

Spring 5-31-2006

Interactions of ionic liquids with uranium and its implications on biotransformation

Chengdong Zhang
New Jersey Institute of Technology

Follow this and additional works at: <https://digitalcommons.njit.edu/dissertations>



Part of the [Environmental Sciences Commons](#)

Recommended Citation

Zhang, Chengdong, "Interactions of ionic liquids with uranium and its implications on biotransformation" (2006). *Dissertations*. 782.

<https://digitalcommons.njit.edu/dissertations/782>

This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at Digital Commons @ NJIT. It has been accepted for inclusion in Dissertations by an authorized administrator of Digital Commons @ NJIT. For more information, please contact digitalcommons@njit.edu.

Copyright Warning & Restrictions

The copyright law of the United States (Title 17, United States Code) governs the making of photocopies or other reproductions of copyrighted material.

Under certain conditions specified in the law, libraries and archives are authorized to furnish a photocopy or other reproduction. One of these specified conditions is that the photocopy or reproduction is not to be “used for any purpose other than private study, scholarship, or research.” If a user makes a request for, or later uses, a photocopy or reproduction for purposes in excess of “fair use” that user may be liable for copyright infringement,

This institution reserves the right to refuse to accept a copying order if, in its judgment, fulfillment of the order would involve violation of copyright law.

Please Note: The author retains the copyright while the New Jersey Institute of Technology reserves the right to distribute this thesis or dissertation

Printing note: If you do not wish to print this page, then select “Pages from: first page # to: last page #” on the print dialog screen



The Van Houten library has removed some of the personal information and all signatures from the approval page and biographical sketches of theses and dissertations in order to protect the identity of NJIT graduates and faculty.

ABSTRACT

INTERACTIONS OF IONIC LIQUIDS WITH URANIUM AND ITS IMPLICATIONS ON BIOTRANSFORMATION

By
Chengdong Zhang

Room temperature ionic liquids (ILs) are pure ionic compounds with melting points below 100 °C, which have drawn great attention due to their many unique properties. They have been widely used in organic synthesis, analytical chemistry, and biochemistry. Although ionic liquids have been used to extract metals and radionuclides, there is no systematic study on the interactions of ILs with uranium and their effects on the biotransformation of uranium.

My research focused on: (1) the interactions between uranium and ionic liquids in aqueous solution; and (2) biotransformation of uranium in the presence of ILs. The interactions between three representative ionic liquids, [EtPy][BF₄] (N-ethylpyridinium tetrafluoroborate), [EtPy][CF₃COO] (N-ethyl pyridinium trifluoroacetate), [BMIM][PF₆] (1-butyl-3-methylimidazolium hexafluorophosphate), and uranium were determined by various analytical techniques, such as UV-Vis spectroscopy, potentiometric titration, LC-MS and X-ray absorption spectroscopy. Extended X-ray absorption fine structure (EXAFS) analysis showed the formation of monodentate complexes between uranium and [BMIM][PF₆], [EtPy][BF₄]; and a bidentate complex with [EtPy][CF₃COO].

The effects of ionic liquids on the growth of anaerobic bacterium *Clostridium* sp. were measured by changes in optical density, pH, and gas production. The ionic liquids inhibited the growth of bacterium to varying degrees. TEM and EDS studies showed that the ILs may affect the cell membrane of the bacterium and thereby its growth.

Biosorption of uranium by *Clostridium* sp. dramatically decreased in the presence of ILs. It decreased in the order of $U > U+[BMIM][PF_6] > U+[EtPy][BF_4]$ and $U+[EtPy][CF_3COO]$.

The bioreduction of U(VI) to U(IV) by *Clostridium* sp. was affected by the presence of ILs. The rate of reduction of U, in the absence or in the presence of the monodentate complexes $U:[EtPy][BF_4]$ and $U:[BMIM][PF_6]$ was similar. However, no reduction was observed in the presence of the bidentate $U:[EtPy][CF_3COO]$ complex. Also ionic liquids showed different affinities toward reduced U(IV) in solution after bioreduction. In the absence of IL, U(IV) precipitated out rapidly; whereas in the presence of $[EtPy][BF_4]$ a substantial amount of U(IV) was present in the solution. This result can be attributed to the complexation of U(IV) with $[EtPy][BF_4]$. Notably, this phenomenon was not observed in the presence of $[BMIM][PF_6]$. The formation of U(IV) in solution and in precipitate were confirmed by UV-Vis spectroscopy, XANES and EXAFS analysis.

Since ILs are relatively resistant to photodegradation and show toxicity at higher concentrations, the persistence in the environment was determined by the investigation of biodegradation of the three ILs. A soil enrichment culture degraded $[EtPy][BF_4]$ and $[EtPy][CF_3COO]$ to innocuous products, glyoxylate and acetic acid. In the presence of uranium, the rate and extent of biodegradation was slowed. N-ethyl-(4-(carboxyamino)but-3-enoic acid, semialdehyde, and (4-(carboxyamino)but-3-enoic acid were accumulated in medium as biodegradation products. Imidazolium-based ILs $[BMIM][PF_6]$ were resistant to biodegradation. This information is useful for the risk assessment of the “environmentally friendly” compounds.

**INTERACTIONS OF IONIC LIQUIDS WITH URANIUM
AND ITS IMPLICATIONS ON BIOTRANSFORMATION**

by
Chengdong Zhang

**A Dissertation
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Environmental Science**

Department of Chemistry and Environmental Science

May 2006

Copyright © 2006 by Chengdong Zhang

ALL RIGHTS RESERVED

APPROVAL PAGE

**INTERACTIONS OF IONIC LIQUIDS WITH URANIUM
AND ITS IMPLICATIONS ON BIOTRANSFORMATION**

Chengdong Zhang

Dr. Sanjay V. Malhotra, Dissertation Advisor
Assistant Professor of Chemistry and Environmental Science, NJIT

Date

Dr. Arokiasamy J. Francis, Committee Member
Group Leader of Molecular Environmental Science at Brookhaven National Laboratory
Associate Director of Center for Environmental Molecular Science at Stony Brook
University /Brookhaven National Laboratory

Date

Dr. Tamara Gund, Committee Member
Professor of Chemistry and Environmental Science, NJIT

Date

Dr. Zafar Iqbal, Committee Member
Research Professor of Chemistry and Environmental Science, NJIT

Date

Dr. Daniel J. Watts, Committee Member
Executive Director of Otto H. York Center for Environmental Engineering and Science
Panasonic Professor of Sustainability

Date

BIOGRAPHICAL SKETCH

Author: Chengdong Zhang
Degree: Doctor of Philosophy
Date: May 2006

Undergraduate and Graduate Education:

- Doctor of Philosophy in Environmental Science,
New Jersey Institute of Technology, Newark, NJ, 2006
- Master of Environmental Science,
Nanjing University, Nanjing, P. R. China, 2001
- Bachelor of Science in Environmental Chemistry,
Nanjing University, Nanjing, P. R. China, 1998

Major: Environmental Science

Presentations and Publications:

Chengdong Zhang and Sanjay V. Malhotra,
“Application of ionic liquids in enzymatic detection of paraoxon,”
228th ACS National Meeting, Philadelphia, August, 2004.

Chengdong Zhang and Sanjay V. Malhotra,
“Enzymatic synthesis of dipeptides in ionic liquids,”
228th ACS National Meeting, Philadelphia, August 2004.

Chengdong Zhang, Sanjay V. Malhotra and Arokiasamy J. Francis,
“Interaction of ionic liquids with uranium and its implication on bioreduction,”
230th ACS National Meeting, Washington D.C., August 2005.

Chengdong Zhang, Sanjay V. Malhotra and Arokiasamy J. Francis,
“Effects of ionic liquids on uranium bioreduction,”
Pacifichem 2005 International Chemical Congress of Pacific Basin Societies,
Honolulu, Hawaii, December 15-20, 2005.

Sanjay V. Malhotra; Chengdong Zhang, and Arokiasamy J. Francis,

“Biocatalysis of uranium in the presence of ionic liquids,”
ACS-Green Chemistry Meeting, Washington D.C., June 2006.

Chengdong Zhang and Sanjay V. Malhotra,
“Increased paraoxon detection by acetylcholinesterase inactivation with ionic
liquid additives,” Talanta, 67(3), pp. 560-563, 15 September 2005.

Chengdong Zhang and Sanjay V. Malhotra,
“Enzymatic synthesis of dipeptides in ionic liquids,” (submitted to Advanced
Synthesis and Catalysis).

Chengdong Zhang, Arokiasamy J. Francis, and Sanjay V. Malhotra,
“Characterization of uranium associated with ionic liquids,” (in preparation)

Chengdong Zhang, Arokiasamy J. Francis, and Sanjay V. Malhotra,
“Effects of ionic liquids on uranium biosorption by *Clostridium* sp.,” (in
preparation).

Chengdong Zhang, Arokiasamy J. Francis, and Sanjay V. Malhotra,
“Effects of ionic liquids on uranium bioreduction by *Clostridium* sp.,” (in
preparation).

Chengdong Zhang, Arokiasamy J. Francis, and Sanjay V. Malhotra,
“Biodegradation of ionic liquids by enriched soil bacteria,” (in preparation).

In memory of my parents

ACKNOWLEDGMENT

I would like to express my deepest appreciation to Dr. Sanjay V. Malhotra, who not only served as my research supervisor, but also constantly gave me support and encouragement. His valuable insight and knowledge will benefit my whole life. I would also give my special gratitude to Dr. Arokiasamy J. Francis for his countless help during my stay at Brookhaven National Laboratory. Special thanks are given to Dr. Tamara Gund, Dr. Zafar Iqbal, and Dr. Daniel J. Watts for actively participating in my committee.

The author is really grateful to Dr. Cleveland J. Dodge for helpful discussion on interpreting EXAFS data and LC-MS analysis; Jeff B. Gillow for assistance in the preparation of cultures and insightful discussion; Dr. Vazquez Gustav for assistance in the uranium analysis.

The author acknowledges the financial support from Chemistry and Environmental Science Department and DOE Laboratory Directed Research and Development program at BNL. The special support from Office of Graduate Studies and Dean Ronald S. Kane are also appreciated.

The author would like to thank fellow graduate student Hao Wang for his kind help during the study.

Particularly thanks to my husband, Shihao Yang, for his loving care and continued support.

TABLE OF CONTENTS

| Chapter | Page |
|---|-------------|
| 1 INTRODUCTION AND OBJECTIVE..... | 1 |
| 1.1 Green Chemistry..... | 1 |
| 1.2 Application of Ionic Liquids in Biocatalysis..... | 2 |
| 1.2.1 Properties of Ionic Liquids..... | 2 |
| 1.2.2 Biocatalysis in Ionic Liquids..... | 6 |
| 1.2.3 Enzymes in Biocatalysis..... | 8 |
| 1.2.4 Limitations of Industrial Application..... | 11 |
| 1.3 Application of Ionic Liquids in Metal Extraction..... | 12 |
| 1.3.1 General Metal Extraction..... | 12 |
| 1.3.2 Application of Ionic Liquids in Actinide Chemistry..... | 15 |
| 1.4 Toxicity Studies of Ionic Liquids | 18 |
| 1.5 Fate of Ionic Liquids in Environment..... | 24 |
| 1.6 Objective..... | 26 |
| 2 CHARACTERIZATION OF URANIUM ASSOCIATED WITH IONIC LIQUIDS | 29 |
| 2.1 Introduction | 29 |
| 2.2 Materials and Methods..... | 30 |
| 2.2.1 Synthesis of Ionic Liquids..... | 30 |
| 2.2.2 Preparation of U-IL Mixture..... | 32 |
| 2.2.3 pH Change and Potentiometric Titration..... | 32 |
| 2.2.4 UV-Vis Spectroscopy Analysis..... | 32 |

TABLE OF CONTENTS
(Continued)

| Chapter | Page |
|--|-------------|
| 2.2.5 Liquid Chromatograph-Mass Spectroscopy Analysis..... | 32 |
| 2.2.6 Speciation of Uranium by XANES and EXAFS..... | 33 |
| 2.3 Results and Discussion..... | 34 |
| 2.3.1 pH Change and Potentiometric Titration..... | 34 |
| 2.3.2 UV-Vis Spectroscopy Analysis..... | 37 |
| 2.3.3 Liquid Chromatograph-Mass Spectroscopy Analysis..... | 39 |
| 2.3.4 X-ray Absorption Near-edge Spectroscopy Analysis..... | 42 |
| 2.3.5 Extended X-ray Absorption Fine Structure Analysis..... | 43 |
| 2.4 Summary..... | 47 |
| 3 EFFECTS OF IONIC LIQUIDS ON GROWTH OF <i>Clostridium</i> sp..... | 49 |
| 3.1 Introduction..... | 49 |
| 3.2 Materials and Methods..... | 49 |
| 3.2.1 Culture Conditions..... | 49 |
| 3.2.2 Chemicals..... | 50 |
| 3.2.3 Analysis of Total Gas Production and pH..... | 50 |
| 3.2.4 LC-MS Analysis..... | 50 |
| 3.3 Results and Discussion..... | 51 |
| 3.3.1 Effects on Optical Density..... | 51 |
| 3.3.2 Effects on pH Change..... | 51 |
| 3.3.3 Effects on Gas Production..... | 54 |

TABLE OF CONTENTS
(Continued)

| Chapter | Page |
|--|-------------|
| 3.3.4 Studies on Morphology of Bacteria..... | 54 |
| 3.3.5 Mass Spectroscopy Analysis of Ionic Liquids Integrity | 56 |
| 3.4 Summary..... | 64 |
| 4 EFFECTS OF IONIC LIQUIDS ON URANIUM BIOSORPTION..... | 65 |
| 4.1 Introduction..... | 65 |
| 4.2 Materials and Methods..... | 66 |
| 4.2.1 Preparation of Uranium and Ionic Liquids Mixture..... | 66 |
| 4.2.2 Biosorption Experiment..... | 66 |
| 4.2.3 Uranium Measurement..... | 67 |
| 4.2.4 Transmission Electro Microscope and Energy Dispersive X-ray Spectroscopy Study..... | 67 |
| 4.3 Results | 68 |
| 4.3.1 Accumulation of U by <i>Clostridium</i> sp. | 68 |
| 4.3.2 Accumulation of U in the Presence of [BMIM][PF ₆]..... | 70 |
| 4.3.3 Accumulation of U in the Presence of [EtPy][BF ₄]..... | 72 |
| 4.3.4 Accumulation of U in the Presence of [EtPy][CF ₃ COO]..... | 74 |
| 4.4 Discussion and Conclusions..... | 76 |
| 5 EFFECTS OF IONIC LIQUIDS ON URANIUM BIOREDUCTION..... | 78 |
| 5.1 Introduction..... | 78 |
| 5.1.1 Bioremediation of Uranium..... | 78 |
| 5.1.2 Effects of Ligands on the Bioreduction..... | 79 |

TABLE OF CONTENTS
(Continued)

| Chapter | Page |
|---|-------------|
| 5.2 Materials and Methods..... | 80 |
| 5.2.1 Comparison of Different Concentrations of Ionic Liquid..... | 80 |
| 5.2.2 Detection Methods of U(IV) and Total Uranium..... | 81 |
| 5.2.3 Comparison of Different Ionic Liquids Effects..... | 82 |
| 5.2.4 UV-Vis Spectroscopy Analysis of U-citrate Extract..... | 82 |
| 5.2.5 XANES and EXAFS Analysis..... | 82 |
| 5.3 Results and Discussion..... | 82 |
| 5.3.1 Effects of Various Concentration of [EtPy][BF ₄] on U Bioreduction..... | 82 |
| 5.3.2 Mass Balance of U in Medium after Bioreduction in the Presence of Various Concentration of [EtPy][BF ₄]..... | 84 |
| 5.3.3 Determination of U Accumulated in Bacteria Cell During Reduction..... | 88 |
| 5.3.4 Effects of Different Ionic Liquids on Bioreduction..... | 98 |
| 5.3.5 Mass Balance of U in Medium after Bioreduction..... | 99 |
| 5.3.6 Comparison of UV-Vis Spectroscopy of U Before and After Bioreduction Extracted by 5mM Citric Acid..... | 100 |
| 5.3.7 XANES and EXAFS Analysis..... | 101 |
| 5.4 Summary..... | 102 |
| 6 BIODEGRADATION OF IONIC LIQUIDS..... | 104 |
| 6.1 Introduction..... | 104 |
| 6.2 Degradation of ILs by <i>pseudomonas fluorescens</i> | 105 |
| 6.2.1 Materials and Methods..... | 106 |

TABLE OF CONTENTS
(Continued)

| Chapter | Page |
|--|-------------|
| 6.2.2 Results and Discussion..... | 107 |
| 6.2.3 Summary..... | 114 |
| 6.3 Degradation of ILs by a Soil Enrichment Culture..... | 115 |
| 6.3.1 Materials and Methods..... | 115 |
| 6.3.2 Results and Discussion..... | 117 |
| 6.3.3 Summary..... | 131 |
| 7 CONCLUSIONS AND RECOMMENDATIONS | 132 |
| 7.1 Conclusions..... | 132 |
| 7.2 Recommendations..... | 133 |
| REFERENCES..... | 135 |

LIST OF TABLES

| Table | | Page |
|--------------|--|-------------|
| 1.1 | Enzymes other than lipase investigated in ionic liquids..... | 10 |
| 1.2 | Toxicity studies on bacteria and cells..... | 22 |
| 2.1 | pH changes of U:[EtPy][CF ₃ COO] mixture..... | 35 |
| 2.2 | EXAFS structure parameters for uranium and ILs mixtures..... | 44 |
| 6.1 | pH change of medium during incubation..... | 119 |

LIST OF FIGURES

| Figure | | Page |
|---------------|--|-------------|
| 1.1 | Commonly used cations and anions..... | 2 |
| 1.2 | Levels of complexity for the evaluation of the biological activity of chemicals..... | 19 |
| 1.3 | Multidimensional risk analysis of [BMIM][BF ₄], [DMIM][BF ₄], and acetone..... | 19 |
| 1.4 | Effects of H ₂ O ₂ on the photodegradation of imidazolium ionic liquids..... | 25 |
| 2.1 | Structures of [EtPy][BF ₄], [BMIM][PF ₆] and [EtPy][CF ₃ COO]..... | 30 |
| 2.2 | Potentiometric titration curve of U : [BMIM][PF ₆]..... | 35 |
| 2.3 | Potentiometric titration curve of U : [EtPy][BF ₄]..... | 36 |
| 2.4 | Potentiometric titration curve of U : [EtPy][CF ₃ COO]..... | 36 |
| 2.5 | UV-Vis spectrophotometry of uranium in water..... | 38 |
| 2.6 | UV-Vis spectrophotometry of U:[EtPy][BF ₄] mixture in water..... | 38 |
| 2.7 | UV-Vis spectrophotometry of U:[BMIM][PF ₆] mixture in water..... | 39 |
| 2.8 | UV-Vis spectrophotometry of U:[EtPy][CF ₃ COO] mixture in water... | 39 |
| 2.9 | Mass spectroscopy of U in water at positive mode..... | 40 |
| 2.10 | Mass spectroscopy of 1:1U:[EtPy][BF ₄] mixture at positive mode..... | 40 |
| 2.11 | Mass spectroscopy of U in water at negative mode..... | 41 |
| 2.12 | Mass spectroscopy of 1:1U:[EtPy][CF ₃ COO] mixture at negative mode..... | 41 |
| 2.13 | Normalized XANES spectra..... | 43 |
| 2.14 | Experimental EXAFS oscillation of uranium and different ILs mixtures..... | 44 |

