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Uranium biomineralization by a metal resistant *Pseudomonas aeruginosa* strain isolated from contaminated mine waste

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ABSTRACT

Uranium biomineralization by a metal-resistant *Pseudomonas aeruginosa* strain isolated from uranium mine waste was characterized for its potential in bioremediation. Uranium resistance, its cellular localization and chemical nature of uranium–bacteria interaction were elucidated. Survival and uranium biomineralization from mine water were investigated using microcosm experiments. The selected bacterium showed U resistance and accumulation (maximum of 275 mg U g^{-1} cell dry wt.) following incubation in 100 mg U L^{-1}, pH 4.0, for 6 h. Transmission electron microscopy and X-ray diffraction analyses revealed that bioaccumulated uranium was deposited within the cell envelope as needle shaped U-phosphate compounds that attain crystallinity only at pH 4.0. A synergistic involvement of deprotonated phosphate and carboxyl moieties in facilitating bioprecipitation of uranium was evident from FTIR analysis. Based on these findings we attribute the localized U sequestration by this bacterium as innocuous complex to its possible mechanism of uranium resistance. Microcosm data confirmed that the strain can remove soluble uranium (99%) and sequester it as U oxide and phosphate minerals while maintaining its viability. The study showed that indigenous bacteria from contaminated site that can survive uranium and other heavy metal toxicity and sequester soluble uranium as biominerals could play important role in uranium bioremediation.

1. Introduction

Environmental contamination with uranium originating from uranium mining, leakage from radioactive storage sites and during phosphate enrichment is a severe public health concern [1]. Apart from radioactivity, toxicity of uranium to all living components poses a tremendous threat to diversity, structure and function of affected ecosystems [2]. As the biogeochemical complexities at radionuclide and metal contaminated mixed waste sites make the remediation a complex process posing significant economical and technological challenges, bioremediation with microorganisms remains potentially the most cost effective viable cleanup technology [3–6]. It has been demonstrated that uranium contaminated sites often harbor viable and metabolically active microorganisms capable of executing various modes of metal–microbe interaction to maintain viability and can dramatically impact the form and distribution of uranium in the environment [7–9]. Such uranium–bacteria interactions include binding of cationic uranyl species to membrane and cell wall anionic ligands, intracellular accumulation and redox transformations resulting in altered uranium mobility and toxicity [1,8–10]. Considering the abundance and diversity of microorganisms in the natural domain, it is of immense importance to identify and characterize such microbial strains with respect to their interactions with uranium [11]. A thorough characterization and understanding of underlying mechanisms will enable us to appraise the potential of microorganism for the development of *in situ* uranium bioremediation strategies [9,12,13]. Optimization of such strategies requires extensive studies on the speciation and chemical nature of toxic metals, their cellular localization and an assessment of the performance of selected microorganisms using real effluent. Furthermore, owing to the complex nature of U mine wastes with concentration of other heavy metals beyond toxic levels, metal resistance and sequestration by indigenous bacteria could be critical, and therefore these attributes must be well characterized for the success of *in situ* remediation efforts.

In spite of considerable recent interest on the assessment of microbial diversity within various uranium and other radionuclides contaminated habitats and the nature of uranium bacteria interaction for their environmental and other biotechnological applications [9,14,15], studies on uranium resistance and accumulation by U resistant bacteria isolated from U contaminated sites remain relatively scanty. There are few reports on U resistance by bacterial strains isolated from uranium mine or radioactive waste

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repositories, which were mostly identified as *Microbacterium* spp., *Arthrobacter* spp., *Rhodococcus globerulus*, *Bacillus* spp. and *Rahnella* sp. [8,9,12]. Uranium sequestration by these resistant strains was investigated employing electron microscopic and spectroscopic techniques to describe the uranium bacteria interaction through cellular ionizable groups (carboxylate and phosphate) present mainly within the cell wall and membrane components or by phosphatase enzyme activity producing excess orthophosphate that biomineralizes uranium [5,12]. Such microbially mediated mineral precipitation by inhabitant U resistant bacteria has been identified as a promising strategy for bioimmobilization of metals and radionuclides during remediation of aerobic contaminated environments [5,12]. In the present study, we examined the uranium resistance and sequestration by a metal resistant and accumulating *Pseudomonas aeruginosa* J007 strain previously isolated from uranium mine water and reported as *Pseudomonas* sp. J007 [11]. Uranium resistance and accumulation capacity of this strain was determined at low pH (pH 4.0). Intracellular localization of accumulated uranium was elucidated by transmission electron microscopy coupled with energy dispersive X-ray analysis and SAED. Chemical characterization of sequestered uranium was done by FTIR and XRD. Performance of this strain in tolerating and sequestering uranium in mine water sample was also evaluated using microcosm based studies.

2. Materials and methods

2.1. Microorganisms and growth conditions

Bacterial strains used in this study were *P. aeruginosa* J007, and *Escherichia coli* JM109. The *P. aeruginosa* J007 was earlier isolated from a uranium mine water tank of Uranium Corporation of India Ltd, Jaduguda, India (GPS location: N 22°39.026′ E 86°20.089′) following growth and enrichment in Tris-buffered minimal medium broth [11]. For routine growth and experiments bacterial strains were grown in Luria Broth or in modified Tris minimal medium at 30 °C with a shaking speed of 170 rpm (New Brunswick shaker, Innova 4230, orbit 19 mm) as appropriate. The composition of Tris-minimal medium used was 6.06 g of tris hydroxymethyl aminomethane hydrochloride, 4.68 g of NaCl, 1.49 g of KCl, 1.07 g of NH_4Cl, 0.43 g of Na_2SO_4, 0.20 g of $MgCl_2{\cdot}6H_2O$, 0.03 g of $CaCl_2{\cdot}2H_2O$ dissolved in 1 L of distilled water, pH adjusted to 7.3–7.4 with HCl or NaOH. Filter sterilized beta-glycerol phosphate (0.432 g/L) and glucose (5 g/L) were added separately after autoclaving [16].

