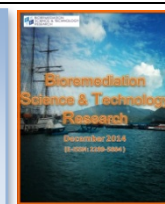




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Isolation, Characterization and Growth Optimization of a Chromate-reducing Bacterium

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ABSTRACT

The application of chromium in industries has introduced anthropogenic release of chromate which is very toxic and a soluble environmental contaminant. The contaminated sites and industrial wastewater containing chromate are treated based on reduction of Cr(VI) to Cr(III) which is insoluble and less toxic to environment. This can be achieved through utilization of Cr(VI)-reducing bacteria which serves more economical and environmental-friendly treatment as compared to conventional chemical treatment method. To fulfill this, a new chromate reducing bacterium was isolated from an agricultural site. This bacterium was identified as *Aeromonas encheleia* using Biolog analysis with 0.603 similarity index. Chromate reduction capability of the bacterium was optimized using Response Surface Methodology with optimum conditions occurring at 250 μ M Cr(VI), temperature 34 °C and pH 7.5 with 99% reduction was achieved. The bacterium showed high tolerance in LB media containing 3.0 mM of K₂Cr₂O₇, but only reduce 1.5 mM Cr(VI) within 24 hours. The presence of metals and metabolic inhibitors in the media did not give significant inhibitory effect on Cr(VI) removal except Hg, NaCN and Co with percentage inhibition 6.7, 80.5 and 90.8 respectively. Based on this study, *Aeromonas encheleia* could be a good candidate in bioremediation of contaminated site or industrial wastewater containing Cr(VI).

INTRODUCTION

Chromium (Cr) is a transition element in periodic Table with oxidation number ranging from (-II) to (+VI). The different of oxidation state display different chemical properties, toxicity, aqueous concentration and its mobility in different geologic environment [1]. Trivalent chromium, Cr(III) is the most stable form in environment as compared to hexavalent chromium, Cr(VI) as it attributed to low water solubility and tend to be precipitated as in hydroxide form or adsorbed onto soil [2]. In addition, trivalent chromium serves as a nutritional enhancement

for glucose metabolism and act as a cofactor for insulin secretion for animal and human [3,4]. When it comes to toxicity, Cr(VI) often reported as most toxic form of Cr i.e., as in the form of chromate (CrO₄²⁻) or dichromate (CrO₄⁷⁻). In addition, the Integrated Risk Information System (IRIS) had classified hexavalent chromium as strong oxidizing agent, mutagen and carcinogen to human health [5]. It has been reported that Cr(VI) can rapidly permeate through biological membranes and subsequent interact with proteins and nucleic acids in the plant system [6]. The toxicity of chromium on human, animal, plant and environment varies, depending on dose and duration of exposure.

Chromium is the vital and critical demanded metal in industry especially in stainless steel, pigment and textile manufacturing, leather tanning, wood preserving and photography [7]. The high usage in selected industry caused unintentional and intentional release of chromium into environment through wastewater, industrial effluent, leachate, sludge and other forms. The excessive chromium concentration in environment had received attention by regulatory bodies in promulgating law, regulation and standard acceptable chromium concentration. Environmental Quality (Industrial Effluent) Regulation 2009 has set a limit of 0.20 mg/L Cr(III) and 0.05 mg/L Cr(VI) of treated industrial effluent to be discharged in water bodies.

Meanwhile, Interim National Water Quality Standard for Malaysia has set a limit of 0.05 mg/L Cr(VI) for class IIA for drinking water. In order to comply statutory standard, the industrial effluent containing chromium usually treated with conventional method through chemical reduction to trivalent chromium followed by precipitation of Cr(OH)₃ using lime, ion exchange, membrane separation and adsorption [7]. These conventional method require large amount of chemicals, energy and costly to remediate the contaminated environment.

Furthermore, the conventional methods are preferable to remove heavy metal at concentration greater than 100 mg/L. As consequences, these techniques might be unsuitable to treat increasing volume of wastewater containing low metal concentration [8]. On the other hand, biological reduction of Cr(VI) to Cr(III) serves more economical value and environmental friendly as compared to existing conventional method [9].

