups on the cell wall (Khan *et al*., 2009). When the cells were grown in the medium containing glucose, metabolism process happen and form biomolecules and produce organic acids. Organic acids act as H source that can be used chromate reductase as electron donor for Cr (VI) reduction.

Cr (VI) reduction by resting cells. Bacterial isolates were grown separately in the minimal medium for 48 hours and cells were harvested by centrifugation thus washed twice with Tris-HCl buffer.

Cell suspension were exposure with 15 ppm Cr (VI). Observation result presented at Table 1 that indicated decreasing drastically of Cr (VI) level within 6 hours first incubation and less at the end of incubation. Cr (VI) remain reached up to 46 % from initial level. Cr (VI) reducing capability of resting cells treatment may be caused of cell in the resting condition thus they were not producing reductase enzyme and reduction preferable derived from biosorption by cell membrane. At the resting cells condition, the treatment used Tris-HCl buffer as medium without electron donor presence. In this experiment, buffer medium has not been supplemented by electron donor so that did not reduced Cr(VI) enzimatically. Glucose was known to promote Cr(VI) reduction by *Agrobacterium radiobacter*, *Bacillus cereus*, *Escherichia coli* ATCC33456, & *Pseudomonas fluorescens* LB300 (Wang and Shen, 1995).

Cr VI) reduction by supernatant. Supernatant derived from cell separation was exposure of 15 ppm Cr(VI), then incubated by shaking for 24 hours. In fact, decreasing in Cr(VI) level drastically as soon as the supernatant mix up with Cr(VI). The highest of decreasing Cr(VI) level was done within 6 hours first incubation, and Cr(VI) completely reduced after 18 hours incubation (Table 1).

Reduction test by growing cells, resting cells and supernatant treatments were designed to clarify the mechanism of Cr(VI) reduction. On Table 3, growing cells and supernatant treatments of Isolate 39 showed that Cr(VI) reduction activity up to 100% after 18 hours incubation. Cr(VI) reduction activity appeared clearly derived from soluble fraction in the supernatant, due to by supernatant treatment showed higher Cr(VI) reduction than resting cells treatment that reached 51%. Supernatant derived from cultures liquid centrifuged and assumed contain the metabolites (organic acids and extracellular enzymes) that be secreted by cell.

Cr(VI) reduction ability by resting cells treatments was less compared than growing cells and supernatant. We presumed that endogenous electron act as electron source on resting cells and organic acids that produced from glucose catabolism act as electron donor in Cr(VI) reduction on growing cells. The Cr(VI) reduction to less toxic Cr(III) is electron consuming process. Cell showed higher Cr(VI) reduction activity when the electron donor is available (Llovera *et al*., 1993).

Based on the pH measuring, the pH decreased during growth and incubation at growing cells and supernatant treatments (Table 2). Initial pH of minimal medium was pH 6.9, by inoculation of this rhizobacterial isolate after 48 hours incubation the pH decreased up to 3.3-6.3. This phenomenon was caused by glucose metabolism that producing organic acids. This case strengthen the reason if Cr(VI) reduction done by enzyme activity that needed electron donor from this compounds.

Table 2
pH of cultures medium during incubation and Cr(VI) treatments

Treatments	Incubation time (hours)			After 12 hours Cr(VI) exposure		
	0	24	48	*Growing*	*Resting*	Supernatant
Isolate 39	6.9	6.5	4.5	3.9	6.3	3.3
Medium control	6.9	6.9	6.8	6.5	-	-
Buffer control	7.0	7.0	7.0	-	7.3	-

3. Chromium Absorption in Maize Plant

On the third stage of this experiment, we used culture medium of Isolate 39 that treated with Cr(VI) for watering the maize plant. Seven days after seeding of maize plant were irrigated with culture medium with or without isolates during the plant growth and at the 30 days after seeding plant were harvested for analyzing the Cr absorption in the root and shoot. Isolate 39 increased the phytostabilization ability of chromium by *Zea mays* at 30 days after seeding 3.8 times compared than control (without isolate) (Table 3).

Phytostabilization ability was estimated by dry weight of plant divided by Cr plant absorption. Isolate 39 decreased the Cr(VI) level by reduction mechanism so less Cr(VI) absorbed by plant. Chromium could enter in the plant tissue via unspesific pathway mechanism (Arslan *et al*., 1987), but like esssential element because it have similirity to SO_4^{2-} structure so making Cr(VI) easy to be absorbed through sulphate system transport (Shanker *et al*., 2005).

Chromium may be absorbed as Cr(III) or Cr(VI) by roots. Cr toxicity to plant varied depends on their type and concentration. The symptoms of Cr(VI) toxicity on maize plant in this experiment were abnormal growth and root system, curling leaves and clorosis, biomass reduction. Since Cr is a non-essential element for plants, there is no uptake mechanism; Cr is taken up along essential elements such as sulfate through sulfate transporters.

Cr accumulation in plants causes high toxicity in terms of reduction in growth and biomass accumulation, and Cr induces structural alterations. Cr interferes with photosynthetic and respiration processes, and water and minerals uptake mechanism. Various enzymatic activities related to starch and nitrogen metabolism are decreased by Cr toxicity either by direct interference with the enzymes or through the production of reactive oxygen species. Cr causes oxidative damage by destruction of membrane lipids and DNA damage (Singh *et al*., 2013).

