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Hexavalent chromium reduction by *Acinetobacter haemolyticus* isolated from heavy-metal contaminated wastewater

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Abstract

Possible application of a locally isolated environmental isolate, *Acinetobacter haemolyticus* to remediate Cr(VI) contamination in water system was demonstrated. Cr(VI) reduction by *A. haemolyticus* seems to favour the lower concentrations (10–30 mg/L). However, incomplete Cr(VI) reduction occurred at 70–100 mg/L Cr(VI). Initial specific reduction rate increased with Cr(VI) concentrations. Cr(VI) reduction was not affected by 1 or 10 mM sodium azide (metabolic inhibitor), 10 mM of PO_4^{3-}, SO_4^{2-}, SO_3^{2-}, NO_3^- or 30 mg/L of Pb(II), Zn(II), Cd(II) ions. However, heat treatment caused significant dropped in Cr(VI) reduction to less than 20% only. *A. haemolyticus* cells loses its shape and size after exposure to 10 and 50 mg Cr(VI)/L as revealed from TEM examination. The presence of electron-dense particles in the cytoplasmic region of the bacteria suggested deposition of chromium in the cells.
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Keywords: Reduction; *Acinetobacter*; Cr(VI); TEM; Heavy-metal

1. Introduction

Bacterial reduction of Cr(VI) can be considered as a mechanism of resistance to Cr(VI). Cr(VI) is a strong oxidizing agent that can easily penetrate the cell membrane of prokaryotic cells such as bacteria. Cr(VI) uptake is carried out by the sulphate transport pathway, hence is competitively inhibited by sulphate [1]. However, the role of sulphate as inhibitor for Cr(VI) uptake is more pronounced in anaerobic cells [2,3] compared to aerobic cells [4,5]. The ability of Cr(VI) anions to overcome the permeability barrier of a prokaryotic cell can be attributed to the chemical similarity between CrO_4^{2-} and SO_4^{2-} ions [6]. Bacterial resistance to Cr(VI) was reported to be plasmid-determined [7]. Cr(VI) resistance was also related to the decrease in Cr(VI) accumulation in resistant cells compared with the sensitive cells [7,8].

The aerobic Cr(VI) reduction is normally associated with a soluble protein fraction utilizing NADH or NADPH as electron donor [9,10] whereas in anaerobic condition, Cr(VI) can act as the terminal electron acceptor through membrane-bound reductase activity [11] which was reported in *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Enterobacter cloacae* by Wang and Xiao [5]. Aerobic reduction is considered to be a detoxification mechanism where normally the reduction of Cr(VI) by the soluble protein fraction takes place either internal or external to the plasma membrane. In the anaerobic respiration of *Enterobacter cloacae* HO1, possible involvement of the respiratory chain in the transfer of reducing equivalents to anionic Cr(VI) compounds through cytochrome *c* was implicated [11]. Anaerobic reduction of Cr(VI) by six strains of Cr-resistant Pseudomonads was also reported [12]. However, it was postulated that energy generated in the anaerobic respiration process is insufficient to sustain cell growth because fermentable organic compounds generated is utilized for cell metabolism [11,13]. Besides that, some of the bacteria were able to reduce Cr(VI) either aerobically or anaerobically. *Pseudomonas ambigua* G-1 and *Pseudomonas putida* PRS2000 were reported to reduce Cr(VI) in both conditions with higher reduction rates under aerobic conditions [13]. However, opposite trend was observed for *Enterobacter coli* ATCC 33456 where Cr(VI) reduction proceeded at higher rate anaerobically [11].

This paper discusses the Cr(VI) reducing properties of an aerobic gram negative environmental isolate, *Acinetobacter*

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Table 1
Summary of heavy metal concentrations in batek effluents

Metal	Concentrations range (mg/L)	Mean (mg/L)	Standard B (mg/L)[a]
Pb	0.63–0.80	1.47 ± 0.14	0.50
As	0.50–0.70	0.60 ± 0.10	0.10
Hg	0.025–0.10	0.07 ± 0.04	0.05
Cu	0.35–0.75	1.49 ± 0.22	1.00
Fe	1.50–3.75	2.55 ± 1.13	5.00
Ni	0.55–2.05	1.19 ± 0.77	1.00
Cd	0.20–0.50	1.35 ± 0.15	0.02
Cr	0.10–0.15	0.12 ± 0.03	0.05

[a] Mean values of triplicate samples; standard B limits the discharging of effluent containing substances with concentration higher than the specified value into any inland waters not within the catchment area [14].

haemolyticus. Cr(VI) reduction was assessed at different Cr(VI) concentrations and in growth-inhibiting conditions. Growth and Cr(VI) reduction in NB and NB added with carbon sources was also investigated. TEM and Langmuir adsorption isotherm was used to evaluate Cr deposition onto bacterial cell. Results obtained are expected to alleviate the realization of using bacterial system in treating toxic environmental contaminants such as Cr(VI).

2. Materials and methods

2.1. Bacteria

In this work, *Acinetobacter haemolyticus* (*A. haemolyticus*) was isolated from effluent of one batek (textile-related) manufacturing premise in Kota Bharu, Kelantan, Malaysia. The batek effluent contains a mixture of heavy metals (Table 1) which was determined(ICP-MS, Toshiba UP6100) to be slightly higher than the permissible discharge limit according to Standard B Malaysian standard [14]. *A. haemolyticus* was isolated using method suggested by Greenberg [15] and identified using the Microlog® Biolog Identification System, as previously described [16] and via the 16S rRNA analysis which was carried out by First BASE Laboratories Sdn. Bhd., Malaysia.

