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Simultaneous Cr(VI) reduction and methylene blue removal by *Bacillus* sp. JH2-2 isolated from mining site soil

Jaehong Shim[a,b], Patrick J. Shea[b], Jeong-Muk Lim[a], Byung-Taek Oh[a,*]

[a]*Division of Biotechnology, Advanced Institute of Environment and Bioscience, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan, Jeonbuk 570752, South Korea, Tel. +1 402 472 1633; Fax: +1 402 472 7904; email: jshim2@unl.edu (J. Shim), Tel. +82 63 850 0838; Fax: +82 63 850 0834; emails: kenge0155@nate.com (J.-M. Lim), btoh@jbnu.ac.kr (B.-T. Oh)*

[b]*School of Natural Resources, University of Nebraska-Lincoln, Lincoln, NE 68583-0817, USA, Tel. +1 402 472 1533; Fax: +1 402 472 7904; email: pshea1@unl.edu (P.J. Shea)*

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ABSTRACT

A *Bacillus* sp. strain (JH2-2), isolated from soil at an abandoned mine site, reduced Cr(VI) to Cr(III) under aerobic conditions. At pH 7, the strain reduced essentially all of the Cr(VI) in M9 minimal medium to Cr(III) at initial concentrations ≤50 mg Cr(VI) L^{-1} within 100 h. The X-ray diffraction pattern of the Cr(III) precipitate matched chromium (III) hydrogen phosphate ($CrH_2P_3O_{10}{\cdot}2H_2O$). The JH2-2 strain showed high tolerance to other heavy metal (loid)s, with minimal inhibitory concentrations in liquid medium of (mg L^{-1}): As (500), Cd (100), Cu (350), Ni (300), Zn (200), and Pb (1800). JH2-2 also promoted decolorization of methylene blue dye. Decolorization was faster in the presence of 10 mg Cr(VI) L^{-1} than in the absence of Cr(VI). A lag in decolorization at 30 and 50 mg Cr(VI) L^{-1} is likely due to initial toxicity and inhibition of bacterial growth. The chemistry of MB is complicated by its reduction to colorless leucomethylene blue, which can reoxidize to MB. However, aeration of the solution did not restore measurable MB, supporting removal of the dye via biosorption. Results indicate the bioremediation potential of *Bacillus* sp. JH2-2 for simultaneous Cr(VI) reduction and methylene blue removal from contaminated water.

Keywords: *Bacillus* sp.; Detoxification; Dye decolorization; Hexavalent chromium; Methylene blue

1. Introduction

Chromium (Cr) has been widely used in stainless steel, chrome plating, leather tanning, dye and pigment production, and other industries. High concentrations of Cr and other heavy metals in soil and water remain a global concern [1,2]. Cr occurs in multiple oxidation states ranging from Cr(II) to Cr(VI) [3] but in environmental media it is typically present as Cr(VI) and Cr(III). Because Cr(VI) is one of the most toxic heavy metals, with the potential to induce genetic mutations and cancer in humans, its removal is very important [4]. Cr(VI) is highly soluble and 100-fold more toxic and more mobile than Cr(III) [5].

Methylene blue (MB) is a phenothiazine dye, which is most commonly used for dyeing textiles. It can cause permanent eye injury in humans and other animals. If

*Corresponding author.

swallowed, MB irritates the gastrointestinal tract with symptoms of nausea, vomiting, and diarrhea [6]. Therefore, dye removal is important in wastewater treatment. While mineralization is possible via aerobic oxidation following anaerobic reduction [7–9], many dyes are not readily degraded and it can be difficult to effectively remove them from effluent [10].

