Cr(VI) reduction in naturally rich growth medium and sugarcane bagasse by Acinetobacter haemolyticus

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ABSTRACT

The potential of agricultural waste as substitute for rich-growth medium for bacteria during Cr(VI) reduction was investigated using the locally isolated Cr(VI) resistant-reducing strain of Acinetobacter haemolyticus and sugarcane bagasse. A. haemolyticus showed higher resistance to 100 mg/L Cr(VI) in Luria Bertani (>10^{10} CFU/mL) compared to sugarcane bagasse (10^6 CFU/mL). Higher Cr(VI) reduction by the SCB-immobilized bacteria (>90%) compared to in LB only (around 25%) clearly indicated the role of SCB in carrying out abiotic reduction of Cr(VI) as well as Cr(VI) adsorption. Nevertheless, Cr(III) was detected on the bacterial surface using Electron Spin Resonance (ESR) and electron microscopy (FESEM–EDX) analysis. The Cr(III) deposition occurred probably via complex formation with either carboxyl, hydroxyl or amide groups present on the bacterial cells surface as suggested from the FT-IR analysis. TEM analysis further showed Cr distribution at the membrane and cytosolic fractions. This work clearly demonstrated the role of bacteria in reducing Cr(VI) to Cr(III) as well as the potential of using agricultural waste material such as SCB to carry out abiotic Cr(VI) reduction.

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1. Introduction

Chromium is one of the toxic heavy metals, which is widely used in various industrial applications such as leather tanning, electroplating, metal finishing, petroleum refining, textile manufacturing, pulp production and others. These activities generate enormous volumes of wastewater which is costly to be notably treated due to the high price for chemical reducing agents such as FeCl₃, Fe₂(SO₄)₃, Na₂S₂O₅ and NaHSO₃. Although Cr is able to exist in several oxidation states (−2 to +6), the most stable and common forms are the trivalent chromium, Cr(III) and hexavalent chromium, Cr(VI) species. However, the solubility of Cr(VI) makes it a very toxic and carcinogenic element as compared to Cr(III) which is much less toxic, less soluble under neutral pH and unable to cross cell membranes. Cr(VI) represents a serious threat to human health, living resources and ecological system as they considered as persistent, bioaccumulative and toxic. Therefore, there is an urgent need to seek alternative treatment methods to remove Cr(VI) which is cost effective and environmental-friendly. One plausible alternative is the use of microbial approach which is cleaner, sustainable and environmentally friendly. A number of chromium resistant bacteria such as Bacillus sp. (Sarangi and Krishnan, 2008), Pseudomonas sp. (Desai et al., 2008), Brucella sp. (Thacker et al., 2007), Achromobacter sp. (Zhu et al., 2008) and Acinetobacter sp. (Srivastava and Thakur, 2007) have been reported to reduce Cr(VI) to Cr(III). Although the use of rich growth medium such as Luria–Bertani or nutrient broth results in fast growth and good growth yields, its high cost limits its application in biological wastewater treatment processes at industrial scale. This makes it imperative to find a useful, cheap and easily available alternative source for culture growth medium. Agricultural waste presents an interesting option based on its abundance and availability. For example, sugarcane bagasse is a residue produced by foods/drinks industries. It consists of mainly lignocellulosic materials as well as other polar functional groups such as alcohols, aldehydes, ketone, carboxylates, phenols and ethers. This work highlights the reduction of Cr(VI) by Acinetobacter haemolyticus in the presence of sugarcane bagasse (SCB) with LB medium used as comparison. Instrumental analysis was also carried out to elucidate mode of interaction between Cr species and the bacterial cells.
2. Materials and methods

2.1. Microorganism and culture conditions

The Cr(VI) resistant-reducing A. haemolyticus (EF369508), an aerobic Gram-negative bacterium, was isolated from Cr(VI)-containing textile dye effluent in Kota Bharu, Kelantan, Malaysia (Zakaria et al., 2007). This bacterium was inoculated into 200 mL of Luria–Bertani (LB) medium consisting of (in g/L): tryptone (10.0), granulated yeast extract (5.0) and NaCl (10.0) followed by overnight cultivation at 200 rpm and 30 °C (B. Braun Certasom®). SCB (pale yellow, wet and cut into 1–2 cm long) was collected from local sugarcane hawkers and was used without any physical or chemical treatments. Some of its characteristics are as follows; (in percentage) moisture = 8.03 ± 0.08, Ash = 20.5 ± 0.25, pH = 3.04 ± 0.04, α-cellulose = 27.70 ± 0.30, lignin = 13.60 ± 0.10, (in mg/kg) sugar content = 139.31 ± 0.22, SO$_4^{2-}$ = 9649 ± 264, PO$_4^{3-}$ = 219 ± 9, NO$_3^-$ = 4150 ± 42, As = 1.365 ± 0.145, Cd = 7.910 ± 0.335, Co = 2.938 ± 0.354, Cr = 0.002 ± 0.001, Cs = 4.677 ± 0.677, Be = 0.796 ± 0.130, Ni = 0.197 ± 0.070 and Pb = 7.278 ± 0.818.