LIST OF FIGURES
(Continued)

| Figure | | Page |
|---------------|---|-------------|
| 2.15 | Proposed structures of U-ILs complexes..... | 47 |
| 3.1 | Effects of ionic liquids on optical density (A) [BMIM][PF ₆]; (B) [EtPy][BF ₄]; and (C) [EtPy][CF ₃ COO] | 52 |
| 3.2 | Effects of ionic liquids on pH change (A) [BMIMPF ₆]; (B) [EtPy][BF ₄]; (C) [EtPy][CF ₃ COO]..... | 53 |
| 3.3 | Effects of ionic liquids on gas production (A) [BMIMPF ₆]; (B) [EtPy][BF ₄]; (C) [EtPy][CF ₃ COO]..... | 55 |
| 3.4 | Effects of [EtPy][BF ₄] on bacterial morphology change..... | 54 |
| 3.5 | Mass spectroscopy of control medium at 0h..... | 56 |
| 3.6 | Mass spectroscopy of control medium after 48h incubation..... | 57 |
| 3.7 | Mass spectroscopy of medium containing [BMIM][PF ₆] (0.1%) at 0h..... | 58 |
| 3.8 | Mass spectroscopy of medium containing [BMIM][PF ₆] (0.1%) after 48h incubation..... | 59 |
| 3.9 | Mass spectroscopy of medium containing [EtPy][CF ₃ COO] (0.1%) at 0h..... | 60 |
| 3.10 | Mass spectroscopy of medium containing [EtPy][CF ₃ COO] (0.1%) after 48h incubation..... | 61 |
| 3.11 | Mass spectroscopy of medium containing [EtPy][BF ₄] (0.1%) at 0h... | 62 |
| 3.12 | Mass spectroscopy of medium containing [EtPy][BF ₄] (0.1%) after 48h incubation..... | 63 |
| 4.1 | TEM of <i>Clostridium</i> sp. treated by uranium..... | 69 |
| 4.2 | EDS of <i>Clostridium</i> sp. treated by uranium..... | 70 |
| 4.3 | Kinetic study of U accumulation by <i>Clostridium</i> sp. in presence of 1% [BMIM][PF ₆]..... | 70 |

LIST OF FIGURES
(Continued)

| Figure | | Page |
|---------------|---|-------------|
| 4.4 | TEM of <i>Clostridium</i> sp. exposed to uranium and 1% [BMIM][PF ₆]... | 71 |
| 4.5 | EDS of <i>Clostridium</i> sp. exposed to uranium and 1% [BMIM][PF ₆]... | 71 |
| 4.6 | Kinetic study of U accumulation by <i>Clostridium</i> sp. in presence of 1% [EtPy][BF ₄]..... | 72 |
| 4.7 | TEM of <i>Clostridium</i> sp. exposed to uranium and 1% [EtPy][BF ₄]..... | 72 |
| 4.8 | EDS of <i>Clostridium</i> sp. exposed to uranium and 1% [EtPy][BF ₄]..... | 73 |
| 4.9 | Kinetic study of U accumulation by <i>Clostridium</i> sp. in presence of 1% [EtPy][CF ₃ COO]..... | 74 |
| 4.10 | TEM of <i>Clostridium</i> sp. exposed to uranium and 1% [EtPy][CF ₃ COO]..... | 75 |
| 4.11 | EDS of <i>Clostridium</i> sp. exposed to uranium and 1% [EtPy][CF ₃ COO]..... | 75 |
| 5.1 | U bioreduction by <i>Clostridium</i> sp. in presence of (A) 0%; (B) 0.1%; (C) 0.5%; and (D) 1% of [EtPy][BF ₄]..... | 84 |
| 5.2 | Mass balance of U after reduction..... | 85 |
| 5.3 | Enhanced U reduction by decrease of biosorption..... | 88 |
| 5.4 | Kinetic study of uranium concentration in solution during bioreduction | 88 |
| 5.5 | Mass balance of U after 24h incubation..... | 89 |
| 5.6 | Whole cell of <i>Clostridium</i> sp. Alone..... | 90 |
| 5.7 | (A) Thin section image of <i>Clostridium</i> sp. alone (24h); (B) EDS analysis (24h)..... | 91 |
| 5.8 | Whole cell of <i>Clostridium</i> sp. in the presence of uranium..... | 91 |
| 5.9 | Thin section of <i>Clostridium</i> sp. exposed to uranium after 24h | 92 |

LIST OF FIGURES
(Continued)

| | | |
|------|---|-----|
| 5.10 | EDS of <i>Clostridium</i> sp. exposed to Uranium..... | 94 |
| 5.11 | Whole cell of <i>Clostridium</i> sp. exposed to Uranium and 1% [EtPy][BF ₄]..... | 95 |
| 5.12 | EDS (in cell) of <i>Clostridium</i> sp. exposed to uranium and [EtPy][BF ₄]. | 96 |
| 5.13 | Thin section image of <i>Clostridium</i> sp. exposed to U and [EtPy][BF ₄]..... | 97 |
| 5.14 | U reduction in presence of various ionic liquids..... | 99 |
| 5.15 | Mass balance of U in medium containing different ionic liquids..... | 99 |
| 5.16 | UV-Vis spectroscopy of precipitates extracted by citric acid (A) before bioreduction; (B) after bioreduction..... | 101 |
| 5.17 | Normalized XANES spectra..... | 102 |
| 5.18 | EXAFS analysis of bio-reduced biomass..... | 102 |
| 6.1 | Effects of ILs on the growth of <i>P. fluorescence</i> | 108 |
| 6.2 | Effects of different concentration of [EtPy][BF ₄] on change of (A) optical density; (B) pH..... | 109 |
| 6.3 | Growth of <i>Pseudomonas</i> in presence of [EtPy][BF ₄]..... | 110 |
| 6.4 | Effect of [EtPy][BF ₄] on citric acid degradation..... | 111 |
| 6.5 | Comparisons of <i>P. fluorescence</i> cell growth in the absence and presence of [EtPy][BF ₄]..... | 111 |
| 6.6 | MS of mixture before biodegradation of [EtPy][BF ₄] at positive mode.. | 112 |
| 6.7 | MS of mixture after 24h biodegradation of a culture sample containing [EtPy][BF ₄] at positive mode..... | 112 |
| 6.8 | Nucleophilic addition and dealkylation of N-ethylpyridinium..... | 113 |
| 6.9 | Degradation of 1.2mM [EtPy][BF ₄] by enriched culture..... | 118 |

LIST OF FIGURES
(Continued)

| Figure | | Page |
|---------------|---|-------------|
| 6.10 | UV-Vis spectroscopy of EtPy ⁺ during incubation..... | 118 |
| 6.11 | Biodegradation of [EtPy][BF ₄] at different concentrations..... | 119 |
| 6.12 | Degradation of different ionic liquids by enriched culture..... | 120 |
| 6.13 | Metabolites during incubation by HPLC analysis..... | 120 |
| 6.14 | HPLC monitoring of metabolites from 0h to 26h..... | 121 |
| 6.15 | HPLC monitoring of metabolites from 26h to 8days..... | 122 |
| 6.16 | LC-ESI/MS analysis of products A and B..... | 123 |
| 6.17 | Proposed degradation pathway..... | 124 |
| 6.18 | Mass spectroscopy of degradation products at positive mode (after 35h)..... | 125 |
| 6.19 | Mass spectroscopy of degradation products at negative mode (after 35h)..... | 125 |
| 6.20 | LC-ESI/MS/MS analysis of product A at 40% collisions energy..... | 126 |
| 6.21 | LC-ESI/MS/MS analysis of product B at 40% collisions energy..... | 127 |
| 6.22 | Uranium concentration in solution during incubation..... | 130 |
| 6.23 | Effects of uranium on biodegradation of [EtPy][BF ₄]..... | 130 |

CHAPTER 1

INTRODUCTION AND OBJECTIVE

1.1 Green Chemistry

Green Chemistry is defined as the practice of chemical science and manufacturing in a manner that is sustainable, safe, and non-polluting moreover one which consumes a minimum amount of materials and energy while producing little or no waste (Manahan, 2004). There are different rules to develop green processes (Anastas, et al., 1998) such as the prevention of waste and by-products, a maximum yield of products, a minimization of the use or generation of hazardous products, a minimal energy requirement, the selection of appropriate solvents and starting materials etc. Meanwhile, products that have to be dispersed into the environment should be designed to breakdown rapidly into innocuous products.

One of the important approaches of practicing green chemistry is to use existing chemicals but within more environmentally-friendly processes, such as using catalysts, or biocatalysts. Also important is the substitution of toxic and volatile organic solvents with more environmentally benign ones. Thus biocatalysis, environmentally benign solvents, toxicity, and biodegradability are the major topics in this study.

The term “biotransformation” or “biocatalysis” is used for processes wherein the starting material is converted to a product in a simple step (using either whole cell or purified enzymes) process. Such reactions are usually fast, and can possibly achieve stereospecific products (Kragl, et al., 2003).

The use of volatile organic compounds represents the bulk of the volatile hazardous substances lost to the atmosphere. These substances cause human health and global warming problems. Pure water, super critical CO₂ and ionic liquids have been tried as alternative media to achieve the goals of green chemistry. Among them, ionic liquids are the neoteric materials, which are gaining popularity in green chemistry.

Ionic liquids are composed of entire ions, which are liquids at ambient temperature. Their unique physical and chemical properties, such as non-volatility, non-flammability, and excellent stability (both chemically and thermally), have made them desirable solvents, which are environmentally-friendly and can substitute for organic synthesis (Zhao, et al., 2002; Xiao, et al., 2004, 2005; Earle, et al., 2000). They are therefore, considered to be “Green Solvents”.

1.2 Application of Ionic Liquids in Biocatalysis

1.2.1 Properties of Ionic Liquids

Ionic liquids are low-melting salts that are comprised entirely of cations and anions. The commonly used cations and anions in ionic liquids are listed in Figure 1.1.

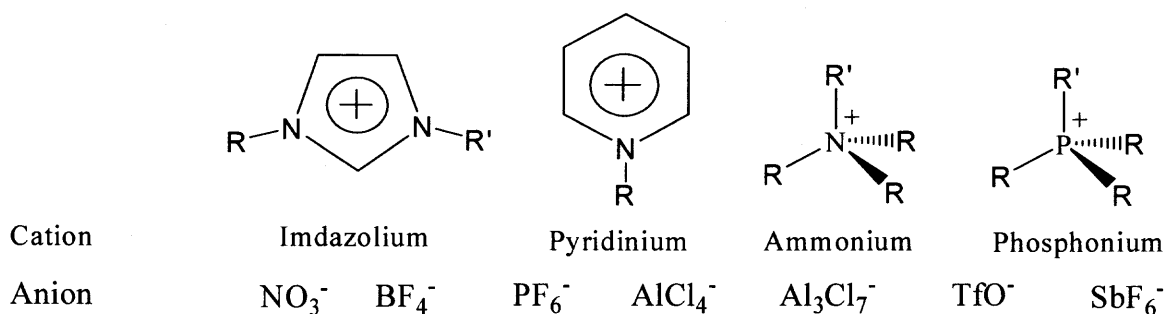


Figure 1.1 Commonly used cations and anions.

Cation modification and anion substitution of ionic liquids produce dramatic changes in the macroscopic physical properties of the ionic liquids. These include

variations in melting points, shear viscosity, conductivity, and glass transition temperatures etc. Their attractive properties have been recently reviewed (Wilkes, 2004; Forsyth, et. al., 2004; Marsh, et al., 2004; Zhao, et al., 2003). These properties are briefly summarized below:

- They have essentially no vapor pressure. Low vapor pressure means they can be used in a high vacuum system without loss and thus facilitate the separation process.
- Normally ionic liquids have low melting points (<100 °C) and remain as liquids in a broad temperature window. The major factors influencing melting points are the charge distribution on the ions, the hydrogen bonding ability, the symmetry of the ions and the van der Waals interaction. For example, the melting points decreased from the methyl substitution to the butyl and hexyl compounds and then increased (Visser, et al., 2001; Dzyuba, et al., 2001).
- They have reasonable thermal stability and are suitable for reactions spanning a great range in temperature.
- They are able to dissolve a wide range of organic and inorganic compounds.
- The solubility of gases, such as CO, H₂ and CO₂, is good. Many reactions now can be performed in ionic liquids and in a super critical CO₂ biphasic system.
- Different combinations of cations and anions give ionic liquids different solubilities, and hydrophobicities, which make them “tunable” solvents (Marsh, et al., 2004).

Reactions in ionic liquids exhibit different thermodynamic and kinetic behaviors, which often lead to improved process performance. Their physical and chemical

properties including hydrophobicity, polarity, viscosity, and solvent miscibility, play important roles in biocatalysis.

Hydrophobicity Materials, which are characteristically “hydrophobic”, have the opposite response to interaction as compared with hydrophilic materials. There is the logP concept that concerns the enzyme activity in an organic solvent. Log P is the logarithm of the partition coefficient of a solvent in an octanol/water mixture. In general, solvents with a log P>3 are less deactivating to enzymes than those with a low log P. For example, hexane (log P=3.9) may be more suitable than ethanol (log P= -0.24) in biocatalysis (Kragl, et al., 2002) because the more hydrophilic solvent is able to strip water from the enzyme and break the hydrogen bond. This bond stabilized the tertiary structure of the protein, thus its breakdown causes enzyme denaturalization. However, this concept is not suitable for ionic liquids. Many ionic liquids show strong hydrophilic properties but can still maintain enzyme activity (Kaar, et al., 2003). Kaar’s group first measured the log P value of several dialkylimidazolium-based ionic liquids according to their absorbance capabilities at 211nm, and yielded extremely low values (around -2.9 and -2.39).

The conventional molecular solvents exhibit interactions, such as hydrogen bonding, dipole-dipole and van der waals interaction. In addition to these features, ionic liquids also have strong electrostatic attraction and charged particle repulsion (Huang, et al., 2001). Hydrogen bonding in ionic liquids is believed to exist between oxygen and halide atoms on the anion, and in the hydrogen atoms on the imidazolium or pyridinium ring of the cations. In order to characterize the solvent properties, Anderson et al. (2002) suggested following equation to identify the interaction in ionic liquids:

$$\log k = c + rR_2 + s\pi_2^H + a\alpha_2^H + b\beta_2^H + l \log L^{16}$$

Numerous factors make ionic liquids the most complex solvents. Also the nature of the anion could change its water miscibility, (e.g. a change from the tetrafluoroborate to hexafluorophosphate anion has a significant effect on hydrophobicity).

Polarity One of the most special properties of ionic liquids is its high polarity. On the normalized polarity scale (E_T^N), set tetramethylsilane at 0 and water at 1, normal ionic liquids fall in the range of 0.6-0.7, which is similar to that of the lower alcohols and formamide (Rantwijk, et al., 2003). Because of their high polarity, they can dissolve both organic and inorganic materials. Ionic liquids manifest obvious potential as reaction media for the biotransformation of polar substrates such as amino acids and nucleotides (Zhao, et al., 2003), whereas, when water is used as the medium there are many limitations.

Viscosity Viscosity is an important factor that affects the mass transfer in reaction system. Ionic liquids are more viscous than organic solvents, which demonstrate their high tendency to form hydrogen bonds and strong van der waals interactions. Normally, a longer alky chain on the cation and a larger anion size lead to a higher viscosity.

Miscibility Ionic liquids are generally immiscible with non-polar solvents, such as hexane, and miscible with solvents, such as tetrafurane and acetone. A relationship with the dielectric constant has been proposed as an indicator of miscibility (Bonhôte, et al., 1996). For example, alcohol, ketones, dichloromethane and THF do mix with

[BMIM][Tf₂N], whereas alkanes and ethers do not. The immiscibility makes them suitable for two phase catalysis.

Ionic liquids form a special structure when mixed with an aromatic compound. For example, a 1-alkyl-3-methylimidazolium cation, when combined with hexafluorophosphate, bis(trifly)amide tetrafluoroborate and chloride anions, can form liquid clathrate with aromatic hydrocarbons (Holbrey, et al. 2003). Similarly, when mixed with benzene, the structure [(1, 3-dialkyl imidazolium hexafluorophosphate)₂ (benzene)] could be trapped. The ionic liquids-rich phase shows a different viscosity and immiscibility with excess aromatic compounds. This unique phenomenon may be useful in the separation of aromatic compounds from hydrocarbons.

1.2.2 Biocatalysis in Ionic Liquids

Normally, enzymatic reactions are carried out in an aqueous solution with an optimized pH level, temperature, and reactant concentration. However, factors such as low solubility of the substrate in water, side reactions promoted by water, and unfavorable thermodynamic equilibrium, limit the application of biocatalysis in pure aqueous solutions. Efforts have been made to use organic solvents with water or pure organic solvents for enzymatic reactions.

Biocatalysis in organic solvents has some unique advantages over the traditional aqueous solution, moreover, enzymes may exhibit dramatically different properties in a non-aqueous medium. The advantages include: (1) an increased solubility of hydrophobic substrates and/or products, thereby diminishing the diffusion barrier and speeding up the reaction; (2) a reversal in the thermodynamic equilibrium of some reactions; (3) an

inhibition of the water-dependent side reactions; (4) a change in the enantioselectivity of the reaction product.

Due to these features, the use of ionic liquids as a reaction medium has been extended to biocatalysis. Ionic liquids can be used in an enzymatic system in three different possible ways: (1) as a co-solvent in an aqueous phase; (2) as a pure solvent; (3) as a two-phase system together with other solvents.