The biological transformation of Cr(VI) by bacteria has been reported since 1970s under aerobic and anaerobic conditions. Many studies have addressed variety of Cr(VI)-resistant bacteria with high potential of reducing Cr(VI) such as *Bacillus*, *Pseudomonas*, *Pannonibacter*, *Pseudochrobactrum*, *Enterobacter*, *Acitenobacter*, *Microbacterium*, *Aeromonas* and other species [2,10,11]. Under aerobic condition, the mechanism of bacterial reduction for Cr(VI) is associated with utilization of soluble enzyme such as chromate reductase. In addition, the reduction of Cr(VI) to Cr(III) also can be attributed by chemical reaction between media and intercellular components such as amino acid, nucleotide, sugar and others [2].

This study was carried out to isolate and characterize a Cr(VI)-tolerance bacterium from an agricultural contaminated site. The effect of temperature, pH, heavy metal and metabolic inhibitor were evaluated on Cr(VI)-reducing bacterium. The effect concentration of Cr(VI) on Cr(VI) reduction and bacterial growth were also elucidated in this study. These findings will provide broad understanding on new isolated bacterium from agricultural contaminated site and provide alternative bacterium to remediate the contaminated environment.

MATERIALS AND METHODOLOGY

Isolation and identification chromate reducing bacteria

Soil from a palm oil plantation at UPM, Serdang Malaysia was collected randomly 15 to 20 cm beneath the surface using sterile spatula and placed in sterile screw-capped falcon tube. Then, 5 g

of sample was diluted in 50 mL nutrient broth (8 gL⁻¹ of nutrient broth) and incubated 72 hours at room temperature on 150 rpm rotary shaker. A loop of primary culture was streaked on nutrient agar plate (14 gL⁻¹ nutrient agar and 8 gL⁻¹ nutrient broth) with supplemented filter-sterilized 100 μM K₂CrO₄ (stock solution 100 mM K₂CrO₄). The plates were incubated at 28 °C and repeatedly streak on chromate agar (100 μM) until pure colonies were obtained. The pure colonies were further screened for chromate reduction at 100 μM K₂CrO₄ in 50 mL nutrient broth. The colony with highest chromate reduction was selected for further studies and identified by Biolog Identification System, which was carried out by Focus Biotech Sdn. Bhd., Petaling Jaya, Malaysia.

Screening of chromate reducing media

In order to get the highest Cr(VI) reduction, the isolated colony was grown overnight aerobically at room temperature in different media (pH 7) which were nutrient broth, Luria Bertani, LB (tryptone, 10 gL⁻¹, NaCl, 10 gL⁻¹, and yeast extract, 5 gL⁻¹), minimal salt media (K₂HPO₄, 0.4 g; KH₂PO₄, 0.2 g; NaCl, 0.1 g; MgSO₄, 0.1 g; MnSO₄.H₂O, 0.01 g; Fe₂(SO₄)₃.H₂O, 0.01 g; Na₂Mo₄.2H₂O, 0.01 g; (NH₄)₂SO₄, 0.4g; yeast extract, 1 g; and glucose, 10 g) and acetate minimal media (NH₄Cl, 1 g; K₂HPO₄, 0.5 g; CaCl₂.H₂O, 0.001 g; MgSO₄, 0.2 g; FeSO₄.7H₂O, 0.001 g; yeast extract, 0.5 g; and sodium acetate, 5 g). All media were autoclaved at 121 °C, 15 psi for 15 min before use. Then, the media were supplemented with 250 μM Cr(VI) and media that showed highest reduction Cr(VI) was selected to be used media in further studies.

Assaying chromate reduction

Chromate reducing activity was measured according to calorimetric changes using 1,5-diphenyl carbazide (Sigma, USA). The calorimetric reagent was prepared by dissolving 0.025 g 1,5-diphenylcarbazine in 100 mL of analytical grade acetone in order to minimize deterioration. The hexavalent chromium in the culture was assayed by reacting 400 μL of culture supernatant with 400 μL of 1 N H₂SO₄ and 200 μL 1,5-diphenylcarbazine solution and further analyzed spectrophotometrically at 540 nm [12].