2.2. Preparation of bacterial cell suspension

Cell suspension of *A. haemolyticus* was prepared by harvesting cells after 24 h of growth. The cells were harvested by centrifugation (SIGMA 2K-15, B. Braun) at 9000 rpm, 5 min and 0 °C. Pellet obtained was then washed twice using 0.85 g NaCl/100 mL solution at 9000 rpm, 3 min and 0 °C and resuspended in the same solution at 17.5% of the original volume of cell. Cell suspension (5 mL) was filtered through hydrophobic-type 0.45 μm Whatman filter paper and dry weight determined after overnight drying at 70–80 °C.

2.3. Determination of minimum inhibitory concentration (MIC) for Cr(VI), As, Pb, Cd and Zn

Fresh NB medium (1.00–3.75 mL) was pipetted into repliplate dish (Sterilin, UK) followed by 0.25–4.75 mL of stock Cr(VI) solution (1000 mg/L) to give Cr(VI) concentrations between 50 and 1000 mg/L. Stock Cr(VI) solution was prepared by dissolving 2.829 g $K_2Cr_2O_7$ (294.18 gmol^{-1}) in 1 L of deionised water. The pH of Cr(VI) solution was adjusted to 7.0 using 0.1 M NaOH or 0.1 M HCL before filter-sterilized using a 0.45 μm Whatman filter paper. A 5% (v/v) inoculum of the 24 h grown cell was then added to the repli-plate and incubated at 30 °C for 7 days (Memmert, USA). Control sets consist of bacterial cells in NB medium, NB medium only and Cr(VI) in NB medium. The first concentration of Cr(VI) that totally inhibits bacterial growth is considered as MIC. MIC or resistant level of bacteria to Cr(VI) was evaluated by measuring percentages of cell survival at OD_{600} (Spectronic 21D) after 7 days of incubation period. The same procedure was repeated with the following metal solutions (prepared at 1000 mg/L each); As: 1.320 g As_2O_3 (197.84 g mol^{-1}); Pb: 1.599 g Pb $(NO_3)_2$ (331.21 g mol^{-1}); Cd: 2.030 g $CdCl_2{\cdot}5/2H_2O$ (228.34 g mol^{-1}); Zn: 4.398 g $ZnSO_4{\cdot}7H_2O$ (287.55 g mol^{-1}). Similar sets of control experiments as Cr(VI) were prepared with As(III), Pb, Cd and Zn replacing Cr(VI). The experiment was performed in duplicates.

2.4. Growth in mixtures of NB and carbon source

Addition of carbon source was made to determine its effect on bacterial growth and Cr(VI) reduction by *A. haemolyticus* which was previously exposed to nitrogen-rich environment only i.e. NB medium. 10 mg cell dry weight was added to a series of 100 mL NB medium in 1 L Erlenmeyer flasks. Then, 0.2% (w/v) of either citrate, lactate, malate, succinate, oxalate, glucose, sucrose or acetate was added from its respective stock solutions (5%, w/v) before incubated at 30 °C, 200 rpm for 48 h. The carbon source stock solutions were filter-sterilized prior to use. Bacterial growth was determined at OD_{600}. Control experiments were NB medium only and NB medium added with respective carbon sources minus the bacteria.

2.5. Growth in salts minimal medium and carbon source

Same procedure as growing bacteria in mixture of NB and carbon source was used except that basal salts (acting as salts minimal medium) replaced NB. Basal salts consist of 3 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L K_2HPO_4 and 0.1 g KCl. Control experiments consist of saline solution only and saline solution added with respective carbon sources minus bacteria.

2.6. Experimental design for Cr(VI) reduction

2.6.1. Cr(VI) reduction in mixtures of NB and carbon source

The reaction mixtures was prepared by adding 0.1–1.0 mL of 1000 mg/L stock Cr(VI) solution into a series of 1 L Erlenmeyer flasks containing 100 mL NB medium. Based on highest bacterial growth, sucrose was chosen as carbon source where 0.2% (w/v) was added from 5% (w/v) stock solution followed

by 10 mg cell dry weight of *A. haemolyticus*. The mixture was then incubated at 30 °C, 200 rpm for 48 h. During the 48 h incubation period, Cr(VI) reduction was determined using the DPC method while cell viability was determined using spread-plate method. Control experiments consist of NB medium only and NB medium added sucrose without bacteria.

2.6.2. Effect of Cr(VI) concentration

Cell suspension (10 mg cell dry weight) was mixed with 100 mL NB medium in 250 mL Erlenmeyer flasks and grown for 12 h. Then, Cr(VI) at 10–100 mg/L was added to the bacterial mixture where Cr(VI) reduction was evaluated at 0, 0.25, 0.50, 1, 2, 4, 8, 12, 24, 36 and 48 h. The hyperbolic equation of Michaelis–Menten, $V = V_{max}[S]/K_m + [S]$, where $[S]$ represents Cr(VI) concentration, V_{max} the maximum reaction velocity and K_m is the Michaelis constant is used to determine the kinetic parameters (K_m and V_{max}) of the reaction. Evaluation was based on the assumption of a pseudo first-order rate reaction. Mixtures of NB medium and Cr(VI) minus the bacterial cell acted as control.

2.6.3. Effect of heavy metals

Stock Cr(VI) solution (0.3 mL) was added to a series of 1 L Erlenmeyer flasks containing 100 mL NB medium. Then, the same volume of respective stock solutions (1000 mg/L) of either Pb, Zn or Cd were transferred into the flasks where final concentration of Pb, Zn and Cd prepared was 30 mg/L. One particular 1 L Erlenmeyer flask containing the mixture of all metals was also prepared. Following this, 10 mg cell dry weight of *A. haemolyticus* was inoculated followed by incubation at 30 °C, 200 rpm for 48 h. At the end of experiment, supernatant obtained from centrifugation (9000 rpm, 5 min, 4 °C) was determined for residual Cr(VI) using the DPC method. Individual heavy metal stock solutions (1000 mg/L) of Pb, Zn and Cd was prepared by dissolving 0.1599 g of $Pb(NO_3)_2$ (331.21 g mol^{-1}, Merck), 0.4398 g of $ZnSO_4{\cdot}7H_2O$ (287.55 g mol^{-1}, Merck) and 0.2030 g of $CdCl_2{\cdot}5/2H_2O$ (228.34 g mol^{-1}, BDH) in 100 mL of deionised water. Sets of NB medium only and NB medium added with respective heavy metals minus the bacterial cell acted as control.

