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Microbial Reduction of Cr (VI) into Cr (III) by Locally Isolated Pseudomonas Aeruginosa

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Abstract. Chromium hexavalent, Cr (VI), has been known as the toxic agent for living organisms. Many methods have been exploited to remove Cr (VI) in the environment, including empowering bacteria as bioremediation agent. Here, this study aims at investigating the influence of Cr (VI) on growth of pseudomonas aeruginosa and its ability to convert Cr (VI) into Cr (III) on leather tannery effluent model. The dissolved of sodium dichromat (K2Cr2O7) was used as source of Cr (VI). The effect of Cr (VI) on Pseudomonas aeruginosa growth was checked by comparing the growth of Pseudomonas aeruginosa in medium containing certain concentration of chromium and in medium without chromium. Spectrophotometer analysis showed that the rate of pseudomonas aeruginosa growth decreased at the concentration of 100 ppm of Cr (VI). In addition, the reduction of Cr (VI) was monitored by growing Pseudomonas aeruginosa in the medium with K2Cr2O7. The result of AAS and spectrophotometer show the decreased of Cr (VI) concentration in the medium from 100 to 5.86 ppm with the conversion efficiency reach to 94.73% during 48 hours of the treatment. High efficiency conversion of Cr (VI) in to Cr (III) indicates the possibility of pseudomonas aeruginosa to be used as a bioremediation agent to reduce Cr (VI) in the polluted environment.

1. Introduction

Rapid growth of industrial sector contributes to the increasing of toxic heavy metals pollution. Chromium is one of the heavy metals whose concentration in the environment found to be increased due to different industrial processes.[1] For instance, the leather industries employ chromium-tanning processes to boost their production and leather quality.[2] Unfortunately, the most of thus residual chromium is discharge in solid or liquid effluent. Indeed, the waste problem gained from chromium cannot be avoided. Naturally, chromium may exist in different oxidation states with the most stable and common forms are the hexavalent chromium [Cr (VI)] and trivalent chromium [Cr (III)].[3] The existence of Cr (VI) in the environment mostly comes from the industrial activities, while Cr (III), naturally predominates in the environment, especially in the acidic conditions. Due to the Cr (VI) strong oxidation properties, significant quantities of Cr (VI) in the environment may constitute toxicological risk to living organisms. Cr (VI) also causes sometimes life-threatening illness including irreversible damage to vital body system. [4]

Many physical and chemical removal-based methods were studied to solve the Cr (VI) waste.[5] The effluents are treated with ferrous sulphate, chemical reduction, followed by either alkaline

precipitation or removal by ion exchange; however, the adsorption that suffers from precipitation and additional treatment methods to remove those is to be sorted.[6] However, these methods require high cost and high energy to conduct the process. Instead of it, the process also sometimes produce non-eco-friendly waste product. In response to this challenge, bioremediation of Cr (VI) using indigenous bacteria emerged as sustainable and an environmentally compatible technology.[7]

Some microbial species have been reported to reduce Cr (VI) under either aerobic or anaerobic condition.[8] The survival of these microorganisms in the Cr (VI) contaminated environment depends on their capabilities on protective mechanisms, such as adsorption, uptake, methylation, oxidation, and reduction.[9] Ilustration model of microabial-based biosorption was shown on Fig. 1. Some bacteria can remove Cr (VI) by uptake it as a nutrient for their metabolism, or convert Cr (VI) into Cr (III), which is less toxic and less mobile compared to Cr (VI). For example, *Acinetobacter haemolyticus* shows an ability to remove Cr (VI) by adsorbing Cr (VI) into their cell membrane, while *Pseudomonas aeruginosa*, *Pseudochrobacterium* sp*., Proteus* sp*., Bacillus* sp*.,* and *Bacillus methylotrophicus* were found to be Cr (VI) resistant and reduce Cr (VI) in the environment by reduction it into Cr (III).[10] Another enzymatic transformation of Cr (VI) into Cr (III) was performed by *Ochrobactrum* sp.[11] Other mechanism which is conducting by bacteria is detoxifying and immobilizing Cr (VI) by converting it into insoluble chromium hydroxides.