Optimization chromate reduction

The optimization of chromate reduction was carried out by using Response Surface Methodology (RSM) with three independent variables namely temperature, pH and concentration Cr(VI) [13]. The three variables were studied according to ranges and level as stated in **Table 1**. In RSM, Box Behnken model was selected to design the 17 optimization experiments.

Table 1. Variable, range and level used in RSM

Independent variable	Unit	Range and level		
		-1	0	1
pH	pH	6	7.5	9
Temperature	°C	28	34	40
Concentration	μM	100	250	400

The experiments were conducted in triplicate according to design as stated in **Table 2** with percentage reduction chromate was used as response of experiment. 50 mL LB media containing

100, 250 and 400 μM Cr(VI) with different initial pH values (pH 6, 7.5 and 9) were inoculated with 1% original culture ($\text{OD}_{600}=1.0$) and incubated for 24 hours [11]. The cultures were incubated at 28, 34 and 40 °C on shaker 150 rpm. Tris buffer (50 mM) and potassium phosphate buffer (50 mM Na_2HPO_4 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) were used to regulate pH media [14]. The media's pH was adjusted using 1 M HCl and 1 M NaOH. After incubation period, 1 mL of sample was withdrawn aseptically, centrifuged 10,000 rpm for 10 minutes and the supernatant is used to determine Cr(VI) reduction.

The statistical significance of the experiment was determined by F-test in ANOVA Table. The fit quality of the second order equation in the experiment was expressed by determination of R^2 . The significance coefficient of the equation and ANOVA was determined using Student's t-test. For the final predictive equation was done using Design expert version 6.0.8 software.

Table 2. Design of Box Behnken

Run	pH	Temperature (°C)	Concentration Cr(VI)(μM)
1	7.5	28	400
2	7.5	28	100
3	9.0	34	100
4	6.0	34	100
5	7.5	34	250
6	9.0	40	250
7	9.0	28	250
8	7.5	34	250
9	6.0	28	250
10	9.0	34	400
11	7.5	40	400
12	7.5	34	250
13	6.0	34	400
14	7.5	40	100
15	7.5	34	250
16	7.5	34	250
17	6.0	40	250

Effect of concentration Cr(VI) on bacterial growth and Cr(VI) reduction

1% of bacterial culture grown overnight aerobically at 34 °C ($\text{OD}_{600}=1$) was inoculated with different concentration of potassium dichromate ranging from 100 μM to 4.5 mM in 50 mL LB containing 50 mM potassium phosphate buffer (pH 7.5) while shaking at 150 rpm. After 24 hours incubation period, 1 mL of bacteria culture was centrifuged at 10,000 rpm for 10 min. The supernatant was assayed and measured at 540 nm. The growth of the bacterium was measured by diluting the pellet with distilled water and measured at 600 nm. LB without inoculant but with the same concentration of Cr(VI) was used as negative control [15].

Effect of heavy metal and metabolic inhibitor on Cr(VI) reduction

The effect of other metal on Cr(VI) reduction was measured through treatment of sterilized standard metal ICPMS on LB containing 100 μM Cr(VI). The selected metals used in this study were cadmium (Cd), cobalt (Co), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn) at final concentration of 50 μM in 50 mM potassium phosphate buffer. Besides that, the effect of

metabolic inhibitor on Cr(VI) reduction was determined through reaction of filter sterilized 1 mM sodium azide (NaN_3), 1 mM sodium cyanide (NaCN) and 10 mM sodium sulphate (Na_2SO_4). LB without inoculant but with the same concentration of Cr(VI) was used as negative control [15]. All the experiments were conducted in triplicates in 50 mL LB containing 50 mM potassium phosphate buffer (pH 7.5) and incubated aerobically at 34 °C, overnight with constant inoculant density ($\text{OD}_{600}=1$) [15].

RESULTS

Isolation, bacterial identification and screening Cr(VI) reducing media

The isolated bacterium was gram negative aerobic bacterium identified as *Aeromonas encheleia*, by using Biolog Identification System with 0.603 similarity index after 24 hours incubation. The similarity index greater than 0.500 indicate genus or species identification [16]. In addition, this identification were accepted as correct if the index was greater than 0.750 at 4 hours and greater than 0.500 after 24 hours incubation [17]. This chromate reducing bacterium showed highest reduction in LB media containing 0.25 mM K_2CrO_4 as compared to nutrient broth, minimal salt media and acetate minimal media (Table 3).