2.6.4. Effect of oxyanions

Stock Cr(VI) solution (0.3 mL) was added to a series of 1 L Erlenmeyer flasks containing 100 mL NB medium. Then, 0.1 mL of 1 M stock solutions of either SO_4^{2-}, SO_3^{2-}, NO_3^- and PO_4^{3-} were added to the 30 mg/L Cr(VI). Following this, 10 mg cell dry weight of *A. haemolyticus* was inoculated followed by incubation at 30 °C, 200 rpm for 48 h. Cr(VI) reduction was determined at the end of the 48 h period using DPC method. SO_4^{2-} was added as Na_2SO_4 (142.04 g mol^{-1}, Merck), SO_3^{2-} as Na_2SO_3 (126.04 g mol^{-1}, Merck), NO_3^- as KNO_3 (101.11 g mol^{-1}, Merck) and PO_4^{3-} added as KH_2PO_4 (136.09 g mol^{-1}, Merck). The stock solutions for SO_4^{2-}, SO_3^{2-}, NO^{3-} and PO_4^{3-} were sterilized via autoclaving at 121 °C for 15 min (Fedegari, Italy). NB medium only and NB medium added with respective oxyanions minus the bacterial cell acted as control.

2.6.5. Effect of cell viability

Two types of cell treatment were carried out i.e. addition of cell metabolite inhibitor and autoclaving at 121 °C for 15 min. Sodium azide (NaN_3) was used to assess possible involvement of the respiratory (electron transport) system of the bacteria as suggested by Philip et al. [20] as the intended site of inhibition for NaN_3 is cytochrome oxidase [11]. Autoclaving of cells was carried out to render the cells non-living. Stock Cr(VI) solution (0.3 mL) was added to a series of 1 L Erlenmeyer flasks containing 100 mL NB medium. Then, 10 mg cell dry weight of autoclaved cells was added before incubation for 48 h, 200 rpm and 30 °C. To determine the effect of sodium azide, 10 mg cell dry weight was first grown for 4 h at 200 rpm, 30 °C in 30 mg/L Cr(VI). Then, 0.1 and 1.0 mL of 1 M stock solution of NaN_3 (65.01 g mol^{-1}, Merck) was added to prepare a 1 and 10 mM NaN_3 solutions, respectively. At the end of experiment, cell viability and Cr(VI) reduction was determined using plate-count technique and OD_{540} respectively. Control consists of NB medium only, NB medium containing 1 and 10 mM NaN_3 and active cells of *A. haemolyticus* in saline.

2.7. Transmission electron microscope (TEM) analysis

Cells of *A. haemolyticus* grown in 10 and 50 mg/L Cr(VI) for 48 h, 200 rpm and 30 °C were centrifuged at 9000 rpm, 4 °C for 7 min. Pellet obtained was prepared for TEM examination (Philips TEM 400 Transmission Electron Microscope) using modified procedure of Bencosme and Tsutsumi [17]. The sample was fixed using 3% (v/v) glutaraldehyde in 0.1 M phosphate buffered saline for 2 h. It was then washed and suspended in 5 mL of the same buffer followed by staining using 1% (v/v) osmium tetraoxide in deionised water for 20 min. After washing in three changes of deionised water, the sample was stained using 2% (v/v) uranyl acetate in deionised water for 10 min. It was then rinsed in deionised water prior to dehydration in 50% (3 min), 70% (3 min), 90% (3 min) and 100% (v/v) ethanol (3 min). Propylene oxide was then added for 5 min, mixture between epoxy resin and propylene oxide (1:1 and 3:1) for 15 min each and epoxy resin only (10 min). The sample was then embedded in a fresh epoxy resin and polymerized at 75 °C (45 min) and 95 °C (45 min). The resultant resin block containing bacterial sample was allowed to cool at room temperature overnight before ultra-thin sectioning (70–80 nm thick) was made using LKB-IV System 2128 (Bromma, Sweden) ultramicrotome. The ultra-thin sections obtained were then viewed using TEM.

2.8. Analytical method

The reduction of hexavalent chromium was determined colorimetrically at 540 nm using the diphenylcarbazide (DPC) method with a detection limit of 5 μg L^{-1} [15]. In a 10 mL volumetric flask, 1 mL of sample was mixed with 9 mL of 0.2 M H_2SO_4. Then 0.2 mL of freshly prepared 0.25% (w/v) DPC in acetone was added to the volumetric flask. The mixture was then vortexed (Maxi Mix-II Thermolyne) for about 15–30 s and let to stand between 10 and 15 min for full colour development. The red-violet to purple colour formed was then measured at OD_{540}

Table 2
Identification of *A. haemolyticus* by 16S rRNA gene sequence analysis

Species as close relatives	Accession no.	Percent similarity
Acinetobacter haemolyticus	X81662	99.5
A. haemolyticus	AY586400	99.5
Acinetobacter sp.	AY639376	99.5
Acinetobacter sp.	AY902243	98.6
Uncultured *Acinetobacter* sp.	AF467306	98.6

The top 20 sequence matches were identified as an *Acinetobacter* of the Moraxellaceae family of the Pseudomonadales order from the nucleotide sequence of 597 bp.

using distilled water as reference. Instrument used was calibrated using 0.4–2.0 mg/L Cr(VI) prepared from Cr(VI) stock solution (1000 mg/L).