Heavy metals such as Cu, Co, and Cr are often used in dye fixation [11] and form stable complexes that resist decomposition [12]. The co-presence of metals and/or dyes in wastewater can complicate treatment, leading to negative health and environmental impacts [13]. While biological removal, adsorption, and photochemical methods have been investigated for removing metals and dyes [14,15], simultaneous removal remains of great interest. Techniques for removing Cr(VI) from water include electrokinetic treatment, chemical precipitation, membrane processes, reverse osmosis, ion exchange, liquid extraction, electrodialysis, evaporation, and adsorption. However, high costs, the possibility of secondary pollution, and the environmentally unfriendly nature of many of these treatments often limit their application [16]. Biological approaches are gaining interest as an alternative for remediating Cr(VI) contamination [17]. Biological remediation can be accomplished or initiated by microbial reduction of Cr(VI) to Cr(III) forms. In contaminated environments, some microorganisms have adapted to toxic concentrations of Cr(VI) by developing tolerant or resistant strains [18]. Genera include *Pseudomonas* [19], *Bacillus* [20], and other bacteria [21,22].

Various microorganisms have been shown to remove single dyes and Cr from wastewater, but little work has been done on simultaneous removal of multiple noxious substances [23,24]. Simultaneous biosorption of dyes and Cr by fungi, bacteria, and algae has been reported, but these processes are slow and biotransformation to less toxic forms is uncertain [11,23,25]. More research is needed in this area. This study characterizes simultaneous reduction of Cr(VI) and removal of MB dye by a bacterial strain isolated from mine site soil.

2. Materials and methods

2.1. Isolation and identification of Cr-tolerant bacteria

A bacterial strain was isolated from soil at an abandoned mine site in the Gapyeong province of the Republic of Korea, using standard procedures. The strain was cultured in M9 minimal medium at 30˚C with shaking at 180 rpm. The medium contained 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 NH_4Cl, 0.5 NaCl, 0.246 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2$ per L The minimal inhibitory concentration (MIC) of Cr(VI) (filter-sterilized solution of analytical grade $K_2Cr_2O_7$) was determined by incubating the strain in Luria-Bertani (LB) agar medium containing 50–1,500 mg Cr(VI) L^{-1} for 24 h at 30˚C. The MIC was the concentration that completely inhibited bacterial growth. The bacterial strain showing the most Cr(VI) reduction, designed JH2-2, was isolated and preserved at −80˚C in LB medium containing 10% glycerol.

To identify the bacteria, pellets were obtained from overnight LB cultures and DNA was extracted with lysis buffer containing 25% sucrose, 25 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris–HCl buffer, and 5 mg lysozyme L^{-1} [26]. The 16S rDNA was amplified using polymerase chain reaction with the universal primers 27 F (5′-AGA GTT TGA TCA TGG CTC AG-3′) and 1492 R (5′-GGT TAC CTT GTT ACG ACT T-3′). The 16S rDNA sequence results were analyzed using the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov). The JH2-2 strain has been deposited in the Korean Agricultural Culture Collection (KACC) under the number of KACC 16578. The sequence (1,433 bp) was submitted to the NCBI databases (accession number JN880445).

2.2. Heavy metal(loid) MICs

Tolerance of the *Bacillus* sp. JH2-2 strain to As ($NaAsO_2$), Cd ($Cd(NO_3)_2{\cdot}4H_2O$), Cu ($CuCl_2{\cdot}2H_2O$), Pb ($Pb(NO_3)_2$), Ni ($NiSO_4{\cdot}6H_2O$), and Zn ($ZnCl_2$) was determined. LB agar plates containing 100–2,000 mg L^{-1} of each heavy metal(loid) were streaked with the strain and incubated at 30˚C for 24 h. The MICs were heavy metal(loid) concentrations that completely inhibited microbial growth.

2.3. Cr(VI) reduction by Bacillus sp. JH2-2

In initial experiments, the optimum pH for Cr(VI) reduction by *Bacillus* sp. JH2-2 was determined by adjusting the pH of 100 mL of M9 minimal medium containing 50 mg Cr(VI) L^{-1}. The pH was adjusted to 3.5, 5.0, 7.0, or 9.0 with 0.1 N H_2SO_4 or 6 N NaOH, inoculated with 100 μL of the strain (4.7×10^8 CFU mL^{-1}), and incubated at 30˚C for 100 h at 100 rpm. Optimum temperature was similarly determined by comparing Cr(VI) reduction at 25, 30, and 35˚C (pH 7). Subsequent experiments were conducted at an initial pH of 7 and at 30˚C in inoculated M9 minimal medium containing 10, 30, 50, or 100 mg Cr(VI) L^{-1}. Samples (1 mL) were periodically removed after incubating up to 100 h and centrifuged at 13,000 rpm for 5 min in preparation for analysis.