In addition to these merits, there are also many other features that make the application of ionic liquids more attractive. They are:

Extend the enzyme activity in polar solvent Although ionic liquids have a relatively high polarity, some reports suggest that an enzyme (and even the whole bacterial cell) can maintain activity in such ILs (Pfruender, et al., 2004) solvents. The polarity of ILs, such as [BMIM][PF₆] or [EMIM][(CF₃SO₂)₂N](1-ethyl-3-methyl imidazolium bis(trifluoromethylsulfonyl)imide) is similar to that of a more polar solvent, such as ethanol or N-methylformamide (Park, et al. 2001; Kim, et al. 2001). The higher polarity of the solvent can increase the solubility of polar reactants such as amino acids and vitamins, and therefore lead to a faster reaction and changes in selectivity (Zhao, et al., 2003; Malhotra, et al., 2005).

Increase enzyme stability and enantioselectivity There are some reports about increasing lipase stability and selectivity in imidazolium based ionic liquids (Noel, et al, 2004; Miyako, et al., 2003; Gubicza, et al., 2003). A marked regioselectivity towards the formation of 4,6-di-O-acetyl-D-glucal was observed in [BMIM][PF₆] by Nara et al.(2004). In this study 84% product was formed after six hours with 98% selectivity in hydrolysis. Similarly 48% product was formed after eight hours with 98% selectivity in

alcoholysis (Nara, et al., 2004). ILs were also found to serve as stabilizing agents in an ionic liquid/supercritical carbon dioxide process. The half-life of the enzyme was improved about 2000-fold in [BMIMP][PF6] compared to that in hexane (Lozano, et al., 2004).

Recyclability Ionic liquids are also considered to be green solvents, because the reaction product or reactants can be extracted or precipitated from the reaction system easily. Therefore, the solvent can be reused after purification. Also, catalysts, immobilized enzymes, or enzymes coated on ionic liquids (Lee, et al., 2002), have been found to maintain catalytic activity after several uses. Roberts et al. demonstrated the ability to re-use the enzyme over 10 reaction cycles (Robert, et al., 2004).

1.2.3 Enzymes in Biocatalysis

Enzymes widely used in industry as biocatalysts include: hydrolases, oxidoreductases, transferases, ligase, lyase and isomerase (Wandrey, et al., 2000; Gianfreda, et al., 2004). Among these nearly 80% of all industrially used enzymes are *hydrolase*. These enzymes catalyze the hydrolytic of C-O, C-N, C-C and P-O bonds in phosphate. Most of these enzymes are used to degrade proteins, carbohydrates, and lipids. *Oxidoreductases* are all cofactor-dependant; thereby they require an efficient means of recycling expensive cofactors if used in industry. These enzymes catalyze oxido-reduction reactions, which mean they act on substrates through the transfer of electrons. *Transferases* are enzymes that transfer a chemical group from one compound (donor) to another (acceptor). *Ligases* catalyze a bond formation between two molecules, coupled with the hydrolysis of a pyrophosphate bond in ATP or similar triphosphate. The bonds formed are C-O, C-S, and

C-N. *Lygases* catalyze the cleavage of C-C, C-O, C-N and a few other bonds differently from hydrolysis. This often leaves double bonds that may be subject to further reactions. *Isomerases* represent a small number of enzymes that catalyze geometric or structural changes within one single molecule and make it possible to employ cheaper substrates to garner high value products.

Because of some of the limitations of ionic liquids in biocatalysis, only hydrolases (protease and lipase) and oxidoreductase (peroxidases and dehydrogenase) have been studied extensively. Table 1.1 summarizes the enzymes other than lipases that have been studied in different ionic liquids.

Table 1.1 Enzymes other than lipase investigated in ionic liquids.

| Enzyme | Ionic Liquid | Reaction System | Reference |
|------------------------|---|---|---------------------------|
| β-Galactosidase | [MMIM][MeSO ₄] | Synthesis of N-acetyllactosamine | Kaftzik et al., 2002 |
| | | Enzymatic condensation reaction | Kaftzik et al., 2003 |
| Mandelate racemase | [BMIM][PF ₆], [BMIMBF ₄] | Ester synthesis | Husum et al., 2001 |
| | [MMIM][MeSO ₄], [BMIM][OctSO ₄] | Deracemization of mandelic acid | Kaftzik et al., 2004 |
| | [OMIM][PF ₆] | | |
| α-chymotrypsin | [EMIM][BF ₄], [EMIM][Tf ₂ N] | Enzymatic ester synthesis | Lozano et al., 2003, 2001 |
| | [BMIM][PF ₆], [BMIM][BF ₄] | | |
| | [BMIM][Tf ₂ N], [mtoa][Tf ₂ N] | | |
| Cellulase | [BMIM]Cl | Inactivation and unfolding study | Turner et al., 2003 |
| GOD oxidase-peroxidase | [BMIM][PF ₆] | Oxidation of sulfides | Okrasa et al., 2003 |
| Peroxidase | [(4-MBP)BF ₄], [BMIM][PF ₆] | Laccase catalyzed oxidation | Glen et al., 2003 |
| | | [BMIM][Tf ₂ N], [BMIM][PF ₆], [OMIM][PF ₆] | Oxidize 2-methoxyphenol |
| Formate dehydrogenase | [MMIM][MeSO ₄] | Enzymatic oxidation of formic acid to carbon dioxide | Kragl et al., 2002 |
| Protease | Imidazolium based ionic liquids | Peptide synthesis | Husum et al., 2001 |
| | [EtPy][CF ₃ COO] | Enzymatic resolution of amino acid esters | Zhao et al., 2002 |
| Epoxide hydrolase | [BMIM][PF ₆], [BMIM][N(Tf) ₂], [BMIM][BF ₄] | Hydrolysis trans-β-methylstyrene oxide | Chiappe et al., 2004 |
| Alkaline phosphatase | [H ₃ NEt][NO ₃] | Enzymatic activity and stability | Magnuson et al., 1984 |

1.2.4 Limitations of Industrial Application

The catalytic activity and stability displayed by enzymes in neat organic solvents are far lower than in water, which limits the applications of ionic liquids in biocatalysis. Because of their ionic nature, ionic liquids may interact with a charged group in the enzymes and cause a change in the enzyme's structure. It has been seen that enzymes usually can maintain activity in ionic liquids containing BF_4 , PF_6 and Tf_2N anions, but lose activity in a medium containing anions such as NO_3^- , CH_3COO^- , CF_3COO^- and CF_3SO_3^- . The possible mechanism could be that an enzyme with a compatible anion shows low hydrogen bond basicity, thus minimizing the interference with the internal hydrogen bonding of the enzyme protein. A low nucleophilicity of anions decreases the potential change of the enzyme's conformation by interacting with positively charged sites in the enzyme structure (Yang, et al., 2005). Generally, the mechanism of organic solvents affecting enzyme performance can produce the following results: (1) a stripping of water from the enzyme, which is essential for catalytic activity; (2) an interaction with the enzyme by changing the protein dynamic, protein conformation, or enzyme-active center; (3) an interaction with the substrate or products.

Low vapor pressure makes ionic liquids environmentally friendly solvents, however, this attractive property always decreases the efficiency of product separation. For hydrophobic ionic liquids, the product can be: (1) washed with water; (2) extracted by volatile organic solvents or even supercritical CO_2 ; (3) precipitated out from the solvent system. The liquid-liquid extraction is always a problem for residual non-volatile products, moreover, the extract solvents may affect the quality of the ionic liquids

dramatically. Furthermore, the dissolution of ionic liquids in an aqueous solution can cause a significant loss and therefore waste-treatment becomes a challenge.

Until now there is no report of a successful product separation and/or solvent recycling in hydrophilic ionic liquids. Detection methods, such as HPLC and GC, moreover, are not compatible for an analysis of ionic liquids. The development of rapid and efficient analysis techniques for ionic liquids will be required if these liquids are to replace the traditional volatile solvents in chemical processes.

When using ILs as solvents factors, such as impurity (which always acts as an enzyme inhibitor or leads to a pH shift or change in physical/chemical properties), reaction with a metal co-factor, and/or hydrolysis (when in presence of water et al.), should be considered. The high viscosity and low solubility of an enzyme in ionic liquids may augment the mass transfer problem.

Despite these rather difficult points, ionic liquids have been applied for analytical purposes (e.g. in HPLC analysis) in the pharmaceutical industry, photochemical processes, in electrochemistry (e.g. as battery electrolytes), and even in nuclear science.

1.3 Application of Ionic Liquids in Metal Extraction

1.3.1 General Metal Extraction

As a replacement for conventional molecular solvents, potential utilities of ionic liquids in a liquid-liquid extraction of heavy metal ions become increasingly attractive (Abbott, et al., 2004, 2005). When ionic liquids are hydrophobic in two phases IL/Water system, a solute can partition between these phases depending on its solubility. Usually aqueous metal ions have poor solubility in an ionic liquids phase, but when coordinated with or

solvated by a hydrophobic organic complex, the complex can be transferred to an ionic liquids phase. It is believed that the complexes formed in ionic liquids are different from the complexes known to exist in molecular solvents.

Visser et al. have reported the applications of functionalized ILs as the extractant in a liquid/liquid extraction of Hg^{2+} and Cd^{2+} and uranium (Visser, et al., 2001). They explored the applications of PAN (1-(pyridylazo)-2-naphthol), TAN (1-thiazoly-lazo)2-naphthol), CN^- , OCN^- , SCN^- and halides extractants for the partition of a metal cation between an ionic liquid phase and an aqueous phase. Wei et al. (2003) used dithizone as a metal chelate to form neutral metal-dithizone complexes with heavy metal. They proposed that ionic liquids may be participating in a liquid ion exchange process in which PF_6^- is replaced by a more hydrophobic metal-anion complex, which is formed in the aqueous phase. Hirayama et al. (2005) reported the high extraction performance of $[\text{BMIM}][\text{PF}_6]$, $[\text{hmim}][\text{PF}_6]$ and $[\text{omim}][\text{PF}_6]$ for divalent metal cations with 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (Htta). In order to study the effects of structural variation in ionic liquids on the metal extraction, the Giridhar group (2004) investigated the effects of an alkyl group in 1-alkyl-3-methylimidazolium hexafluorophosphate ionic liquids on the extraction of uranium by tri-n-butylphosphate diluted with ionic liquids. Their results show that the increase of the alky chain also increased the viscosity of TBP/IL. The extraction of uranium by 1.1M TBP/OMIMPF₆ was marginally less than the extraction by 1.1M TBP/BMIMPF₆. Moreover, the efficiency of the extraction was influenced by the concentration of nitric acid and uranium in the aqueous phase.

However, a study by Dietz et al. (2005) found that in contrast to the extraction of Sr crown ether (CE) complex into molecular organic solvents, extraction by

dicyclohexano-18-crown-6 (DCH₁₈C₆) from acidic nitrate media into various 1-alkyl-3-methylimidazolium-based ionic liquids takes place predominantly via a mechanism in which the cationic 1:1 metal-CE complex is exchanged for the cationic constituent of the ionic liquids.

Since increased dissolution of the ionic liquids in the aqueous phase cannot be regarded as “green”, more task-specific ionic liquids have been designed to overcome this problem. Mercury (II) and cadmium (II) were targeted in a study by Visser (2001). The modified ILs were 1-alkyl-3-methylimidazolium, C_nmim⁺ (n = 4, 6, 8) salts of PF₆⁻ which form two phase systems with water. ILs that incorporated thiourea, thioether and urea into derivatized imidazolium cations were prepared. These when combined with PF₆⁻ anion, functioned as both the hydrophobic solvent and the metal ion extractant in a liquid/liquid separation.

Another method involves the addition of neutral organophosphorus reagents, tri-*n*-butyl phosphate (TBP), to function as synergists in the extraction of alkali and alkaline earth cations by crown ethers into 1-alkyl-3-methylimidazolium-based ionic liquids (Stepinski, et al., 2005). They demonstrated that synergistic interactions between extractant molecules can occur in ionic liquids. Moreover, these interactions may yield significant improvements in both metal ion extraction efficiency and selectivity.

The development of environmentally benign metal ion separation systems through the use of ionic liquids requires an improved understanding of the mechanism by which ions partition into these solvents and an understanding of the metal coordination environment in ILs.

1.3.2 Application of Ionic Liquids in Actinide Chemistry

The extensive usage of uranium and other actinides in the nuclear fuel industry make actinide contamination a major concern. Uranium and its compounds are highly toxic and may cause progressive or irreversible renal injury and acute cases may lead to death. It is important to explore the advanced techniques to concentrate and recover uranium from the environment (Rao, et al., 2006).

Liquid–liquid extraction Liquid- liquid extraction is a commonly used method to separate analytes of interest between two immiscible solvents. Different organic ligands are used to form various metal-complexes and to facilitate their transport to the organic layer. There are disadvantages to this method, however, including the large volume of organic solvent it requires and means of further separation prove problematic.

Liquid membrane / ion exchange / extraction chromatography All these methods are based on the ion exchange mechanism. For the liquid membrane, the transport of uranium is induced by electro dialysis through a cation-exchanging membrane. The disadvantage is the formation of metal hydroxide, which clogs the membrane. Ion exchange normally means the exchange of an ion between an aqueous solution and a solid insoluble body. Extraction chromatography consists of a buffer solution as a mobile phase and an organic extractant as a stationary phase. The chelate or ion pair formed is soluble in the stationary phase. The disadvantages of these methods include its high cost and the partial removal of certain ions.

Reverse Osmosis / Ultrafiltration Reverse Osmosis and Ultrafiltration are based on membrane technology. In reverse osmosis, heavy metals are separated by a semi-permeable membrane at a pressure greater than the osmotic pressure caused by the

dissolved solids in waste water. This method is relatively expensive. Ultrafiltration is a pressure-driven membrane operation that uses porous membranes for the removal of heavy metals. The main disadvantage is the generation of sludge.

Chemical Precipitation The precipitation of metals is obtained by the addition of coagulants such as alum, lime, iron salts and/or other polymers. This process will produce a large amount of sludge that contains toxic materials.

The tunable properties of ionic liquids make them attractive media/systems for the purification of actinides from water or mixtures.

Ionic liquids as molten salts Electrochemical pre-refining of metal from a high temperature molten salt is a new and effective way for the recovery of actinides. The lower melting temperature, lower operation temperature, and use of less corrosive ionic liquids may reduce the production costs for such processes. Hardacre (2004) studied the species dissolved in molten salts and ionic liquids by EXAFS. They demonstrated the possibility of using EXAFS to analyze the coordination chemistry of uranium with ionic liquids in an aqueous solution. Oldham et al. (2002) tried to develop ionic liquids with extended cathodic stability, so that these liquids can be used as an alternative to molten salts in an electrochemical process. Ionic liquids containing either acyclic or cyclic quaternary ammonium cations combined with the $(\text{SO}_2\text{CF}_3)\text{N}^-$ anion show favorable viscosity and conductivity properties and excellent electrochemical stability.

Uranium in AlCl_3 -1-ethyl-3-methylimidazolium Chloride Mixture The interaction between $\text{UO}_2\text{Cl}_4^{2-}$ and a solvent mixture of ionic liquids 1-ethyl-3-methylimidazolium chloride and aluminum chloride (60:40) has been identified by optical absorption, emission spectrum and FT raman (Hopkins, et al., 2001). The mixture

of AlCl_3 and EMIMCl exhibits widely varying Lewis acid-base properties depending on their composition. Ionic liquids containing more than 50% AlCl_3 are acidic due to the formation of Lewis acidic species Al_2Cl_7^- . Those containing less than 50% AlCl_3 are basic because of free chloride ions. These ionic liquids do not undergo a solvolysis reaction, which is commonly seen in an aqueous solution. They also form a chloride complex with uranium more readily than in a molecular solvent such as acetonitrile or methanol (Anderson, et al., 1991). Therefore, basic AlCl_3 -1-butylpyridinium chloride and AlCl_3 -1-ethyl-3-methylimidazolium chloride have been used to study the electrochemistry and spectroscopy of transition-metals, lanthanide, and actinide chloride complexes. Their results show hexachloro anionic metatolate complexes are not present in the ionic liquid as simple anions but are associated with one or more EMIM cations. Another study by Sheng Dai and her co-worker (Sheng, et al., 1997), suggests that the linear oxo group (UO_2^{2+}) is directly involved in the hydrogen bonding via the uranyl group ($\text{U}=\text{O}$) with the H-X group (e.g. H-O, H-N) of the solvent. In 1-ethyl-3-methylimidazolium chloride ionic liquids, a hydrogen bond is formed between the positive charged C(2) of EMIC and uranyl solute.

Uranyl Coordination Environment in fluorinated acid The complexation capability of fluorinated acid F^- , BF_4^- , PF_6^- and Tf_2N^- toward a uranyl ion in an aqueous solution was reported by Gaillard et al. (2005). By EXAFS and TRES analysis, they showed that Tf_2N^- does not complex with uranyl at all, whereas other anions such as BF_4^- and PF_6^- form inner sphere complexes with U(VI). However, the limitation on this study is that the complex-ability of an anionic part cannot be considered the same as the whole

ionic liquid. They behave as an ion pair in an aqueous solution and may not completely dissociate into cation and anion parts.

Uranyl Coordination Environment in hydrophobic ionic liquids Visser et al. (2003) show that the complexes formed between uranyl nitrate and octyl-phenyl-N,N-diisobutylcarbamoylmethylphosphine oxide show a difference in the hydrophobic ionic liquids, [C₄min][PF₆] and [C₈mim][N(SO₂CF₃)₂]. A liquid/liquid extraction for uranyl in both ILs indicates a net stoichiometry of UO₂(NO₃)(CMPO)⁺. Their study suggests that in order to prevent a cation exchange mechanism from occurring upon a metal ion coordination, a more hydrophobic cation is desired.

1.4 Toxicity Studies of Ionic Liquids

Although ionic liquids have been regarded as environmentally friendly solvents because of their low vapor pressure, we have little information about their toxicity to organisms, their effects on the ecosystem, and their fate in the environment.

Ionic liquids could prove to have numerous applications. However, before that can happen, many questions need to be answered, such as (1) How toxic are the ILs in the environment? (2) Is there any reasonable model to predict the toxic effect when ILs are released to the environment? (3) Is there any difference between the two different testing systems?

As described by Jastorff et al. (2003) the whole testing system is based on four levels: enzymes, whole cells, organisms, and the ecosystem (Figure 1.2). Based on fundamental studies, an SAR (Structure-Activity-Relationship) or QSAR (Quantitative

Structure Activity Relationship) model will be set up. All evaluated data should be compared with said predicted models.

Jastorff's study concerns a multidimensional risk analysis (Figure 1.3). The risk analysis system includes five factors: Release (R), Spatiotemporal Range (S), Bioaccumulation (B), Biological activity (A), and Uncertainty (U). In comparison with the organic solvent acetone, the very high uncertainty of ILs, [BMIM][PF₆] and [BMIM][BF₄], yield them high risk scores.