3. Results

3.1. Bacteria

Result from the 16S rRNA gene sequence analysis for *A. haemolyticus* is shown in Table 2. To date, *A. haemolyticus* has not been reported as either chromium-resistant or chromium-reducing bacterium.

3.2. MIC for Cr(VI), As, Pb, Cd and Zn

A. haemolyticus has slightly higher resistance towards Cd, As, Pb and Zn compared to Cr(VI) (Fig. 1). The general order of resistance is as follows; Cr(VI) < Zn < Cd < As(III) < Pb which was based on LC_{50} values for As which is 750 mg/L, Cd at 400 mg/L, 200 mg/L for Zn and 70 mg/L for Cr(VI). Percentages of cell survival in the presence of Pb remain more than 50% even at 1000 mg Pb/L. For Cr(VI), cell survival was reduced to less than 10% at 200 mg/L. The fact that Pb being the most tolerated metal and Cr(VI) being the least, can be attributed to degree of solubility and affinity towards potential complexing agents such as organic compounds.

3.3. Growth in mixtures of nb and carbon source

In this study, citrate, lactate, malate, succinate, oxalate, glucose, sucrose and acetate at 0.2% (w/v) were evaluated for its role as electron donors in bacterial growth. Results obtained is shown in Fig. 2.

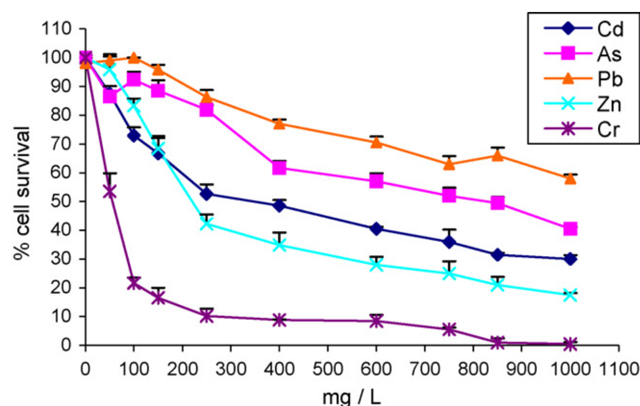

Fig. 1. MIC of *A. haemolyticus* towards Cd, Pb, Zn and Cr(VI).

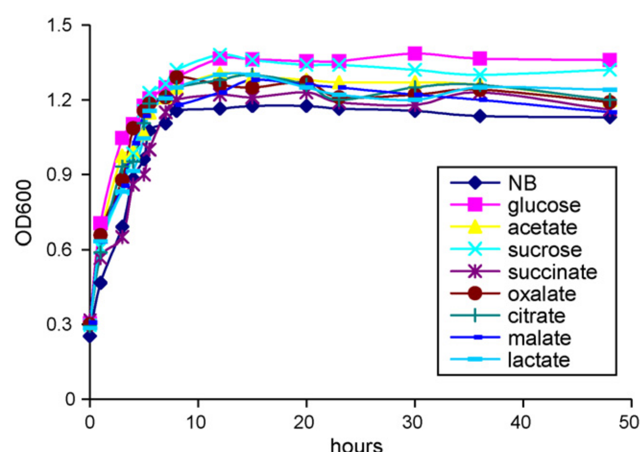

Fig. 2. Growth profile of *A. haemolyticus* in NB and extra carbon source.

The presence of extra carbon source increased overall growth of *A. haemolyticus*, suggesting utilization of these carbon sources in metabolism. Overall, preference of *A. baumanni* for carbon source can be represented in increasing order as follows; sucrose > glucose > lactate > acetate, succinate, oxalate, malate, citrate (OD_{600} values ranging from 1.10 to 1.20). Maximum cell yield for *A. haemolyticus* was also increased from 1.16 when grown in NB medium only to 1.38 in sucrose, 1.36 (glucose) and 1.31 (acetate).

3.4. Growth in salts minimal medium and carbon source

A. haemolyticus was able to use all carbon sources based on cell growth percentages of around 13 to 53%, relative to initial OD_{600}. Sucrose was the preferred carbon source utilized by *A. haemolyticus* with highest percentages of cell growth of 53.13%, followed by lactate (44.15%), citrate (40.51%), glucose (39.76%) and acetate (37.37%). This prompts the selection of sucrose in the following Cr(VI) reduction studies. Succinate, malate and oxalate were the least preferred carbon source with growth percentages of less than 20% each. Maximum growth of *A. haemolyticus* was greatly reduced in the absence of NB as growth medium. For example, growth of *A. haemolyticus* in the presence of NB and sucrose yielded maximum OD_{600} of 1.38. However, the value dropped to 0.53 when basal salts was used instead of NB.

3.5. Cr(VI) reduction in mixtures of nb and carbon source

The addition of 0.2% (w/v) sucrose as extra carbon source for *A. haemolyticus* did not increase Cr(VI) reduction (Fig. 3) but causes an increase in cell concentration (CFU/mL) (Fig. 4). However, such metabolic activity did not result in increased or even prolonged Cr(VI) reduction, as observed for *A. haemolyticus* in this study.

3.6. Effect of Cr(VI) concentration

A. haemolyticus was able to completely reduce Cr(VI) up to initial Cr(VI) concentrations of 50 mg/L (Fig. 5). Around 8, 13

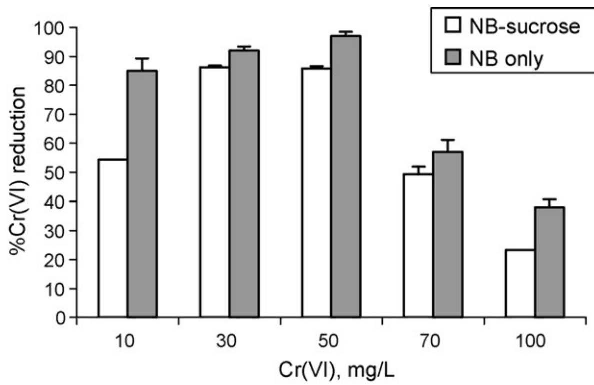

Fig. 3. Reduction of Cr(VI) in mixtures of NB—0.2% (w/v) sucrose and in NB medium only.

