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Toxicity of ionic liquids to *Clostridium* sp. and effects on uranium biosorption

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HIGHLIGHTS

- Three ILs showed varying degrees of toxicity to *Clostridium* sp.
- In the presence of ILs uranium biosorption by the bacteria decreased.
- The decreased biosorption was due to membrane damage induced by ILs.
- Intracellular accumulation of uranium was also impacted.

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ABSTRACT

As green solvents ionic liquids (ILs) show high potential in nuclear industry for extraction and purification of actinides. However, to date relatively little information has been gained on ILs application in microbial processes, for example biosorption of radionuclides. We investigated the effects of three ILs, 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$), N-ethylpyridinium trifluoroacetate ($EtPyCF_3COO$) and N-ethylpyridinium tetrafluoroborate ($EtPyBF_4$) on the growth and biosorption of uranium by *Clostridium* sp. The ILs affected the growth of the bacterium as evidenced by decreases in optical density, total gas production, and organic acids production from glucose metabolism. The $IC_{50\text{-}48\ h}$ of three ILs decreased in the order of $BMIMPF_6$ (8.26 mM) > $EtPyBF_4$ (7.04 mM) > $EtPyCF_3COO$ (4.05 mM). Uranium biosorption by the bacterial cells decreased by 75% in the presence of 1% (v/v) $BMIMPF_6$ and by about 90% with 1% (v/v) $EtPyBF_4$ or $EtPyCF_3COO$, in comparison to the control without ILs. The diminished biosorption may be attributed to the membrane damages induced by $EtPyBF_4$ and $EtPyCF_3COO$, which can be visualized by Transmission Electron Microscope (TEM) analysis. Energy-dispersive X-ray spectroscopy (EDS) analysis revealed the accumulation of uranium inside peripheral membrane of the cells exposed to uranium alone or with $BMIMPF_6$, while little or no accumulation was observed in the presence of $EtPyBF_4$ and $EtPyCF_3COO$. These results imply that potential toxicity of ILs towards microorganisms is a particularly important issue in limiting its biotechnological applications.

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1. Introduction

Ionic liquids (ILs) are a class of organic salts with relatively low melting points and unique combinations of solubility-related properties dependent upon the constituent cations or anions which could be separately modified [1,2]. ILs have attracted great interest as an environmentally-benign source for alternative solvents. In comparison with many studies about ILs applications in organic synthesis, fewer studies have been performed about their applications in biotechnology. Among them whole-cell biocatalysis in the presence of ILs draws much attention [3,4].

The use of ILs in whole-cell processes has been mainly focused on "extractive fermentation", which is based on the addition of a second liquid phase (for example, immiscible ILs phase) for the in situ removal of toxic metabolic products in order to avoid cell inhibition [5]. Sendovski et al. [6] reported that the addition of 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide increased 2-phenylethanol production by *Saccharomyces cerevisiae* up to five times relative to controls without IL. However, up to now very few studies have

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investigated the effect of ILs on other whole-cell processes, such as biosorption and bioreduction [7]. Meanwhile, most of whole-cell process involved the use of hydrophobic ILs while little information has been gained on the application of hydrophilic ILs in bioprocess. Nevertheless, the toxicity of ILs is so far the major drawback for further application of ILs in whole-cell process. Toxic effects of ILs towards yeasts and bacteria have been reported in some studies [8,9]. Thus, careful studies to reveal the toxic mechanism and novel strategies to improve IL biocompatibility are necessary to expand their potential applications in extractive fermentation and other whole-cell processes.

Uranium is one of the most toxic naturally occurring radioactive heavy elements. Its widespread contamination from the front- and back-end of the nuclear fuel cycle is a major environmental concern. Uranium contamination of soils, sediments, and groundwater is primarily due to milling, nuclear fuel and weapons production, and nuclear waste processing and disposal [10]. Potential application of ILs in nuclear industry due to their solvent stability as means of extraction and concentration of radionuclides from waste stream has been pursued [11,12]. Allen et al. [13] demonstrated that ILs were significantly more stable than mixtures of tri-n-butylphosphate and kerosene which is widely used solvent systems in the PUREX process for the separations of actinides from spent nuclear fuel. Recently, we demonstrated the potential application of ILs in the bioreduction of uranium under anaerobic condition [7]. Nevertheless, there is paucity of information on microbial adsorption of uranium in the presence of various ILs. Biosorption show many advantages in the bioremediation of uranium waste stream, such as efficiency, simplicity, analogous operation to conventional ion exchange technology, and availability of biomass [14,15].