Cr(VI) reduction was measured using the diphenylcarbazide method [27] with Cr(VI) quantification by UV–Vis spectrophotometry (8453 UV–Vis spectrophotometer, Agilent, Santa Clara, CA, USA). Total Cr was determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500a, Agilent Technologies Inc.) after digesting 2 mL samples with 18 mL of 50% concentrated HCl in water on a shaker for 1 h, filtering and washing with dilute HCl [28].

2.4. Characterization of JH2-2 cells and associated Cr(III) product

The bacterial cells and reduced Cr product were characterized by scanning electron microscopy (SEM; SM6400, JEOL, Tokyo, Japan), X-ray diffraction (XRD; Philips X'Pert Pro MPD, Eindhoven, Netherlands), SEM-energy dispersive spectroscopy (SEM-EDS), and Fourier transform infrared spectroscopy (FTIR; Spectrum GX, Perkin Elmer, MA, USA). JH2-2 cells were prepared by culturing for 2 d at 30˚C in M9 minimal medium containing 30 mg Cr(VI) L^{-1}. Cells were removed from the medium by centrifugation as previously described and washed three times with sodium carbonate buffer (pH ~ 8). The cells were fixed with 3% glutaraldehyde, washed with Tris–HCl buffer, and three times with deionized water. Samples were dried using ethanol and mounted on aluminum stubs and coated with gold for examination by SEM-EDS at 200 kV. For FTIR and XRD analyses, samples were prepared by washing the cells several times with 0.2 M phosphate buffer (pH 7.0) and air-drying at 50˚C [28].

2.5. Decolorization and removal of MB by JH2-2

Tests were conducted to determine the capacity of the JH2-2 strain to decolorize and remove MB from solution. MB was obtained from Sigma-Aldrich (St. Louis, MO) and used as received. An experiment was conducted to determine MB removal at 100 mg L^{-1} in the presence of 0, 10, 30, and 50 mg Cr(VI) L^{-1}. MB was added to 100 mL of M9 minimal medium in 250 mL flasks and inoculated with the strain (4.7×10^8 CFU mL^{-1}). Flasks were incubated at 30˚C by agitating at 100 rpm and sampled periodically for 96 h. Samples were centrifuged at 8,000 × g for 5 min and MB concentration in solution determined at 660 nm (λ_{max}) with a UV–Vis spectrophotometer. The pH and oxidation–reduction potential (ORP) were determined using a HANNA pH/ORP meter model HI991003 immediately after sampling.

Because bacterial cells can act as a biosorbent for MB [29], removal by live and dead (heat-treated) cells was compared. JH2-2 cells were grown for 1 d in M9 minimal media (with shaking at 180 rpm) at 30˚C, collected by centrifuging for 30 min at 4˚C, and washed several times with deionized water. Live bacteria were prepared by resuspending the cell pellet in deionized water. Dead cells were obtained by heating at 100˚C for 60 min before resuspension. MB solutions were prepared and pH was adjusted to 7.0 with 0.1 N H_2SO_4 or 6 N NaOH. The prepared live or heat-treated biomass (0.05 g dry weight) was added to flasks containing 50 mg MB L^{-1} in the presence or absence of 10 mg Cr(VI) L^{-1}. Aliquots were periodically removed from the flasks, centrifuged, and MB concentration was measured at 660 nm. Decolorization efficiency was determined from the change in MB concentration in solution.

Because reduction of MB produces leucomethylene blue (LMB), LMB was monitored at 256 nm (λ_{max}). Reoxidation of LMB produces MB, so MB regeneration was checked by purging the solution with air (100 mL min^{-1}) for 1, 3, and 10 min, centrifuging, and measuring absorbance at 660 nm.