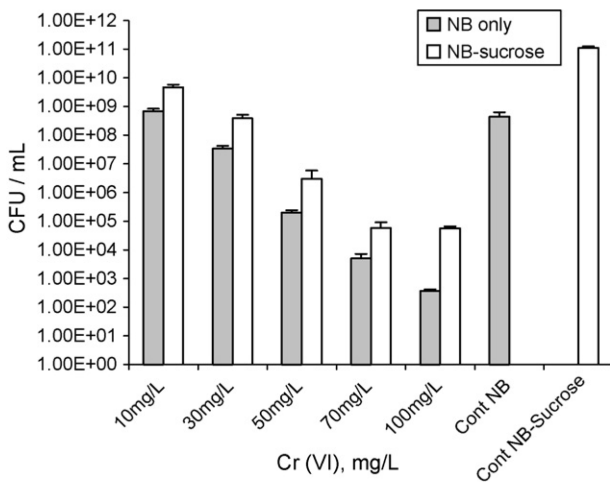

Fig. 4. Effect of initial Cr(VI) concentration on cell viability of *A. haemolyticus* in 0.2% (w/v) sucrose.

and 36 h was needed to completely reduce 10, 30 and 50 mg/L Cr(VI), respectively. Apparent Michaelis–Menten constant, K_m of 77.52 mg/L CrO_4^{2-} (18.29 mM) and a maximum velocity, V_{max} of 2.26 μg CrO_4^{2-} L^{-1} min^{-1} mg^{-1} cell dry weight were obtained from Lineweaver–Burke plots. The values were derived from the assumption that with excess carbon source in NB

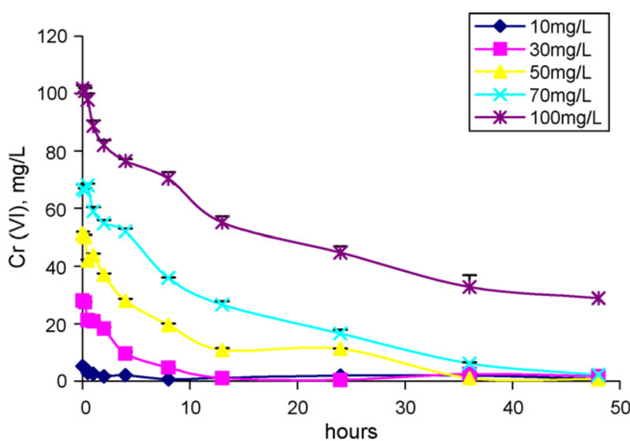

Fig. 5. Effect of Cr(VI) concentrations on Cr(VI) reduction rate by *A. haemolyticus*.

Table 3
Initial specific reduction rate for Cr(VI) at different initial Cr(VI) concentration

Initial Cr(VI) concentration (mg/L)	Initial specific reduction rate (mg Cr(VI) h^{-1} g^{-1} cell dry wt.)
10	254.21
30	264.33
50	359.77
70	404.39
100	397.43

medium, the reaction would be pseudo first order and the initial reaction rate would be independent of the substrate i.e. Cr(VI) concentration [13].

The reduction rate decreased gradually at Cr(VI) concentrations higher than 50 mg/L. At initial Cr(VI) concentration of 70 mg/L, 88% of Cr(VI) was reduced while the value further decreased to 75% at 100 mg/L Cr(VI) after 48 h reaction time. The rate of Cr(VI) reduction decreased with time and eventually ceased at higher Cr(VI) concentrations. Another point to note is that, even though complete Cr(VI) reduction was not observed, initial specific reduction rate increased with Cr(VI) concentrations as shown in Table 3.

3.7. Effect of heavy metals

The effect of heavy metals on Cr(VI) reduction by *A. haemolyticus* is shown in Fig. 6. Neither the presence of 30 mg/L of Pb(II), Zn(II), Cd(II) or mixture of all, resulted in significant decrease in Cr(VI) reduction by *A. haemolyticus* with more than 90% of Cr(VI) reduced. However, Cr(VI) reduction slightly decreased to 82.54% in the presence of Pb.

3.8. Effect of oxyanions

The role of oxyanions such as PO_4^{3-}, SO_4^{2-}, SO_3^{2-} and NO_3^{-} as competing electron acceptors in Cr(VI) reduction were investigated. SO_4^{2-} is of particular importance as Cr(VI) is

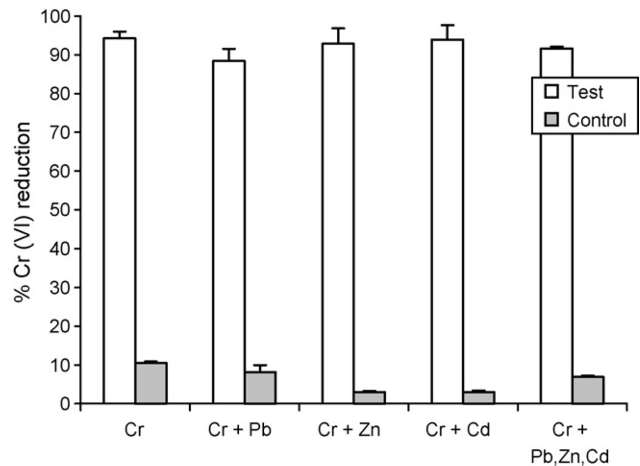

Fig. 6. The effect of 30 mg/L heavy metals on reduction of 30 mg/L Cr(VI) by *A. haemolyticus*.

