Journal of Hazardous Materials 177 (2010) 481–486

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Heavy metal resistance in *Arthrobacter ramosus* strain G2 isolated from mercuric salt-contaminated soil

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ARTICLE INFO

Article history:
Received 14 August 2009
Received in revised form
10 December 2009
Accepted 10 December 2009
Available online 21 December 2009

Keywords:
Bioaccumulation
Chromate reductase
Mercuric reductase
MerA
Nano-LC–MS/MS

ABSTRACT

Present study describes isolation of a multiple metal-resistant *Arthrobacter ramosus* strain from mercuric salt-contaminated soil. The isolate was found to resist and bioaccumulate several metals, such as cadmium, cobalt, zinc, chromium and mercury. Maximum tolerated concentrations for above metals were found to be 37, 525, 348, 1530 and 369 μM, respectively. The isolate could also reduce and detoxify redox-active metals like chromium and mercury, indicating that it has great potential in bioremediation of heavy metal-contaminated sites. Chromate reductase and mercuric reductase (MerA) activities in protein extract of the culture were found to be 2.3 and 0.17 units mg^{-1} protein, respectively. MerA enzyme was isolated from the culture by $(NH_4)_2SO_4$ precipitation followed by dye affinity chromatography and its identity was confirmed by nano-LC–MS/MS. Its monomeric molecular weight, and optimum pH and temperature were 57 kDa, 7.4 and 55 °C, respectively. Thus, the enzyme was mildly thermophilic as compared to other MerA enzymes. K_m and V_{max} of the enzyme were 16.9 μM $HgCl_2$ and 6.2 μmol min^{-1} mg^{-1} enzyme, respectively. The enzyme was found to be NADPH-specific. To our knowledge this is the first report on characterization of MerA enzyme from an *Arthrobacter* sp.

1. Introduction

Although several heavy metals serve as essential trace elements for life, they are toxic at higher concentrations. These metals have been abundant on the earth since the beginning of life. Hence, several microbes have evolved resistance systems towards them [1]. These resistance systems also play important role in global cycling of several metals. Recent anthropogenic activities have led to severe heavy metal pollution of the environment. This is not only ecologically threatening, but also inhibits removal of other pollutants by inhibiting microbial activity. This has led to increased interest in the heavy metal resistance mechanisms as means of remediating metal-contaminated sites [2].

Mercury, the sixth most toxic substance on earth, has no beneficial function like some other heavy metals [3]. Microbial resistance to Hg is based on its unique properties, such as very low redox potential (0.851 V) and high vapour pressure (0.0018 mm of Hg). Thus, microbes are able to reduce toxic Hg(II) to relatively non-toxic volatile Hg(0), which leaves the cell by passive diffusion [4]. The Hg resistance determinants are normally clustered in an operon (mer operon). The operon is often a component of a transposon located on plasmids or chromosomes [5]. The key enzyme of the operon is mercuric reductase (MerA), which catalyzes reduction of Hg(II) using NADPH as electron donor. Activity of this enzyme has been exploited in several bioreactors for removal of Hg from wastewater [6,7]. It has also been expressed in yeast [8] and plants [9] to increase their Hg resistance and promote bioremediation.

MerA has been characterized from several organisms. Usually it is a dimer or a trimer and contains FAD as prosthetic group [10,11]. However, it has not been isolated from any *Arthrobacter* sp. so far. *Arthrobacter* are widespread soil Actinobacteria that show typical features like snapping fission and rod to coccus transition during batch growth. They exhibit great metabolic versatility and can degrade/detoxify several anthropogenic xenobiotics and pollutants, such as heavy metals. Hence, there is lot of interest in application of *Arthrobacter* for bioremediation purpose [12].