2.6. Statistical analyses

Statistical analyses were performed using SigmaPlot (San Jose, CA, USA). Data were subjected to an analysis of variance. Significant treatment differences were determined using Duncan's multiple range test (DMRT) at $p < 0.05$. All experiments were conducted in triplicate; values are presented as means ± standard deviation (SD).

3. Results and discussion

3.1. Isolate identification and heavy metal(loid) tolerance

Eighteen strains isolated from the mining site soil were evaluated for tolerance to Cr(VI). A highly tolerant (MIC of 1,500 mg L^{-1}) strain (JH2-2) was identified as *Bacillus* sp. Growth of the strain exhibited a 6 h lag phase, reduced about 50% at 50 mg Cr(VI) L^{-1}, and minimal at 100 mg L^{-1} (Fig. 1). Inhibition of growth can be attributed to Cr toxicity [30]. At 50 mg Cr(VI) L^{-1} the pH of the medium decreased slightly (from 6.3 to 6.0) after 72 h. The JH2-2 strain also tolerated other heavy metals, with MICs of 500 mg As, 100 mg Cd, 350 mg Cu, 300 mg Ni, 1,800 mg Pb, and 200 mg Zn per L.

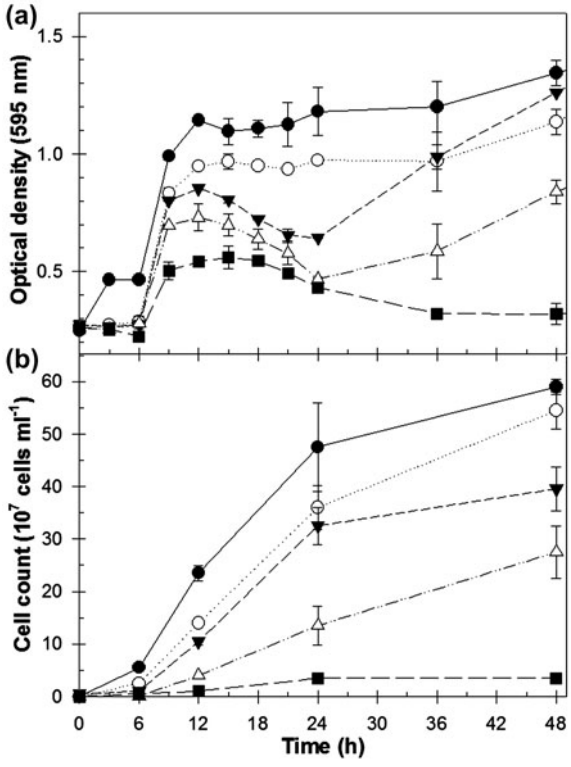

Fig. 1. *Bacillus* sp. JH2-2 growth curve (a) and CFU (b) in M9 minimal medium (at pH 7 and 30°C) containing no Cr(VI) (●), 10 mg Cr(VI) L^{-1} (○), 30 mg Cr(VI) L^{-1} (▼), 50 mg Cr(VI) L^{-1} (▽), and 100 mg Cr(VI) L^{-1} (■). Error bars indicate SDs; where absent bars fall within symbols.

3.2. Reduction of Cr(VI)

Initial experiments indicated that Cr(VI) reduction after 72 h in media containing *Bacillus* sp. JH2-2 was the greatest (93.0 ± 0.8%) at pH 7 and 30°C (Table 1). No Cr(VI) reduction was detected in the absence of

Table 1
Initial screen of the effect of pH and temperature on Cr(VI) reduction after 100 h in M9 minimal medium containing 50 mg Cr(VI) L^{-1} in the absence and presence of *Bacillus* sp. JH2-2

Conditions		Cr(VI) reduction (%)[a]	
pH	Temp. (°C)	Without JH2-2	With JH2-2
3.5	30	0	1.1 ± 0.7
5.0	30	0	8.2 ± 0.2
7.0	25	0.3 ± 3.8	61.5 ± 3.9
7.0	30	4.6 ± 2.4	93.0 ± 0.8
7.0	35	18.1 ± 1.2	76.0 ± 0.4
9.0	30	3.4 ± 0.1	75.7 ± 0.4

[a]{[Cr(VI) initial conc. - Cr(VI) final conc.]/Cr(VI) initial conc.} × 100.