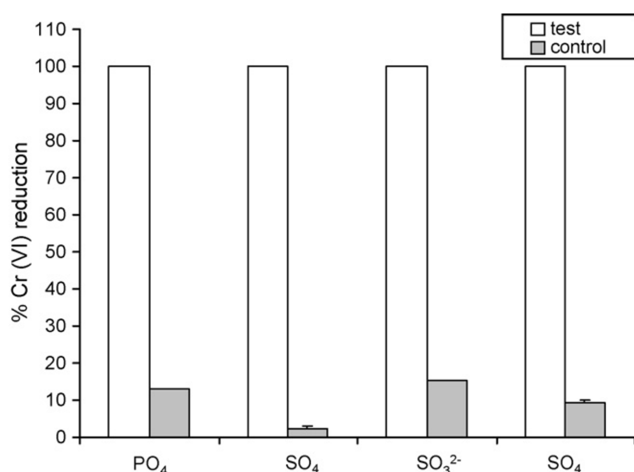

Fig. 7. Reduction of 30 mg/L Cr(VI) in 10 mM oxyanions by *A. haemolyticus*.

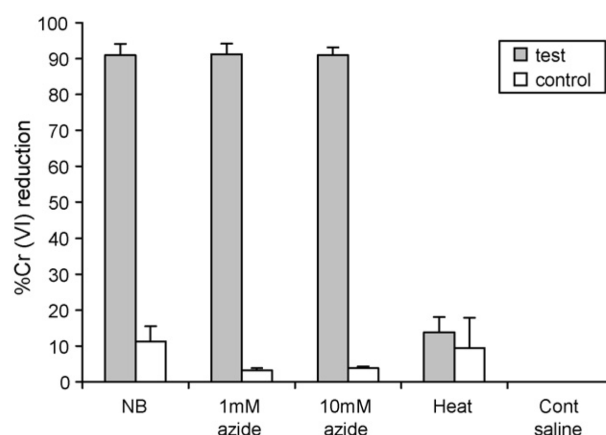

Fig. 8. Effect of NaN_3 and heat treatment on the reduction of 30 mg/L Cr(VI) by *A. haemolyticus*. Control for azide is NB + azide only.

known to enter the bacterial cell via the sulphate uptake pathway [18,19]. Results obtained is shown in Fig. 7.

Cr(VI) reduction by *A. haemolyticus* was unaltered in the presence of 10 mM of phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), sulphite (SO_3^{2-}) and nitrate (NO_3^{-}) indicating Cr(VI) as better electron acceptor than these oxyanions, a statement supported by Philip et al. [20]. The ability of *A. haemolyticus* to withstand 10 mM of SO_4^{2-} indicated that Cr(VI) uptake via the sulphate uptake pathway may not be interfered by SO_4^{2-}. A point to note is that the insensitivity of *A. haemolyticus* towards PO_4^{3-}, SO_4^{2-}, SO_3^{2-} and NO_3^{-} suggested that Cr(VI) reduction process was solely aerobic.

3.9. Effect of cell viability

The addition of 1 mM and 10 mM azide after 4 h of cell incubation, did not interfere with the reduction of 30 mg/L Cr(VI) by *A. haemolyticus* with more than 90% Cr(VI) reduced (Fig. 8). The result is comparable to Cr(VI) reduction in NB only (>90% Cr(VI) reduced). However, heat treatment caused significant dropped in Cr(VI) reduction to less than 20% only. Since *A. haemolyticus* cannot withstand high temperature as shown by no colony isolated on NA plates, the 20% Cr(VI) reduction observed may be attributed to the complexation of Cr(VI) with the denatured organic matter from the biomass [20].

3.10. Transmission electron microscope (tem) analysis

The TEM analysis was carried out to assess possible intracellular Cr precipitation by *A. haemolyticus* during Cr(VI) reduction and results obtained are as shown in Fig. 9a–c.

When *A. haemolyticus* was grown without Cr(VI) (Fig. 9a), the cells appeared as rod and cocci with inner and outer sections of bacteria clearly discernible. However, upon exposure to either 10 (Fig. 9b) or 50 mg Cr(VI)/L (Fig. 9c), the cells loses its shape and increases in size. The cells also remain intact and did not rupture from partial decompression. One interesting observation is cell wall for control cells i.e. cell grown without Cr(VI), appears thicker than cells exposed to Cr(VI). For example, exposing the cells to 10 mg/L Cr(VI) decreased cell wall thickness to 50–100 nm compared to 100–300 nm for control cells. Fine electron - dense particles (indicative of Cr precipitation) was observed for *A. haemolyticus* grown in 50 mg/L Cr(VI) only.

4. Discussion

A wide array of technologies is available for the removal of chromium from wastewaters. Treatment of Cr(VI) waste usually consists of a two-stage process i.e. chemical reduction of Cr(VI) to Cr(III) using sulphur dioxide, sodium bisulphite or sodium metabisulphite followed by precipitation of Cr(III) using either