The unique ability of each starin bacteria in reducing Cr (VI) may use as promising bioremediation agent of Cr (VI). The utilization of *Pseudomonas* strain bacteria as an agent for bioremediation model due to its largerly available, inexpensive, and harmless. This bacterium has also a rapid cultivation rate, requires minimum nutrient to grow, and well established as an effective agent for bioremediation.[12] The previous research found the ability of *Pseudomonas aeruginosa 99* and *Pseudomonas aeruginosa 78* to reduce the 10 ppm of Cr (VI) into Cr (III). In the present study, the capability of local *Pseudomonas* to convert the higher concentration of Cr (VI) was evaluated at concentration of 70 ppm, seven times higher than the previous study.[13] Here, an indigenous Cr (VI) resistant bacteria, *Pseudomonas Aeruginosa* was characterized and the effects of Cr (VI) on its growth and Cr (VI) reducing ability were determined by spectrophotometer and AAS analysis. The result found the decreased of *Pseudomonas aeruginosa* growth rate at the medium with 100 ppm of Cr (VI). It was monitored the decreased of Cr (VI) from 100 ppm to 5.86 ppm with the efficiency reach to 94.73% during 48 hours of the treatment. These findings indicate the potential of local *Pseudomonas aeruginosa* to be further applied as bioremediation agent in Cr (VI) contaminated environment.

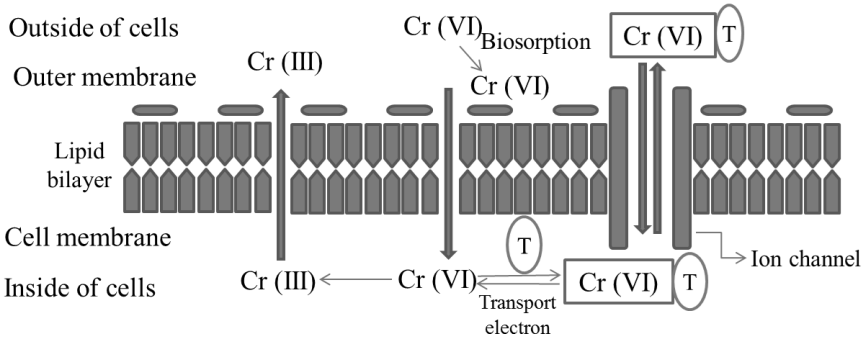

Figure 1. Model of Cr (VI) reduction into Cr (III) by *Pseudomonas aeroginosa*

2. EXPERIMENTAL METHODS

2.1. Raw Materials

Isolat of *Pseudomonas aeruginosa* (from Microbiology laboratory, Institut Teknologi Bandung), ethanol 70%, King's B medium, Potasium dichromate ($K_2Cr_2O_7$) (p.a., Merck), 1,5-difenilkarbazida

(DPC p.a., Merck) and de-ionized water (PT. Bratachem, Indonesia). All purchased raw material were used without further purification.

2.2. Bacterial Growth Conditions

Isolated *Pseudomonas aeruginosa* was inoculated onto nutrient agar which is supplemented with 100 ppm of Cr (VI). A filter-sterilized solution of $K_2Cr_2O_7$ was used as the source of Cr (VI), which was added to the sterile molten nutrient agar to prevent problems associated with autoclaving chromate-containing solutions. The inoculated plates were incubated at 37ºC for 24 h.

2.3. Determination of Minimum Inhibitory Concentration (MIC) of Cr (VI)

The minimum inhibitory concentration (MIC) of chromium at which no colony growth occurred was determined by broth agar dilution method. The isolates were inoculated individually into 10 mL nutrient agar medium and incubated at 37ºC for 24 h. Nutrient agar plates containing different concentrations of Cr (VI) (20–200 ppm) were inoculated aseptically from the exponential growing cultures of each bacterial strain. These plates were incubated at 37ºC for 24 h. The MIC was considered to be the lowest concentration of Cr (VI) at which no visible growth occurred.