Table 3. Screening Cr(VI) reducing media

Media	Cr(VI) reduction (%)
Luria bertani	90.9
Nutrient broth	88.5
Acetate minimal media	9.9
<u>Minimal salt media</u>	
Fructose	5.6
Galactose	2.6
Glucose	10.0
Glycerol	6.0
Maltose	4.9
Mannitol	4.0
Potassium acetate	10.1
Raffinose	5.6
Sodium acetate	10.7
Starch	11.6
Sucrose	4.0

Optimization by Response Surface Methodology (RSM)

The 17 optimization experiments in Box Behnken design were conducted in order to obtain a quadratic model of biotransformation Cr(VI) to Cr(III). The result of three independent variables; pH, temperature and concentration Cr(VI) on Cr(VI) reduction are presented in Table 4 along with the mean predicted and observed response. The following equation is derived from multiple regression analysis in order to explain Cr(VI) reduction by *Aeromonas encheleia*:

$$Y = 99.16 + 9.62A - 23.51B - 33.71C - 43.18A^2 - 21.00B^2 - 1.70C^2 - 19.40AB - 0.47AC - 23.07BC$$

where Y is predicted Cr(VI) reduction and A, B and C are the coded variables for pH, temperature and concentration respectively. The ANOVA was carried out to determine

significant effect of individual variable and combined variables on Cr(VI) reduction. Based on ANOVA analysis, it distinguished that the single variable of B (temperature), C (concentration) and combined variable of A², B², BC were statistically significant on Cr(VI) reduction (p<0.05). The "Lack of Fit F-value" of 2757.91 (p<0.0001) implies the Lack of Fit is significant (Table 5). The regression model was significant (p<0.05), with R² value of 0.937. The adjusted R² value was 0.856 indicated that only 14.4% variation of Cr(VI) reduction could not be explained by this model. The regression value and adjusted R² were close to 1 and it signified a good correlation between actual and predicted response of Cr(VI) removal [18]. The adequate precision measured the signal to noise ratio and the value of 9.089 (greater than 4) indicated adequate signal. The optimization experiment by RSM approach is proven can increase and enhance Cr(VI) reduction from 90.9% (under condition 28 °C, pH 7, 250 μM) to 99% (under optimum condition 34 °C, pH 7.5, 250 μM) (Table 6). The three-dimensional response surface curves were plotted for further understanding on interaction of combined variables on Cr(VI) reduction (Fig. 1).

Table 4: Box Behnken design variables along with observed and predicted response

Run	pH	Temperature (°C)	Concentration (μM)	Cr(VI) reduction (%)	
				Observed Response ± SEM	Predicted value
1	7.5	28	400	99.57 ± 0.07	89.32
2	7.5	28	100	97.69 ± 0.75	110.61
3	9.0	34	100	99.69 ± 0.59	98.10
4	6.0	34	100	99.47 ± 0.52	77.90
5	7.5	34	250	99.04 ± 0.15	99.16
6	9.0	40	250	10.36 ± 1.99	1.70
7	9.0	28	250	98.84 ± 1.07	87.51
8	7.5	34	250	99.01 ± 0.24	99.16
9	6.0	28	250	20.82 ± 9.34	29.47
10	9.0	34	400	8.15 ± 1.71	29.72
11	7.5	40	400	9.09 ± 0.72	-3.83
12	7.5	34	250	98.52 ± 0.22	99.16
13	6.0	34	400	9.82 ± 2.20	11.42
14	7.5	40	100	99.47 ± 0.79	109.73
15	7.5	34	250	99.74 ± 0.17	99.16
16	7.5	34	250	99.51 ± 0.20	99.16
17	6.0	40	250	9.93 ± 1.46	21.25