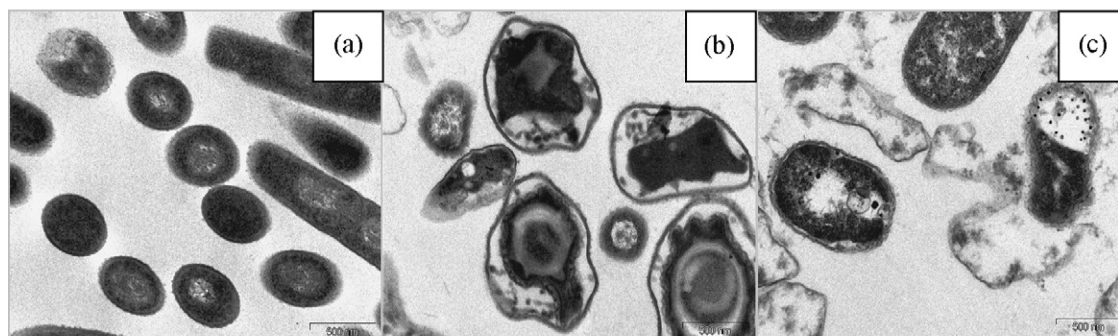

Fig. 9. *A. haemolyticus* grown for 24 h in nutrient broth; (a) without Cr(VI); (b) in 10 mg/L Cr(VI); (c) in 50 mg/L Cr(VI); bar: 500 nm.

lime, caustic soda or sodium bicarbonate [21]. Even though the process is efficient, large volumes of sludge generated the release of dangerous gases and expensive cost of the chemical reducing agents, makes it imperative to look into safer and cheaper alternative where, biological system seems to be a suitable approach. Numerous reports have demonstrated the feasibility of using biological processes for the treatment of Cr(VI) industrial effluents by either pure culture or a consortium of Cr(VI) reducing bacteria [22–26].

In this study, besides the apparent Cr(VI) toxicity, low tolerant level of *A. haemolyticus* to Cr(VI) can be attributed to *A. haemolyticus* being an obligate aerobic organism. In the repliplate technique, the plate was incubated in stationary mode i.e. without shaking, resulting in limited O_2 diffusion into the culture medium, hence limiting cell metabolic activity. Complex organic medium constituents such as yeast extract, proteose peptone and amino acids (especially cysteine and glutamic acids) are known binders of Hg^{2+}, Pb^{2+}, Ag^{+}, Cu^{2+} and to a lesser extent, Cd^{2+} [27]. The MIC value obtained may not be absolute as it is dependant on the growth medium used [28]. MIC values obtained in rich medium such as NB are usually two to five times higher than in salts minimal medium such as TRIS due to the possible complexation of Cr(VI) by components in the medium especially organic substances and phosphate [29]. However, Pattanapipitpaisal et al. [30] contends that since Cr(VI) resistance is usually associated with exclusion of Cr(VI) rather than bioreduction, the role of organic compounds in rich medium to complex Cr(VI) was probably less important. In this study, this possibility has been taken into account from the abiotic control set consisting of mixtures between NB and Cr(VI) where OD_{600} values obtained were used during calculating actual percentage of cell survival. This condition where *A. haemolyticus* resist Cr(VI) in a strictly aerobic condition have also been reported for other bacteria such as *Enterobacter cloacae* [7], *Bacillus* sp. [11] and *Arthrobacter* sp. [31].

Carbon source was added to the N-rich NB medium to assess its effect on time needed to achieve maximum cell yield. This knowledge is advantageous especially in large-scale bacterial fermentation where factors such as cost and time are of extreme importance. Even though *A. haemolyticus* is not known to utilize wide range of carbon source, some strains of *A. haemolyticus* were reported to utilize glucose, D-ribose, D-xylose, D-arabinose, ethanol, D-malate, DL-lactate and citrate during metabolism processes [32]. This could account for the preference of *A. haemolyticus* towards glucose, sucrose and lactate. This situation however may be species dependent as other bacteria such as *P. aeruginosa*, *B. circulans* and *B. coagulans* prefer acetate, succinate, oxalate, malate and citrate as electron donor compared to glucose or sucrose. *B. coagulans* was able to utilize intermediate products formed during the Krebb's cycle such as acetate, succinate, oxalate, malate and citrate much easier compared to glucose that need to be catabolized into pyruvate before entering the Krebb's cycle [20].

A. haemolyticus showed good growth in the absence of NB and Cr(VI). However, maximum growth achieved i.e. OD_{600} of 0.53 was less than growth in NB and sucrose i.e. OD_{600} of 1.38. This indicates the important role played by nitrogen containing groups present in NB medium such as 'Lab-Lemco' powder, yeast extract and peptone. Even though *A. haemolyticus* was able to use NH_4^+ from basal salts for metabolism, the concentration may not be sufficient to sustain bacterial growth. Therefore, the presence of 'Lab-Lemco' powder, yeast extract and peptone should suffice N-requirement for *A. haemolyticus*. The ability of *A. haemolyticus* to utilize wide range of carbon and energy sources indicates its potential application to support *in situ* remediation of Cr(VI). These carbon sources can act as alternative to complex medium such as NB whose high cost might become prohibitive in actual field applications. The use of cheap industrial grade molasses (USD 67.50/tonnes) with high sugar content such as beet molasses (66.5% sugar of which approximately 95% is sucrose), might provide a more cost effective alternative to any of the carbon and energy sources examined [33]. It is noteworthy that the rate of sucrose consumption was not directly measured in this study. However, since the purpose of this work was to determine the most suitable concentration to yield maximum cell density, optical density measurement (OD_{600}) was deemed sufficient as an indicator of the change in cell concentration. Similar approach was suggested by Rege et al. [34] where OD_{600} measurement was used as an indicator for the growth of *Enterobacter cloacae* in sucrose.