2.2. Uranium sensitivity tests

Uranium sensitivity was tested by monitoring cell viability following exposure to uranium with a modified protocol adapted from Suzuki and Banfield [8]. Bacterial cells were grown in minimal medium with 2% inoculum density and monitored for growth at regular intervals by measuring absorbance at 660 nm in a UV–vis spectrophotometer (Carry 50, Varian). Mid log phase cells (0.5–0.6 OD) were harvested by centrifugation (2360 × *g*, 15 min), washed and transferred to NaCl solution (0.1 N) at pH 4.0 with or with out uranium [100 mg U L^{-1} as uranyl nitrate solution $UO_2(NO_3)_2{\cdot}6H_2O$] and incubated at 30 °C with continuous shaking (170 rpm). Timed (1 and 6 h) aliquots were withdrawn, diluted appropriately and CFU counts were determined using nutrient agar medium following incubation at 30 °C for 12 h. All viability test experiments were performed thrice with CFU determination in triplicate.

2.3. Uranium uptake experiments

Minimal medium grown mid exponential phase cells of *P. aeruginosa* J007 were harvested by centrifugation, washed, and resuspended in uranium solution (100 mg L^{-1}, pH 4.0). Uranium uptake by the bacterium was monitored for 6 h with continuous shaking at 30 °C. This time period was chosen based on initial uptake experiments that showed saturation of uranium uptake by 6 h with no further metal accumulation following prolonged incubation. Samples were withdrawn at selected intervals, cells were removed, and supernatant was used for uranium estimation. The cell pellets were washed and lyophilized for spectroscopic analysis. Acid digested cell biomasses were also used for cell bound uranium assay. In each set, samples without bacterial cells were used as control. Dissolved uranium was determined by the arsenazo III method [17] and inductively coupled plasma-mass spectrometry (ICP-MS) (Varian 820 MS).

2.4. Transmission electron microscopy, energy dispersive X-ray (EDX) and select area electron diffraction (SAED) analyses

Transmission electron microscopy was done following the methods described previously [11]. Bacterial cells recovered following incubation with or without uranium were washed in 0.1 M sodium cacodylate buffer (pH 7.2). Fixed in 2.5% glutaraldehyde for 2 h at 4 °C and then washed twice with the same buffer. Cell pellets were treated with 1% OsO_4 for 30 min at 4 °C, followed by dehydration with acetone and embedding in epoxy resin (araldite CY 212). Ultra thin sections (70–80 nm) cut by a Reichert Ultracut E Ultramicrotome were loaded in formvar carbon coated copper grids and examined in a FEI Philips Morgagni 268D transmission electron microscope at an operating voltage of 80 kV. Images were digitally acquired by using a CCD camera (Megaview III, Fei Company) attached to the microscope. EDX analysis of selected spots was carried out using Oxford-$INCA_X$ EDX system (M/S Oxford Instruments Analytical, Oxfordshire, UK) attached with HRTEM JEOL JEM 2100 high resolution Transmission Electron Microscope to obtain qualitative data on elemental compositions of control cell sectors and uranium-bearing solid phases associated with cells. The instrument was routinely calibrated using the standard supplied by M/S Microanalysis Consultants Limited, Cambridgeshire, UK through Oxford Instruments, Oxfordshire. Select area electron diffraction (SAED) pattern of selected electron dense region was done using a HRTEM JEOL JEM 2100 in the diffraction mode with a camera length of 1000 mm and an exposure time of between 15 and 20 s. The diffraction pattern was analyzed manually by measuring the diameter of the Debye rings. The formula used is: Rd = Camera constant (CC), where R is the radius of the ring and d is the interplanar spacing.

2.5. X-ray powder diffraction (XRD) analysis

XRD pattern of lyophilized powder samples of control and U loaded bacterial cells were recorded in a Philips X'Pert Pro Panalytical PW3040/60 powder diffractometer with Cu source radiation ($\lambda = 1.54$) over the range of 10–90 (2θ), step length of 0.05°. The chemical nature of U crystals was determined based on comparison with the powder diffraction standard files (Search Manual for Selected Powder Diffraction Data for Metals and Alloys, JCPDS, International Centre for Diffraction Data, PA, USA, 1978).

2.6. Fourier Transform Infra Red spectroscopy (FTIR)

For FTIR spectroscopic analysis bacterial cells were incubated with or without uranium for 6 h, recovered by centrifugation (3680 × *g*, 10 min), washed in 0.1 N NaCl solution and lyophilized (ELYLA-FDU 1200 lyophilizer). One milligram lyophilized cell mass was intimately ground with solid KBr (FTIR grade, Sigma) in mortar pestle and a thin pellet was made with a die using a hydraulic press (SPECAC) and placed in the IR beam for analysis. Infrared spectra of uranium free and loaded cells were recorded within a range

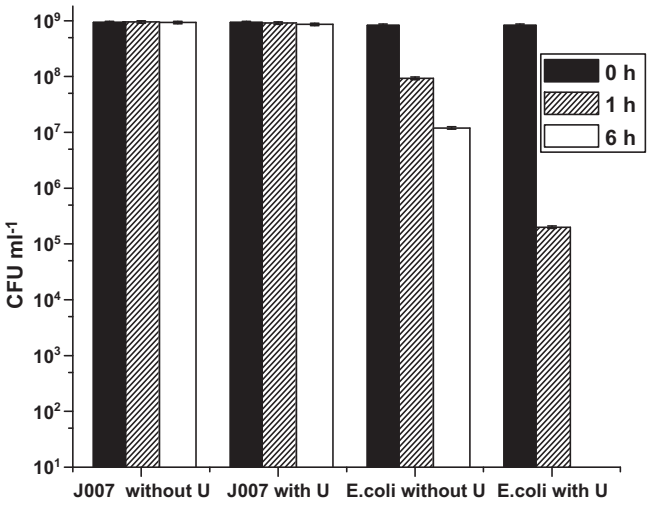

Fig. 1. Uranium resistance as determined by viability of *P. aeruginosa* J007 and *E. coli* (JM109) following exposure to U-free and U-containing [100 mg U L^{-1} as uranyl nitrate solution] 0.1 N NaCl solution at pH 4.0 for 0, 1 and 6 h.