Table 5: Analysis of variance Table (ANOVA) for RSM

Source	Sum of squares	DF	MeanSquare	FValue	P>F
Model	28143.78	9	3127.09	11.60	0.0019*
A(pH)	741.10	1	741.10	2.75	0.1413
B(Temperature)	4421.69	1	4421.69	16.40	0.0049*
C(Concentration)	9092.78	1	9092.78	33.73	0.0007*
A ²	7849.06	1	7849.06	29.12	0.0010*
B ²	1857.56	1	1857.56	6.89	0.0342*
C ²	12.22	1	12.22	0.05	0.8375
AB	1504.83	1	1504.83	5.58	0.0501
AC	0.89	1	0.89	0.00	0.9557
BC	2128.27	1	2128.27	7.90	0.0262*
Residual	1886.95	7	269.56		
Lack of Fit	1886.04	3	628.68	2757.91	<0.0001
Pure Error	0.91	4	0.23		

*P > F less than 0.05 =statistically significant

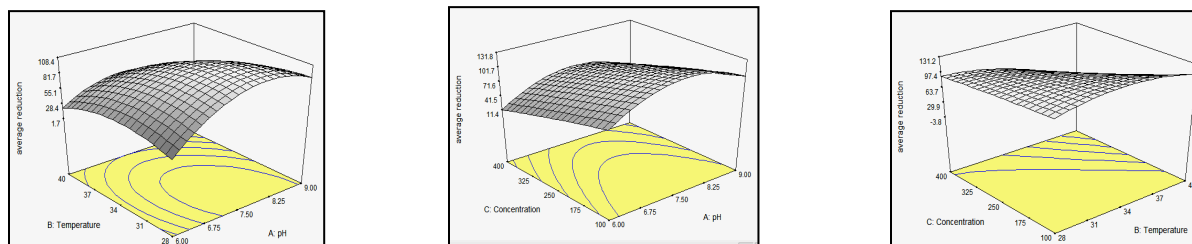


Fig. 1. Three dimensional surface curve on interaction between temperature vs pH, temperature vs concentration and pH vs concentration.

Table 6: Comparison preliminary and optimization experiment on Cr(VI) reduction

Experiment	Temperature (°C)	pH	Cr(VI) reduction (%)
Preliminary	28	7.0	90.9
Optimization	34	7.5	99.0

Effect of concentration Cr(VI) on bacteria growth and Cr(VI) reduction

The bacterium grew in LB media containing 3.0 mM K₂Cr₂O₇ and the growth was gradually decreased with increasing concentration of K₂Cr₂O₇. The bacterium was capable to reduce 90-100% of 0.1-1.0 mM of K₂Cr₂O₇ within 24-hour incubation period. At concentration 1.5 mM K₂Cr₂O₇, 40% removal of Cr(VI) was achieved. However, the Cr(VI) reduction declined rapidly as the concentration of K₂Cr₂O₇ increased at 2.5 to 4.0 mM (Fig.2). The effect of Cr(VI) concentration were further studied by measuring bacterial growth and Cr(VI) reduction every 2 hours from initial point of experiment (Fig. 3). The bacterial growth kept increasing and doubling from initial point until 12 hours of incubation (log phase). The bacterial growth undergo stationary phase for the next 12 hours of incubation, this might be due to growth limiting factors such as formation organic acid and depletion of nutrient in media. The culture reduced 4.37±0.03% within 2 hours of incubation time. On the next 14 hours, the culture achieved 100% Cr(VI) reduction and remain constant for the next 8 hours incubation.

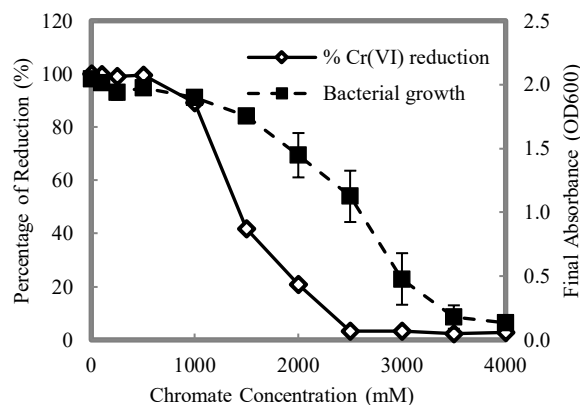


Fig. 2. Effect of concentration Cr(VI) on bacterial growth and percentage Cr(VI) reduction.