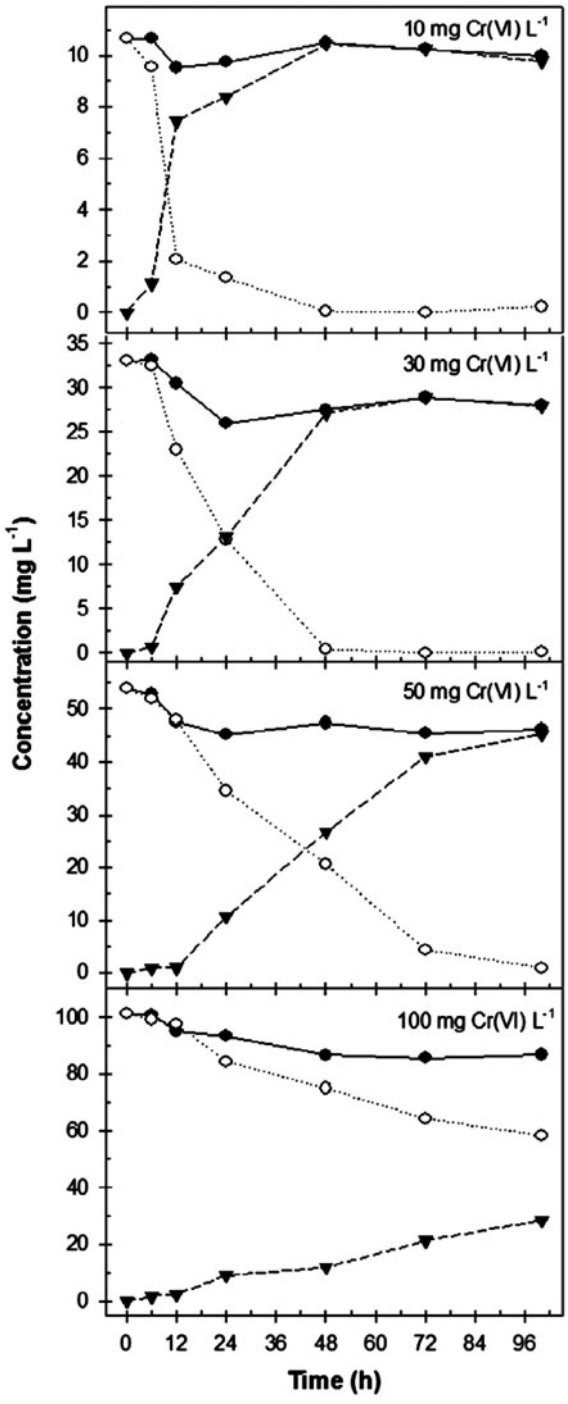

Fig. 2. Total Cr (●), Cr(VI) (○), and Cr(III) (▼) in M9 minimal medium after incubating with *Bacillus* sp. JH2-2 at 30°C and pH 7.0 in the presence of 10, 30, 50, and 100 mg Cr(VI) L^{-1}. Error bars indicate SDs; where absent bars fall within symbols.

the JH2-2JH2-2 strain at pH 3.5 and 5, and reduction was minimal in the absence of the strain at pH 7 and 9. Cr(VI) reduction by *Bacillus* sp. has been

Table 2
Changes in pH and ORP with time in M9 minimal medium at 30°C in the absence or presence of *Bacillus* sp. JH2-2 and 10 mg Cr(VI) L^{-1}

	Cr(VI) only			JH2-2 only			JH2-2 + Cr(VI)		
	0 h	48 h	72 h	0 h	48 h	72 h	0 h	48 h	72 h
pH	7.00	6.94	6.84	7.00	7.50	7.99	7.00	6.84	6.70
ORP (mV)	229	163	137	229	112	84	229	120	51