400–4000 cm^{-1} using a Thermo Nicolet NEXUS-870 FTIR Spectrometer. Spectra were viewed in OMNIC software and peak markings were done followed by analysis.

2.7. Microcosm studies on survival and uranium sequestration of P. aeruginosa J007

Ability of the *P. aeruginosa* J007 to survive and remove uranium from U-mine water was investigated using laboratory microcosm systems. Triplicate shake flasks containing 100 ml of either (i) mine water or (ii) mine water amended with extra 5000 μg L^{-1} uranium (as uranyl nitrate) or (iii) 0.1 N NaCl solution with 5000 μg L^{-1} of uranium (as uranyl nitrate) were bioaugmented with the test bacterium. The fourth set of microcosm was maintained as an abiotic control for each of the above three conditions without any bioaugmented cells. *P. aeruginosa* J007 cells were pre-grown in minimal medium up to the mid exponential phase, harvested, washed twice in isotonic saline, and inoculated at a concentration of 10^9 cells ml^{-1} within the microcosm systems. Mine water was filter sterilized prior to experimentation to remove the background microbial cells. The mine water used in this experiment was collected freshly from the mine water discharge tank at uranium mine Jaduguda. All flasks were incubated at 30 °C, with continuous shaking (170 rpm) and subsamples were aseptically removed periodically to determine: (i) number of viable cells by CFU counts on nutrient agar plates (ii) soluble U concentrations by ICP-MS (VARIAN 820 MS) and (iii) chemical nature of sequestered uranium by XRD. For XRD analysis cells harvested after 1, 7 and 21 days were washed, lyophilized and the pellets were used to generate the X-ray diffraction pattern (details as described in previous section).

All data represent the mean of three independent experiments. Standard deviations and error bars are indicated wherever necessary. All statistical analyses were done using Origin Pro, Version 8.0.

3. Results and discussion

3.1. Uranium-resistance and -uptake by P. aeruginosa J007

Uranium sensitivity of the test bacterium was determined by CFU counts following incubation in uranium free or amended low pH (pH 4.0) NaCl solution (Fig. 1). CFU counts obtained after exposure to metal free condition indicated that the strain was capable to withstand the nutrient deprived acidic condition without any loss of cell viability. Noticeably, incubation along with uranium did not exert any lethal effect on the test bacterium as evident from insignificant change ($P > 0.05$) in cell viability up to an exposure of 6 h, thus indicating resistance to uranium toxicity. Under similar conditions *E. coli* cells used for comparison, showed high U sensitivity with nearly 3 orders of magnitude lowering in cell counts followed by complete death with increased incubation periods (data not shown), thus confirming that sensitivity to the chemical toxicity of uranium was readily detected in our experiments. The bacterium also showed uranium accumulation capacity with a maximum of 275 mg U g^{-1} cell dry wt. within an exposure time of 6 h at 100 mg L^{-1} metal concentration (pH 4.0).

Uranium has no known biological function and it is considerably more toxic compared to trace metals and even thorium [7]. Our finding on the viability of *P. aeruginosa* J007 in presence of uranium at low pH is highly pertinent as uranium at this pH is primarily present as uranyl (UO_2^{2+}), the most toxic aqueous species [8]. Previous studies on uranium toxicity by bacterial strains like *R. globerulus*, *Arthrobacter* spp., *Rahnella* sp. and *Bacillus* spp. isolated from U contaminated sites revealed that *Arthrobacter* strains (S3 and FRC) were most tolerant to uranium (80 mg L^{-1}) up to 1 h [8,12]. Since the toxic effect of uranium to microbes is manifested mainly due to its chemical properties that interfere with vital cellular processes rather than its radioactivity (short microbial cell cycle and long half life of U), the strain that showed resistance must possess intrinsic mechanism to affect local concentration of this ion in cell interior. Along with its resistance, the ability of the test bacterium to accumulate large amount of (>98%) uranium and precipitate it within the cell envelope region (as evident from TEM analysis present in Section 3.2) could be implicated as a mechanism to withstand U toxicity. Previously, with respect to uranium mine isolated *Microbacterium* spp. and *B. sphaericus* that showed elevated U resistance, a selective deposition within the cell surface (membrane and wall) have been observed [1,9]. Recently, Martinez et al. [12] have explained U bioadsorption or sequestration as possible cellular mechanism for uranium tolerance to various bacteria [12,18]. The later process originally reported by Suzuki and Banfield [8] could result in intracellular accumulation of U precipitates possibly as a means to limit U toxicity. In line with these reports our findings on the *P. aeruginosa* J007 in accumulating high uranium as precipitates correlated well with its resistance. Precipitation of accumulated uranium within the cell envelope as uranium phosphate compounds (as evident by TEM and XRD studies) could play important role in protecting biologically important molecules in the cytoplasm.