2.4. Growth Kinetics Measurement

Growth of *Pseudomonas aeruginosa* was studied in 250 mL flasks containing 100 mL of medium supplemented with 100 ppm potassium dichromate as a source of Cr (VI). Flasks were inoculated with 0.1 mL of freshly prepared inoculum and incubated at 37°C with 150 rpm shaking for 24 h. Samples were drawn at regular 2 h time intervals. The Optical density changes of the culture during growth were recorded at 600 nm using a spectrophotometer UV (Shimadzu 1700, Shimadzu Corp., Japan). Culture media without chromium treatment served as a control for the growth experiment.

2.5. Reduction of Cr (VI) by isolated Pseudomonas aeruginosa

Pseudomonas aeruginosa was inoculated at broth medium, supplemented with sterilized Cr (VI) (100 ppm), and incubated overnight at 30°C in a shaker at 150 rpm. 10 ml aliquots were withdrawn at regular 8-hours intervals up to 48 hours and centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant obtained from the centrifugation was then analyzed for chromium reduction. The Cr (VI) uptake and reduction during growth was followed by measuring the absorbance of the purple complex of Cr (VI) with 1,5-diphenilcarbazide (DPC, Merck) at 540 nm using Spectrophotometer UV-Vis (Shimadzu 1700, Shimadzu Corp., Japan), the increase concentration of Cr (III), and total chromium, Cr_T. The total concentration Cr (VI) in the media was determined spectrophotometrically using 1,5-diphenilcarbazide as complexing agent. Total chromium [(Cr (VI) + Cr (III)] was determined using Perkin Elmer Analyst 1000 atomic absorption spectrophotometer (AAS), while Cr (III) was calculated from Cr_T and Cr (VI), [Cr_T-Cr (VI)]. Uninoculated media containing the same concentration of Cr (VI) were used as controls.

2.6. Characterization

The number of grow colonies was performed using a viable counting technique and monitored by digital colony counter (Bantex Colony Counter 920; Bantex, Burlingame). The Cr (VI) reduction by *Pseudomonas aeruginosa* was measured using Spectrophotometer UV-Vis (Shimadzu 1700, Shimadzu Corp., Japan), while the chromium total was analyzed using Perkin Elmer Analyst 1000 atomic absorption spectrophotometer (AAS).

3. Results and Discussion

3.1. Minimum Inhibitory Concentration of Cr (VI) on Pseudomonas aeruginosa

Variation concentrations of Cr (VI) were prepared for analyzing the minimum concentration of Cr (VI) that could inhibit the growth of *Pseudomonas aeruginosa*. The results of the first MIC experiment

were shown in the Table 1. At the first MIC experiment, we used higher concentration (100-200 ppm) of Cr (VI) to evaluated the highest concentration of Cr (VI) which can be accepted by *Pseudomonas aeruginosa*. According to the results resumed in the Table 1. It was shown that no visible colonies are growth at the concentration of Cr (VI) of 100, 150, and 200 ppm. These results indicate that local *Pseudomonas aeruginosa* could survive up to 50 ppm but less than 100 ppm. The growth of colonies of *Pseudomonas aeruginosa* on the solid medium of King's B were detected with white colonies and the alteration of medium King'B from yellow to greenish yellow due to the production of pyoverdines or pseudobactins pigment by *Pseudomonas aeruginosa*.

Table 1. Resistant analysis of Pseudomonas aeruginosa to Cr(VI) at range of 0-200 ppm

Parameters	Cr (VI) (ppm)					
	0	**20**	**50**	**100**	**150**	**200**
Medium color	Yellow transparent	Yellow greenish transparent	Yellow greenish transparent	Yellow transparent	Yellow transparent	Yellow transparent
Colony density	Countless	Countless	Countless	None	None	None
Colony morphology	Round shape	Round shape	Round shape	NI	NI	NI
Colony color	White	White	White	NI	NI	NI
Colony distribution	Random	Random	Random	NI	NI	NI
Number of colony	>300	>300	>300	None	None	None

Note : NI: not identified; means no visible colonies was growth, therefore the identification of colony's morphology, colony's color, colony's distribution and number of colony cannot be determined.