Here we report the effects of ILs on growth and biosorption of uranium by *Clostridium* sp. (ATCC No. 53464). The bacterium was isolated in our laboratory from coal-cleaning waste. The organism is a strict anaerobic, Gram positive, spore-forming, fermentative bacterium, known to reduce Tc(VII) to Tc(IV), U(VI) to U(IV), and Pu(IV) to Pu (III) under anaerobic condition [16,17]. Three ILs we chose, 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$), N-ethylpyridinium trifluoroacetate ($EtPyCF_3COO$) and N-ethylpyridinium tetrafluoroborate ($EtPyBF_4$), are commonly used in chemical synthesis and catalysis [18]. $BMIMPF_6$ is hydrophobic (with the solubility of 2.2% (wt) in water [19]) and two pyridinium-based ILs are hydrophilic. This investigation helps to lay the foundation for elucidating the interaction between ILs and bacteria; how it affects biosorption process; and thus it would aid the design of biocompatible ILs for application in biotreatment of waste stream.

2. Materials and Methods

2.1. Chemicals

$BMIMPF_6$, $EtPyCF_3COO$ and $EtPyBF_4$ were synthesized and purified according to Zhao and Malhotra [20]. The ILs were >98% pure as tested by NMR and FTIR. Uranyl nitrate ($UO_2(NO_3)_2{\cdot}6H_2O$) was obtained from BDH Chemicals, Analar, Poole, England.

2.2. Bacterial culture and growth medium

Clostridium sp. was grown in a mineral salts medium composed of glucose (5.0 g), glycerol phosphate (0.3 g), $MgSO_4{\cdot}7H_2O$ (0.2 g), $FeSO_4{\cdot}7H_2O$ (2.8 mg), $CaCl_2$ (0.5 g), peptone (0.1 g), and yeast extract (0.1 g) in one liter distilled water. The pH was adjusted to 6.5. The medium was pre-reduced by boiling and purging with N_2 gas for 15 min to remove dissolved oxygen. The medium was then allowed to cool and transferred to an anaerobic glove box filled with 95% N_2 and 5% H_2. Forty milliliters of the medium was dispensed into a 60 mL serum bottle. The bottle was then fitted with a butyl rubber stopper, sealed with an aluminum cap and autoclaved.

2.3. Toxicity of ILs

To determine the effect of ILs on the growth of *Clostridium* sp. we dispensed the following concentrations of each ionic liquid, i.e., 0.1%, 0.25%, 0.5% and 1% (v/v), into the serum bottles of the autoclaved medium in the anaerobic glove box. Two parallel samples

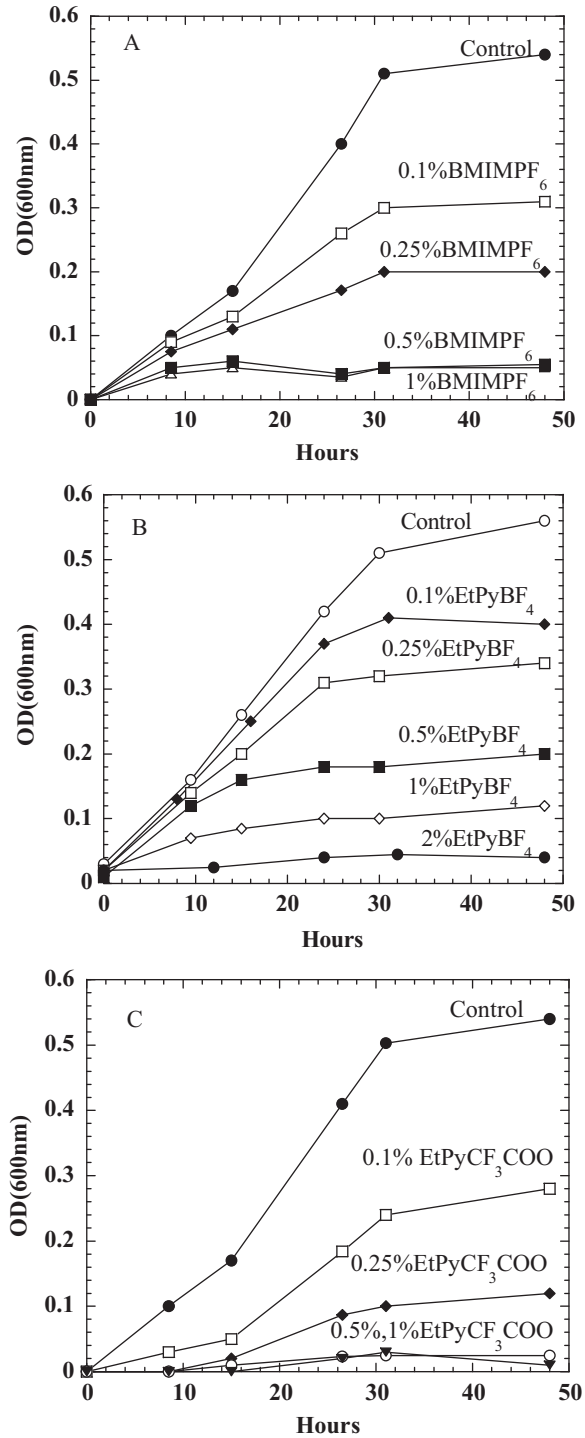

Fig. 1. Effects of various ionic liquids on the growth of *Clostridium* sp. (A) $BMIMPF_6$; (B) $EtPyBF_4$; (C) $EtPyCF_3COO$.

were prepared for each concentration: One was used for a kinetic study; the other was kept intact to measure the gas produced by the bacterium.