Along with resistance to uranium, its uptake by the test bacterium is important from the potential application of this bacterium as a candidate for uranium removal/recovery from contaminated environments. The rapid U uptake as shown by this bacterium could be attributed mainly to electrostatic interaction between anionic sites (carboxyl, phosphoryl groups present on lipopolysaccharides and parts of cell envelope region) for which uranium have a very strong affinity [19,20]. Uranyl ions initially sorbed to the cell surface gradually formed complexes (most often with phosphate moieties) resulting in microprecipitation in and around the cells [21,28]. In corroboration to this study, there are previous reports on very rapid, high uranium uptake in bacterial strains isolated from uranium contaminated sites [21].

3.2. Transmission electron microscopy, EDX and SAED analyses

Transmission electron microscopy (TEM) was used to investigate the cellular localization of accumulated uranium (Fig. 2). Compared to metal free *P. aeruginosa* J007 control cells showing relatively diffuse cell boundary and homogenous cytoplasm

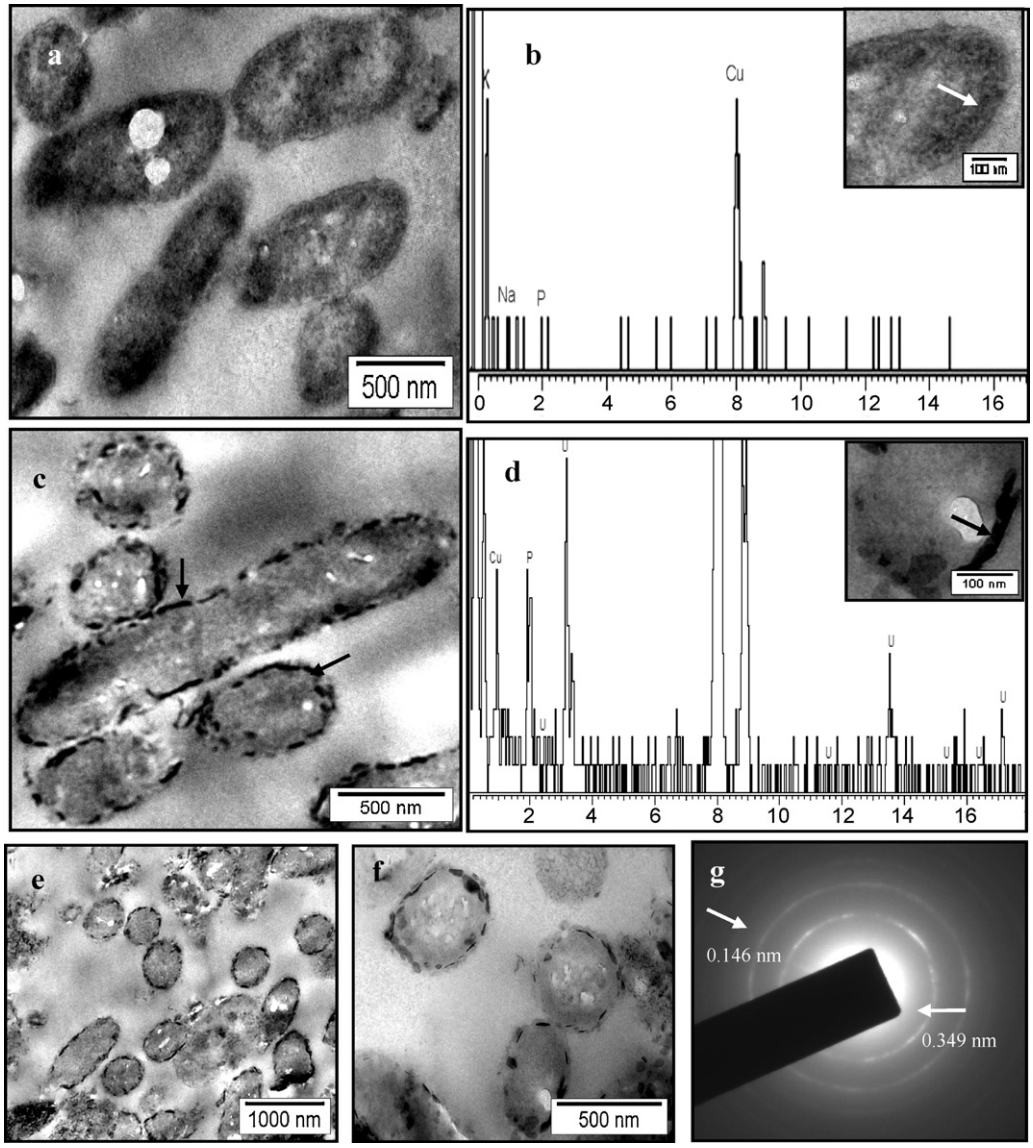

Fig. 2. TEM micrograph (a), EDX spectra (b) of thin sections of *P. aeruginosa* J007 control cells. TEM micrographs (c, e, f) and EDX spectra (d) of uranium loaded *P. aeruginosa* J007 cells. SAED pattern obtained from the uranium containing crystalline deposits in cell envelope region (g). Uranium deposits in the cell envelope region are indicated by arrows. Cell envelope regions where EDX and SAED were targeted are shown as inset within figure b (control cells) and d (U-loaded cells).

and few electron dense granules (Fig. 2a), uranium loaded cells presented distinct features highlighting the site of uranium sequestration. Bacterial cells exposed to uranium for 6 h showed presence of dark electron dense needle shaped and/or granular deposits throughout the cell envelope region (Fig. 2c). Noticeably, no cytosolic deposition of similar nature was apparent. No sign of cell lyses or destruction was apparent even after 6 h exposure corroborating our previous observation about the ability of these cells to withstand U toxicity. Conclusive identification of the deposited element was achieved by EDX and SAED coupled with high resolution transmission electron microscope (HRTEM). Compared to metal free control sample, presence of specific peak for uranium in U loaded sample confirmed the presence of accumulated radionuclide (Fig. 2b and d). The SAED pattern obtained from the needle shaped deposits present in cell envelope region showed crystalline nature with diffraction spots having *d*-values of 0.349 nm and 0.146 nm (Fig. 2g). The matching of *d*-value of 0.349 nm to d_{200} of chernikovite ($HUO_2PO_4{\cdot}4H_2O$) [8,22], a U(VI) compound belonging to autunite group of minerals, indicated the possible chemical identity of the cell bound crystalline uranium deposits.