The resistance of the isolated Pseudomonas aeruginosa towards the bactericidal action of Cr (VI) was studied at varying concentration of Cr (VI) (Table 1). The Pseudomonas aeruginosa was monitored to be resisted up to 50 ppm of Cr (VI), and totally killed at 100 ppm of Cr (VI) and higher concentrations in the solid medium of King's supplemented by Cr (VI). Since the range 50-100 ppm of Cr (VI) concentration too broaden, the resistance analysis was elaborated to see the effect of Cr (VI) on cell viability at 50-90 ppm. The results of this experiment were shown in the Table 2. It was observed that the Pseudomonas aeruginosa shows their resistance up to 70 ppm of Cr (VI). This was basically due to the inhibitory effect of Cr (VI). The more higher concentration of Cr (VI) the more inhibitory effect of Cr (VI) on the growth of organisms. As the initial age of the inoculums remains fixed at 24 hours, the acclimatization period at varying Cr (VI) concentrations would not remain the same. Higher concentrations of Cr (VI) also found to lead the extreme cells lysis.

Table 2. Resistant analysis of Pseudomonas aeruginosa to Cr(VI) at range of 0-90 ppm

Parameters	Cr (VI) (ppm)					
	0	**50**	**60**	**70**	**80**	**90**
Medium color	Yellow transparent	Yellow greenish transparent	Yellow greenish transparent	Yellow greenish transparent	Yellow transparent	Yellow transparent
Colony density	countless	Countless	Countless	Low	None	None
Colony morphology	Round shape	Round shape	Round shape	Round shape	NI	NI
Colony color	White	White	White	White	NI	NI
Colony distribution	Random	Uniform	uniform	Random	NI	NI
Number of colony	>300	>300	>300	>30	None	None

Note : NI: not identified; means no visible colonies was growth, therefore the identification of colony's morphology, colony's color, colony's distribution and number of colony cannot be determined.

3.2. Effect of Cr(VI) on Pseudomonas aeruginosa Growth Rate

The effect of Cr (VI) on growth of *Pseudomonas aeruginosa* was determined by comparing the growth profile of *Pseudomonas aeruginosa* at temperature of 37°C and pH 6.8 in the medium with and without supplementation of Cr (VI) as can be seen in the Fig. 2. Though the MIC experiment in the solid medium found no colonies grow in the solid medium at 70 ppm of Cr (VI), the application of this bacteria at the higher concentration of Cr (VI) is possibly to be conducted. This study found the ability of *Pseudomonas aeruginosa* to reduce Cr (VI) at 100 ppm (mg/L) of Cr (VI). However, it was found that the presence of 100 ppm of Cr (VI) slightly delay the bacterial growth (Fig. 2). This indicates that the growth of *Pseudomonas aeruginosa* was considerably inhibited due to the toxicity of Cr (VI). This observation is in agreement with the previous studies which are reported that the growth rate of *Bacillus cereus* decreased as the concentrating of Cr (VI) concentration in the culture medium increased (Singh, *et al*., 2013; Sony *et al*., 2013). Other previous study found that the growth of *Pseudomonas aeruginosa* and *Serratia marcescens* decreased with increases of Cr (VI) concentration.

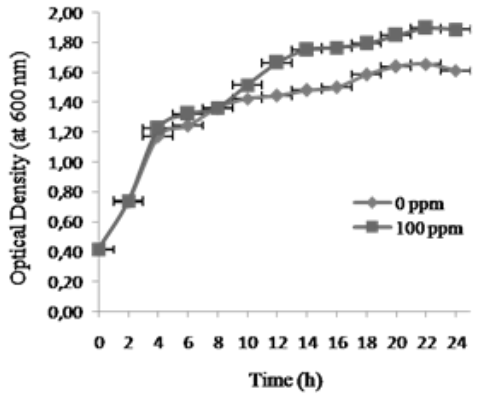

Figure 2. Growth Profile of Pseudomonas aeruginosa in the absence and in the presence of 100 ppm of Cr(VI).

3.3. Bioreduction of Cr(VI) into Cr(III) by Pseudomonas aeruginosa

Biotransformation of Cr (VI) was determined by growing the Pseudomonas aeruginosa in the medium with the presence of Cr (VI) at the concentration of 75 ppm for 24h of observation. The reduction of Cr (VI) was evaluated for each 8 h of observations as can be seen in the Fig.3.