The effect of extra carbon source on Cr(VI) reduction by *A. haemolyticus* was also investigated. The addition of 0.2% (w/v) sucrose as extra carbon source for *A. haemolyticus* did not increase Cr(VI) reduction but cell concentration (CFU/mL). This same condition was also reported for *P. fluorescens* LB300 [24] and *P. ambigua* [35]. In both reports, Cr(VI) reduction occurs with simultaneous utilization of substrate. However, such metabolic activity did not result in increased or even prolonged Cr(VI) reduction, as observed for *A. haemolyticus* in this study. Cr(VI) reduction decreased progressively and eventually ceased. This phenomenon can be attributed to the mutagenic effects of Cr(VI) towards bacterial cells [4]. Komori et al. [2] reported mutations in *Enterobacter cloacae* as a result from DNA damaging due to interactions with Cr(VI). Wang [4] reported that the damaging of DNA would lead to disruption of normal cell replication, resulting in mutated replicate of the affected cells. However, mutant cells may be more resistant to Cr(VI) [4,5,35]. The ability of *A. haemolyticus* to withstand high concentrations of azide might be of interest because most studies reported uses lower concentrations of azide with varying inhibitory effect as summarized in Table 4.

Partial inhibition of Cr(VI) reduction was observed for *Bacillus subtilis* when 4.0 mM of sodium azide was added after 6 h of

Table 4
Effect of sodium azide, NaN_3 on bacterial reduction of Cr(VI)

NaN_3 (mM)	Strain	Effect	Reference
4.0	*Bacillus subtilis*	Partial inhibition	[9]
1.0	*Bacillus* sp. ES 29	Not inhibitory	[10]
1.0	*Streptomyces griseus*	Partial inhibition	[36]
1.0	*E. coli* ATCC 33456	Not inhibitory	[11]
1.0	*Shewanella putrefaciens* MR-1	Inhibitory	[37]
1.0, 10.0	*A. haemolyticus*	Not inhibitory	This study

bacterial cultivation [9]. The same condition was observed for 1.0 mM azide for *Streptomyces griseus* [36]. In both situations, bacterial growth and reduction slowed down after the addition of azide. However, heat treatment caused significant drop in Cr(VI) reduction to less than 20% only. Since *A. haemolyticus* cannot withstand high temperature as shown by no colony isolated on NA plates, the 20% Cr(VI) reduction observed may be attributed to the complexation of Cr(VI) with the denatured organic matter from the biomass [20]. This is supported from the fact that around the same amount of Cr(VI) was reduced in NB only. Laxman and More [36] also reported the impairment or loss of the reduction ability of 20 mg/L Cr(VI) by *Streptomyces griseus*, a Gram-positive bacterium, upon increasing the experimental temperature to 50 °C. The effect of heat was also instrumental in immediate inhibition of Cr(VI) reduction by *Bacillus subtilis* [20].

TEM analysis (Fig. 2) showed that when *A. haemolyticus* were grown without Cr(VI), the cells appeared as rod and cocci forms with inner and outer sections of bacteria clearly discernible. However, upon exposure to either 10 or 50 mg Cr(VI)/L, the cells loses its shape and increases in size. The cells also remain intact and did not rupture from partial decompression. One interesting observation is cell wall for control cells i.e. cell grown without Cr(VI), appears thicker than cells exposed to Cr(VI). For example, exposing the cells to 10 mg/L Cr(VI) decreased cell wall thickness to 50–100 nm compared to 100–300 nm for control cells. *P. putida* was reported to experience marked change in size upon exposure to Cr(VI) even though no explanatory statements were included [38]. It was also stated that at 15 mM or 1560 mg/L Cr(VI), the cells became both longer and wider. However, further increase to 30 mM or 3120 mg/L Cr(VI) led to reduction in cell size i.e. smaller than the cells grown without Cr(VI). However, this condition was not observed for *A. haemolyticus*, probably due to small difference between the two concentrations used i.e. 10 and 50 mg/L compared to 1560 and 3120 mg/L [38]. Fine electron-dense particles (indicative of Cr precipitation) was observed for *A. haemolyticus* grown in 50 mg/L Cr(VI) only. This precipitates having rough diameter of between 5 and 20 nm should account as Cr(III) based on its absence for the cells grown without Cr(VI).

The ability of a gram negative aerobic bacterium such as *A. haemolyticus* to intracellularly reduce Cr(VI) was also reported for *P. putida* [39] where majority of cells contained intracellular precipitates in the form of round globules with less amount being precipitated on the cell wall or cell surface. These electron-dense precipitates were absent in *P. putida* not exposed to chromium. The formation of round globules was reported to be due to the cross linking of reduced Cr(III) with DNA. These intracellular and extracellular electron-dense precipitates were confirmed as chromium through examination using electron energy loss spectroscopy (EELS) where Cr precipitated as Cr(III) and not Cr(VI).

McLean and Beveridge [13] reported the precipitation of Cr of various sizes to cells of *Pseudomonads*. Largest precipitates were slightly rounded which was predicted as amorphous Cr(III) hydroxide, $Cr(OH)_3$. Cr(III) was uniformly adsorbed to surface of the cells resulting in the formation of precipitates. Bacteria are excellent nucleation sites for fine-grained mineral formation due to their high surface area-to-volume ratio and the presence of electronegative functional groups such as carboxyl, phosphoryl and hydroxyl groups. As Cr(VI) is reduced, Cr(III) is free to bind stoichiometrically to these sites and once bound, will act as a template for further heterogenous nucleation and crystal growth. Daulton et al. [40] reported that precipitation of Cr was restricted to outer surface of *Shewanella oneidensis* only. This was based on the absence of electron-dense precipitates in the inner region of the bacterial cell. Precipitates ranged in size between 10 and 200 nm. Selected area diffraction indicated that the grains were predominantly amorphous due to high degree of hydration. The cells are often having thickness of between 30 and 40 nm with distinct cell line, indicating cell boundary became saturated with absorbed element of heavy mass i.e. Cr. However, this situation was not observed for *A. baumanii*.

5. Conclusion

As a conclusion, *A. haemolyticus* was demonstrated to be a potential biological reducing agent for Cr(VI). This finding is important as this gives early indication on its potential use in the industry. However, further studies using real Cr(VI)-containing industrial wastewater and sugar-rich and cheap industrial waste as feed for the bacteria needs to be carried out.

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