Putative *merA* gene has been identified in the genomes of *Arthrobacter* sp. FB24 and *Arthrobacter aurescens* strain TC1 based on 59% identity to MerA protein from *Streptomyces* sp. strain CHR28 (GenBank accession numbers CP000454, CP000455, CP000456, CP000457) [12]. However, the *merA* gene of *Streptomyces* strain CHR28 itself was identified based on sequence similarity to *merA* from *Streptomyces lividans* [13], which in turn, was identified by its similarity to *merA* from other bacteria [14]. Thus, in this long series of similarity-based predictions, there is no direct experimental evidence for the presence of *merA* gene or enzyme.

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doi:10.1016/j.jhazmat.2009.12.058

Present study describes characterization of heavy metal resistance in an *Arthrobacter ramosus* strain isolated from mercuric salt-contaminated soil, followed by purification and characterization of its MerA enzyme. The enzyme was found to be similar to the putative MerA from *A. aurescens* based on nano-LC–MS/MS analysis. In our knowledge it is the first study to provide the missing experimental support for presence of MerA in *Arthrobacter*. Also, the isolated *Arthrobacter* strain was found to resist and bioaccumulate other metals like cadmium, cobalt, and zinc. It also possessed chromate reductase activity responsible for conversion of highly toxic and soluble Cr(VI) to less toxic and insoluble Cr(III) form. Thus, it may be a suitable candidate for bioremediation of heavy metal-contaminated sites.

2. Experimental

2.1. Isolation of the culture

Mercuric salt-contaminated soil was obtained from an industrial site in India and analyzed for Hg content by acid digestion followed by cold vapour atomic absorption spectrometry [15]. Aerobic Hg-resistant bacteria were isolated from the soil by plating it on nutrient agar plates (composition in $g\,l^{-1}$: peptone, 10; NaCl, 5; beef extract, 3; agar, 15) containing progressively higher concentrations of mercuric chloride. The colony showing the highest Hg tolerance was selected for further study. It was Gram stained and identified by 16S rDNA sequencing. Genomic DNA was isolated from it as described previously [16] and purified using Ultrapure prep kit (Bangalore Genei, India). 16S rRNA gene was amplified from the extracted DNA using universal primers [17] and the amplified product was cloned in T-vector (Bangalore Genei) using competent *E. coli XL1-blue* cells (Stratagene, USA) as host. Sequencing was carried out with M13 primer on an automated DNA sequencer (ABI 3100, Applied Biosystems, USA). Presence of any plasmids in the culture was checked by standard midiprep method using lysozyme pre-treatment step [16]. A plasmid-containing Hg-resistant strain of *Bacillus sphaericus* was used as the positive control.

2.2. Determination of metal and antibiotic resistance profile

Resistance of the isolate towards different heavy metal salts (cadmium acetate, cobalt chloride, zinc sulfate, potassium dichromate and mercuric chloride), and antibiotics (ampicillin and tetracycline) was determined. Phenylmercuric acetate (PMA) was also used to detect the resistance towards organic mercuric compounds. Briefly nutrient agar plates containing different concentrations of respective metals (1–1000 μM) or antibiotics ($1–10\,mg\,l^{-1}$) were plated with OD_{600} 0.1 culture of the isolate. The plates were incubated at 25 °C for 48 h before recording the maximum tolerated concentration (MTC), i.e. the maximum concentration at which culture could grow. All further experiments were carried out at 50% of MTC, referred to as sublethal concentrations hereafter (Table 1). Firstly, effect of above metal salts on growth curve of the culture was studied at sublethal concentrations. For this nutrient broth flasks containing appropriate concentrations of different metal salts were inoculated with OD_{600} 0.1 culture of the isolate. The flasks were incubated on shaker at 25 °C and growth was quantified in terms of OD_{600}.

2.3. Bioaccumulation of Cd, Co and Zn

Overnight culture of the isolate was diluted to OD_{600} of 0.1 in nutrient broth flasks containing sublethal concentrations of respective metal salts (Table 1). After 24 h of growth, the cultures were centrifuged and cell pellets washed with saline (0.85% NaCl). The cells were then digested with acid for metal content analysis by ICP-AES (inductively coupled plasma-atomic emission spectrometry) [15].