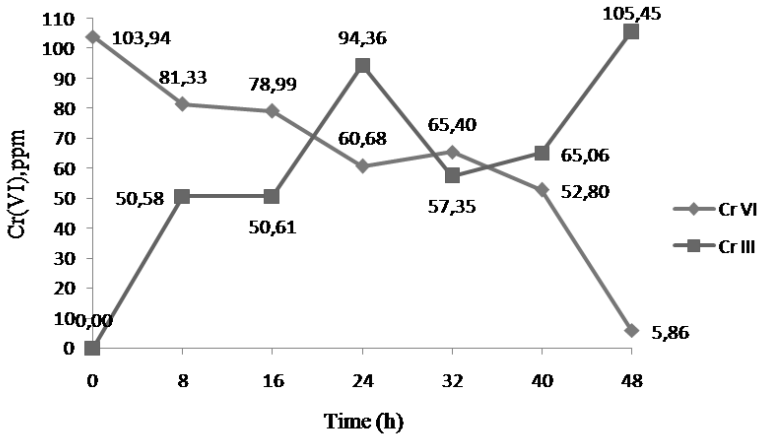

Figure 3. Bioreduction of Cr(VI) by Pseudomonas aeruginosa

Bioreduction evaluation of Cr (VI) by Pseudomonas aeruginosa shows the decreased of Cr (VI) concentration with the time. These results are in agreement with previous work. Biotransformation efficiency of Cr (VI) into Cr (III) at a higher concentration applied was shown in Fig. 4. Based on the line curve of Fig. 4, it can be seen that at an initial Cr (VI) concentration of 103.94 ppm, nearly 60% of Cr (VI) was reduced after 24h of reaction, while the value further decreased to 94% after 48h of reaction time (Fig. 4). This research shows the ability of indigenous Pseudomonas aeruginosa to reduce the higher concentration of Cr (VI) in the medium.

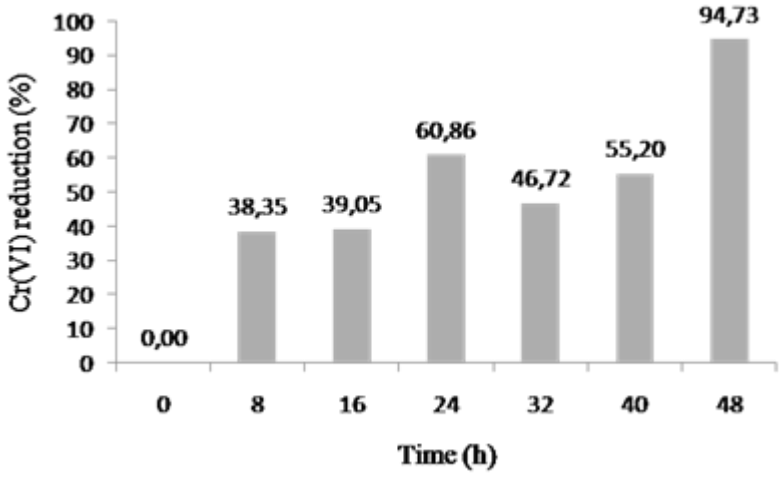

Figure 4. Effeciency of Cr (VI) reduction by Pseudomonas aeruginosa.The initial concentration of Cr(VI) in the medium is 103.94 ppm.

4. Conclusion

Minimum inhibitory analysis (MIC) analysis of local *Pseudomonas aeruginosa* shows that Cr (VI) is a toxic compound for this bacterium and totally inhibits its growth at the high concentration of Cr (VI) (greater than 100 ppm). In the current study also found that local *Pseudomonas aeruginosa* completely reduced Cr (VI) into Cr (III) with the conversion efficiency reach to 94% after 48h treatment. This

highest efficiency conversion of Cr (VI) indicates that this local bacterium can be used as an effective bioremediation agent for polluted environment with Cr (VI). Further understanding of the proper mechanism of Cr(VI) biotransformation and factors affecting Cr(VI) reduction should assist toward its application in chromium bioremediation processes.

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