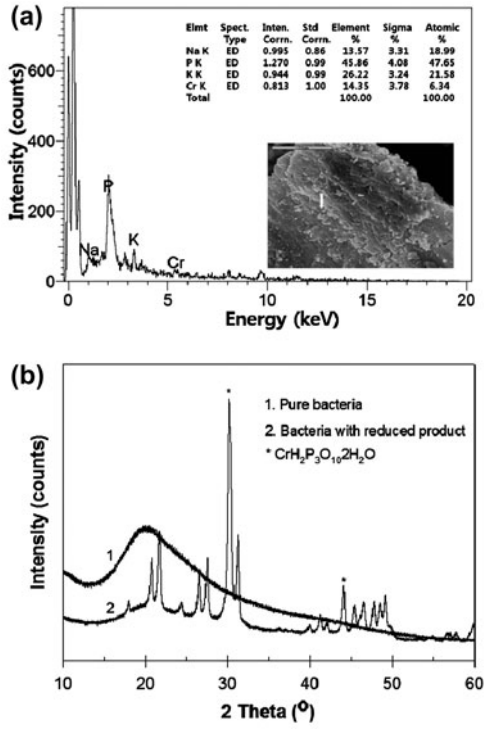

Fig. 3. (a) SEM image (insert) and SEM-EDS spectra for chromium associated with the cell surface and (b) XRD of the reduced product formed by *Bacillus* sp. JH2-2 after incubating with Cr(VI) for 72 h in M9 minimal medium.

attributed to chromate reductase [20] and although not assayed was likely associated with Cr(VI) reduction in media containing the JH2-2 strain. Previous research also shows that pH and temperature are major factors in Cr(VI) reduction by species of *Bacillus* and other bacteria [28,31]. In the present study little JH2-2 growth was observed at pH 3.5. While cell growth was the greatest at pH 7 and 35°C, chromate reductase activity is optimum at 30°C [32]. Some chromate reductase activity may be observed in the absence of Cr(VI), but activity is greater when the bacteria are grown in the presence of Cr(VI) [11,23,25,30].

Visual MINTEQ 3.0 (KTH Royal Inst., Stockholm, Sweden) [33] estimated >80% of the Cr(VI) in M9 minimal medium at pH 7 was initially present as CrO_4^{2-} and >15% as $HCrO_4^-$. At pH 7, JH2-2 reduced essentially all of the Cr(VI) in the medium to Cr(III) at initial concentrations ≤50 mg L^{-1} within 100 h (Fig. 2). The lower ORP in the presence of JH2-2 would facilitate Cr(VI) reduction (Table 2). Less efficient Cr(VI) reduction at 100 mg Cr(VI) L^{-1} can be attributed to reduced bacterial growth, as shown in Fig. 1. Reduction of Cr(VI) to Cr(III) is the major mechanism reducing toxicity [24].

Fig. 3(a) shows SEM images and SEM-EDS analysis of the bacterial cells before and after reducing Cr(VI). CrK compounds formed on the cell surface after reduction; potassium and phosphorus produced PK compounds. Chromium and phosphorus contents of the cell surface were 6 and 48%, respectively. Powder XRD patterns show Cr associated with the bacterial cells after growing with Cr(VI) in M9 minimal medium (Fig. 3(b)). *D*-values closely matched those of chromium (III) hydrogen phosphate ($CrH_2P_3O_{10}·2H_2O$; JCSPD No. 28-0367), which is the product identified by Dhal et al. [28] from Cr(VI) reduction by a *Bacillus* sp. strain isolated from Indian chromite mine soil.