Table 1
Resistance profile of *A. ramosus* strain G2.

Growth medium	Metal/antibiotic	MTC[a] (μM)	Sublethal concentration (μM)
Nutrient agar	Cd acetate	37	18.5
	$CoCl_2$	525	262.5
	$ZnSO_4$	348	174
	$K_2Cr_2O_7$	1530	765
	$HgCl_2$	369	184.5
	PMA	S	–
	Ampicillin	S	–
	Tetracycline	S	–
Minimal mineral medium	$K_2Cr_2O_7$	408	204
	$HgCl_2$	37	18.5

[a] S: sensitive at $1\,mg\,l^{-1}$ concentration of the metal/antibiotic. See text for further details.

2.4. Resistance to Cr

Since resistance to Cr(VI) is known to be mediated by its reduction in several bacteria, this activity was also checked in our isolate. However, constituents of nutrient broth interfered with Cr(VI) estimation. Hence, a minimal mineral medium (composition in $g\,l^{-1}$: Na_2HPO_4, 1.264; KH_2PO_4, 0.326; NH_4Cl, 1; $MgSO_4$, 0.098; $CaCl_2$, 0.044; glucose, 0.1) was used. The MTC of $K_2Cr_2O_7$ in the minimal medium was estimated and experiment was carried out at sublethal concentration (Table 1). The culture was diluted to OD_{600} of 0.1 in minimal medium containing $K_2Cr_2O_7$ and samples were removed after 1–3 days. A control flask without the culture was also maintained. Cr(VI) content of the samples was obtained by analyzing both supernatant medium and cell pellet by 1,5-diphenylcarbazide method [15]. % Cr reduction was determined by comparing Cr(VI) content of the samples with that of control.

To demonstrate the chromate reductase activity in cell free extract, the culture was induced by growth in the presence of $K_2Cr_2O_7$. A very low concentration of $15\,mg\,l^{-1}$ of $K_2Cr_2O_7$ was used for induction, as high concentrations can inhibit culture growth and reduce the reductase yield. The resulting biomass was resuspended in 100 mM phosphate buffer (pH 7), followed by disruption by sonication. Cells debris was removed by centrifugation and supernatant was used as the crude cell extract. The chromate reductase assay mixture consisted of 100 mM phosphate buffer (pH 7), 0.1 mM $K_2Cr_2O_7$, 0.1 mM NADH and 0.1 ml of crude cell extract in a total volume of 1 ml. To study the effect of electron donor, NADH was replaced by NADPH in the assay mixture. One unit of enzyme activity was defined as the amount of enzyme that reduces 1 nmol of Cr(VI) min^{-1} at 25 °C [18].

2.5. Resistance to Hg

Resistance to Hg(II) is usually mediated by its reduction and volatilization [4]. To test this, the culture was diluted to OD_{600} of 0.1 in nutrient broth containing sublethal concentration of $HgCl_2$ (Table 1) and samples were removed after 1–3 days. A control flask was also maintained to determine abiotic volatilization. Hg content was analyzed in both biomass and supernatant medium fractions by acid digestion followed by cold vapour atomic absorption spectrometry [15]. % Hg reduction was determined by comparing total Hg content of the samples with that of control.

For further confirmation, reduction of Hg(II) was also tested in the minimal mineral medium. For this, MTC of $HgCl_2$ in mini-

mal medium was determined and experiment was carried out at sublethal concentration (Table 1). Samples were removed after 1, 3 and 5 days, as growth of the isolate was comparatively slower in minimal medium. Results were expressed as % Hg reduction as above.