We transferred, by a syringe needle, 2 mL of an 18 h-old logarithmic growth phase of a *Clostridium* sp. culture grown in the mineral salts medium (without ILs) into each of the serum bottles. The samples were incubated at 28 °C. Periodically, aliquots of the culture sample were withdrawn to determine the growth of the bacterium and changes in the pH of the medium. Growth was measured at 600 nm by UV-vis spectrometer (Hewlett Packard Model 8453 UV-vis spectrometer). pH was determined by Mettler Toledo MP 220 pH meter. Total gas production in the headspace was determined via a pressure transducer with a needle (Model 665-D/030,Wallace and Tiernan). All experiments were performed in triplicates.

2.4. Preparation of IL-U mixture

We mixed 0.25 mM uranium and 1% (v/v) of each ionic liquid (48 mM for $BMIMPF_6$, 45 mM for $EtPyBF_4$ and 54 mM for $EtPyCF_3COO$) in 50 ml sterilized de-ionized water, ionic strength was adjusted to 20 mM by KCl. After equilibrium overnight, the solutions were adjusted to pH 3.5 by adding HCl or KOH.

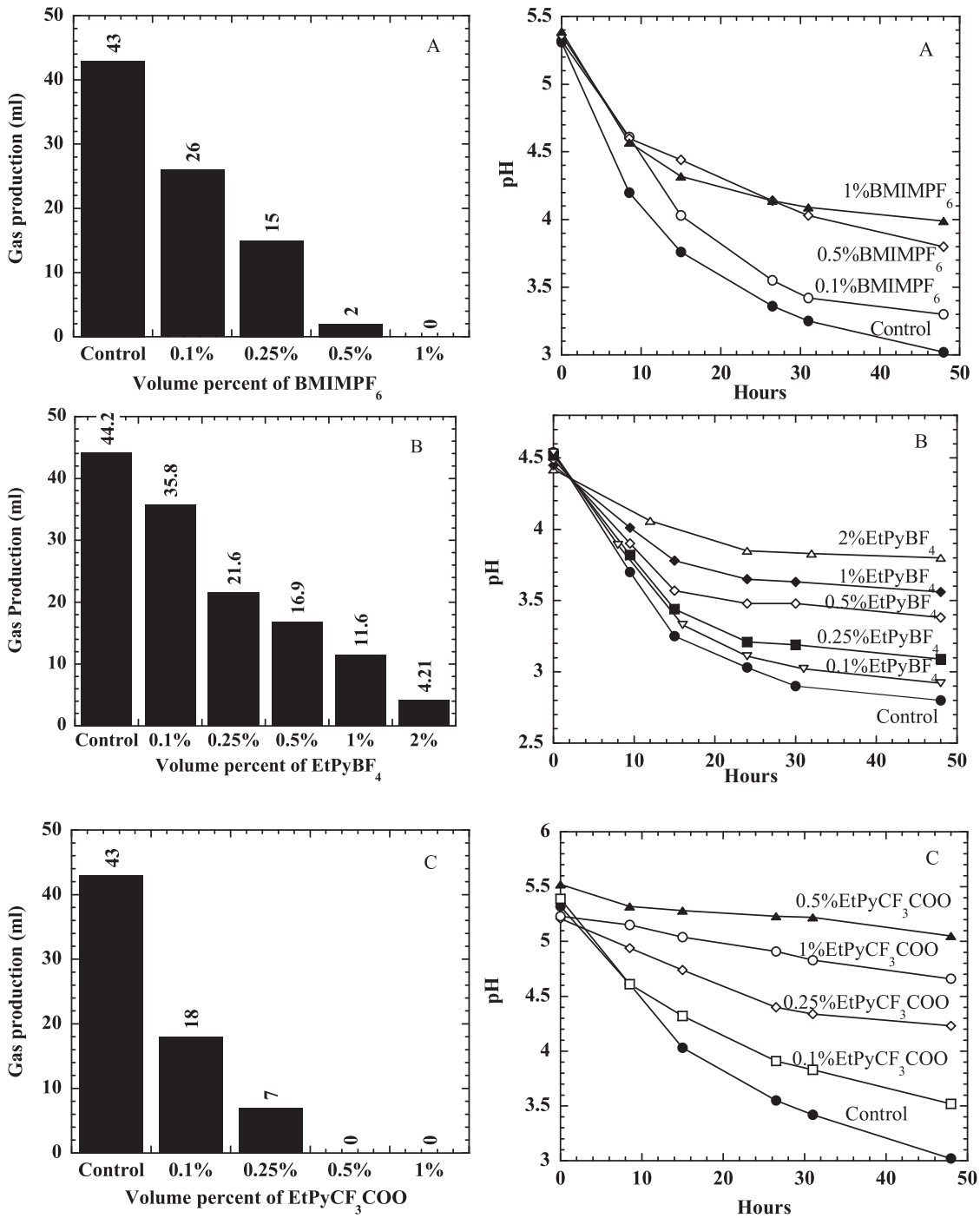

Fig. 2. Effects of various ionic liquids on the gas production (left panel) and pH change (right panel) during *Clostridium* sp. growth in the presence of (A) $BMIMPF_6$; (B) $EtPyBF_4$; (C) $EtPyCF_3COO$.

2.5. Biosorption experiment

Cells were grown in mineral salts medium for 18 h at 28 °C. Cells were then harvested at early stationary growth, pelleted by centrifugation at 5000 g for 25 min. After measuring the wet weight, 5 mL of sterilized KCl solution (pH 3.5, 20 mM) was added to the centrifuge tube and the cells were resuspended. The bacterial cell suspension was mixed with U-ILs mixture (50 mL). Two milliliters of the samples were withdrawn every 10 min for up to 30 min, after which sample was taken every 30 min up to 2 h. Control experiment was performed in the same manner, except in the absence of bacteria, to test for a loss of uranium due to precipitation or adsorption onto the reaction vessel. The biosorption experiment was performed under aerobic condition, to avoid reductive precipitation of uranium due to bioreduction during incubation. All experiments were carried out in triplicates.

2.6. Uranium analysis

Each sample was filtered by 0.45 μm syringe filter, and then diluted with filtered de-ionized water. After acidifying each with 2.5 M sulfuric acid, samples were analyzed by kinetic phosphorescence analyzer (Chemcheck, WA) for uranium concentration in solution.