FTIR spectra (Fig. 4(a)) of JH2-2JH2-2 show functional groups typical of gram-positive bacteria, which have cell walls containing a thick layer of peptidoglycan linked with teichoic acid and polysaccharides. The peptidoglycan polymer contains *β*–(1,4) Linked *N*-acetylglucosamine, *N*-acetylmuramic acid, and amino acids. Teichoic acid contains glycerol/ribitol phosphate linked by phosphodiester bonds. Metals can bind to amino, carboxy, phosphate, and sulfate groups in the cell wall of *Bacillus* sp. [34]. Broad overlapping bands for N–H and O–H stretching are present at 3,200–3,600 cm^{-1}; the strong band at 2,500–3,800 cm^{-1} is due to –OH in the carboxyl group. The band at 1,550–1,660 cm^{-1} is attributed to –NH bending of amides from the peptide bond coupled with the carboxylate anion; peaks at 1,384 and 1,410 cm^{-1} are due to the symmetrical stretching of the carboxylate anion and carboxylic acid, respectively. The peak at 1,090 cm^{-1} corresponds to the –C–O bond of polysaccharides (Fig. 4(b)).

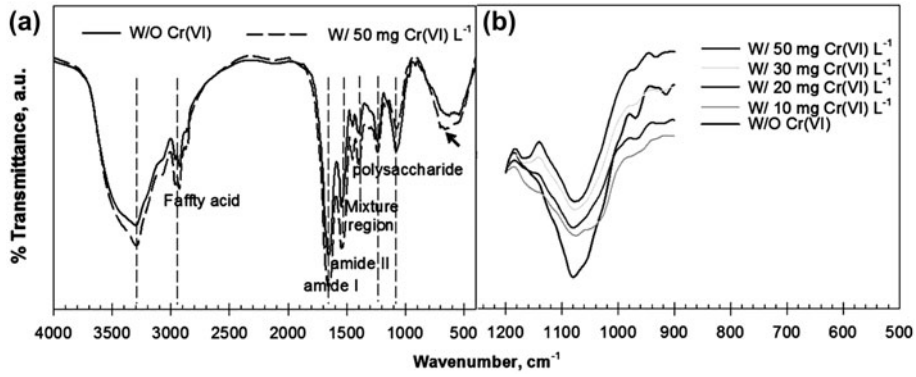

Fig. 4. FTIR spectra of (a) *Bacillus* sp. JH2-2 after 72-h growth in M9 minimal medium with/without Cr(VI) (50 mg L^{-1}) and (b) the phosphate band at 1,200–900 cm^{-1} with increasing Cr(VI) concentration.

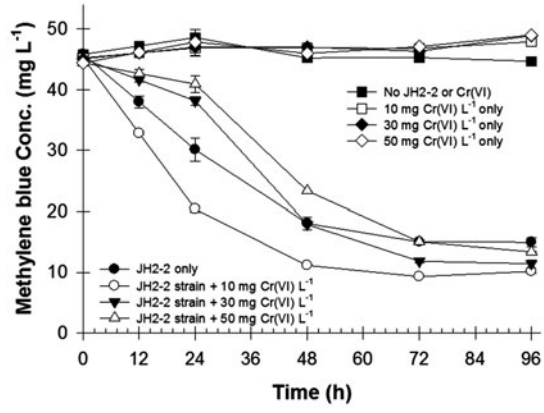

Fig. 5. Effect of initial Cr(VI) concentration on decolorization of MB in the absence or presence of *Bacillus* sp. JH2-2 at 30˚C and pH 7.0 during a 96-h incubation. Error bars indicate SDs; where absent bars fall within symbols.

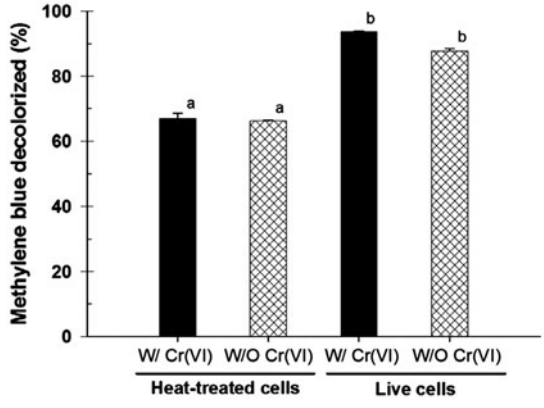

Fig. 6. Decolorization of MB by live and heat-treated *Bacillus* sp. JH2-2 cells. Error bars indicate SDs; where absent bars fall within symbols. Bars with the same letters are not significantly different as determined by DMRT at $p < 0.05$.