The peripheral deposition of uranium within the cells of *P. aeruginosa* J007 corroborates well with similar observations on several other bacterial strains isolated from radioactive disposal sites [8,9,23]. Association of uranium with bacterial cell surface is considered to be primarily due to interactions with functional groups present on bacterial cell wall, exopolymers, proteins, and lipids, which formed complexes with uranium. Similar cell surface accumulation profiles have been reported earlier in several organisms like *P. fluorescens*, *Sphingomonas*, *Acidithiobacillus*, *Arthrobacter*, *Microbacterium* and *Bacillus* strains where uranium deposition took place within the entire cell envelope region owing to strong interaction with lipopolysaccharide, phospholipids, peptidoglycan and inner membrane complex (for Gram negative bacteria) or functional groups of peptidoglycan and teichoic acid (for Gram positive bacteria) [1,8–10]. Considering the fact that strains isolated from uranium contaminated habitats mostly showed sequestration of accumulated uranium within the cell surface/envelope, we suggest this as a possible survival strategy of such microorganisms inhabiting in radionuclide/uranium contaminated habitats. Deposition of accumulated uranium in the form of U phosphate compound(s)

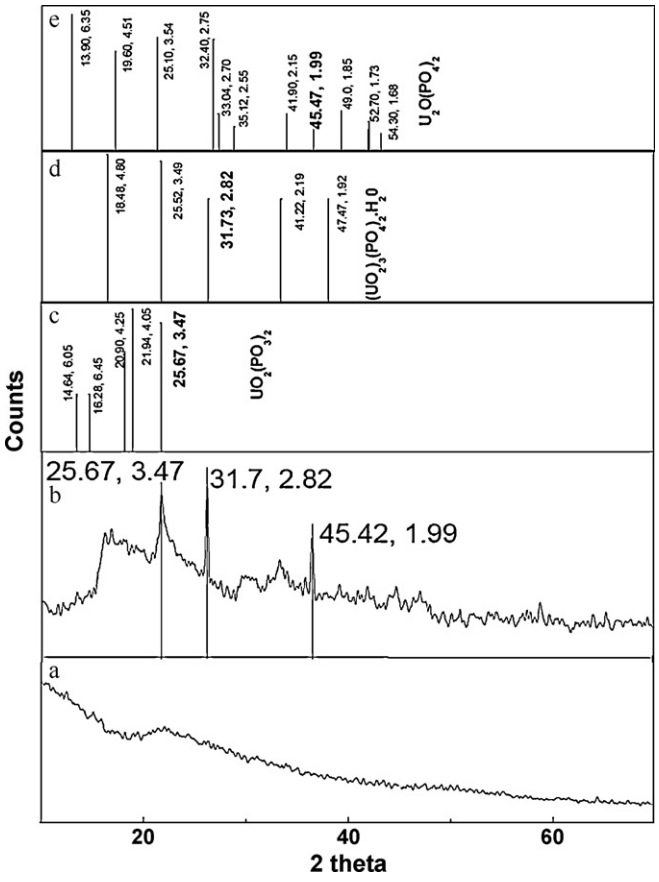

Fig. 3. X-ray powder diffraction spectra before (a), and after uranium accumulation (initial concentration 100 mg U L^{-1} at pH 4.0, time 6 h) (b) by *P. aeruginosa* J007, and comparison with reference database (vertical lines) for uranyl phosphate compounds, $UO_2(PO_3)_2$ (c), $(UO_2)_3(PO_4)_2 \cdot H_2O$ (d), and $U_2O(PO_4)_2$ (e).

within the cell envelope region as indicated by electron microscopic studies coupled with EDX and SAED analyses corroborated well with our FTIR results (presented in Section 3.4) that clearly revealed major involvement of cellular phosphate groups in interaction with uranium.

3.3. X-ray powder diffraction (XRD) analysis

The chemical nature of sequestered uranium was ascertained by X-ray powder diffractometry (Fig. 3). In contrast to the amorphous nature of metal free biomass, spectrum for the uranium loaded cells showed distinct reproducible patterns typical for well characterized materials. The XRD pattern of the later biomass showed three distinct peaks at 2θ, 25.67, 31.7 and 45.42 corresponding to *d*-spacing values of 3.47 Å, 2.82 Å and 1.99 Å, respectively. A comparison of these *d*-spacing values with data files of known compounds (JCPDS), showed satisfactory correlation with lines of uranium phosphate compounds [uranyl phosphate $\{UO_2(PO_3)_2\}$, uranyl phosphate hydrate $\{(UO_2)_3(PO_4)_2 \cdot H_2O\}$ and uranium oxide phosphate $\{U_2O(PO_4)_2\}$] (Fig. 3c–e). Noticeably, uranium accumulation at lower pH (pH 1.0–3.0) did not result any crystalline metal deposit as indicated by absence of any distinct peak (data not shown). The crystalline uranium phosphate formation following uranium accumulation indicates possible complexation of such metal with cellular phosphate groups facilitating metal nucleation and precipitation in a crystalline state [24]. Previous studies on molecular level speciation of uranium complexes within the cells of *Stenotrophomonas maltophilia* JG-2, *M. oxydans* SW-3 and *Sphingomonas* sp., isolated from uranium mill tailings by EXFAS