2.2. Cr(VI) resistance and reduction studies

Cr(VI) resistance by A. haemolyticus was evaluated using LB and SCB-adapted cells. In LB, 10% (v/v) of A. haemolyticus was inoculated into a series of 1 L Erlenmeyer flasks containing 100 mL of LB and 10–100 mg/L of Cr(VI) (from Cr(VI) stock solution) followed by incubation at 200 rpm, 30 °C for 48 h. The adaptation of A. haemolyticus in SCB was carried out as follows; 10% (v/v) of cells (grown in NB) were transferred into a 1 L Erlenmeyer flask containing a mixture of 10% (w/v) of SCB, 5% (v/v) of denatured ethanol and NB followed by incubation at 200 rpm, 30 °C for 24 h. Then, the bacterial culture was inoculated into a fresh mixture containing 20% (w/v) of SCB followed by similar process. The adaptation sequence was repeated until the bacterial cells were able to grow in 100% (v/v) of SCB. The SCB-adapted cells were then evaluated for its Cr(VI) reduction capacity using 100 mg/L of Cr(VI) and incubation condition of 200 rpm, 30 °C and 48 h. At regular time intervals, aliquots (4 mL) were withdrawn and analyzed for OD$_{600}$, CFU/mL, Cr(VI) and total Cr concentration. The samples were centrifuged at 5000 rpm for 10 min where the resulting supernatants were analyzed for residual Cr(VI) concentration using the DPC method and total chromium analysis by AAS. The percentage of Cr(VI) reduction was calculated as follows; \[ \left( \frac{C_0 - C_f}{C_0} \times 100 \right) \] where $C_0$ is the initial Cr(VI) concentration (mg/L) while $C_f$ represents residual Cr(VI) concentration in solution (mg/L). To evaluate the effect of abiotic condition on Cr(VI) reduction i.e. control experiment, similar experimental setup was carried out in the absence of A. haemolyticus. All experiments were carried out in triplicates. The stock Cr(VI) solution (1000 mg/L) was prepared by dissolving 2.829 g K$_2$Cr$_2$O$_7$ (294.18 g/mol, BDH–AR) in 1 L of deionized water. The pH of Cr(VI) solution (pH 3–4) 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.

2.3. Analysis

2.3.1. FT-IR

The interaction between Cr species and functional groups present on the bacterial cells surface and SCB was investigated using a Fourier Transform Infrared spectrometer (Perkin Elmer: Spectrum One FT-IR Spectrometer). The bacterial cell pellets and SCB samples were obtained from mixtures containing 100 mg/L Cr(VI). The samples were first washed in subsequent changes of 0.85% (v/v) NaCl and deionized water prior to oven drying at 50 °C for 8 h (Pei et al., 2009). The dried pellet and SCB were ground with KBr (spectroscopic grade) with a ratio of 1:100 (pellet:KBr) in a mortar before pressed into 10 mm diameter disks under 6 tonnes of pressure. It was then analyzed using 16 scans at a resolution of 4 cm$^{-1}$ between 450 and 4000 cm$^{-1}$.

2.3.2. Electron Spin Resonance (ESR)

ESR was employed to verify the oxidation state of the Cr species bound to the bacterial cells surface and A. haemolyticus-adapted SCB. The cell pellets and SCB were washed with deionized water several times followed by freeze-drying in a vacuum freeze dryer (Christ Alpha 1–2). The freeze-dried pellet and SCB were ground in a mortar and purged under N$_2$ gas for about 10 min to remove remaining oxygen. Reference compound used for Cr(III) and Cr(VI) were CrCl$_3$·6H$_2$O (Fluka) and K$_2$Cr$_2$O$_7$ (BDH–AR) respectively. The samples were then analyzed using Electron Spin Resonance (ESR) spectrometer (JEOL, JES–FA100). Analysis were carried out in a 5 mm diameter glass tube at 25 °C, 1 mW, 9.01 GHz, 360 ± 250 mT, 300 mT modulation amplitude, 0.2 mT modulation width, 30 s scan time and 0.3 s time constant (Myers et al., 2000; Li et al., 2008; Suksabye et al., 2009; Xu et al., 2011).