Bioreduction of Cr(VI) produces Cr(III) and binding of Cr(III) to cell wall constituents results in IR peak shifts and changes in intensity [34]. The reduced intensity of the phosphate band at 900–1,200^{-1} (Fig. 4(b)) can be attributed to Cr(III) adsorption, while the more intense C=O peak at 1,654 cm^{-1} is indicative of metal binding (–C–O–M) [28].

3.3. Decolorization and removal of MB in the presence of Cr(VI)

Fig. 5 shows the effects of Cr(VI) on MB concentration (decolorization) in M9 minimal medium in the absence and presence of *Bacillus* sp. JH2-2. No decolorization was observed in the absence of the bacteria. Decolorization was initially faster in media containing 10 mg Cr(VI) L^{-1} than in media without Cr(VI), although decolorization was similar after 96 h. Low heavy metal(loid) concentrations can enhance biological activity [35] and we previously reported a hormetic effect of Cr(VI) on indole-3-acetic acid biosynthesis by *Bacillus* sp. JH2-2 [36]. The lag in MB decolorization observed at 30 and 50 mg Cr(VI) L^{-1} in the present study is likely due to initial toxicity and inhibition of bacterial growth [11]. This appeared to be overcome as Cr(VI) was reduced to Cr(III).

Because Ayla et al. [29] showed efficient biosorption of MB to *B. subtilis*, decolorization was compared in the presence of dead and living JH2-2 cells. Approximately 67% of the MB in M9 minimal medium was decolorized after incubating for 6 h with heat-killed bacteria in the presence or absence of 10 mg Cr(VI) L^{-1} (Fig. 6). Blue-colored cell pellets at the bottom of the culture indicate MB removal via adsorption. With live cells, decolorization increased to 87% in the absence of Cr(VI) and to 93% with 10 mg Cr(VI) L^{-1}. In contrast to dead cells, the color of the

surface of live cells remained unchanged. Color differences may reflect the chemistry of MB, which is complicated by its chemical reduction to colorless LMB, which can lead to misinterpretation of experimental data based on measurement at 660 nm (MB λ_{max}) alone [37]. While a loss of blue color indicates decreasing MB concentrations, LMB may be present and can reconvert to MB when oxygen levels increase.

Results suggest that biosorption was the primary mechanism of MB dye removal in the present study. MB carries a positive charge and can be expected to bind to phosphate and carboxyl groups of teichoic acid on the surface of the JH2-2 bacteria. In contrast to MB, LMB has several protonated and deprotonated forms [38]. At the pH of the present study (pH 6.7–6.8), neutral (uncharged) LMB will exist in equilibrium ($pK_a = 5.9$) with dissociated (negatively charged) LMB. As neutral LMB molecules are adsorbed to constituents of the bacterial cell wall, dynamic equilibrium of the LMB species promotes sustained removal from solution. ORP decreased from 180 to 38 mV after incubating JH2-2 in M9 minimal medium containing 50 mg MB L^{-1}. While the decrease in ORP favors LMB formation, aeration of the solution did not restore measurable MB, supporting its removal from solution.

4. Conclusion

Bacillus sp. JH2-2 effectively reduced Cr(VI) and removed both MB and LMB from liquid media. Growth of the strain was not affected by Cr(VI) up to 50 mg L^{-1} and Cr(VI) was completely reduced to Cr(III) after 100 h of incubation. Cr(VI) reduction was optimum at pH 7, 30˚C, and 100 rpm shaking speed. Results indicate that the JH2-2 strain can convert water soluble and highly toxic Cr(VI) to less soluble and less toxic Cr(III). SEM-EDX analysis showed Cr associated with the bacterial cells and XRD analysis indicated precipitation as chromium (III) hydrogen phosphate. These findings suggest the potential use of *Bacillus* sp. JH2-2 for treating water containing both MB dye and Cr(VI).

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