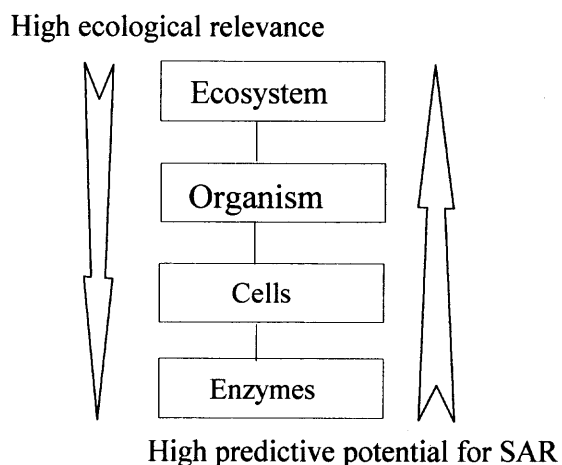


Figure 1.2 Levels of complexity for the evaluation of the biological activity of chemicals. (Jastorff, et al., 2003)

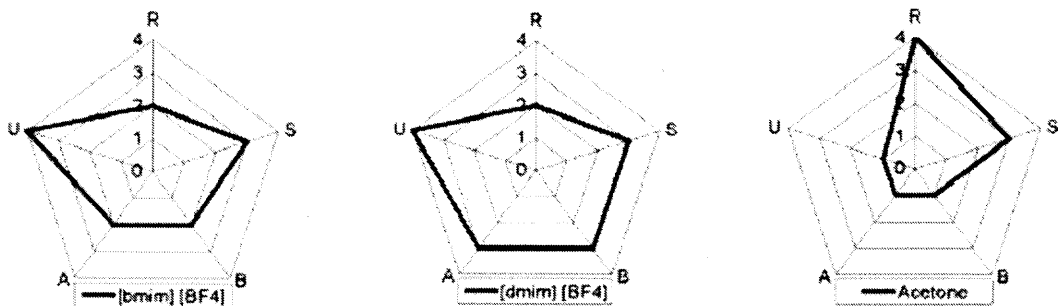


Figure 1.3 Multidimensional risk analysis of [BMIM][BF₄], [DMIM][BF₄] and acetone. (Jastorff, et al., 2003)

Based on this study, I summarize below the toxicity studies that have been done on different levels.

Enzyme Level The only report on the effect of ionic liquids on acetylcholinesterase inhibition suggested a simple QSAR model based on structural properties of ionic liquids to predict toxicity (Stock, et al., 2004). They use rapid screening methods and get a concentration-response curve and EC_{50} value. The relationship between the logarithms of the EC_{50} and the number of the carbon chain lengths of pyridinium or imidazolium ring was observed. This suggests that a longer chain length results in a stronger inhibition of the enzyme.

Madeira et al. (2004) reported the change in the lipase conformation in the ionic liquids [BMIM][PF₆] and [BMIM][BF₄], which may be the reason for the loss of enzymatic activity in ILs. In Jastorff's study (2003) on imidazolium-based ILs, the most toxic among them exhibits about the same toxicity as the least toxic of the four most common organic solvents (i.e. methanol, acetone, acetonitrile, and MTBE). For a complete understanding of the toxicity, many other factors also should be considered, such as different test systems, the chronic effects, etc.

Bacterial Level A similar trend of toxicity related to alkyl chain length was also observed in cell cultures. Usually rapid screening methods, such as a cell viability assay, and a luminescence inhibition test are applied (Table 1.2). There are few reports on the toxicity of ionic liquids used on the bacterial level as summarized below. There are also several problems extant, such as differing testing systems, differing toxicity mechanisms et al., which make the results less credible.

Antimicrobial activities have been observed by Docherty and co-workers (2005). They examined the antimicrobial effects with 1000 ppm of butyl, hexyl and octyl imidazolium and pyridinium-based ionic liquids on the growth of a group of microorganisms. Generally hexyl and octyl imidazolium, and pyridinium ILs exhibited higher inhibitive effects than those ILs containing a butyl chain.

The testing of glucose consumption and the survival rate of microbes (CFU) was suggested by the Michiaki Matsumoto group (Matsumoto, et al., 2004) as a means to measure the toxicity of imidazolium-based ILs on the lactic acid producing bacteria, *Lactobacillus Rhamnosus*. In comparison to the organic solvents, toluene and hexane, the authors conclude that the ILs show a relatively low toxicity for an in situ extractive fermentation.

The biological effects of imidazolium-based ILs on the luminescent bacterium, *Vibrio fischeri*, as well as on the IPC-81 (leukemia cells) and the C6 (glioma cells) rat cell lines were described by Ranke, et al. (2004). The toxicity was expressed as cell viability and luminescent inhibition. They observed that as the n-alkyl chain length increased a higher toxicity was found. They suggested that imidazolium ILs show structures similar to cationic surfactants. For representative surfactants, the membrane permeability could be increased with an increased chain length. A difference was also found among the test systems, with the cell line IPC-81 showing higher sensitivity than that of the rat glioma cell line, C6.

The anion effect was found in a cytotoxicity study of imidazolium ILs by using a human cell line (Stepnowski, et al. 2004). The lowest EC₅₀ value was found for tetrafluoroborate.

Toxicity studies on the bacterial level are listed in Table 1.2.

Table 1.2 Toxicity studies on bacteria and cells

| Bacterial | Ionic liquids | Analysis Content | Reference |
|---------------------------------------|---|--|--------------------------|
| <i>Lactobacillus Rhamnosus</i> | Imidazoliu-based | Glucose consumption and survival rate of microbes | Matsumoto et al., 2004 |
| <i>Vivrio fischeri</i> and WST-1 cell | Imidazolium ILs with varying chain lengths | Cell viability assay and luminescent bacterial acute toxicity test | Ranke et al., 2004 |
| Cell, algae , fish, crustacean | Pyridinium chloride | LC ₅₀ , EC ₅₀ , IC ₅₀ | Grabinska et al., 2003 |
| Lela human cell line | Imidazolium ILs | Concentration effect and anion effect on EC ₅₀ | Stepnowski, et al., 2004 |
| Rods, cocci, fungi | [(C ₃₋₅ OM) ₂ IM][BF ₄] [(C ₃₋₈ OM) ₂ IM][NTf ₂] | Antimicrobial activity | Ezdihar, et al., 2002 |
| J774A.1 Macrophage cell | [BMIM]Cl | Cellular viabilities | Pernak, et al., 2004 |

Organism Level Low level organisms, such as the freshwater crustacean *Daphnia magna* (Bernot, et al., 2005), Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana* (Latala, et al., 2005), are an important link between microbial and higher trophic levels. Since many ILs are hydrophilic, they could potentially be released into the aquatic ecosystem. Therefore, few studies have been done on their toxic effects on the aquatic organism. The mechanisms of IL toxicity to organism are still unknown, but potential modes of actions include enzyme inhibition, disruption of membrane permeability and structural change of DNA.

Couling (2006) tested the toxicity of various ILs on aquatic organisms (*Vibrio fischeri* and *Daphnia magna*), and established a quantitative model structure property relationship model. Toxicity is expected to show the trend with cation type of ammonium < pyridinium < imidazolium < triazolium < tetrazolium. Also, toxicity is expected to decrease with ring methylation and with an increase in the number of negatively charged atoms in the cation.

However, the toxicity of ionic liquids is reduced in more saline waters, which could be important in their fate assessment in marine environments (Latala, et al., 2005). Lower toxicity was thought to be due to the reduced permeability of the ionic liquids cations through the algal cell walls. A high chloride concentration offers a good ion pairing environment for imidazolium cation, which competes with the hydroxyl (green alga) or silanol (diatom) functional groups in the cell wall structures.

The differential of toxic effect was also seen in Grabinska's study (2003) of pyridinium chlorides. This study was based on the organism levels: algae, crustacean and fish. The absorption of a substance by the algae cells and the reaction with algae cells were suggested as the reasons for the high toxic effects.

The only results that have been reported on a higher organism level are the effects of ionic liquids on the survival, movement, and feeding behaviors of the freshwater snail, *PHYSA ACUTA* (Bernot, et al., 2005). The median lethal concentrations (LC_{50}) of imidazolium and pyridinium based cations and Br and PF_6 anion vary from 1 to 325 mg/L. Longer alky chains attached to both pyridium and imidazolium ring yielded higher toxicity. Individual fitness and food web interactions were also potentially affected in the presence of different IL concentrations.

1.5 Fate of Ionic Liquids in the Environment

There are five ways suggested to understand the fate of ILs in the environment.

Sorption The adsorption of [BMIM]Cl by gram positive soil bacteria, *Bacillus subtilis* has been studied by Corman, et al.(2004). Along with biosorption, the absorptions onto gibbsite, quartz, and Na-montmorillonite were measured. In this study, their results indicate that the [BMIM]Cl has little affinity/adsorption to alumina or silica mineral surface sites or to the gram-positive bacteria surface. However, [BMIMP][PF₆] does adsorb to activated carbon (Anthony, et al., 2001). The distribution coefficient (K_D) plays an important role in this process. The hydrophobicity of [BMIM]Cl is not enough to drive adsorption on the bacterial surface nor is the electrostatic force strong enough to cause adsorption on those surfaces. Similarly a study on the sorption behavior of alky imidazolium cations to soils and sediments shows a strong interaction with soils. Depending upon the IL structure, the sorption coefficients may vary between 6.9 and 226ml/g. The electrostatic interaction has been suggested as the major contribution to the sorption of imidazolium cations (Sepnowski, et al. 2005).

Biodegradation In order to make ionic liquids more applicable on a large scale, it is important that these materials be biodegradable. Therefore, it is more desirable to design biodegradable ionic liquids if possible. Results reported so far are based on the introduction of a biodegradable side chain on a cation, such as (1) the potential side for enzymatic hydrolysis; (2) the introduction of a hydroxyl, aldehyde or carboxylic acid group; or (3) the unsubstitution of linear alky chains or phenyl rings (Boethling, 1996). Similarly, Gathergood et al. (2004) measured the biodegradability of ILs by inoculating

them with wastewater microorganisms and detecting the evolution of CO₂. However, the degradation mechanism is not yet clear.

Photodegradation Stepnowski Pitotr et al. (2004) have compared three common advanced oxidation processes: UV, UV/H₂O₂, and UV/TiO₂. Among them the UV/H₂O₂ process was found to be the most effective in the degradation of imidazolium-based ionic liquids. Again, the stabilities of those ILs are structure-related. Elongating the 3-methyl substitute significantly decreases its degradability (Figure 1.4). However, product-identification is problematic.

Oxidation A KMnO₄ aqueous solution has been used to oxidize the ionic liquids (Pernak, et al., 2004). The permanganate index (I_{Mn}) was estimated as the degradation ability. Under the reported experimental conditions, the cation was ultimately oxidized to carbon dioxide, while the anion was not oxidized.

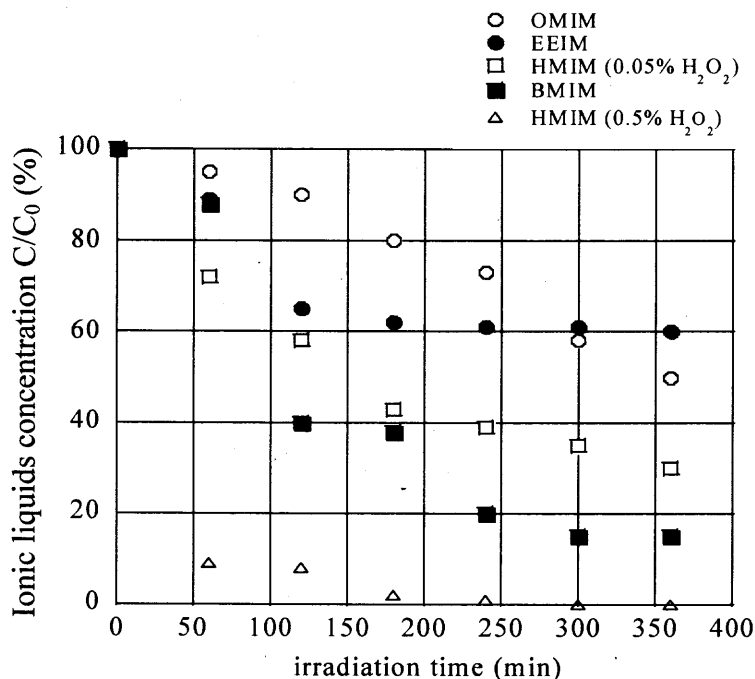


Figure 1.4 Effect of H₂O₂ on the photodegradation of imidazolium ionic liquids. (Stepnowski, et al., 2004)

Thermodegradation Imidazolium cation-based ionic liquids are found to degrade and yield volatile products at an elevated temperature (Baranyai, et al., 2004). Most of the cation degradation products were neutral mono-N-alkylated imidazoles obtained by a dealkylation process, and a small percent of the product was from rearrangement. Also, volatile products were released from anion degradation.

Hydrolysis Swatloski (2003) has reported hydrolysis of the hydrophobic ionic liquid [BMIM][PF₆] during the purification process, which can release the corrosive product HF. Therefore, it is suggested that when synthesizing ILs, environmentally acceptable anions, such as inorganic anions -- Cl⁻, Br⁻, PO₄³⁻, and the organic anions— acetate, succinate, glycolate, lactate, should be considered.

1.6 Objective

The literature and reports suggest that ionic liquids could be very useful for biocatalysis, metal extraction, and the separation process. Therefore, the objective of my dissertation research is to systematically study the applications of ionic liquids in the separation of heavy metal uranium through a microbial process. These ionic liquids namely, [EtPy][BF₄] (N-ethylpyridinium tetrafluoroborate), [EtPy][CF₃COO] (N-ethyl pyridinium trifluoroacetate), and [BMIM][PF₆] (1-butyl-3-methylimidazolium hexafluorophosphate), will be investigated. A basic scientific understanding of the uranium-IL interaction will be developed through the following studies:

1. Interactions between ionic liquids and uranium in an aqueous solution

The chemistry of uranium (VI) is nearly dominated by the linear dioxo cation UO₂²⁺ in water. Many organic / inorganic compounds can form stable complexes with

uranium and increase their solubility and leaching capabilities. The elucidation of the uranium-ionic liquids interaction is important in estimating the mobility in the environment and in the use of microorganisms to remediate a contaminated water system. The special properties of ionic liquids may change the coordination chemistry of uranium in water. Therefore, we will systematically study U-ILs interactions by using various analytical techniques, such as potentiometric titration, UV-Vis spectroscopy, liquid chromatograph-mass spectroscopy, XANES and EXAFS analysis.

2. Effects of ionic liquids on uranium biosorption

Depending on the pH and the properties of the ligands, the complexation of the uranium species may change, which could either increase or decrease the metal ion sorption. So far, literature studies have focused on organic substances with high chelating abilities, such as NTA and EDTA, while fewer studies are reported with ligands having low chelating abilities. Furthermore, there is no report on the effects of ionic liquids on uranium biosorption. Therefore, we will study the effect of U-ILs complex on uranium biosorption and also its effect on the bacterium cell.

3. Effects of ionic liquids on the uranium bioreduction

Among the different separation techniques, bioremediation is a cost-efficient means. With their unique properties, ionic liquids may show remarkable advantages in the biocatalysis process. Thus, with a change in the coordination chemistry of uranium, ionic liquids may affect the bioremediation process dramatically. We will study the toxicity of ionic liquids on the whole cell and the Structure-Activity relationship will be elucidated. A suitable concentration of ionic liquid will be chosen for the bioreduction of

uranium. Different U-ILs complexes will also be compared to illuminate the changes in uranium bioavailability.

4. Biodegradation of ionic liquids by enriched soil bacterium

The extent of contamination and the ultimate recovery of soils, surfaces and ground waters impacted with ionic liquids will particularly depend on the ability of microorganisms to degrade these compounds. Therefore, the microbiological biodegradation of N-ethylpyridinium ionic liquids by local soil bacteria will be investigated. The study of the relative rates of the biotransformation of these compounds, and the final degradation products yielded will help to understand of the mechanism.

This research will provide fundamental results concerning the coordination environment of uranium with ionic liquids in an aqueous solution, which will be useful in the design of task-specific ionic liquids and the achievement of maximum separation efficiency. A change in the coordination structure could affect the bioavailability of uranium. By designing appropriate ionic liquids, there is high potential to achieve a successful bioremediation and recovery of uranium from the environment and a recycling of the ionic liquids as a chelating agent. The biodegradation of ionic liquids will provide very important information for the environmental risk assessments of these new materials.

CHAPTER 2

CHARACTERIZATION OF URANIUM ASSOCIATED WITH IONIC LIQUIDS

2.1 Introduction

The chemistry of uranium (VI) has garnered a lasting interest since its discovery. Numerous studies have been conducted on the exploration, exploitation, nuclear fuel production, the reprocessing, and the disposal of nuclear waste. Its unique linear axial bonds may provide a great potential insight into the fundamental studies of *f*-element separations and associated solution chemistry.

The exploration of the various actinides separation processes has been driven by the need for technologies, which will successfully recover large quantities of aqueous low-level nuclear waste. Volatile or semi-volatile organic compounds have been widely used in the liquid/liquid extraction process, which introduces the risks associated with the solvent's toxic and flammable nature. As described in the Introduction, there have been only a few studies using ionic liquids as an alternative solvent for the liquid/liquid extraction of actinides from water (Visser, et al., 2003; Giridhar, et al., 2004). It is important to investigate the actinide coordination environment in ILs to elucidate the separation mechanism.

This study focuses on the coordination-ability of three commonly used ionic liquids with uranium (VI) in an aqueous system, and provides the fundamental results for their use in separation chemistry and bioremediation research.

2.2 Materials and Methods

The three ionic liquids employed in this study were [EtPy][BF₄], [EtPy][CF₃COO], and [BMIM][PF₆] (Figure 2.1). The uranium was introduced as UO₂(NO₃)₂·6H₂O. N-ethylpyridinium bromide was purchased from the AVOCADO Company; 1-butyl-3-methylimidazolium chloride was obtained from fluka. HBF₄, HPF₆, HCF₃COO and silver (I) oxides were obtained from the Aldrich Company. All solutions were prepared with ultra-pure deionized water (Milli-Q plus, Millipore).