2.3.3. Cell fractionation and localization of chromate reductase activity

Cell fractionation of A. haemolyticus was carried out using modified methods of Desai et al. (2008) and Elangovan et al. (2010) as follows; cell suspensions of A. haemolyticus in LB (1 g cell wet weight:5 mL buffer) were placed in an ice bath and disrupted using a Sonics Vibra Cell 500 Ultrasonic Probe (amplitude — 9%, 50 W, pulse — 9 s, 1 s off-mode, 35 min). The sonicated fraction was then centrifuged (L8–70 M Ultra-centrifuge, Beckman) at 40 000 rpm for 40 min at 4 °C to obtain the cytosolic fraction (supernatant) which represents the crude cell-free extracts, CFE (containing intracellular enzyme) while the resulting cell pellet (representing the cell membrane fraction, MF) were also collected and resuspended in the same buffer. Spent LB broth was centrifuged at 5000 rpm for 10 min to collect the extracellular enzyme fraction. All fractions collected were analyzed for protein content and chromate reductase activity.

2.3.4. Electron microscopy

The morphology of A. haemolyticus cells grown in a mixture of 100 mg/L of Cr(VI) in LB and SCB was analyzed using FESEM–EDX and TEM. For cells grown in LB, the bacterial cell pellet was obtained from a 48 h-grown culture via centrifugation at 9500 rpm, 4 °C for 30 min. The resulting cell pellet and air dried sample of SCB immobilized/adapted with A. haemolyticus was then fixed using 2.5% (v/v) glutaraldehyde (Sigma, USA) for 1–2 h at room temperature. After washing with deionized water, the cell pellet was post-fixed with 2% (v/v) osmium tetroxide (Fluka, Switzerland) for 1 h. The pellet and A. haemolyticus-adapted SCB were subsequently dehydrated using increasing concentrations of ethanol (10–100% v/v, 5 min each) and left to dry overnight at 60 °C in a desiccator (Pei et al., 2009). The specimens were then mounted onto the sample holder with carbon-conductive adhesive tapes and coated with platinum using a sputter coater (Bio-Rad) prior to viewing using a Field Emission Scanning Electron Microscope (Zeiss Supra 35VP) equipped with EDX analysis. The distribution of chromium in A. haemolyticus cells grown in LB medium was investigated via Transmission Electron Microscopy (Philips TEM 400 Transmission Electron Microscope). The cell pellet was first fixed using 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 2 h. Fixed cells were washed twice with deionized water, and post-fixed with 2% (v/v) osmium tetroxide (Fluka, Switzerland) for 1 h. The pellets were subsequently dehydrated using 10–100% (v/v)
ethanol for 5 min each followed by 2 min washes in 100% (v/v) acetone. The cell pellet was then embedded in 50 and 100% (v/v) of epoxy resin (Ted-Pella, California) in acetone for 15 min each. Then, the cell pellet was infiltrated a second time with fresh 100% (v/v) epoxy resin and cured at 60 °C overnight (Pei et al., 2009). The ultra-thin sections obtained were then viewed using TEM.

3. Results and discussion

3.1. Cr(VI) resistance and reduction studies

In both LB and SCB, growth of A. haemolyticus decreased with increasing Cr(VI) concentration. A. haemolyticus showed higher Cr(VI) resistance in LB (10^9–10^10 CFU/mL) compared to SCB (10^7–10^2 CFU/mL), which is expected based on the richer nutrient content in LB. However, the extent of cells Cr(VI) reduction does not correlate with cells Cr(VI) resistance. A similar situation was reported for Brucella sp. (Thacker et al., 2007), Bacillus sp., Arthrobacter sp. (Megharaj et al., 2003) and Pseudomonas isolates (Ohtake et al., 1987). Even though, higher cells survival was recorded in LB, A. haemolyticus was able to reduce 21–67% of 10–100 mg/L Cr(VI) with abiotic reduction accounting for 1–10% Cr(VI) reduction (Fig. 1A). This finding differs with Cr(VI) reduction in SCB where a much higher Cr(VI) reduction between 92 and 95% was recorded with more than 40% abiotic reduction (Fig. 1B). The significant role of SCB in carrying out abiotic reduction of Cr(VI) can be attributed to its rich lignocellulosic and sugar content. Although the concentration of sugars present in SCB may not be sufficient to yield maximum cells concentration, potential use of SCB in scaling up processes is still economically advantageous due to its abundance and cost. The interaction between A. haemolyticus–SCB and Cr(VI) can also be substantiated from the FT-IR analysis (Fig. 2). Upon contacting with 100 mg/L of Cr(VI), band shifts were observed at 3400–3422 cm⁻¹ indicative of −OH stretching, probably due from the oxidation of sugar moieties present in SCB during the Cr(VI) reduction process. Apart from this, the −C=O absorption peaks were also shifted from 1252 to 1261 cm⁻¹ and 1048 to 1037 cm⁻¹. A similar observation was reported by Garg et al. (2007) reporting on the involvement of carboxyl groups from sugarcane bagasse for Cr reduction.