2.7. Transmission Electronic Microscopy (TEM) and Energy Dispersive X-ray Spectroscopy (EDS) study

The cells after incubation with uranium and ionic liquids for 2 h, were collected by centrifugation at 5000 g for 25 min. They were washed twice with 0.05 M Hepes buffer (pH 6.8) and suspended overnight in 2% Glutaraldehyde (EM grade). The samples were then washed three times with Hepes buffer. Each sample was enrobed in 2% Noble agar and dehydrated through a graded ethanol series (25, 50, 75, 95 and 2 × 100% ethanol) for 15 min. The samples were then suspended in 50/50 ethanol/LR White resin for 1 h, and then in pure LR White resin for 1 h. Afterwards they were put into gelatin capsules containing pure LR White and polymerized at 60 °C for 1 h. Sections (of approximate 80 nm thickness) were cut on a Reichert Jung Ultracut E and mounted on copper TEM supports. For imaging we used the LEO 912 AB TEM, which has a 1 K ProScan CCD camera and a SIS EsiVision software package. This is an energy filtered TEM (EFTEM) instrument that operates at 120 kV. Images were taken at 0 eV. EDS was performed in spot mode (approximate 200 nm diameters) on a Philips 400 T at 100 kV with an EDAX, Sapphire detector using the Phoenix software.

3. Results and Discussion

3.1. Toxicity of ILs

Figure 1 shows the effect of three ILs on the growth of *Clostridium* sp. All three ILs affected bacterial growth and at higher concentrations growth was almost completely inhibited. The extent of inhibition depended on the type of IL; with the addition of 0.1% $BMIMPF_6$, the optical density (OD) decreased from 0.54 (control, without IL) to 0.30 while the OD values reached 0.40 and 0.28 in the presence of 0.1% $EtPyBF_4$ and $EtPyCF_3COO$, respectively. Growth was completely inhibited at 0.5% of $BMIMPF_6$ and $EtPyCF_3COO$.

The total gas (consisting of CO_2 and H_2) production from fermentation of glucose by *Clostridium* sp. is another indicator that demonstrates the effect of ILs on bacterial metabolism. Fig. 2 (left panel) shows the total volume of gas produced by *Clostridium* sp. after 48 h of incubation. It followed a similar trend to the OD. All three ILs showed inhibitory effects and that the higher the concentration of ILs, the lower was the growth and production of gas.