To demonstrate mercuric reductase activity in cell free extract, the culture was induced by growth in the presence of $HgCl_2$. A very low concentration of 15 mg l^{-1} of $HgCl_2$ was used for induction, as high concentrations can inhibit culture growth and reduce the reductase yield. The resulting cells were resuspended in buffer A (20 mM phosphate buffer, pH 7.4, 0.5 mM EDTA, 0.1% β-mercaptoethanol) before disruption by sonication. Cell debris was removed by centrifugation and the crude cell extract was assayed for MerA activity. The assay mixture consisted of 80 mM phosphate buffer (pH 7.4), 1 mM β-mercaptoethanol, 0.1 mM $HgCl_2$, 0.2 mM NADPH and 0.1 ml of crude cell extract in a total volume of 1 ml. Oxidation of NADPH was followed spectrophotometrically at 340 nm. A control was also tested to determine Hg-independent oxidation of NADPH. One unit of enzyme activity was defined as the amount of enzyme capable of catalyzing Hg-dependent oxidation of 1 μmol of NADPH min^{-1} at 25 °C [10].

2.6. Purification and characterization of mercuric reductase enzyme

Crude cell extract was prepared as above. Its MerA activity was found to precipitate in 40–60% saturated $(NH_4)_2SO_4$ fraction at 4 °C. The precipitate was dissolved in minimum volume of buffer A and dialyzed against the same to remove residual $(NH_4)_2SO_4$. It was then loaded on cibracon blue agarose column (1 cm × 6 cm, Bangalore Genei, India) at a flow rate of 0.1 ml min^{-1}. The column was washed with buffer A at 1 ml min^{-1} until all the unbound proteins were eluted out. MerA was then eluted with 0.1 mM NADPH. Active fractions were pooled and dialyzed against buffer A to remove the NADPH. During purification, protein concentration of the samples was estimated by Bradford method [19].

The final enzyme preparation was run on 10% SDS-PAGE gel to confirm its purity and determine the subunit molecular weight by comparing its relative migration (R_f) to those of standard molecular weight marker proteins. Optimum working conditions of the enzyme were determined by carrying out the assay at different pH and temperature values. Similarly enzyme kinetics was studied by varying $HgCl_2$ concentration. To study the coenzyme specificity, NADPH was replaced by NADH in the assay. Effect of addition of FAD or FMN on the enzyme activity was also studied. Presence of any flavin prosthetic group in the enzyme was checked spectrophotometrically at 450 nm as well as by thin layer chromatography [11].

The enzyme was further characterized by nano-LC–MS/MS. The enzyme was digested with trypsin, and the resulting peptides were separated on reverse phase C_{18} column and subjected to ESI-MS using ion trap mass spectrometer (Agilent 1100 series). The data was analyzed by Mascot search engine using MSDB (mass spectrometry protein sequence database) database [20].

3. Results

A mercuric salt-contaminated soil, containing 37 mg Hg kg^{-1} soil, was analyzed for presence of Hg-resistant bacteria. The culture showing the highest level of Hg resistance was selected for further study. It was found to be Gram positive with rod to coccoidal cell transition during batch growth, a feature typical of the genus *Arthrobacter*. It was further identified as *A. ramosus* by 16S rRNA gene sequencing (GenBank accession number FJ240439).

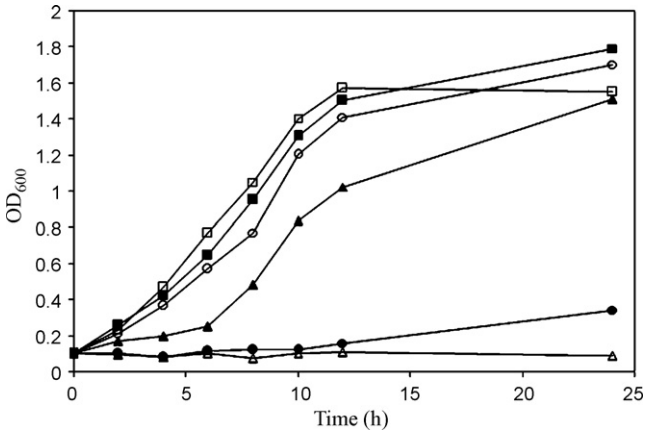

Fig. 1. Growth of *A. ramosus* strain G2 in nutrient broth containing sublethal concentrations (50% of MTC) of metal salts. (■) Control, (▲) Cd-acetate 18.5 μM, (●) $CoCl_2$ 262.5 μM, (○) $ZnSO_4$ 174 μM, (□) $K_2Cr_2O_7$ 765 μM and (△) $HgCl_2$ 184.5 μM.