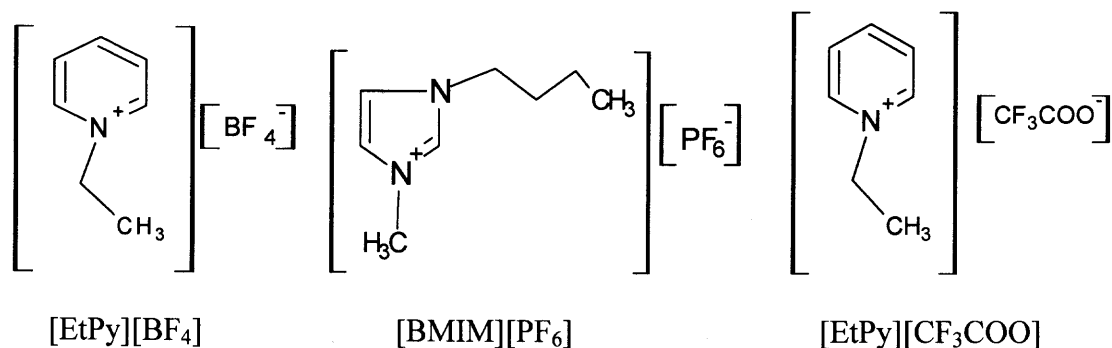


Figure 2.1 Molecular structures for ionic liquids [EtPy][BF₄], [BMIM][PF₆] and [EtPy][CF₃COO].

2.2.1 Synthesis of Ionic Liquids

Ionic liquids were synthesized according to the methods described below:

2.2.1.1 Synthesis of [EtPy][BF₄] The ionic liquids [EtPy][BF₄] were synthesized according to the methods of Zhao et al. (2002). Tetrafluoroborate acid (0.23mol) was slowly added to a stir slurry of silver (I) oxide (0.115mol) in 60ml distilled water over 10 minutes. To avoid photodegradation of the silver (I) oxide, the reaction mixture was fully covered with aluminum foil. When the silver (I) oxide reaction was complete, a solution of N-ethylpyridinium bromide (0.23mol) in 150ml distilled water was added to the reaction mixture; it was then stirred at room temperature for 2h. The resulting yellow

precipitate was filtered off, and water was removed at 65 °C under vacuum to obtain 29ml of product. (Yield is 92%).

2.2.1.2 Synthesis of [BMIM][PF₆] Synthesis and purification were carried out according to Carda-Broch, et al. (2003). 1-butyl-3-methylimidazolium chloride (34.9g, 0.2mol) was dissolved in 100ml distilled water. Hexafluorophosphoric acid (34.5ml, 0.24mol, 60%) was added drop wise. The amount of hexafluorophosphoric acid was about 17% (v/v) in excess of the amount of [BMIM]Cl. The solution was stirred for 3 hours. A viscous pale yellow liquid, which formed in the bottom layer was removed. The ionic liquid was first washed several times with water. Then it was washed with saturated sodium bicarbonate until the pH of the washed decant was about 7. The halide in the water was tested using the silver nitrate method. The product was dried at 60° C under vacuum overnight. (Yield is 80%.)

2.2.1.3 Synthesis of [EtPy][CF₃COO] The Synthesis of [EtPy][CF₃COO] was modified as below (Zhao, et al., 2002): Trifluoroacetic acid (13.3ml, 0.1726mol) was slowly added to a stirred slurry of silver (I) oxide (20g, 0.0863mol) in 50ml distilled water for 10 minutes. To avoid photodegradation of the silver (I) oxide, the reaction mixture was fully covered by aluminum foil. Following the completed reaction of the silver (I) oxide, a solution of N-ethylpyridinium bromide (32.46g, 0.1726mol) in 150ml distilled water was added to the reaction mixture and the solution was stirred at room temperature for 2 hours. The yellow precipitate was filtered off, and the solvent was removed at 65 °C under vacuum. Thirty ml of product was obtained. (Yield is 91%).

2.2.2 Preparation of Uranium-IL Mixture

1:1 U(VI)-IL mixture (5.78mM) were prepared by mixing continuously an equal molar of U(VI) ion and IL solution in a 25ml volumetric flask. It was allowed to stabilize for 24 hours in darkness to avoid photodegradation. Similar methods were used to prepare the 1:2 U(VI) – IL mixture.

2.2.3 pH Change and Potentiometric Titration

The pH of the mixture was measured at the onset of mixing and after 24 hours using the Mettler Toledo MP 220 pH meter. Before titration, the ionic strength was adjusted to 0.1M due to the addition of KCl. The change in pH of 0.29mM 1:1 and 1:2 U(VI)-IL mixture upon the incremental addition of 0.01M NaOH was measured at 26 °C using a Mettler Toledo DL57 titrator. The glass electrode was calibrated at pHs 4, 7, and 10 before each titration.

2.2.4 UV-Vis Spectroscopy Analysis

The absorption spectra of the 5.78mM U(VI)-IL mixtures were determined from 190 to 1100 nm using a Hewlett Packard 8453 diode array scanning UV-Vis spectrophotometers with a 1.0 cm quartz cell.

2.2.5 Liquid Chromatograph-Mass Spectroscopy Analysis

After diluted in water, the U-ILs mixtures were analyzed by LCQ Advantage ESI-MS (Electrospray Ionization Mass Spectrometry) in both positive and negative modes. The sample was introduced into the analyzer using a syringe pump under the following

conditions: sheath gas: N₂; flow rate: 69 liter/h; I spray voltage: 4.5kV; capillary temperature: 325°C; capillary voltage: 35V.

2.2.6 Speciation of Uranium by XANES (X-ray absorption near-edge spectroscopy) and EXAFS (extended X-ray absorption fine structure analysis)

A Twenty millimolar U- ILs mixture was prepared in water and stabilized overnight in darkness. The solution samples were prepared by sealing approximately 2ml of each solution in a polyethylene tube (5 mm i.d.). The solution sample tubes were then mounted on a plastic sample positioner with Scotch tape and measurements were made on the X-11B beam line at the National Synchrotron Light Source, Brookhaven National Laboratory. Uranium was analyzed at the U L_{III} absorption edge (17.166 Kev) using fluorescence detection. The data were collected in both transmission and fluorescence modes. At least five scans were performed for each sample to minimize the signal to noise ratio. The XANES spectra were background-subtracted and normalized to the edge jump, and the oxidation state of uranium in the samples were determined by comparing the energy position at the inflection point with that from the tetravalent uranium dioxide and hexavalent uranyl acetate dihydrate [(UO₂)(OAc)₂·2H₂O] (Atomergic Chemicals, NY). Fourier-transformed EXAFS data, a pseudo-radial distribution function (PRDF) representing the radial coordination shells of the near-neighbor atoms surrounding the metal, were obtained using a multi-step height followed by Fourier transformation of the k³-weighted (2-12Å⁻¹) EXAFS spectra. We used the theoretical EXAFS modeling code, FEFF6, to calculate the back-scattering phase and the amplitude information for the

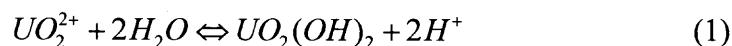
individual neighboring atoms. The amplitude reduction factor (S_0^2) was fixed at 1.0 for all the fits. The r factor evaluated goodness of the fit.

2.3 Results and Discussion

The results of the various characterization studies of uranium and ionic liquids are summarized below.

2.3.1 pH Change and Potentiometric Titration

Potentiometric titration has been widely used in determining the end point detection, precipitation titration, complex formation titration, oxidation/reduction titration, equilibrium constant and dissociation constant determination. The Figure below shows the effects of the addition of 0.01 N sodium hydroxide on the complexation of the uranyl ion with the IL's. Titration of uranium nitrate showed the inflection point at 2 mM OH⁻/mM U at pH 6.4. The release of 2 protons is due to the association of the uranium ion with 2 OH⁻ in water (equation 1).



The coincidence of the titration curves for the U:[BMIM][PF₆] (Figure 2.2) and the U:[EtPy][BF₄] (Figure 2.3) complexes with uranyl nitrate alone indicate no complex formation is evident between the metal and the ILs. A slight pH shift in Figure 2.2 may be due to the hydrolysis of PF₆⁻. Also the addition of one-fold excess IL had no effect on the complexation. However, the titration of the U in the presence of [EtPy][CF₃COO] (Figure 2.4) shows a release of one more proton into the medium as indicated by the increase in the inflection point at 3 mM OH⁻/mM U. These phenomena demonstrate that there is an interaction of the uranium with the [EtPy][CF₃COO]. The interaction increases with

addition of one-fold excess [EtPy][CF₃COO] (more acid released). The pH changes were monitored during incubation as shown in table 2.1. The obvious decrease of pH is only observed in the U: [EtPy][CF₃COO] mixture. After 24 hours of incubation, the pH of the mixture dropped from 3.3 (uranium alone) to 2.3, which also indicates the release of acid during complexation.

Table 2.1 pH changes of U:[EtPy][CF₃COO] mixture

| Solution | 0h | 24h |
|-----------------------------|------|------|
| U | 3.34 | 3.34 |
| [EtPy][CF ₃ COO] | 3.50 | 3.36 |
| 1:1 mixture | 2.95 | 2.30 |
| 1:2 mixture | 2.73 | 2.19 |

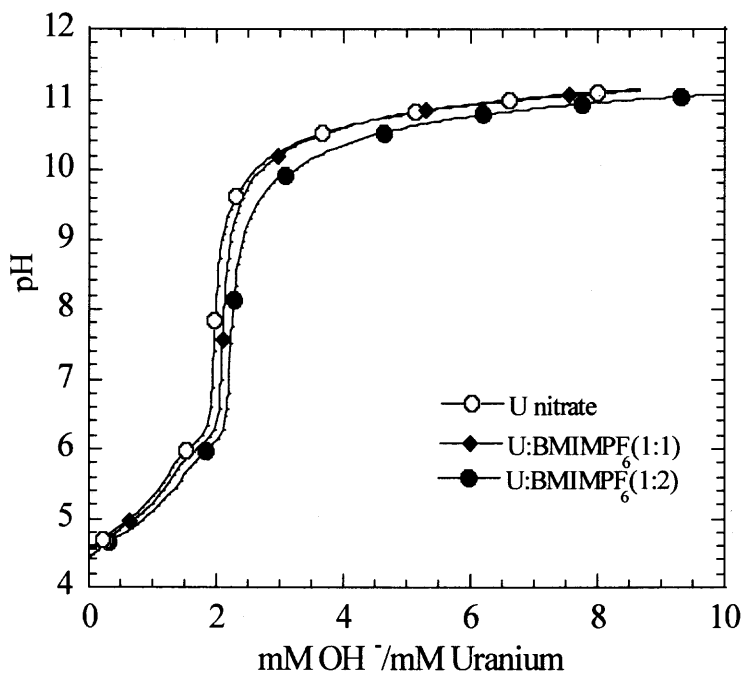


Figure 2.2 Potentiometric titration curve of U: [BMIM][PF₆].

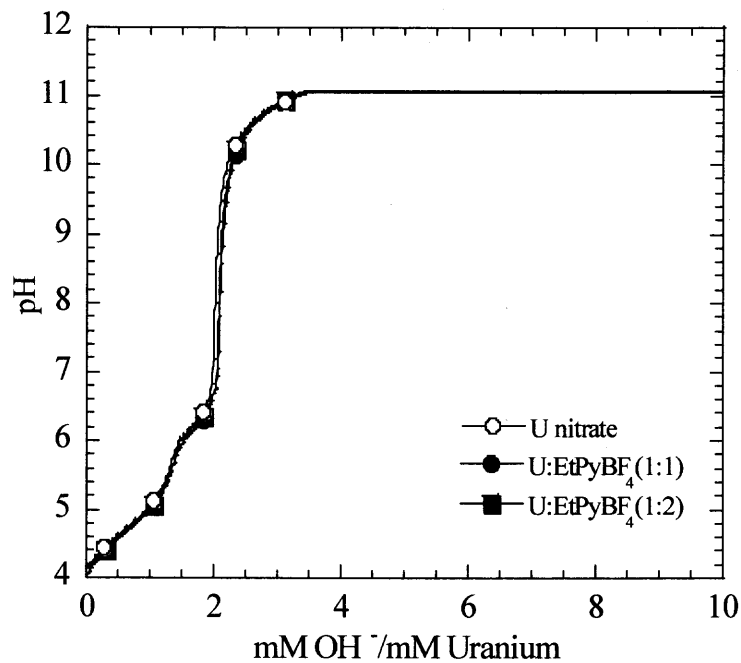


Figure 2.3 Potentiometric titration curve of U: [EtPy][BF₄] mixtures.

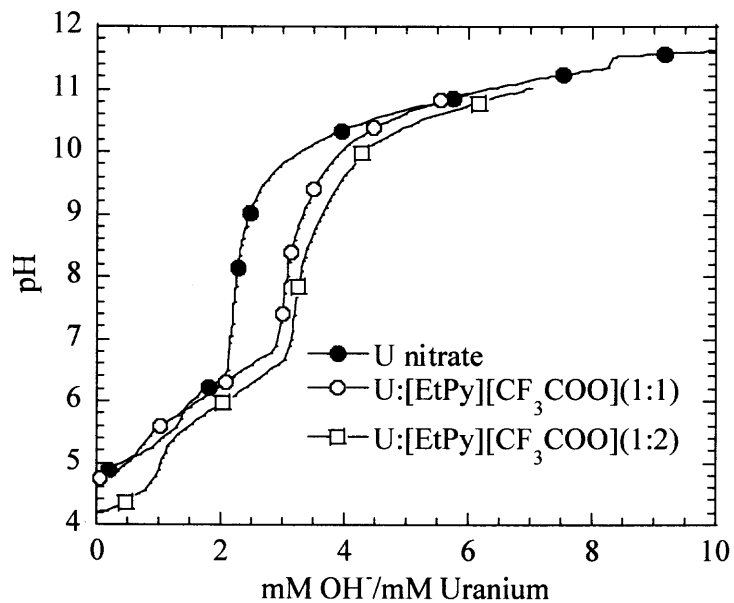


Figure 2.4 Potentiometric titration curve of U:[EtPy][CF₃COO] mixture.

2.3.2 UV-Vis Spectrophotometric Analysis

The UV-Vis absorption spectrum of the free uranyl ion in the range of 300 nm to 500 nm is shown in Figure 2.5. The spectrum shows a weak absorption band in the range of 480 nm and 350 nm with a characteristically fine structure. However, no additional absorption bands were observed above 480 nm. The analysis carried out after 24 hours of incubation shows no change in uranium absorbance. In each case the maximum absorbance was observed at 413nm, with two shoulder peaks at 402 nm and 425 nm.

The UV absorbance change for the U:[EtPy][BF₄] (1:1) and U:[EtPy][BF₄] (1:2) mixtures are shown in Figure 2.6. The spectra are similar to that found for U(VI)-nitrate, indicating no evidence of complexation of the [EtPy][BF₄] with uranium.

Both 1:1 and 1:2 of U:[BMIM][PF₆] mixtures have the same absorbance as uranium itself (Figure 2.7). This indicates that no complexation occurs in the presence of uranium. On the other hand, in the case of the U:[EtPy][CF₃COO] mixture, the maximum absorbance shifted to 408 nm and 419 nm (Figure 2.8). In order to elucidate the pH effect on UV absorbance, the pH of the uranium nitrate solution was adjusted to 2.12 (similar to the U: [EtPy][CF₃COO] mixture) by 0.1N nitric acid (Figure 2.5). The results show that the pH change did not alter the shape of the absorbance spectrum and that a decrease in absorbance was due to the addition of the nitric acid. So the absorbance shift is due to the structure change during complexation; it is not a result of the pH shift.

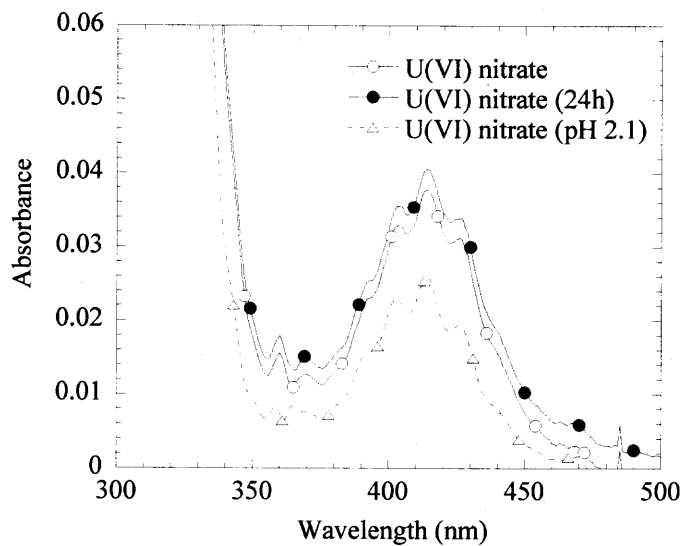


Figure 2.5 UV-Vis spectrophotometry of uranium in water.

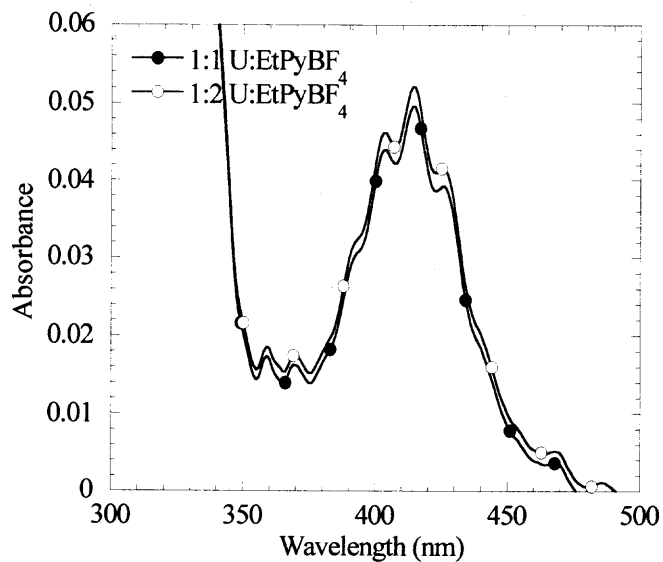


Figure 2.6 UV-Vis spectrophotometry of U: [EtPy][BF₄] mixture (after 24h) in water.

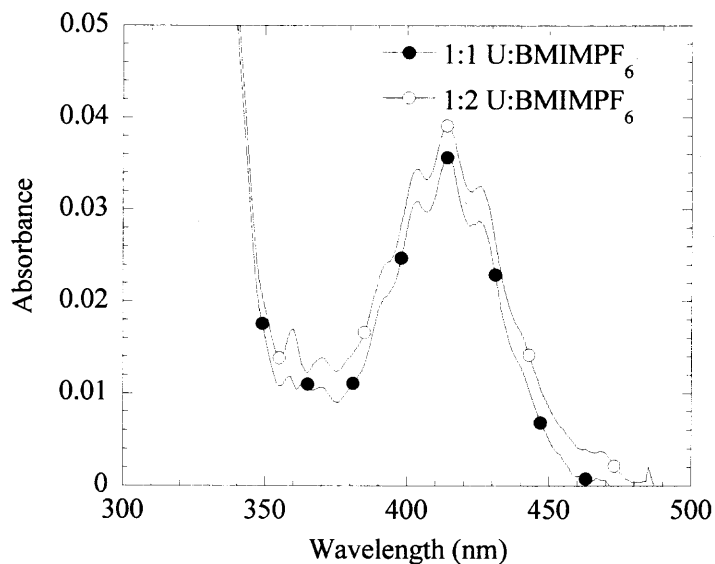


Figure 2.7 UV-Vis spectrophotometry of U: [BMIM][PF₆] mixture (after 24h) in water.