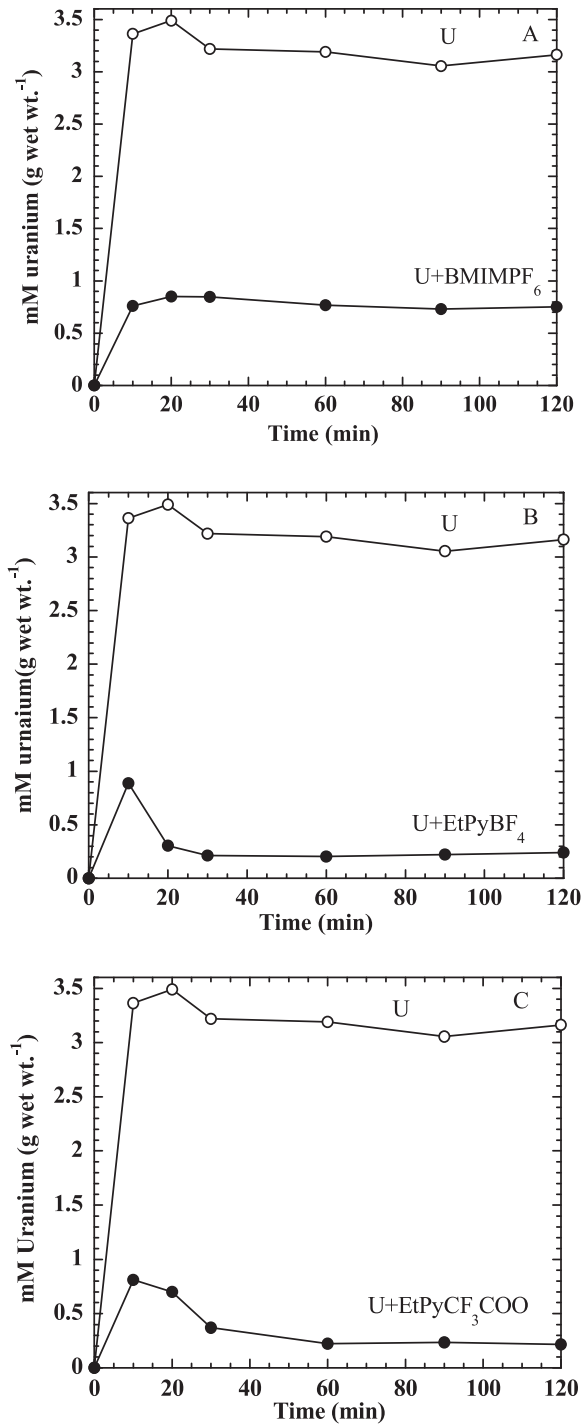

Fig. 3. Effects of various ionic liquids (1% v/v) on the biosorption of uranium by *Clostridium* sp. (A) $BMIMPF_6$; (B) $EtPyBF_4$; (C) $EtPyCF_3COO$.

For instance, the gas production significantly declined with the increase in concentration of $EtPyBF_4$. Compared with about 44 mL of gas generated by the control, 36 mL gas was produced at the 0.1% level, 22 mL at the 0.25% level, 17 mL at the 0.5% level, 12 mL at the 1% level and only 4 mL at the 2% level (Fig. 2B).

Figure 2 (right panel) also shows the change in pH during growth of *Clostridium* sp. The fermentation of glucose generated acetic and butyric acid [21], causing decrease in pH. It was noticed that the pH decrease at both 0.5% and 1% level of $BMIMPF_6$ while no growth was observed (Fig. 1A). And pH change was larger at 1% level of $EtPyCF_3COO$ than that at 0.5% level even though no