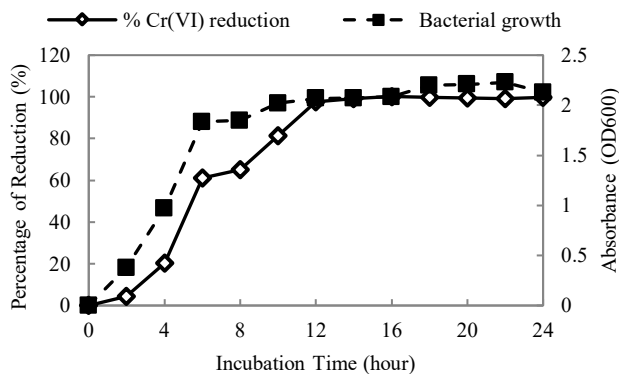


Fig. 3. Effect of 100 μM Cr(VI) on bacterial growth and percentage Cr(VI) reduction (every 2 hours)

Effect of heavy metal and metabolic inhibitor on Cr(VI) reduction

The effect of other heavy metals on Cr(VI) reduction were investigated in LB media with 50 mM potassium phosphate buffer. All of the tested metals were compared with negative control (LB with 100 μM Cr(VI) concentration). The presence of metal like Cd^{2+} , Cu^{2+} , Ni^{2+} , Pb^{2+} and Zn^{2+} showed slightly inhibition effect on Cr(VI) reduction, in the range from 96 to 99%. The presence of Hg^{2+} significantly inhibited bacterial ability to reduce 100 μM concentration of Cr(VI) within 24 hours incubation period. Apart from that, effect of metabolic inhibitors also were investigated in order to ensure the Cr(VI) is actually transported into the cell for reduction process [15]. The presence of Na_2SO_4 and NaN_3 did not significantly inhibit Cr(VI) reduction process ($p > 0.05$). Meanwhile, the presence of 1 mM NaCN inhibit 20% Cr(VI) reduction (Fig. 4).

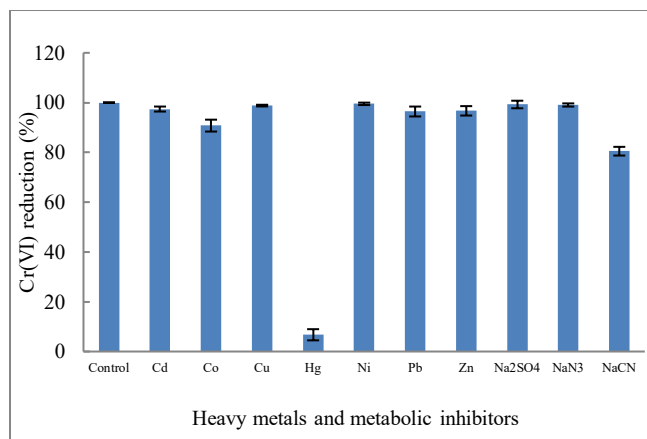


Fig. 4. Effect of selective metals and metabolic inhibitor on 100 μM Cr(VI) reduction by *Aeromonas encheleia*. Error bars represent the standard deviation of triplicate samples.

DISCUSSION

Industrialization had introduced and released anthropogenic hexavalent chromium into environment, which is very toxic and high mobility in aqueous phase. The presence of Cr(VI) is common in industrial effluent and require specific treatment either

physicochemical or biological treatment. However the physicochemical treatment is less preferable due to high-energy consumption, high cost of operation and maintenance, generation of unnecessary sludge and formation of byproduct. In order to overcome this limitation, bioremediation is preferable to be applied as an alternative treatment for biotransformation and reduction of toxic Cr(VI) to stable Cr(III), due to low cost and environmental friendly treatment. In this present investigation, chromate-reducing bacterium identified as *Aeromonas encheleia* had been isolated from palm oil plantation.