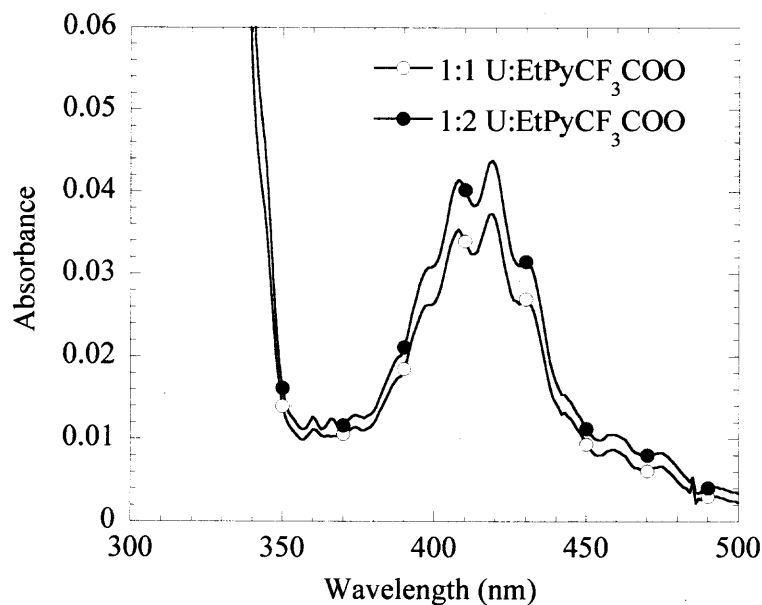


Figure 2.8 UV-Vis spectroscopy of U: [EtPy][CF₃COO] mixture (after 24h) in water.

2.3.3 Liquid Chromatograph-Mass Spectroscopy Analysis

An ESI-MS analysis of the solution of uranium nitrate in water is shown in Figure 2.9. The peaks at 270 (m/z), 287 (m/z) and 305 (m/z) were a result of the uranium ion and its association with the hydroxyl ions in water. In the MS of 1:1 U: [EtPy][BF₄] (Figure

2.10), 108 (m/z) is the molecular ion peak of the N-ethyl pyridinium cation. The small peak at 418 (m/z) may result from the anion exchange between uranium nitrate and [EtPy][BF₄] (equation 2). This anion exchange mechanism has been suggested by Jenson (2003) as a useful application of IL for the extraction of metal ion.

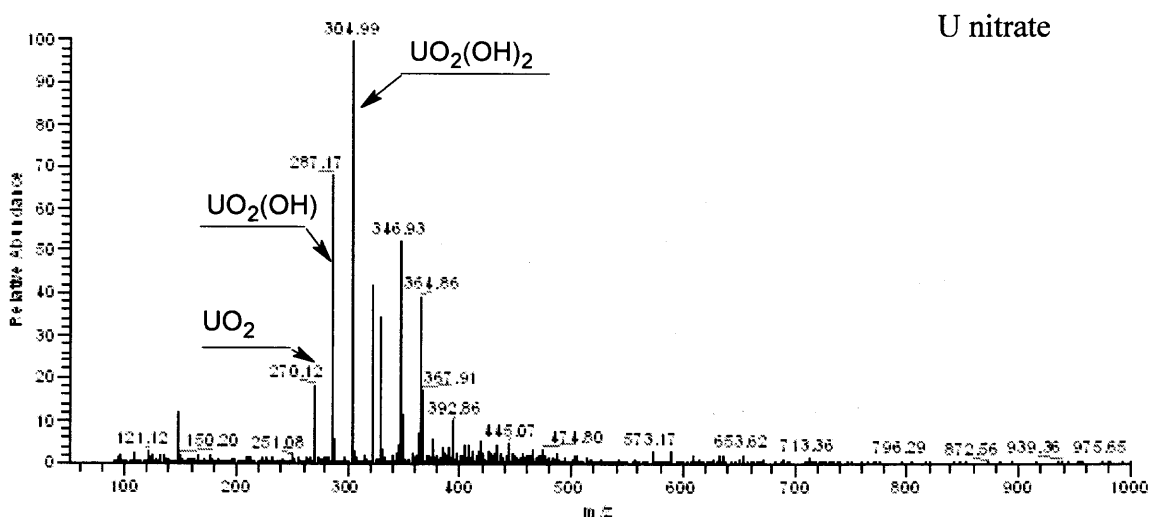
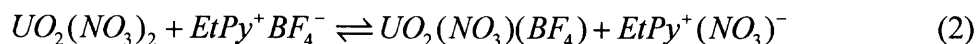


Figure 2.9 Mass spectroscopy of uranium nitrate in water at positive mode.

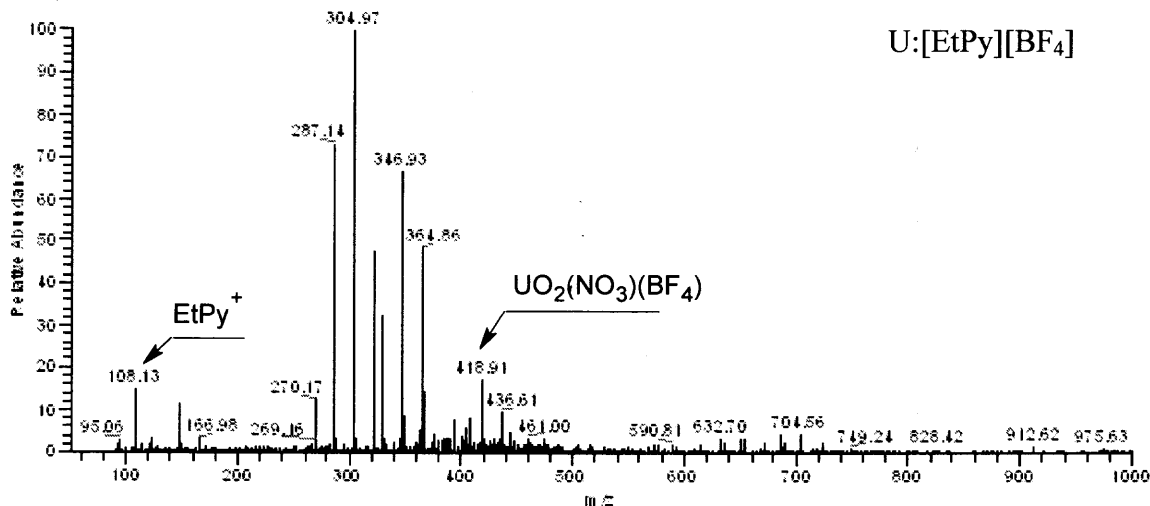


Figure 2.10 Mass spectroscopy of 1:1 U: [EtPy][BF₄] mixture at positive mode.

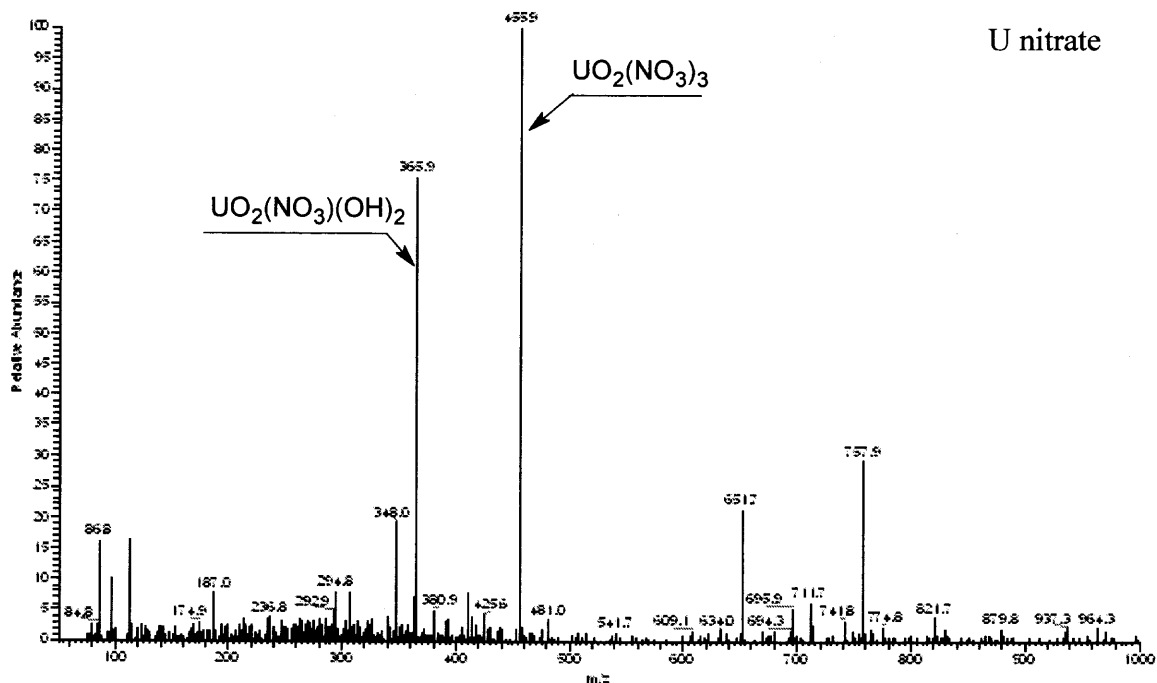


Figure 2.11 Mass spectroscopy of uranium nitrate in water at negative mode.

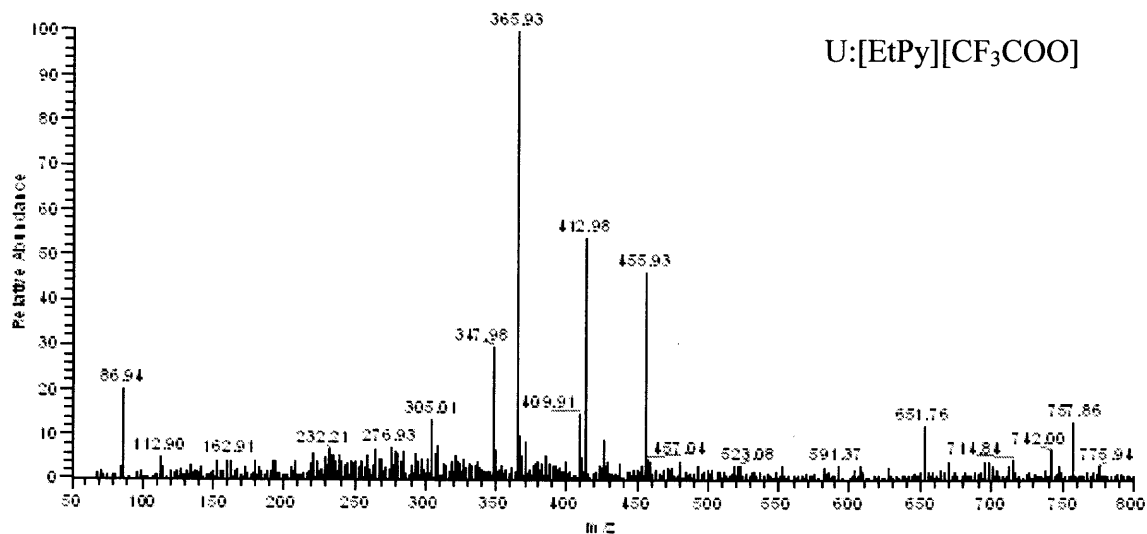
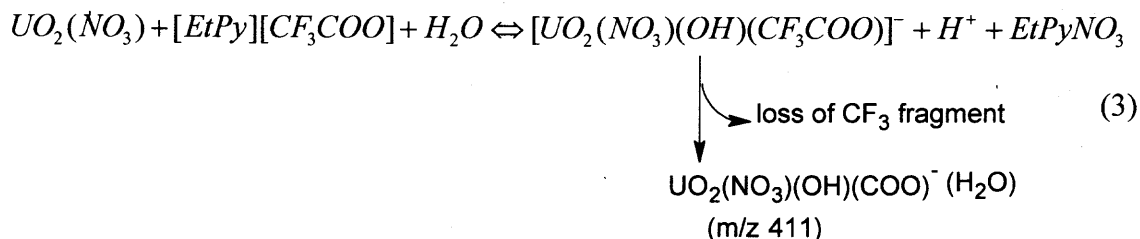


Figure 2.12 Mass spectroscopy of 1:1 U: [EtPy][CF₃COO] mixture at negative mode.

U:[EtPy][CF₃COO](1:1) mixture shows difference at negative mode. We believe that the appearance of 413 (m/z) is due to reaction below:



Water in the aqueous solution of U and [EtPy][CF₃COO] mixture also seems to play an important role. Therefore, besides the anion exchange, water is part of the reaction. The release of H⁺ causes a decrease in pH during complexation. Also, there is no difference between 1:1 and a 1:2 mixture, which demonstrates that an increase in the [EtPy][CF₃COO] concentration does not impact the complex structure.

2.3.4 X-ray Absorption Near-edge Spectroscopy Analysis (XANES)

XANES analysis measures the oxidation state of the central atom by determining the shift in the absorption edge as compared to a known standard. Analysis of the absorption edge energies for U(VI)-nitrate and mixtures of U(VI)-nitrate and various ILs reveals that the absorption edge energy (17175 eV) is the same (Figure 2.13). This confirms that the U(VI) added to the IL's solution was present as U(VI) species and not reduced to U(IV).

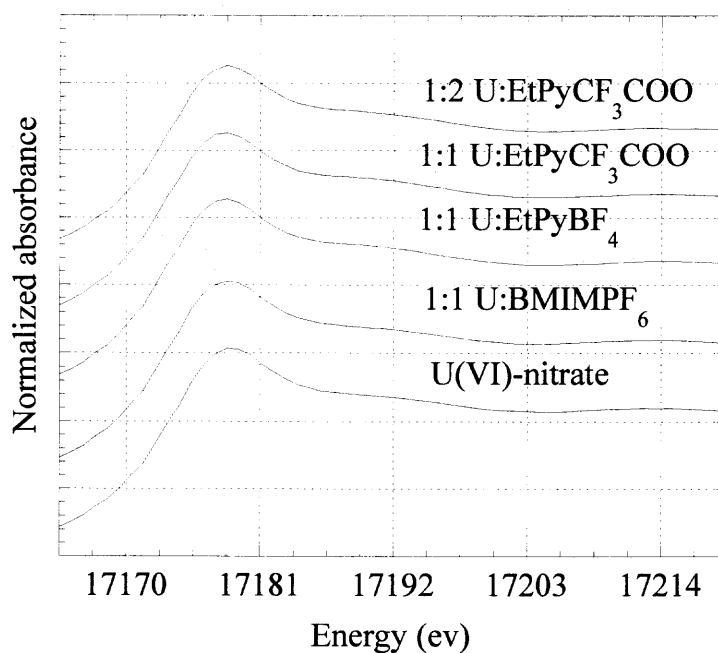


Figure 2.13 Normalized XANES spectra showing uranium nitrate and mixture of U:[BMIM][PF₆], U:[EtPy][BF₄] and U:[EtPy][CF₃COO].

2.3.5 Extended X-ray Absorption Fine Structure (EXAFS) Analysis

Extended X-ray absorption fine structure (EXAFS) spectroscopy measures the X-ray absorption as a function of energy and determines the local arrangement of atoms around a given absorbing atom. Analysis of the EXAFS data yields the type, the number of neighboring atoms, and the distance from the scattering atom.

EXAFS measurements at the complexed metal absorption edge can distinguish differences between functional groups. A number of uranium EXAFS studies have investigated the interaction between U (VI) and different ligand solutions (Allen, et al., 1995, 1996, 1997; Dodge, et al., 2002, 2003).

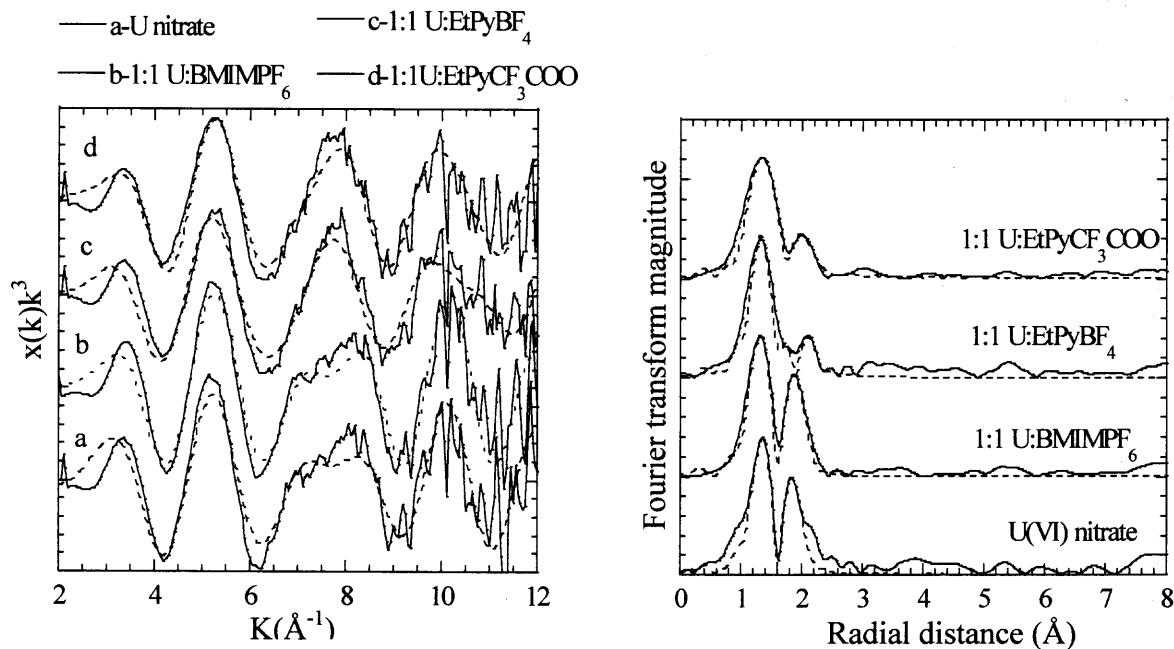


Figure 2.14 Experimental EXAFS oscillation of uranium and different ILs mixtures. experimental data (-); fitted data(--).