The FT-IR spectra of A. haemolyticus grown in SCB and LB medium in the presence and absence of Cr(VI) indicated the involvement of the hydroxyl and amine groups from A. haemolyticus during Cr(VI) reduction. Upon incubation in 100 mg/L Cr(VI), slight band shifts were observed at 3291.85 cm⁻¹−3291.78 cm⁻¹ (indicative of −OH and −NH stretching), 1655–1650 cm⁻¹ and 1545–1546 cm⁻¹ (indicative of C=N stretching and −NH deformation). Similar situations were reported by Park et al. (2005), Das and Guha (2007) and Lameiras et al. (2008). Another significant point to note is the slight changes observed in the 1404−1406 cm⁻¹ and 1088−1089 cm⁻¹ region which are closely related to the carboxylic groups. This may suggest the interaction between Cr(VI) with protein molecules present in A. haemolyticus as suggested by Pandi et al. (2009) for Cr−cyanobacteria interaction. For sugarcane bagasse, strong absorption peak at 3400 cm⁻¹ indicates the presence of the O−H stretching groups, 2924 cm⁻¹ − C−H stretching vibration (probably from carboxylic acids), 1634 cm⁻¹ − C=C stretching and 1048.0 cm⁻¹ − OCH₃ group (both can be attributed to the lignin aromatic groups), 1376 cm⁻¹ − O−H bending, 1164 cm⁻¹ − C=O antisymmetric bridge stretching (one of the fingerprint regions for polysaccharides). Polysaccharides found in SCB consist of biopolymers having many hydroxyl and/or phenolic groups (Navarro et al., 1996). The peak at 898 cm⁻¹ originated from glycosidic C=O deformation with ring vibration contribution and O−H bending, which is characteristic of β-glycosidic linkage between glucose in cellulose (Viera et al., 2007).

3.2. Valence and state of Cr upon contact with A. haemolyticus

The ESR analysis was carried out to determine the valence state of Cr on the bacterial cells (possible adsorption of Cr(VI) or Cr(III) after reduction) surface as well as in the spent broth (Cr(III) excreted from the bacterial cytoplasmic region after Cr(VI) reduction and from the abiotic reduction by SCB). ESR analysis of A. haemolyticus incubated for 48 h in 100 mg/L of Cr(VI) (Fig. 3) revealed a broad signal centered at a g factor of 1.978 for LB medium and 1.953 for SCB which could be attributed to the Cr(III) paramagnetic signal (Xu et al., 2011). Compared with the two standard model compounds namely CrCl₃·6H₂O (control Cr(III)) and K₂Cr₂O₇ (control Cr(VII)), this condition strongly indicates the interaction between Cr(III) and bacterial cells surfaces. A planar profile for the dichromate sample was due to the absence of paramagnetism for Cr(VI) as well as other paramagnetic Cr species such as Cr(V).

From the TEM analysis, A. haemolyticus appeared as coccobacilli with the inner and outer sections clearly discernible (Fig. 4a) as well as the presence of electron opaque particles (indicative of Cr deposition) at the cell wall, membrane fraction and within the cytoplasmic region (Fig. 4b). This finding contrasted the report by Pei et al. (2009) where most of the Cr were deposited in the cytoplasmic region of A. haemolyticus. Hence, it can be deduced that upon entering the cell, Cr(VI) will be reduced to Cr(III) due to the reducing environment and enzymes present inside the cell. Subsequently, Cr(III) formed would be free to bind to ionic sites and, once bound, will act as a template for further heterogeneous nucleation and crystal growth. Srivastava and Thakur (2007) reported the presence of circular electron dense precipitates

![Fig. 1. Cr(VI) resistance-reduction study for the growth of A. haemolyticus grown in two different medium, (a) LB medium and (b) SCB medium.](image-url)
within the cell cytoplasm for *Acinetobacter* sp. PCP3 when contacted with Cr(VI)-containing tannery effluent.