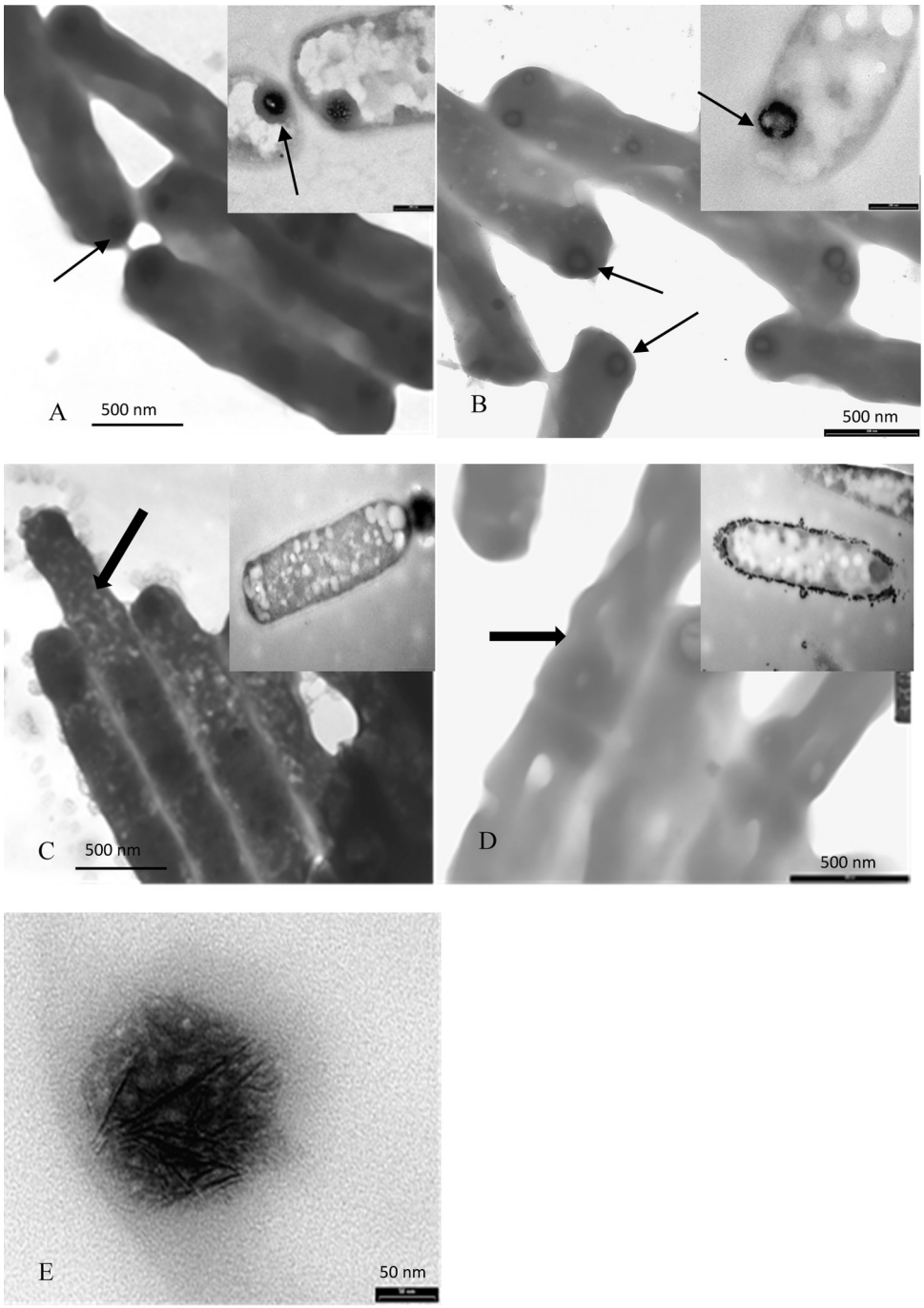

Fig. 4. TEM images of *Clostridium* sp. in the presence of various ionic liquids (1%, v/v) and uranium (0.25 mM): (A) uranium nitrate alone; (B) $BMIMPF_6$; (C) $EtPyBF_4$; (D) $EtPyCF_3COO$; (E) thin-sectioned bacteria of (A) in small scale showing the needle like uranium inside cell. The insert pictures show thin-sectioned bacteria under various treatments. The narrow arrows indicated the presence of U intracellularly and the wide arrows showed the changes of surface structure.

growth was found at both levels (Fig. 1C). This may be caused by hydrolysis of $BMIMPF_6$ and $EtPyCF_3COO$ resulting in pH drop [9,22,23]. The higher vulnerability to hydrolysis (i.e. low chemical stability) and forming of corrosive acid could be responsible for the higher cytotoxicity of some ILs [24]. Although we didn't observe obvious pH drop due to hydrolysis of $EtPyBF_4$, the hydrolysis of BF_4^- to $[BF_3OH]^-$ and HF has been reported elsewhere [23,25].

We quantified the toxicity of each IL by determining the $IC_{50\text{-}48\ h}$; this notation represents the concentration of ILs that induces 50% of the maximum inhibitory response after 48 h exposure. These results show that the $IC_{50\text{-}48\ h}$ of three ILs decreased in the order of $BMIMPF_6$ (8.26 mM) > $EtPyBF_4$ (7.04 mM) > $EtPyCF_3COO$ (4.05 mM). The results were in agreement with other studies that the high viscosities and densities of some ILs (such as $BMIMPF_6$ used in this study) may restrict their bioavailability to bacteria, thereby showing less toxicity. With the same cation of $EtPy^+$, the anion may contribute substantially to the overall toxicity. While in association with H^+, CF_3COO could hydrolyze protein and interact with cell membrane significantly [26]. Previously we showed that $EtPyCF_3COO$ exhibited higher toxicity to *Pseudomonas fluorescens* in comparison with $EtPyBF_4$ [27].