3.1. Determination of metal and antibiotic resistance profile

The MTC values (in μM) for different metal salts are given in Table 1. The culture was least resistant to Cd out of the tested metals. It was also found to be sensitive to very low concentration of 1 mg l^{-1} of antibiotics like ampicillin and tetracycline, and organomercurials like PMA. Thus, the culture displayed narrow-range Hg resistance, i.e. resistance towards inorganic Hg only. Further, it did not contain any plasmid indicating that the Hg resistance determinants were located on the chromosome. When the culture was grown in the presence of sublethal concentrations of above metals, Zn and Cr were found to have virtually no effect on the growth curve, while Co and Hg were found to be the most inhibitory (Fig. 1). The culture was found to bioaccumulate these metals during growth. Accumulation of Cd, Co and Zn is shown in Fig. 2, while accumulation of Cr and Hg is shown in Fig. 3. Bioaccumulation of Cr was studied in minimal mineral medium, as constituents of nutrient broth interfered with its estimation, and the bioaccumulation was found to be negligible (Fig. 3). Cr resistance in the culture was found to be mediated by reduction of Cr(VI) to Cr(III) in the growth medium (Fig. 3). Chromate reductase activity in the crude cell extract of the culture was found to be 2.3 units mg^{-1} protein. It required NADH as the electron donor, and was non-functional with NADPH. Also, the culture was found to reduce and volatilize Hg(II) in both nutrient broth as well as minimal mineral medium (Fig. 3).

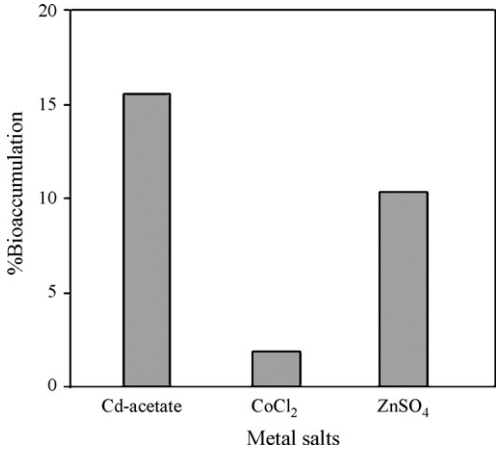

Fig. 2. Bioaccumulation of Cd, Co and Zn in 24 h. Metal salt concentrations as in Fig. 1.