FESEM analysis was carried out to observe any morphological changes to *A. haemolyticus* upon exposure to Cr(VI) and its attachment to SCB while EDX was used to estimate the elemental composition of both *A. haemolyticus* and SCB as well as to determine the presence of Cr element. Fig. 5a and c show the original morphology of *A. haemolyticus* (without Cr(VI)) as coccobacilli with rough surface and cell diameter ranging from 0.580 to 0.636 and 0.648 to 0.782 μm in LB and SCB respectively. However, upon cultivation in 100 mg/L of Cr(VI) for 48 h, the cells became shorter in both media (cell diameter in LB: 0.468–0.513 μm, in SCB: 0.156–0.240 μm) with some wrinkles formed on the surface due to the environmental stress from Cr(VI). However, no microstructures resembling Cr precipitates were observed on the cell surface of *A. haemolyticus* implying a Cr adsorption process rather than Cr reduction (followed by precipitation). Nevertheless, Cr species adsorbed onto the bacterial cells surface is anticipated to be in the Cr(III) form due to the inability of Cr(VI) to bind with the electronegative functional groups commonly found on Gram-negative envelopes (McLean and Beveridge, 2001). The effect of Cr(VI) on inducing wrinkle appearance on the bacterial cells surface was also reported for *Arthrobacter* K-2 (Lin et al., 2006) while its effect on reducing the size of bacteria was reported for *Streptomyces* sp. MCI (Polti et al., 2009) and *Bacillus cereus* SJ1 (He et al., 2010). An interesting point to note is that the finding from this study contradicts that of Pei et al. (2009) where cells of *A. haemolyticus* becomes elongated when exposed to LB agar supplemented with 30 mg/L of Cr(VI) for 24 h. The EDX spectra for *A. haemolyticus* grown in SCB showed a higher C:O percentage compared to growth in LB medium which was due to the higher carbon content in SCB (45% cellulose, 28% hemi-cellulose, 18% lignin, Garg et al., 2007). Nevertheless, a higher Cr content (7.81% weight)
was detected on the cell pellets of *A. haemolyticus* (grown in LB) compared to *A. haemolyticus* grown/immobilized on SCB (<1% weight). This suggests the possible direct contribution from *A. haemolyticus* on Cr(VI) removal besides its primary Cr(VI) reduction capabilities. Similar Cr deposition on the surface of bacteria from Cr(VI) reduction process was also reported for *Streptomyces sp.* MC1 (Polti et al., 2009), *Leucobacter sp.* CRB1 (Zhu et al., 2008) and *Pseudomonad sp.* CRB5 (McLean and Beveridge, 2001).

3.3. Localization of chromate reductase activity

Chromate reductase activity of *A. haemolyticus* was detected in three cell fractions assayed namely whole cells, membrane fraction and crude cell free extract. The involvement of intracellular enzymes in Cr(VI) reduction was strengthened from the absence of chromate reductase activity in the spent broth (Fig. 6). Highest chromate reductase activity was determined in the whole cells (4.73 pmol/min/mg of protein, 20% Cr(VI) reduction) followed by membrane fraction (3.67 pmol/min/mg of protein, 11.4% Cr(VI) reduction) and the crude CFE fraction (1.10 pmol/min/mg of protein, 2.4% Cr(VI) reduction). Specific reasons for the high chromate reductase activity determined in the membrane fraction for an obligate aerobe such as *A. haemolyticus* is currently being investigated as normally aerobic bacterial Cr(VI) reduction takes place in the cytoplasmic fraction i.e. CFE (Desai et al., 2008; Elangovan et al., 2010).
4. Conclusion

This study demonstrated that *A. haemolyticus* could be used as a potential agent to decontaminate Cr(VI) contamination in wastewater systems using agricultural waste as nutrient. The high Cr(VI) to Cr(III) conversion from the action of both bacteria and abiotic reduction promises efficient and cost effective treatment system for Cr(VI) compared to the current chemical-intensive system.

Novelty statements

1) Comparison of Cr(VI) resistance and reduction levels of *A. haemolyticus* in rich medium and agricultural waste
2) Elucidation on the mode of interaction between Cr-
   *A. haemolyticus* and Cr-SCB using instrumental analysis such as
   FT-IR, ESR, TEM and FESEM – EDX
3) Cell fractionation and localization of chromate reductase ac-
   tivity of *A. haemolyticus*

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