At the early stage of study, we investigated the effect of various media such as LB media, NB media, acetate minimal media (AMM) and minimal salt media (MSM) on Cr(VI) removal. Within 24 hour incubation period, *Aeromonas encheleia* able to reduce up to 90% of 250 μM Cr(VI) in LB media. This finding can be supported by other recent studies reported greatest Cr(VI) reduction in LB media under aerobic condition [9,11,15]. Besides, study conducted by Zakaria et al. [19] also revealed that the growth of Cr(VI) reducing bacterium such as *Acinetobacter haemolyticus* was greater in LB media than minimal salts media. The use of complex media like LB media is less preferable in some studies to determine bacterial resistance to Cr(VI) due to probability of masking effect complex formation with organic component in the media [12]. However the use of acetate minimal media and minimal salt media for Cr(VI) reducing bacteria require longer incubation time for complete Cr(VI) reduction [20]. In addition, study conducted by Pattanapitpaisal et al. [21] revealed that *Microbacterium* sp. did not reduce 100-500 μM Cr(VI) in AMM within 24, 48, and 72 hours under aerobic condition but able to biotransform Cr(VI) anaerobically at expense of acetate as electron donor but without significant bacterial growth.

Response Surface Methodology (RSM) is an empirical statistical tool used to design experiment, modeling and determining the relationship of individual factor and recombinant factors on desired response. The application of RSM can increase process efficiency of Cr(VI) reduction, reduce the time of experiment and determine influence of individual factor of each parameters [22]. In this present study, the individual factor (pH, temperature and concentration) and the recombinant factors were investigated on Cr(VI) reduction. Based on the finding, only single variable of B (temperature), C (concentration) and combination variable of A₂, B₂, BC are significant model term. The Cr(VI) reduction increased as temperature and pH increased, however the Cr(VI) removal declined as the concentration kept increasing. The media containing 250 μM Cr(VI) can achieve up to 99% Cr(VI) removal under optimum condition pH 7.5 and 34 °C.

It has been reported that bioremediation by bacteria are controlled by environmental factor such as pH, temperature, nutrient content, metal content and others factor [23]. The effectiveness of Cr(VI) removal by bacteria can be achieved through optimum pH and temperature [14]. Most chromate-reducing bacteria can reduce efficiently at pH 7 to pH 7.8 and temperature range 30 to 35 °C under aerobic condition. Due to the reasons, we decided to investigate the effect of pH 6 to pH 9 and temperature range 28 to 40 °C on Cr(VI) removal. The strong acidic pH was not investigated due to Cr(VI) reduction may contributed by organic compound present in the medium [24]. Extreme alkaline pH (pH 10 and above) was not investigated since the extreme pH value may restrict bacterial growth and Cr(VI)

reduction [25]. It also has been reported in many studies that the growth of aerobic bacteria are inhibited at temperature exceed 40 °C [11,14]. The finding on optimum condition for bacterium *Aeromonas encheleia* in this study is similar to bacterium *Aeromonas* sp. KS-14 which grew optimally at temperature 30 °C and pH 7.2 [15]. Since Cr(VI) reduction is attributed by enzymatic activity, changes of pH and temperature will significantly affect degree of ionization of enzyme, protein structure and enzymatic reaction [25,26]. Furthermore, the biotransformation of Cr(VI) occurs near a neutral pH and over a moderate range of temperatures indicates that there are no requirement of costly chemicals and extensive energy inputs [27].

The study of effect Cr(VI) concentration indicates tolerance level of the bacteria on chromium, which strongly affects bacterial growth and Cr(VI) reduction [25]. In this present study, *Aeromonas encheleia* can tolerate 3.5 mM of Cr(VI), however the bacterial growth kept decreasing as the concentration Cr(VI) increased. This bacterium was also able to reduce 40% of 1.5 mM Cr(VI) within 24 hours of incubation period. Surprisingly, this finding on Cr(VI) tolerance and reduction is higher than other studies of the same genera. As instance, bacterium *Aeromonas* sp. KS-14 in study conducted by Alam and Ahmad [15] able to grow in 1 mM Cr(VI) and took 56 and 88 hours to reduce 125 and 250 µM. Meanwhile, study conducted by Sinhaet et al. [28] reported that bacterium *Aeromonas* sp. which isolated from lake sediment only can tolerate 1.36 mM of Cr(VI) and 1.6 mM in study by Huddleston et al. [29]. The capability of *Aeromonas encheleia* in Cr(VI) removal still considered low as compare to *Ochrobactrum intermedium* which can reduce 90% of 3.4 mM within 24 hour [30]. Other bacteria such as *Pseudochrobactrum* sp., *Bacillus* sp. and *Microbacterium* sp. also known as good Cr(VI) reducing bacteria [9,11,25]. The poor Cr(VI) removal at high concentration of Cr(VI) may be attributed to the inhibition of chromate reductase and thus reduce the function of enzyme to synthesize Cr(VI) [22].