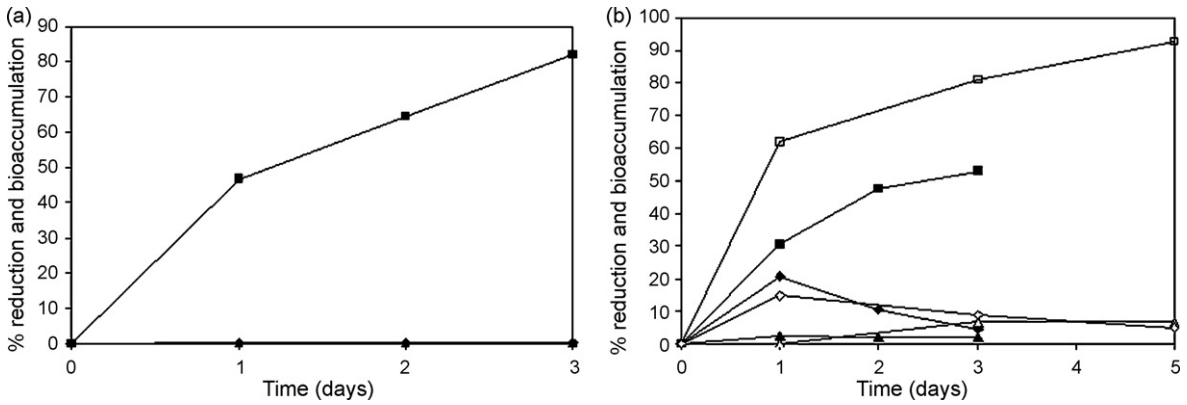

Fig. 3. (a) % reduction (■) and % bioaccumulation (●) of Cr(VI) in minimal mineral medium containing 204 μM $K_2Cr_2O_7$ (50% of MTC). (▲) Abiotic control. (b) % reduction (■) and % bioaccumulation (●) of Hg in nutrient broth containing184.5 μM $HgCl_2$ (50% of MTC), and % reduction (□) and % bioaccumulation (○) of Hg in minimal mineral medium containing 18.5 μM $HgCl_2$ (50% of MTC). (▲) and (△) Abiotic controls in nutrient broth and minimal medium, respectively.

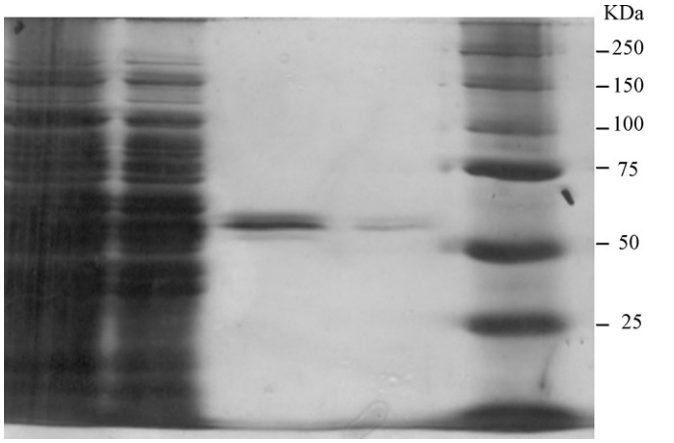

Fig. 4. SDS-PAGE analysis of protein fractions during purification of MerA enzyme. Lane 1: crude cell extract; lane 2: $(NH_4)_2SO_4$ precipitated fraction; lanes 3 and 4: affinity purified fractions; lane 5: molecular weight marker.

The mercuric reductase activity in crude cell extract of the culture was found to be 0.17 units mg^{-1} protein.

3.2. Purification and characterization of mercuric reductase enzyme

MerA enzyme was isolated from the crude extract by $(NH_4)_2SO_4$ precipitation followed by dye affinity chromatography. The process resulted in 25-fold purification and yielded about 0.5 mg of pure enzyme per liter of culture medium. Monomeric molecular weight of the enzyme was found to be approximately 57 kDa by SDS-PAGE analysis (Fig. 4). Optimum pH and temperature of the enzyme were found to be 7.4 and 55 °C, respectively. K_m and V_{max} of the enzyme were 16.9 μM $HgCl_2$ and 6.2 μmol min^{-1} mg^{-1} enzyme, respectively (Fig. 5). When NADPH was replaced with NADH as electron donor, the enzyme activity was abolished. Thus, the enzyme seems to be specific for NADPH. Additionally, the purified enzyme was found to contain FAD as prosthetic group, as shown by presence of an absorbance peak at 450 nm and relative mobility in thin layer chromatography (Fig. 6). Addition of external FAD or FMN did not have any effect on enzyme activity. This is unlike *Azotobacter chroococcum*, where MerA is stimulated by FAD [21].

Table 2
Tryptic peptides of MerA enzyme identified by nano-LC–MS/MS.

Position[a]	Sequence	Molecular weight	Mascot score
39–54	STVGGTCVNTGCVPSK	1623	63
316–326	IWAAGDVTGHR	1181	45
359–382	VTFTSPALAAVGMTDKEANEAGIR	2448	41
387–395	VLPLEYVPR	1084	42
408–416	IVANNSTGR	931	12

[a] According to the *A. aurescens* putative MerA sequence.