Table 2.2 EXAFS structure parameters for uranium and ILs mixtures.

| Sample | pH | Atom | N | R(Å) | σ^2 | <i>r</i> factor |
|--|------|-------------------|---------|-----------|-------------|-----------------|
| Uranium Nitrate(aq) | 1.66 | U-O _{ax} | 2 | 1.77±0.01 | 0.002±0.001 | 0.11 |
| | | U-O _{eq} | 4.5±1.5 | 2.33±0.01 | 0.006±0.002 | |
| U:[BMIM][PF ₆] (1:1) | 1.54 | U-O _{ax} | 2 | 1.75±0.01 | 0.001±0.001 | 0.06 |
| | | U-O _{eq} | 4.9±1.4 | 2.35±0.01 | 0.005±0.001 | |
| U:[EtPy][BF ₄] (1:1) | 1.37 | U-O _{ax} | 2.6±0.3 | 1.76±0.01 | 0.002±0.001 | 0.08 |
| | | U-O _{eq} | 3.5±1.3 | 2.45±0.02 | 0.005±0.002 | |
| | | U-F | 1.4±0.4 | 2.22±0.01 | 0.004±0.002 | |
| U:[EtPy][CF ₃ COO] (1:1) | 1.24 | U-O _{ax} | 2.4±0.2 | 1.77±0.03 | 0.001±0.001 | 0.03 |
| | | U-O _{eq} | 4.6±2.5 | 2.40±0.03 | 0.014±0.003 | |
| | | U-C | 1.4±0.8 | 2.92±0.02 | 0.004±0.007 | |

(N) coordination number, (R) interatomic distance, (σ^2) disorder parameter and (*r* factor) reliability factor.

The best fit model for hydrated uranyl sample consists of 2 axial oxygen (O_{ax}) atoms at 1.78 Å and approximately 4 to 6 equatorial oxygen (O_{eq}) at 2.35Å, which would be the oxygen atom from the waters of hydration for the hydrated uranium (Kelly, et al., 2002; Antonio, et al., 2001). Table 2.2 shows the uranium in water consisted of 2 axial oxygen at $1.77 \pm 0.01 \text{Å}$, and $4.5 \pm 1.5 O_{eq}$ at 2.33Å (Figure 2.15a), which are consistent with anticipated values.

In the U:[BMIM][PF₆] mixture, the best-fit model is almost the same as the hydrated uranyl moiety. In our experiment, due to the low solubility of [BMIM][PF₆] in an aqueous solution, less PF₆⁻ is present in water, and no obvious complex formations were observed by EXAFS. Similar results were also obtained by Gillard and co-workers (2005). Their study found that due to the low complexation constant, the complex concentration was too small to get a detectable signal. However, the formation of a UO₂PF₆⁺ complex was evidenced by time resolved emission spectroscopy (TRES). They also suggested that PF₆⁻ could form a complex with a uranyl ion as shown below (Figure 2.15b), and with a hydrogen bond associated with a water molecule thus forming a six member ring. TRES also shows that PF₆⁻ dissociation leads to the formation of F⁻, which can interfere in the complexation process by forming a stronger fluorinated ligand with uranyl. These phenomena were not observed in this study.

The data clearly confirmed the complex formed between U and [EtPy][BF₄] and [EtPy][CF₃COO]. Compared to UO₂²⁺_{aq}, slight differences are observed on the EXAFS oscillation between 6 and 9Å⁻¹ for the samples of U:[EtPy][BF₄] and U:EtPy][CF₃COO]. The axial oxygen atoms (O_{ax}) are not influenced by the ligands as indicated by the identical peak height in the FT's. However, the shape and height of the equatorial oxygen

are strongly influenced by complexation with weak coordination ligands (BF_4^- and CF_3COO^-). In a 1:1 $\text{U}:[\text{EtPy}][\text{BF}_4]$ mixture, 3.5 ± 1.3 equatorial oxygen were found at $2.45 \pm 0.02 \text{ \AA}$. 1.4 ± 0.4 fluoride atom were found at $2.22 \pm 0.01 \text{ \AA}$, which suggested the presence of a monodentate complex. Similar results were obtained in Gaillard's study (2005), where UO_2BF_4^+ complex, $\text{U}-\text{O}_{\text{eq}}$ was at 2.45 \AA , and the $\text{U}-\text{F}$ bond length was present at $2.24 \pm 0.02 \text{ \AA}$. As suggested by Gaillard, the boron atom could not be detected due to its low backscattering amplitude. There is no obvious U peak appears between 3 and 4 \AA indicating that the complex is mononuclear. These results, combined with the data obtained by MS, suggest that the structure of a uranyl ion and $[\text{EtPy}][\text{BF}_4]$ may be as demonstrated in Figure 2.15c.

In the $\text{UO}_2(\text{OH})_2(\text{CF}_3\text{COO})^-$ complex $2.4 \pm 0.1 \text{ O}_{\text{ax}}$ were found at $1.77 \pm 0.03 \text{ \AA}$ with $4.6 \pm 2.5 \text{ O}_{\text{eq}}$ atom at $2.40 \pm 0.03 \text{ \AA}$, and $1.4 \pm 0.8 \text{ C}$ atom at $2.92 \pm 0.02 \text{ \AA}$, which is similar to the $\text{U}-\text{C}$ length found in the 1:1 uranyl acetate complex (1.3 C at 2.91 \AA) (Jiang, et al., 2002). Normally for a monodentate complex the O_{eq} distance is from 2.30 to 2.35 \AA , whereas for bidentate carboxylate bonding the distance is from 2.40 to 2.50 \AA . Hence, the 2.40 \AA in our experiment is typical of a bidentate complex. Due to the low complex constant or to the dissociation of $[\text{EtPy}][\text{CF}_3\text{COO}]$, a further addition of $[\text{EtPy}][\text{CF}_3\text{COO}]$ would not change the U complex formation. Based on the ESI-MS and EXAFS results we propose the following structure (Figure 2.15d):

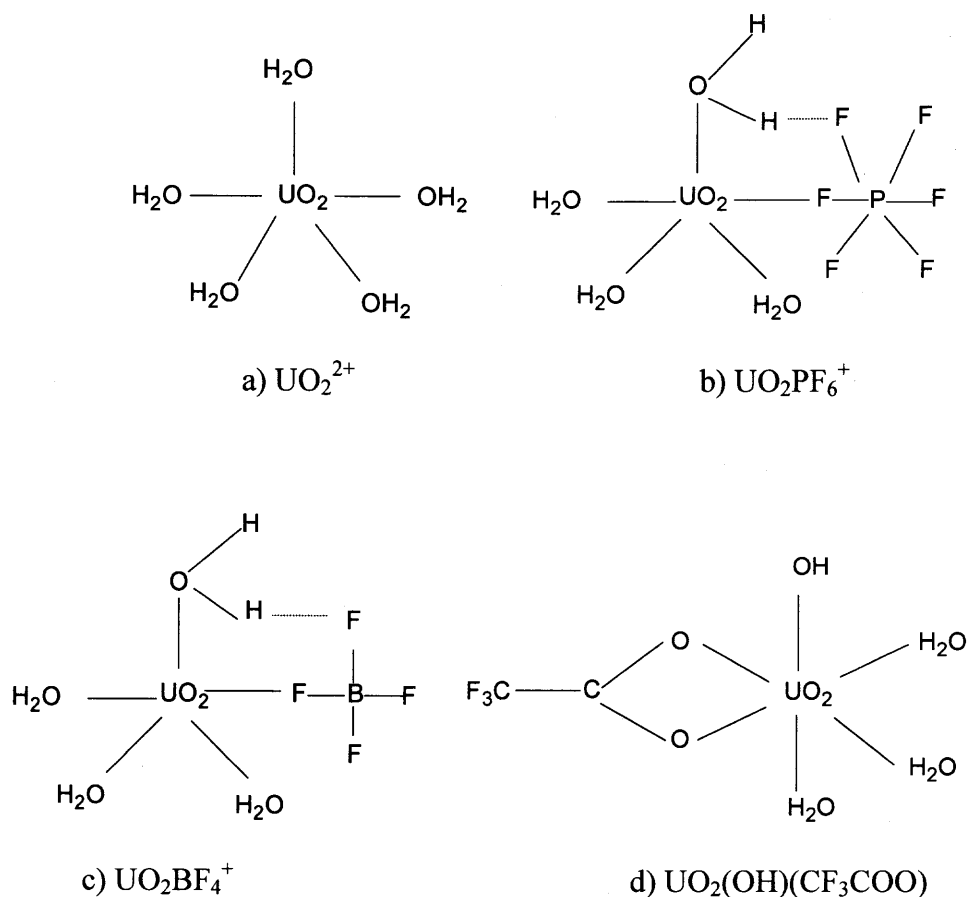


Figure 2.15 Proposed structures of U-ILs complexes.

2.4 Summary

We have combined various analytical techniques to identify the complexes formed between uranyl and three ionic liquids: [EtPy][BF₄], [EtPy][CF₃COO], and [BMIM][PF₆]. A Monodentate complex formed between uranyl and BF₄⁻, PF₆⁻, while a bidentate complex formed between uranyl and the CF₃COO anion. Acid release and UV absorbance changes were only observed in the mixture of U and [EtPy][CF₃COO].

Our study demonstrates the interactions between uranium and ionic liquids under acidic conditions in order to provide useful information about the applications of ILs as solvents for use in uranium extraction processes. We show that the complexability of ILs

with uranium could cause a loss of hydrophobic ionic liquids to an aqua phase. Additionally, our study confirms the possibility for specifically-designed ILs to be used not only as solvents but also as chelating agents in metal extractions.

CHAPTER 3

EFFECTS OF IONIC LIQUIDS ON THE GROWTH OF *Clostridium* sp.

3.1 Introduction

Ionic liquids possess various antimicrobial activities against strains of Gram-positive and negative bacteria and fungi. The antimicrobial activity is dependent on alkyl chain lengths, ILs with a longer alkyl chain show greater activity (Pernak, et al., 2003). Most studies are focused on imidazolium-based ionic liquids, and under aerobic conditions. On the other hand, pyridinium-based ionic liquids represent another group of commonly used ILs. *Clostridium* sp. is a Gram-positive, spore-forming rod. These anaerobic bacteria are ubiquitous in nature and are most often found in soil and wastes. They play an important role in the bioremediation of uranium waste. In order to investigate the effects of U-ILs complex on uranium biocatalysis, it is important to test the toxicity of ILs on the *Clostridium* sp.

3.2 Materials and Methods

3.2.1 Culture Conditions

A gram-positive, rod shaped, spore-forming, nitrate reductase-negative, N₂-fixing *Clostridium* sp. (ATCC 53464) was isolated from coal-cleaning residues. This bacterium is able to reduce Fe(III) to Fe(II), Mn(IV) to Mn(II), Tc(VII) to Tc(IV), and U(VI) to U(IV) (Francis, et al., 1994; 1998; 2002). The organism was grown in a medium composed of glucose (5.0g), glycerol phosphate (0.3g), MgSO₄·7H₂O (0.2g), FeSO₄·7H₂O (2.8mg), CaCl₂·2H₂O (0.5g), peptone (0.1g), yeast extract (0.1g), and

distilled water (1,000ml) (pH 6.8). The medium was pre-reduced by boiling and purging with N₂ gas for 15min to remove the dissolved oxygen. It was then cooled under N₂ atmosphere in an anaerobic glove box and dispensed in 40ml quantities into 60ml serum bottles. The bottles were closed with butyl rubber stoppers, sealed with aluminum caps, and then autoclaved. At the beginning different concentrations (0.1%, 0.5%, 1%, 2% (V/V)) of various ILs were added to the medium. The medium was inoculated with 2 ml of an 18h old culture (OD₆₀₀ 0.64) and incubated at 24 °C. The growth of bacteria was measured at 600 nm using a Bausch and Lomb Spectronic-20 spectrophotometer.

3.2.2 Chemicals

Ionic liquids were purged by N₂ for 10 min before use. Deionized water was pre-reduced before use by boiling while purging with ultra-high-purity N₂. All solutions and chemicals were maintained under anaerobic condition until further use.

3.2.3 Analysis of Total Gas Production and pH

A gas gauge measured the total volume of gas in the head space of the intact sample bottles. Two milliliter samples were withdrawn at different times after filtration by a 0.45µm filter, the pH levels were measured by a pH electrode.

3.2.4 Liquid Chromatograph-Mass Spectroscopy Analysis

The integrity of ILs was monitored by LC-MS. For each analysis 10 microliters of the sample were diluted in 1 ml water. (See previous description of the instrumentation information.)

3.3 Results and Discussion

3.3.1 Effects on Optical Density

With [BMIM][PF₆], the optical density decreased with the increase in [BMIM][PF₆] concentration (Figure 3.1A). Similar results were also seen with [EtPy][BF₄] and [EtPy][CF₃COO] (Figure 3.1 B and C). Compared with [EtPy][BF₄], the two ionic liquids, [BMIM][PF₆] and [EtPy][CF₃COO], showed more inhibitory effects on the growth of *Clostridium* sp. With the addition of 0.1% of [BMIM][PF₆] or [EtPy][CF₃COO], the optical density decreased to 0.3 and 0.25 respectively. As the concentration of these two ionic liquids increased beyond 0.5% (V/V), no growth of bacteria was observed.

3.3.2 Effects on pH Change

Clostridium sp. consumes glucose as its carbon source and produces organic acids (acetic acid and butyric acid) during incubation (Francis, et al., 1994). After 48 hours of incubation, the pH of the medium decreased from 5.5 to 3. However, in presence of various ionic liquids, the growth was inhibited, and even less glucose was consumed, thus less acid was produced. As a result, the pH changed slowly in all ILs treated medium (Figure 3.2). The decrease in pH during incubation at high concentrations of ionic liquids could be due to the hydrolysis of ionic liquids.

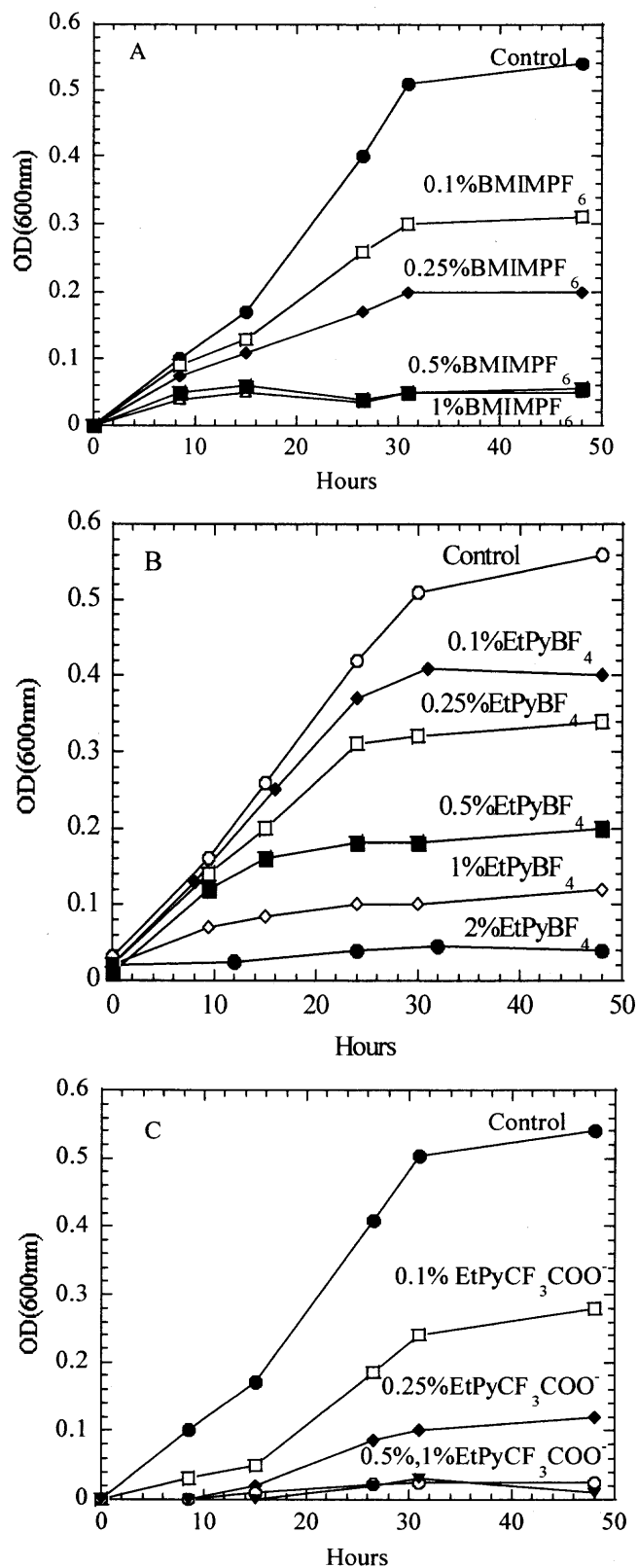


Figure 3.1 Effects of ionic liquids on optical density (A) [BMIM][PF₆]; (B) [EtPy][BF₄]; (C) [EtPy][CF₃COO].