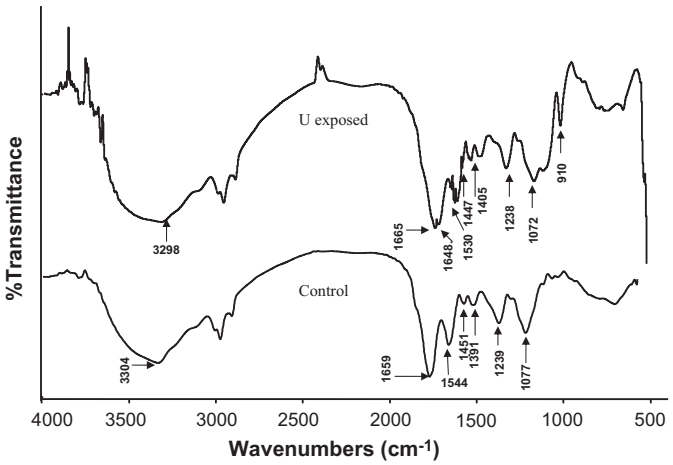

Fig. 4. Fourier transformed Infra Red spectra of *P. aeruginosa* J007 biomass: before and after uranium uptake (initial concentration 100 mg U L^{-1} at pH 4.0, time 6 h).

revealed that at higher pH (pH 4.5) cell bound uranium biomineralize as U phosphate compounds [1,25]. The above observations not only indicate the role of bacterial PO_4^{2-} groups in uranium binding, but also illustrate the effect of pH on crystallinity of the cell bound U deposits. Corroborating the earlier investigators it may be suggested that at lower pH uranium is coordinated to organophosphate groups of the cell surface (which remained protonated and do not favor crystallization of the sequestered U), whereas at pH 4.0 deprotonated phosphate groups play a major role in biomineralization of uranium. Phosphate groups available from intracellular phosphates or from cell membrane and wall materials may act as primary metal binding sites. Initial binding of uranium to these reactive sites may serve as nucleation sites for further precipitation of metals forming large metal deposits [24]. Unlike the role of acid phosphatase or release of cellular phosphates due to cell lyses following U toxicity as observed previously in *Microbacterium* spp., *Citrobacter* sp. [9,26,27] and *D. radiodurans* [8] in precipitating uranium as mineral phase, we attribute the role of naturally synthesized polyphosphate bodies (cytosolic electron dense granules evident from TEM at low magnification), which could be degraded and released phosphates may be used to subsequently immobilize uranyl phosphate on the cell envelope. A similar report of polyphosphate mediated uranium binding has been studied by over expressing polyphosphate kinase (*ppk*) gene in *P. aeruginosa* where the observed uranyl phosphate precipitation was attributed to initial uranium sorption as uranyl hydroxide, followed by its crystallization as uranyl phosphate [28].

3.4. FTIR analysis of uranium loaded bacterial cells

To elucidate the chemical groups involved in uranium binding, FTIR spectra were recorded for control and uranium loaded cells between 4000 cm^{-1} and 400 cm^{-1} (Fig. 4). Analyzing the highly complex IR spectra, certain characteristic peaks can be assigned to the involvement of the main functional groups present in the bacterial biomass. The N–H stretching peak occupied by a broad and strong band in 3200–3600 cm^{-1} region was due to the presence of γO–H of the hydroxyl groups which undergoe change in peak position in uranium loaded spectrum suggesting the involvement of amino and hydroxyl groups in metal binding to bacterial surface [29,30]. In the spectra for uranium loaded biomass the peak around 2400 cm^{-1} could be attributed to the P–O stretching vibrations suggesting possible role of phosphate metabolism (and release of phosphate groups) facilitating cellular biomineralization of uranium. The spectra for both control and uranium

loaded samples revealed protein related bands. The appearance of γC=O of amide I and δNH/γC=O combination of the amide II bonds at ~1659 cm^{-1} and 1544 cm^{-1}, respectively were predominant in the control spectrum. Following U binding the amid I absorption peak mainly accounted for α-/3_{10}-helical secondary structure of proteins and amino sugars (with N-acetyl/glucuronamide groups) was split in to two minor peaks at 1665 cm^{-1} and 1648 cm^{-1}. Similarly, the intense amid II band was shifted to a lower energy level (1530 cm^{-1}) following interaction with uranium. The observed change in peak positions and relative intensities most likely indicate some alteration in the secondary structure of cellular proteins as a result of uranium binding/accumulation [24]. In the control spectrum, sharp peaks in between 1400 cm^{-1} and 1500 cm^{-1} were due to the presence of the carboxyl groups [31]. Particularly, the strong peak at 1451 cm^{-1} which was characteristic of the scissoring motion of CH_2 groups [32] undergo a shift to lower energy level (1447 cm^{-1}) after uranium binding. Following uranium uptake a clear shift of the peak at 1391 cm^{-1} to 1405 cm^{-1} due to symmetric stretching of COO– vibration strongly indicated role of carboxyl groups in metal binding [29]. In the control spectrum, the strong peaks at 1239 cm^{-1} and 1077 cm^{-1} were observed due to vibrations of carboxyl (–COOH) and phosphate groups (P=O and P–O of the C–PO_3^{2-} moiety) [32]. These groups mostly belong to various cellular components like peptidoglycan, cell associated polysaccharides, phospholipids, peptides and are able to complex different metals [33]. Following uranium exposure, a clear shift of these peaks to 1238 cm^{-1} and 1072 cm^{-1} suggests interaction of bound metals with carboxyl and phosphates groups. A gradual shift of the peak in control spectra at 1239 cm^{-1} because of asymmetric stretching modes of protonated polyphosphates and $PO_{uncomplexed}$ in phosphate diesters to 1238 cm^{-1} in uranium loaded samples indicated the weakening of the P=O character as a result of metal binding to the phosphates [32]. Changes in peak position and intensity at 800 cm^{-1} to 400 cm^{-1} region could be assigned to the formation of intense δ(M–O) and δ(O–M–O) bonds (M = metal ion) [30]. In the uranium loaded sample, the distinct peak at 910 cm^{-1} and changes in peak positions and intensity around 550–1000 cm^{-1} region could be assigned to asymmetric stretching vibration of $\upsilon_3UO_2^{2+}$ and stretching vibrations of weekly bonded oxygen ligands with uranium ($\upsilon U–O_{ligand}$) [24]. The overall IR spectroscopic analysis suggests that carboxyl, amide and phosphate groups of bacterial cell are the dominant functional groups involved in bacteria-uranium interaction.