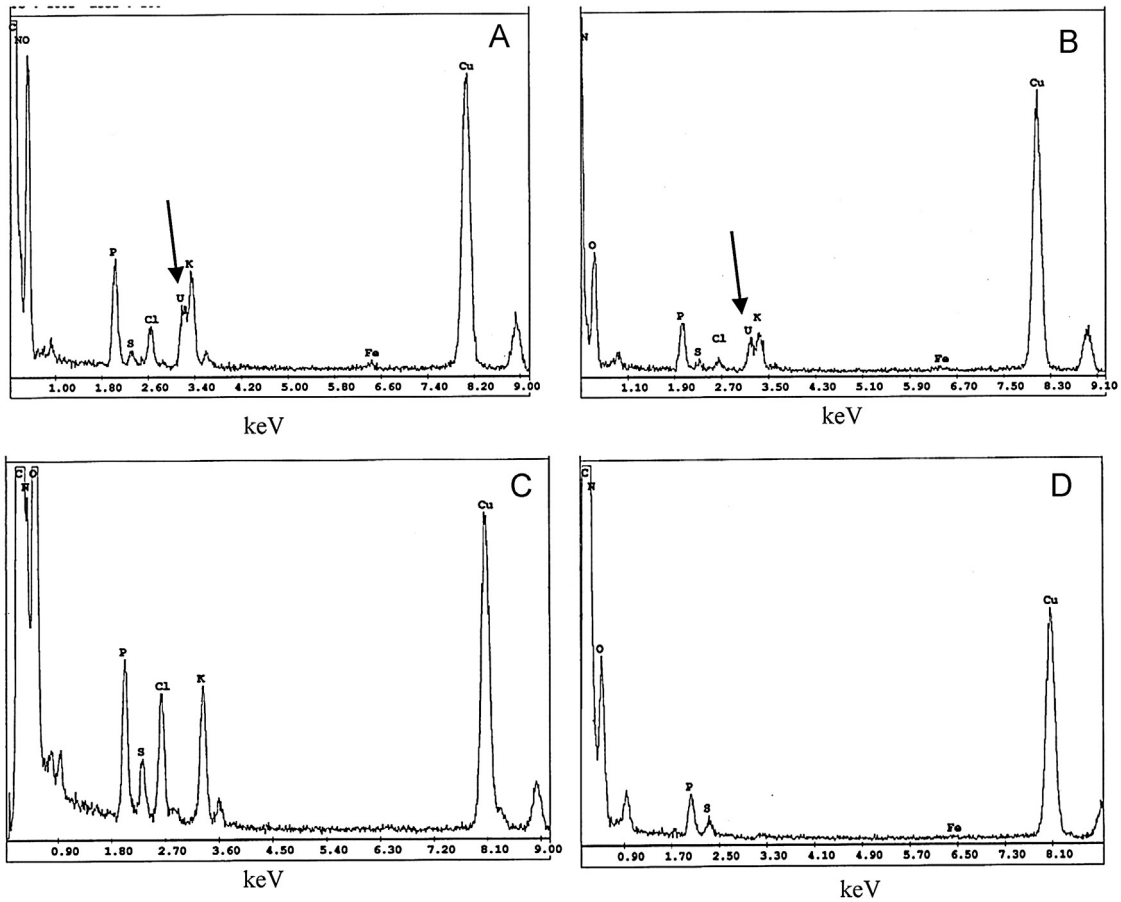

Fig. 5. EDS of *Clostridium* sp. after 2 h incubation with various ionic liquids (1% v/v) and uranium (0.25 mM): (A) uranyl nitrate; (B) $BMIMPF_6$; (C) $EtPyBF_4$; (D) $EtPyCF_3COO$. The arrows highlighted the presence of U inside cell.

3.2. Effects of ILs on uranium biosorption

The biosorption of uranium by *Clostridium* sp. is shown in Fig. 3. In the absence of ILs, uranium absorption in 0.02 M KCl reached the maximum in 20 min and remained associated with the cells. The initial rapid biosorption is due to the existence of several binding sites on the surface of bacterium that are capable of forming extracellular uranium complexation [28,29]. The rapid kinetics observed with *Clostridium* sp. has an important advantage for its application to uranium contaminated-water treatment systems.

However, the uranium absorption in 0.2 M KCl solution containing 1% (v/v) of $BMIMPF_6$ reached equilibrium within 20 min, but the amount of absorption was 75% less than that of without IL (Fig. 3A). In the presence of 1% (v/v) of $EtPyBF_4$ (Fig. 3B) and $EtPyCF_3COO$ (Fig. 3C), the absorption reached maximum in 10 min, and then decreased drastically in the next 30 min. At the end of incubation the amount absorbed decreased by 90% in comparison with that of uranium alone.

3.3. TEM and EDS analysis

TEM analysis revealed changes in the morphology of bacterial cell surface, while the EDS spectra showed the elements associated with the cells. Comparison of morphology of cells exposed to uranium (Fig. 4A), no obvious membrane damage was observed with cells in the presence of 1% (v/v) $BMIMPF_6$ (Fig. 4B). However in solution containing 1% (v/v) $EtPyBF_4$ many tiny pores were noticed on the membrane (Fig. 4C). In particular with the incubation of 1% (v/v) $EtPyCF_3COO$ the structure of the cellular membrane was severely damaged (Fig. 4D). The extent of membrane damage increased in the order of $BMIMPF_6 < EtPyBF_4 < EtPyCF_3COO$ and these observations were in agreement with above toxicity results. Gal et al. [30] suggested that interactions of ILs with the cellular membrane constituted a primary culprit for toxicity. They proposed that $BMIM^+$ showed membrane toxicity due to the short alkyl chains in the cationic structure and overall small dimension of the molecule which might account for its deeper insertion of the compound into the lipid bilayer. They also suggested that membrane interactions of ILs were most likely universal phenomena, even for ILs that were patently hydrophilic. The more serious membrane damage caused by $EtPyBF_4$ and $EtPyCF_3COO$ may be attributed to the release of corrosive acid, for instance HF and trifloroacetic acid, during anion hydrolysis [27].