The effect of heavy metal on Cr(VI) reduction will signify the role of metal in stimulate or inhibit bacterial growth, and associate with enzyme synthesis. The low concentration of certain metals play vital role for bacterial growth, morphology and essential to support metabolic activities. In this present investigation, Cr(VI) reduction by *Aeromonas encheleia* is slightly affected due to addition of metal such as Cd, Cu, Ni, Pb and Zn. Meanwhile the presence of Co and Hg had inhibit 6.7% and 90.8% ability of this bacterium to reduce 100 µM Cr(VI). The finding is similar to study conducted Zakaria et al. [31], as Pb, Zn and Cd showed significant decrease 30 mg/L Cr(VI) removal by *Acinetobacter haemolyticus* in nutrient broth media. The effect of metal on Cr(VI) reduction is might be attributed to the growth medium used, in other word the complete media used in this present study, Zakaria et al. [31], Ge et al. [11], has reduced the possibility complexation effect by component in the media. In addition, Vi et al. [32] had reported that the use of TRIS minimal media reduced the heavy metal tolerance as compared to other studies that use complete and rich media. High tolerance of heavy metal does not mean the bacteria can have high Cr(VI) reduction, as instance *Aeromonas* sp. KS-14 exhibit high tolerance to Ni, Cu, Zn and Cd but have poor Cr(VI) reducing ability as compare to other strain used in study by Alam and Ahmad [15]. This may reflect the terminology of tolerance itself, Gadd [33] defined

tolerance as ability of bacteria to survive and hinder metal toxicity by their intrinsic properties and modification of toxicity.

Apart from that, the effect of metabolic inhibitors on Cr(VI) reduction has been investigated in order to determine intercellular movement of Cr(VI) during reduction process and to determine inhibition specific sites of the electron transport chain [15,34]. Based on previous studies, it has been revealed that metabolic inhibitor like NaCN, NaN₃ and Na₂SO₄ exhibit different effect on Cr(VI) reducing bacteria [15,31]. The differences may be attributed to different type of bacterial strain, characteristic of bacteria and pH. In this study, the presence of NaN₃ and Na₂SO₄ showed no significant inhibitory effect on Cr(VI) reduction, which is similar to study conducted by Rajwade et al. [35], Bae et al. [36] and Zakaria et al. [31]. In contrast, *Stenotrophomonas* sp., *Staphylococcus* sp., *Pontoea* sp. and *Aeromonas* sp. are highly inhibited by 1 mM NaN₃ and NaCN, meanwhile partially inhibited in the presence of Na₂SO₄ [15]. The presence of 1 mM NaCN in the LB media in this study declined 20% ability of bacterium to reduce Cr(VI), meanwhile the same concentration of NaCN did not impose any significant effect on Cr(VI) reduction by *E. coli* ATCC 33456 [37]. The strong hindering effect on metabolic inhibitors may indicate the possibility of Cr(VI) reductase utilize endogenous electron donors for Cr(VI) reduction [37]. Further study on Cr(VI) reductase shall be conducted in future in order to determine the role and mechanism of enzyme for Cr(VI) reduction in *Aeromonas encheleia*.

CONCLUSION

In this study, the isolated bacterium of *Aeromonas encheleia* possess and exhibit strong Cr(VI) reducing ability although being exposed in high concentration Cr(VI), other metals and metabolic inhibitors. The bacterium showed high tolerance in LB media containing 3.0 mM of K₂Cr₂O₇, but only reduce 1.5 mM Cr(VI) within 24 hours of incubation time under optimum conditions at 34°C and pH 7.5. The presence of metals and metabolic inhibitors in the media did not give significant inhibitory effect on Cr(VI) removal except mercury, sodium cyanide and cobalt with percentage inhibition 6.7, 80.5 and 90.7 respectively. Hence, it is expected that the bacterium can be used in bioremediation of chromium contaminated site and biological treatment of wastewater.

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