3.5. Microcosm based studies on survival and U sequestration by *P. aeruginosa* J007 in uranium mine water

Uranium tolerance and its removal and biomineralization abilities of the test bacterium were finally evaluated using U mine water based laboratory microcosm systems. CFU counts obtained at various time intervals through out the incubation period (30 days) indicated no adverse effect of mine water on the survival of the test bacterium (Fig. 5). Although there was a slight fall (0.3 log units) in CFU counts within first few days, it increased subsequently in next 4 days and stabilized thereafter with a value close to the initial counts (0 day). The second set of microcosm containing mine water supplemented with additional 5 mg U L^{-1} showed initial lowering of cell counts by one order of magnitude at the end of 7th day but remained unchanged thereafter. Concomitant to its survival, the bioaugmented *P. aeruginosa* cells could remove U from the microcosms. Within 24 h of incubation, soluble uranium in mine water, or mine water with added U showed 35% or 24% decline, respectively. This was followed by steady removal in both microcosms up to 14 days and beyond that a slower rate resulting 99% uranium removal. Microcosm with heat killed cells or the abiotic control showed insignificant or no change in soluble uranium concentration, respectively (Fig. 5). Chemical nature of microbially sequestered uranium within the mine water microcosms was ascertained by X-ray powder diffraction pattern of the cell biomass recovered at different time intervals. Unlike the cells in uptake solution (5 mg U L^{-1} in 0.1 N NaCl) that showed appearance of distinct peaks at 31.65 and 45.4 2θ values corresponding to crystalline uranium deposits immediately after 24 h (Fig. 6, Panel A), sample recovered from the mine water microcosm did not show any distinct diffraction pattern at an early phase of incubation (Fig. 6, Panel B). For mine water microcosm derived samples, appearance of distinct peaks that could be assigned to crystalline U complexes was noticed after 7 days of incubation. Interestingly, following an extended period of 21 days, XRD data of mine water microcosm derived biomass revealed the presence of crystallinity with distinct peaks at the same 2θ values of 31.65 and 45.4 as observed for cells exposed only to uranium (5 mg L^{-1}) in 0.1 N NaCl solution (Fig. 6). Line matching of these two peaks corresponding to *d*-spacing of 2.82 Å and 1.99 Å confirmed their identity to uranyl phosphate hydrate $\{(UO_2)_3(PO_4)_2{\cdot}H_2O$ and uranium oxide phosphate $[U_2O(PO_4)_2]$ compounds, respectively. Compared to the cells recovered from U solution (5 mg U L^{-1} in 0.1 N NaCl), mine water microcosm derived biomass, however, showed the presence of an additional peak at 2θ 25.9. The later peak could be attributed to calcium uranium oxide ($CaU_5O_{15.4}$) or uranium hydrogen phosphate $[U(HPO_4)_2]$ with 100% or 70% intensity match, respectively.

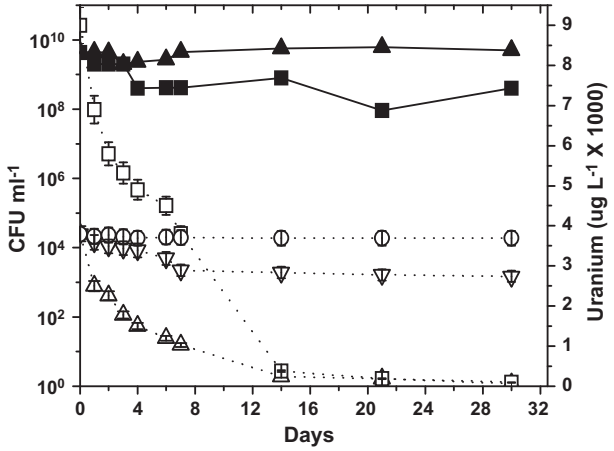

Fig. 5. Uranium tolerance and removal by bioaugmented *P. aeruginosa* J007 cells within mine water microcosms. U tolerance was estimated as CFU counts in mine water (▲) or mine water added with 5 mg U L^{-1} (■). Removal of uranium from the mine water was expressed as lowering of soluble uranium (mg L^{-1}) from mine water (△), mine water added with 5 mg U L^{-1} (□), heat killed cells (▽) and abiotic control (○).

Microcosm studies were performed to evaluate the suitability of the test bacterium in field application for uranium bioremediation. Survival results obtained here corroborated well with our previous experiments showing the superior uranium tolerance by the test bacterium. Although the mine water used here contained high level of soluble uranium (3800 μg L^{-1}) as compared to maximum contaminant level (MCL) of 30 μg L^{-1} set by U.S. Environmental Protection Agency (USEPA), it was still sufficiently low relative to the concentration (100 mg L^{-1}) used for *in vitro* sensitivity tests. However, owing to the simultaneous presence of other toxic metals and actinides within the mine water this survival capacity is very significant. Previously, various bacterial strains deployed for bioremediation of mixed waste sites were reported to be more sensitive to other metallic co-contaminants than to toxicity of actinides that ultimately limit their growth and activity at such sites [12,34]. In respect to this, the present