Figure 4A also shows uranium was accumulated by *Clostridium* both extracelluarly and intracellularly. The thin-sectioned image shows uranium to be accumulated intracellularly as needle-like fibrils, having a size of around 50 nm (Fig. 4E). Extracellular accumulation of uranium with bacterial cell surfaces is likely due to physical and chemical interactions involving adsorption and ion exchange [31]. However, intracellular precipitation is suggested as a result of enzymatic reduction or interaction with intracellular phosphate [28]. Similar precipitations of uranium inside bacteria were also reported in *Halomonas* sp. which accumulated uranium as fine-grained crystals in the periplasm along its plasma and outer membranes [31].The precipitation of uranium inside cell was also confirmed by EDS analysis. Fig. 5A shows the presence

of Oxygen(O), Nitrogen(N), Carbon(C), Phosphorus(P), Sulfur(S), Chlorine(Cl), Potassium (K) and Uranium (U) inside cell. The high copper (Cu) peak was from the EM grid used to support the specimen.

The deposition of uranium inside cell was also impacted in the presence of various ILs. Due to the relatively less membrane damage both intracellular and extracellular deposition of uranium were detected with the incubation with $BMIMPF_6$ (Fig. 4B and Fig. 5B). Nevertheless, only extra-cellular accumulation of uranium was observed in the presence of $EtPyBF_4$ (Fig. 4C and Fig. 5C) and $EtPyCF_3COO$ (Fig. 4D and Fig. 5D). The absence of uranium accumulation inside cell may be due to following reasons. First, the membrane has an important function in maintaining the optimal internal conditions for metabolism and energy transduction. Many membrane proteins facilitate and regulate membrane ion transport, for example, the gated ion-selected channels, ionic pumps, or other hydrophilic transport pathways [32]. Therefore, any effect on the integrity of the membrane will influence the ion transfer process significantly. Second, intracellular accumulation by living cells is carried out through a variety of specific and non-specific metabolism-dependent transport systems. The inhibition of fermentation metabolism (evidenced by decrease of cell growth, gas and acid production) would influence the ion transport, and consequently bioaccumulation and bioreduction by cells. Third, as described by Bashford et al. [33] that leakage was likely to be through those smaller, different distortions or "leaky patches" of the plasma membrane structure. Progressively greater damage can lead to the leakage of larger molecules, such as phosphorylated metabolites and (eventually) cytoplasmic proteins. As mentioned before, the intracellular uranium precipitations mostly exist as complex with phosphate. Herein, the decrease of intracellular phosphorylated metabolites would diminish the deposition of uranium inside cell. For bacteria incubated with $EtPyCF_3COO$, EDS spectrum also showed that almost no K and/or Cl were detected inside cell. A high intracellular K^+ concentration is necessary for the growth of living cells. The K leakage usually is an early indicator of membrane damage [34]. Meanwhile, the change of EDS spectrum on Figure 5C might suggest the alteration of cellular chemistry under the treatment of $EtPyBF_4$.

Herein, all above results demonstrated that cell membrane was damaged to various degrees in the presence of different ILs, which may be primarily responsible for the decreased biosorption of uranium. Meanwhile, the complex formation between uranium and IL may also impact the biosorption greatly. Bacterial cell walls, exopolymers, and lipids contain carboxyl, hydroxyl, amino, sulfhydral and phosphate functional groups which can complex metal ions, resulting in immobilization. The competition for metal binding between the functional groups of the cell biomass and ionic liquid may affect the biosorption. Previously we [7] showed that uranium formed a monodentate complex with the anion BF_4^- and PF_6^- of $EtPyBF_4$ and $BMIMPF_6$, respectively; and a bidentate complex with carboxylate of $EtPyCF_3COO$. The binding strength decreased in the order of $EtPyCF_3COO > EtPyBF_4 > BMIMPF_6$, which corresponded with the decrease in trend of biosorption.

4. Conclusion

In summary, this study revealed membrane interactions of three ILs exhibiting diverse structural and biologically inhibitory properties. Hydrophobic IL may affect membrane integrity to less extent due to limited bioavailability, while hydrophilic ILs shows higher toxicity due to anion hydrolysis. Future investigation about the relationship between the extent of membrane disruption and ILs structure properties will provide more useful information in designing biocompatible ionic liquids. And a rational tuning of ILs would improve and expand the current applications of ILs in biotechnology.

Acknowledgments

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