The protein sequence of the enzyme was characterized by nano-LC–MS/MS. Mascot search of the peptide mass fingerprint of the enzyme identified 5 unique peptides from putative MerA of *A.*

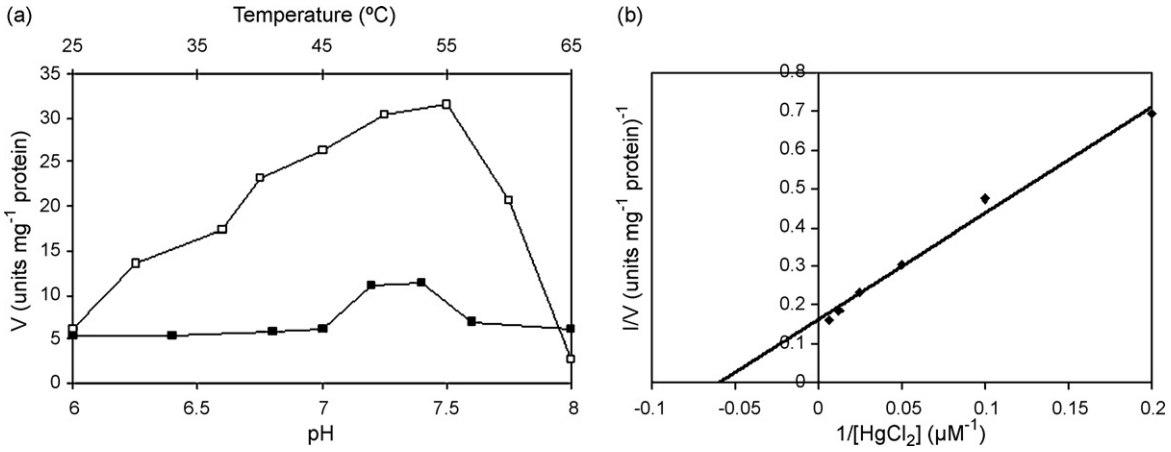

Fig. 5. (a) Effect of pH (■) and temperature (□) on MerA activity; (b) reciprocal plot for kinetic analysis of MerA enzyme.

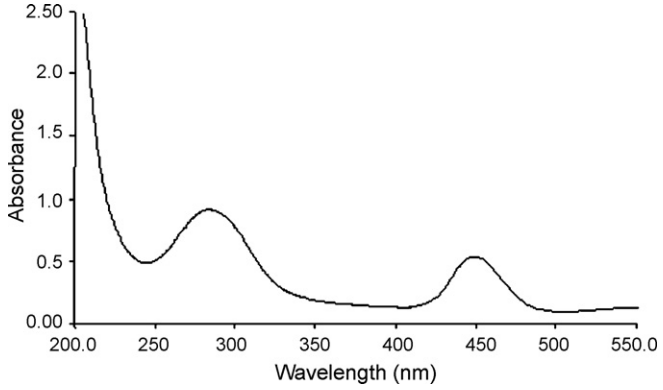

Fig. 6. UV–vis spectrum of purified MerA enzyme.

aurescens (TrEMBL accession number Q6SKF3) with Probability-Based Mowse Score of 199 (Table 2). The peptides identified from the enzyme represented 14% of the amino acid sequence of Q6SKF3, confirming the identification and sequence of MerA enzyme of our isolate.

4. Discussion

MerA enzyme has not been isolated from any *Arthrobacter* sp. so far, although it is known to be a homodimeric or a homotrimeric flavoprotein, containing FAD as prosthetic group, in other organisms [10,11]. Putative *merA* genes have been identified in plasmid from *A. aurescens* strain TC1 and both plasmid and chromosome from *Arthrobacter* sp. FB24, based on similarity to other putative *merA* genes (GenBank accession numbers CP000454, CP000455, CP000456, CP000457) [12]. However, the functional and structural properties these enzymes have not been experimentally characterized. Hence, there was a need to isolate and characterize MerA enzyme from an *Arthrobacter* sp.