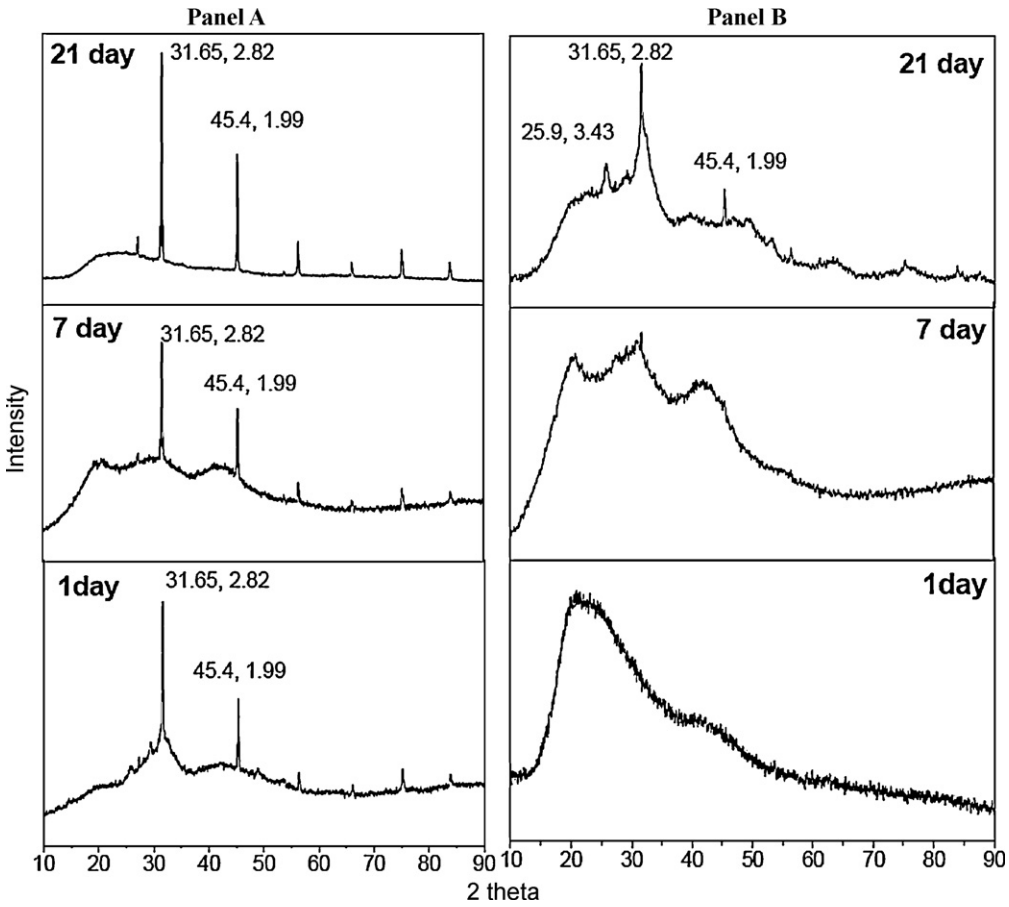

Fig. 6. X-ray powder diffraction pattern of the *P. aeruginosa* J007 cell biomass recovered from microcosms at different time intervals of 1, 7 and 21 days [Panel A (bioaugmented *P. aeruginosa* J007 cells recovered from microcosm with 0.1 N NaCl solution containing 5000 μg L^{-1} of uranium (as uranyl nitrate); Panel B (bioaugmented *P. aeruginosa* J007 cells recovered from mine water microcosm)].

P. aeruginosa strain being resistant to uranium along with several heavy metals seems to be a suitable candidate for application in bioremediation of contaminated mixed waste sites. In contrast to microbial reductive precipitation where dissimilatory metal and sulfate reducing bacteria have been shown to reduce U(VI), which require anaerobic conditions, biomineralization of U(VI) can occur in oxic conditions where U is usually present as the highly soluble uranyl ion (UO_2^{2+}) [18]. In recent years, bacteria mediated U precipitation driven by a redox independent mechanism is identified as possible remediation strategy for radionuclides in contaminated groundwater and oxygenated subsurface zones [18]. Overall, this study demonstrated that bioaugmentation with *P. aeruginosa* J007 cells stimulated nearly 99% removal of soluble uranium under *in situ* conditions. The laboratory scale shake flask microcosm study presented here prior to field experimentation exhibited that introduction of uranium resistant *Pseudomonas* strain to contaminated effluent could deplete uranium under *in situ* conditions. Thus, this study showed the potential of uranium and other metal resistant bacteria in developing bioaugmentation based remediation process for clean up of contaminated sites prevalent in uranium and other mines.

4. Conclusions

P. aeruginosa J007 strain isolated from uranium mine exhibited intrinsic abilities to withstand and accumulate (275 mg g^{-1} dry wt.) uranium at low pH (pH 4.0). As evident from TEM, EDX and SAED analyses, bioaccumulated uranium was sequestered as crystalline needle shaped U phosphate compounds within the cell envelope region. A possible correlation between the localized U sequestration as innocuous complex to U resistance may be implicated. FTIR analysis further indicated a synergistic involvement of deprotonated phosphate and carboxyl moieties in facilitating bioprecipitation of uranium. XRD data confirmed that the test bacterium sequestered uranium as crystalline U phosphate [$UO_2(PO_3)_2$, $(UO_2)_3(PO_4)_2{\cdot}H_2O$ and $U_2O(PO_4)_2$] compounds. Microcosm studies conducted to evaluate the potential of this bacterium for *in situ* applications revealed that *P. aeruginosa* J007 cells can survive well within the contaminated mine water while removing aqueous uranium efficiently as cell bound uranium biominerals. Such biomineralization potential indicated the suitability of U resistant *P. aeruginosa* J007 strain for *in situ* uranium bioremediation.

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