The role of rhizobacterial isolates in Cr phytostabilization particularly seems on well performance of plant growing on Cr contaminated soil. By phytostabilization mechanism, rhizobacteria help plant makes Cr could be precipitated in the rhizosphere or at least the Cr absorbed by root but not as be poison for plant shoot.

Table 3
The ability of Isolate 39 in Cr phytostabilization at growing cells, resting cells and supernatant treatments

Treatments	Plant dry weigth (gram)	Cr Absorbtion in the root (ppm)	Cr Absorbtion in the shoot (ppm)	Cr Absorbtion in plant (ppm)	Plant dry weigth / Cr Absorbtion in plant	Treatment/control
Medium (control)	0.70 ± 0.20	7.24 ± 2.31	11.86 ± 1.32	19.10 ± 1.21	0.036 ± 0.06	1.00
G-I_{39}	1.00 ± 0.46	5.78 ± 2.49	1.31 ± 0.04	7.10 ± 0.23	0.140 ± 0.03	3.84
S-I_{39}	2.53 ± 0.67	5.90 ± 5.28	12.06 ± 1.32	17.97 ± 1.58	0.141 ± 0.05	3.84
Buffer (control)	1.93 ± 0.35	203.27 ± 16.87	12.83 ± 0.14	216.10 ± 10.27	0.008 ± 0.00	1.00
R-I_{39}	2.63 ± 0.32	199.88 ± 8.61	3.12 ± 0.39	203.00 ± 6.63	0.012 ± 0.00	1.45

Notes: These values are average of 3 replications
G=growing cell; R=resting cell; S=supernatant

4. Identification and characterization of Rhizobacterial Isolate

Based on characteristics of physiology, Isolate 39 has similarity to *Agrobacterium tumefaciens* AGL1 (Table 4). Isolate 39 has been identified by amplifying and sequencing of their 16S rRNA genes. Partially base sequence of gene 16S rRNA to Isolate 39 was read 772 bp. The base sequencing result was compared by genes 16S rRNA deposited at gene bank data on www.ncbi.nih.nlm.gov/Blast.cgi using Neighbor-joining method. Comparing base sequence of Isolate 39 by data from *gene bank* revealed that significant similarity of those nine isolates with strains of *Agrobacterium* sp (similarity 99%). Figure 3 presented a phylogenetic tree of Isolate 39 amongst related microorganisms based on 16S rRNA genes sequence.

Table 4.
The comparison of physiology characteristics of Isolate 39 by *Agrobacterium* and *Rhizobium* bacteria (Rini, 2009)

Characteristics	Isolate 39	*Rhizobium japonicum* USDA 122	*Agrobacterium tumefaciens* AGL1
Aerobicity	Aerob	Aerob	**Aerob**
Growing at 40°C	+	+	+
KOH test	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Urea hydrolysis	+	+	+
Gelatin hydrolysis	-	-	-
Indol production	-	-	-
Growing at Hofer's medium	+	-	+
3-ketolactosa production	+	-	+
Growing at NaCl 2 %	+	-	+
Acid production from glucose	+	-	+

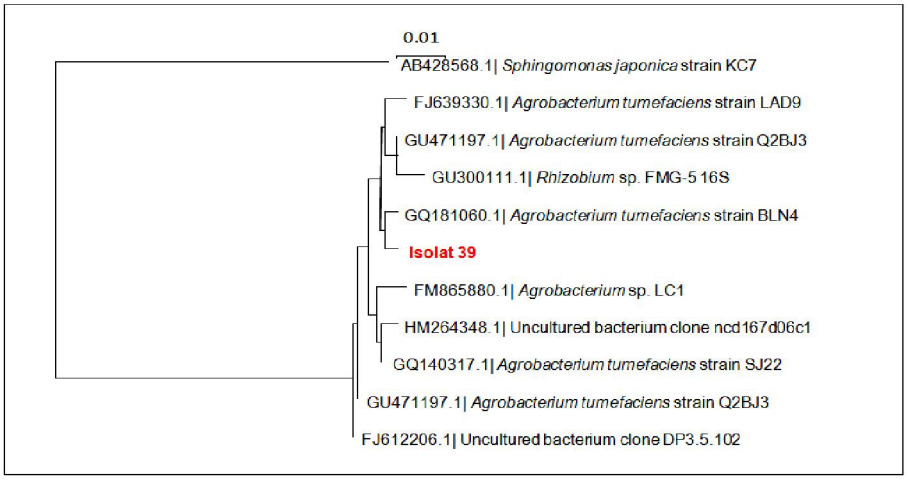

Figure 3
Phylogenetic tree construction of Isolate 39 showed relatedness with several closely related members based on 16S rRNA genes sequence comparison. The phylogenetic tree was constructed using aligned sequences by the neighbor joining using Clustal X and NJ Plot programs.

CONCLUSION

Isolate 39 was able to grow both on LB and minimal medium containing Cr (VI). Isolate 39 has ability to reduce Cr (VI) both at growing cells and resting cells conditions. Isolate 39 increased the phytostabilization ability of chromium by *Zea mays* at 30 days after seeding 3.8 times compared than control. Based on physiological characteristics and partial sequencing of 16S rRNA gene, Isolate 39 was identified as *Agrobacterium* sp. Isolate 39 has demonstrated the ability to effectively reduce Cr (VI) to Cr (III) fermentatively and therefore play a potential role in Cr (VI) remediation at contaminated site.

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