Present study describes isolation of multiple metal-resistant *A. ramosus* strain G2, which exhibited narrow-range Hg resistance, i.e. resistance towards inorganic Hg only. This is consistent with the absence of putative organomercury lyase gene in *A. aurescens* strain TC1 and *Arthrobacter* sp. FB24 (GenBank accession numbers CP000454, CP000455, CP000456, CP000457) [12]. Since the current isolate did not contain any plasmid, the Hg resistance genes must be chromosomally located. The isolate was found to be sensitive to antibiotics like ampicillin and tetracycline, further implying that it may not contain any resistance plasmid. In addition to Hg, the culture was also found to resist other heavy metals like Cd, Co, Zn and Cr, though MTC of Cd was comparatively low (Table 1). Sublethal concentrations of Zn and Cr had virtually no effect on the growth of culture in nutrient broth, while Cd had only moderate effect (Fig. 1). A fraction of all metals, except Cr, was found be bioaccumulated by the culture during growth (Figs. 2 and 3). Resistance to Cr was found to be mediated by reduction of highly toxic Cr(VI) to relatively non-toxic Cr(III). The culture could detoxify more than 80% of sublethal concentration of Cr (204 μM) in minimal mineral medium (Fig. 3). Cr(VI) reduction could also be observed in crude protein extract obtained from the cells, indicating that it was mediated by the soluble cytoplasmic chromate reductase enzyme. It was found to require NADH as the electron donor.

The narrow-range resistance to Hg was confirmed by observing Hg volatilization in the culture medium (Fig. 3). The culture was found to reduce and volatilize more than 50% and 90% of sublethal concentrations of Hg in nutrient broth (184.5 μM) and minimal mineral medium (18.5 μM), respectively. MerA enzyme was then purified from the culture by dye affinity chromatography. It gave a single band of approximately 57 kDa on SDS-PAGE gel, confirming its purity (Fig. 4). It seems to be relatively protease-resistant, as MerA enzymes from some other sources have been reported to show two bands on SDS-PAGE gel due to proteolytic processing [10]. The enzyme was found be a flavoprotein containing FAD as prosthetic group (Fig. 6), and required NADPH as the electron donor for Hg reduction. Optimum pH of the enzyme was 7.4, similar to the values reported for other MerA enzymes, while optimum temperature was comparatively higher at 55 °C (Fig. 5). MerA from other organisms do not have optimum temperature above 45 °C [11,21]. Thus, the enzyme seems to be different from previously reported mesophilic MerA enzymes in being mildly thermophilic. Protein sequence of the enzyme was analyzed by nano-LC–MS/MS. It generated a peptide mass fingerprint, which unambiguously identified *A. aurescens* putative MerA sequence upon database searching (Table 2). To the best of our knowledge, this is the first report to experimentally confirm the putative sequence and characteristics of MerA enzyme in an *Arthrobacter* sp.

5. Conclusions

A. ramosus strain G2 isolated in the present study was found to be resistant to several heavy metals. It has great potential in remediation of heavy metal-contaminated sites as it could not only bioaccumulate the heavy metals, but also reduce and detoxify redox-active metals like Cr and Hg. Since heavy metals can inhibit degradation of other pollutants in their environment, the current isolate may also be a suitable candidate for removal of pollutants from heavy metal co-contaminated sites. To the best of our knowledge, this is also the first report to isolate and characterize mercuric reductase enzyme from an *Arthrobacter* sp.

Acknowledgements

Amit Bafana is grateful to the Council of Scientific and Industrial Research (CSIR), India for the award of Shyama Prasad Mukherjee Fellowship. The authors are thankful to Dr. Sukumar Devotta, Ex-Director, NEERI for providing necessary facilities for carrying out the research work.

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