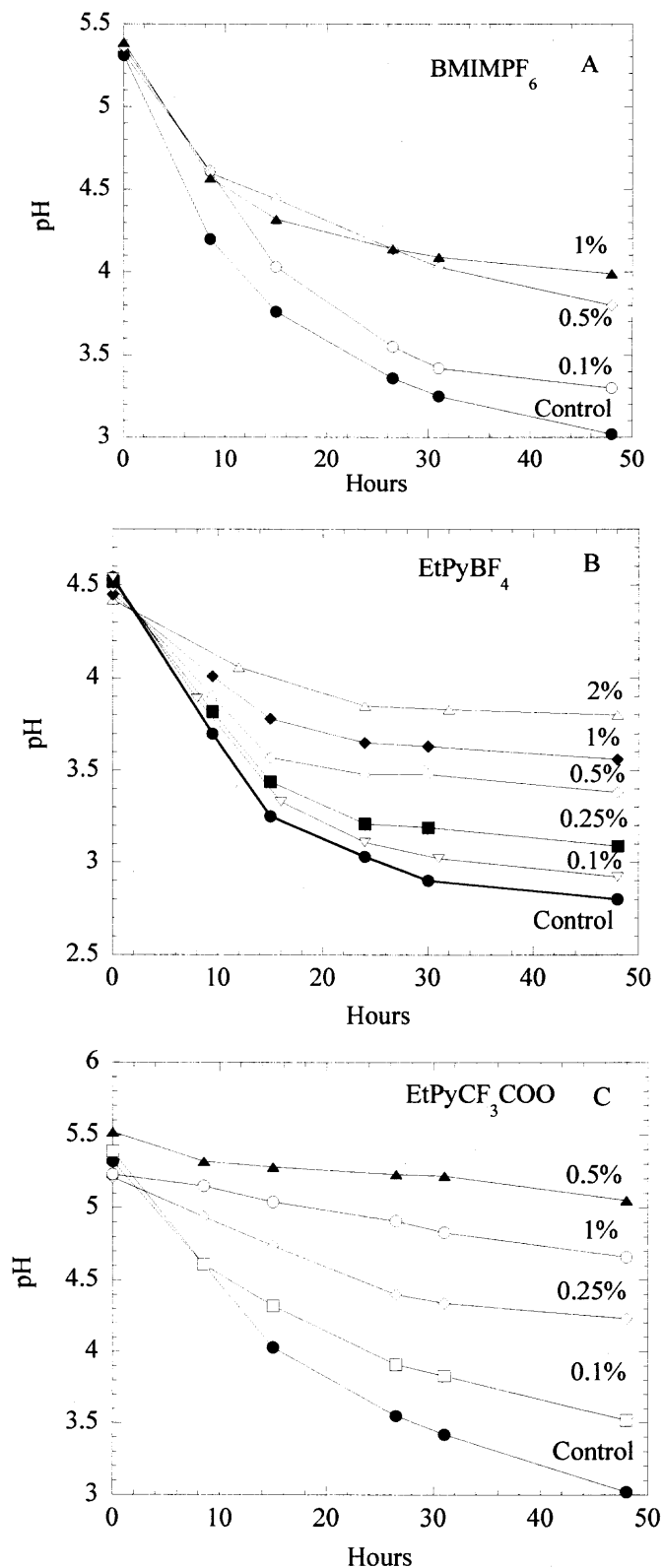


Figure 3.2 Effects of ionic liquids on pH change during incubation (A) [BMIM][PF₆]; (B) [EtPy][BF₄]; (C) [EtPy][CF₃COO].

3.3.3 Effects on Gas Production

During incubation, *Clostridium* sp. produced a large amount of CO₂, H₂. In the presence of different ionic liquids, the growth was retarded and less gas was produced (Figure 3.3). Little growth was observed in the presence of 2% of [EtPy][BF₄]. (About 4ml of gas was produced after 48h incubation.) Whereas with the addition of 0.5% of [EBMIM][PF₆] and [EtPy][CF₃COO], there were only 2 ml and 0 ml of gas produced respectively. These results confirm the high inhibitory effects of ILs on *Clostridium* sp.

3.3.4 Studies on Morphology of Bacteria



Figure 3.4 Effects of [EtPy][BF₄] on bacteria morphology change.

After treatment with ionic liquids, the shape of *Clostridium* sp. stayed almost the same. However, the reaction to the Gram Stain was different. The bacterial cells were poorly stained in the presence of IL. Also fewer spores were visible. As described in Coulding's study (2006), ILs are similar to cationic surfactants, which have a hydrophobic head (cation with long alkyl chain) and a hydrophilic tail (water soluble anion). These surfactants are known to induce polar necrosis due to their ability to be incorporated into biological membranes.

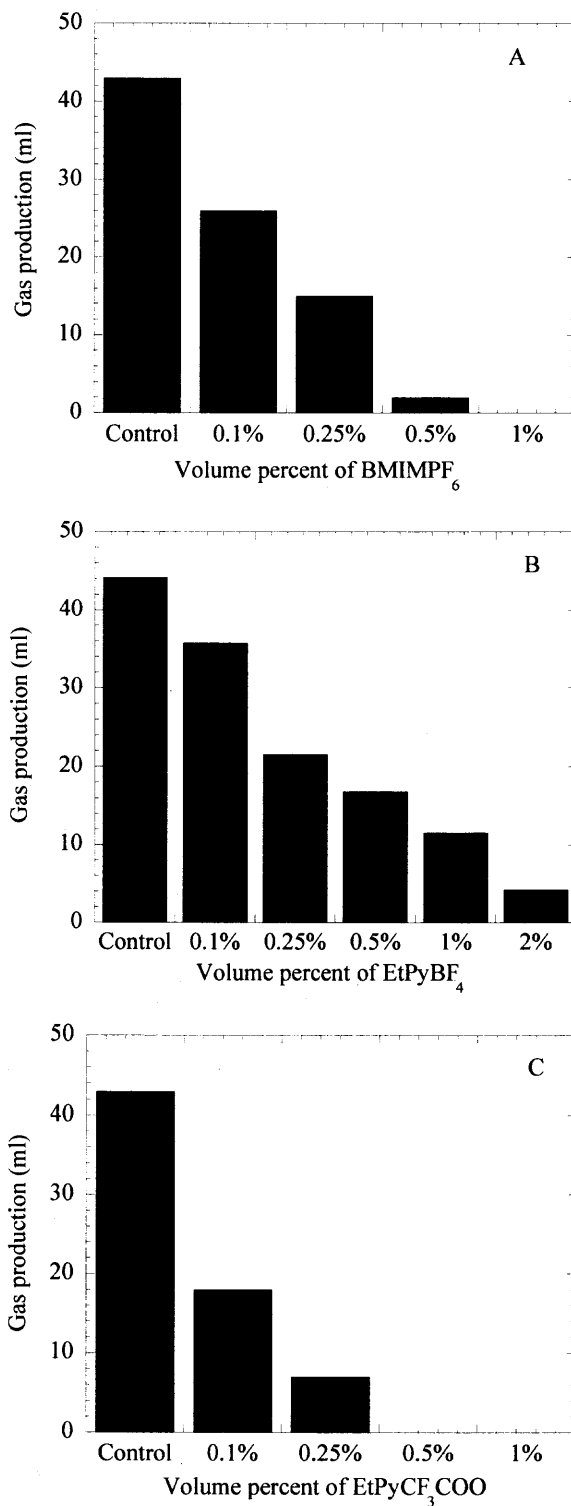


Figure 3.3 Effects of ionic liquids on gas production during incubation (A) [BMIM][PF₆]; (B) [EtPy][BF₄]; (C) [EtPy][CF₃COO]

3.3.5 Mass Spectroscopy Analysis of ILs Integrity

Ionic liquids were monitored by ESI-MS to test their integrity during incubation. Cations were monitored at positive mode, and anions were shown at negative mode. A medium without the addition of ionic liquids was used as background.

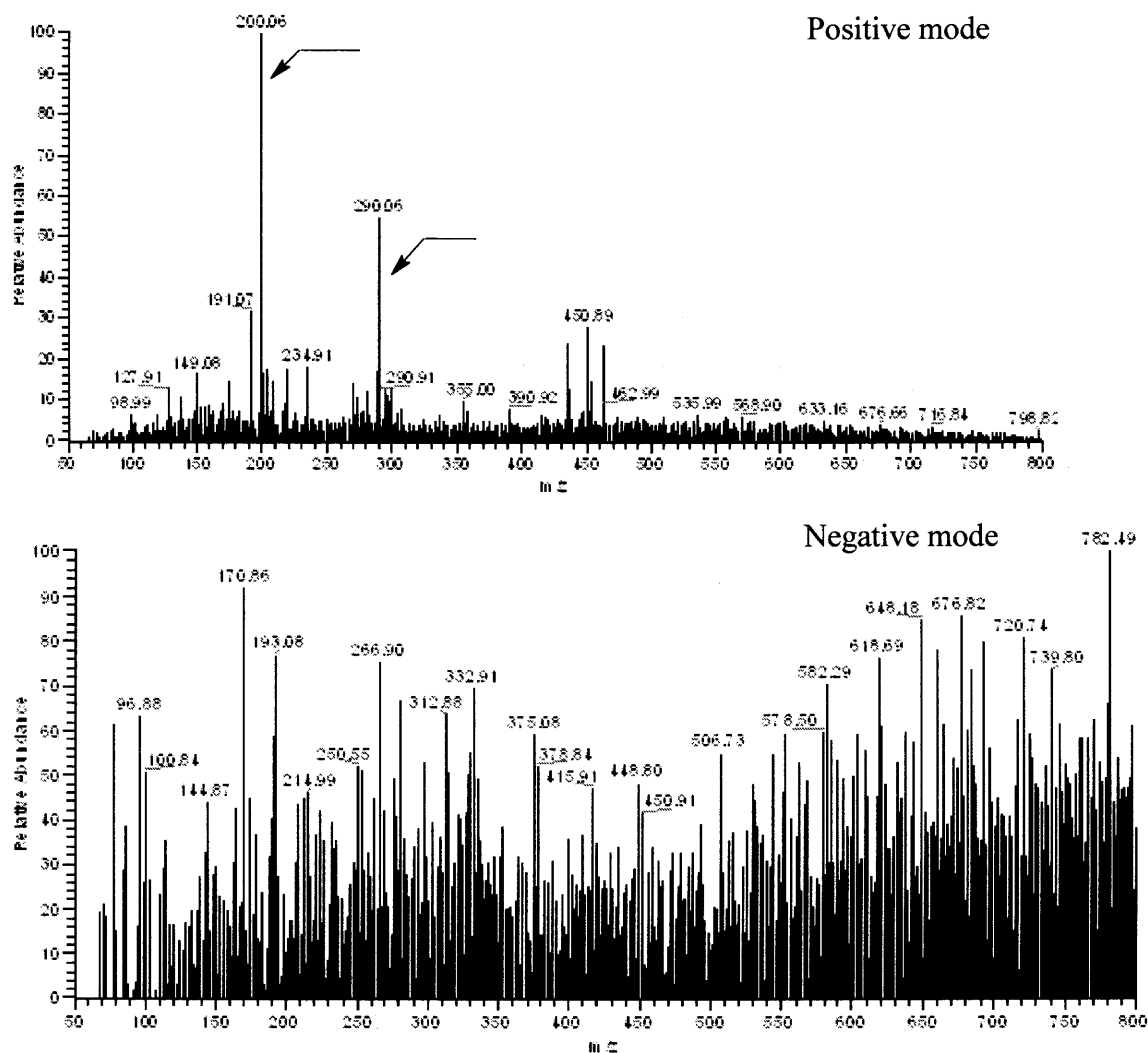


Figure 3.5 Mass spectrometry of control medium at 0h.

Figure 3.5 shows the MS of pure medium before incubation. At positive mode, peaks of 200 (m/z), and 290 (m/z) were due to the medium components. The MS of the negative mode is noisy because no anion is predominant.

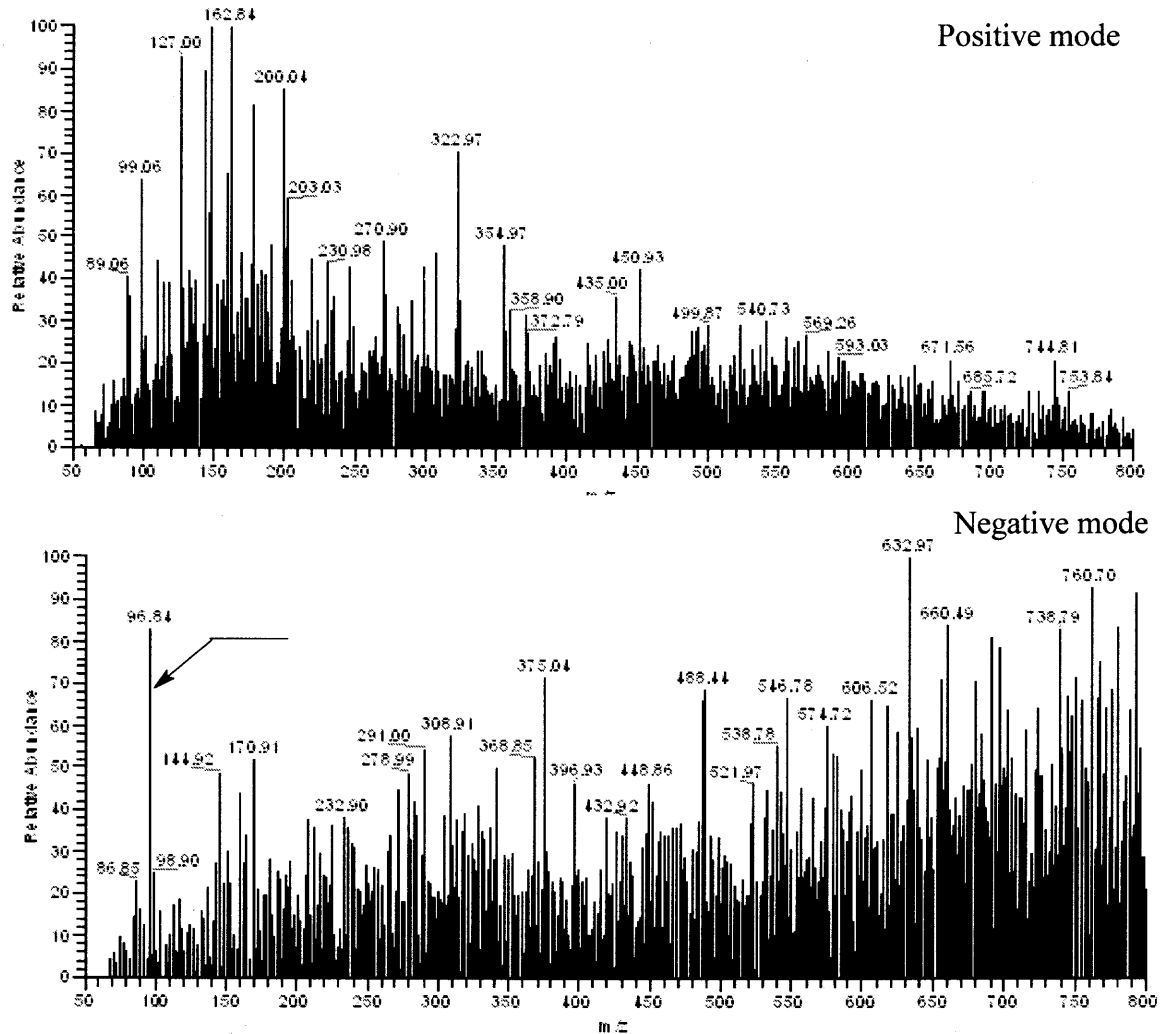


Figure 3.6 Mass spectrometry of control medium after 48h incubation.

Figure 3.6 shows the medium after 48 hours of incubation. At the positive mode, two major components were consumed by microorganisms and produced series of metabolites. No much change at negative mode was observed. The 96.8 (m/z) peak was due to the medium component containing SO_4^{2-} .

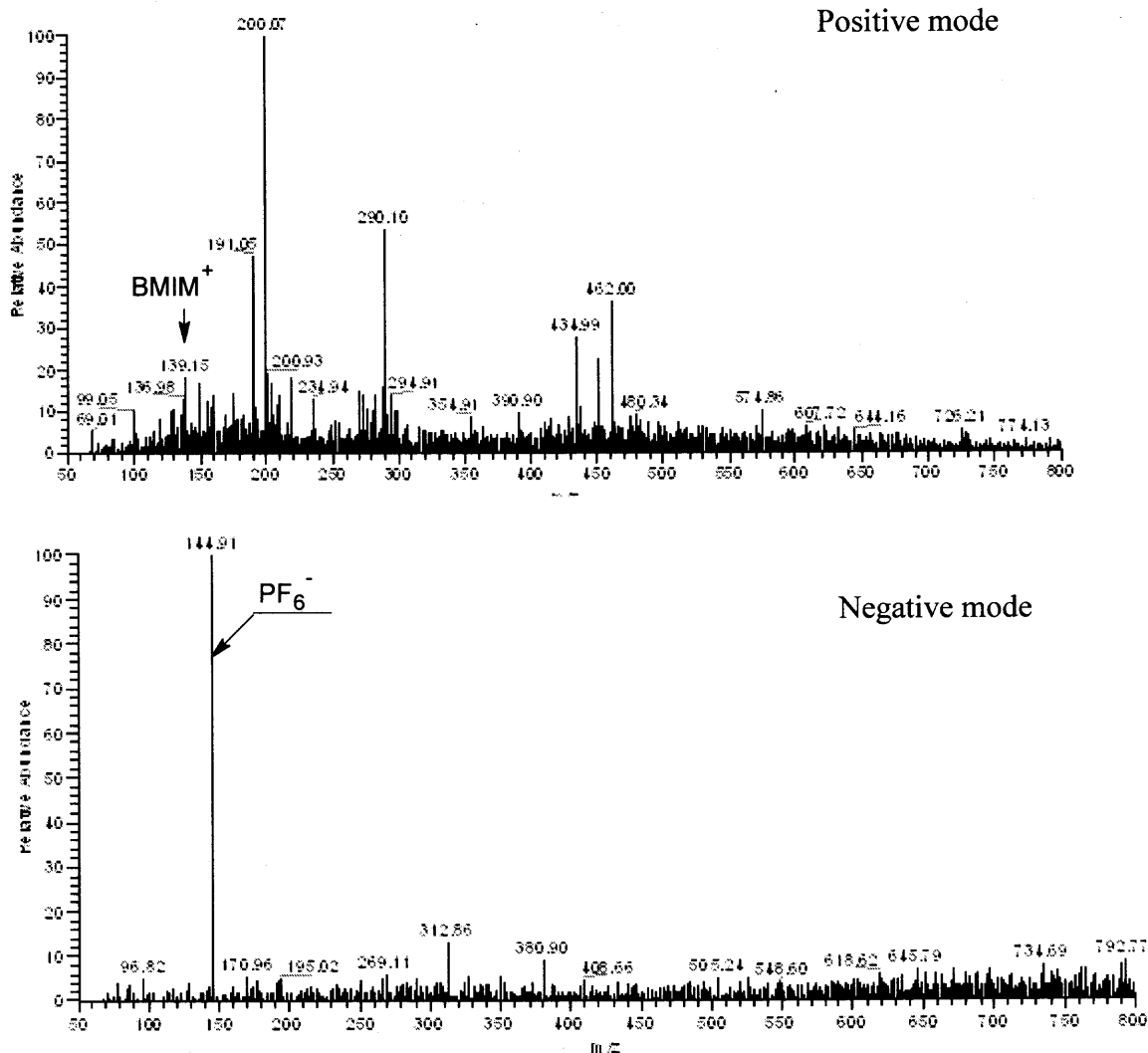


Figure 3.7 Mass spectroscopy of medium containing [BMIM][PF₆] (0.1%) at 0h.

With the addition of 0.1% [BMIM][PF₆] to the medium, the BMIM⁺ cation (139 m/z) was shown at positive mode. PF₆⁻ anion became predominant at negative mode, giving a peak of 144.9 (m/z).

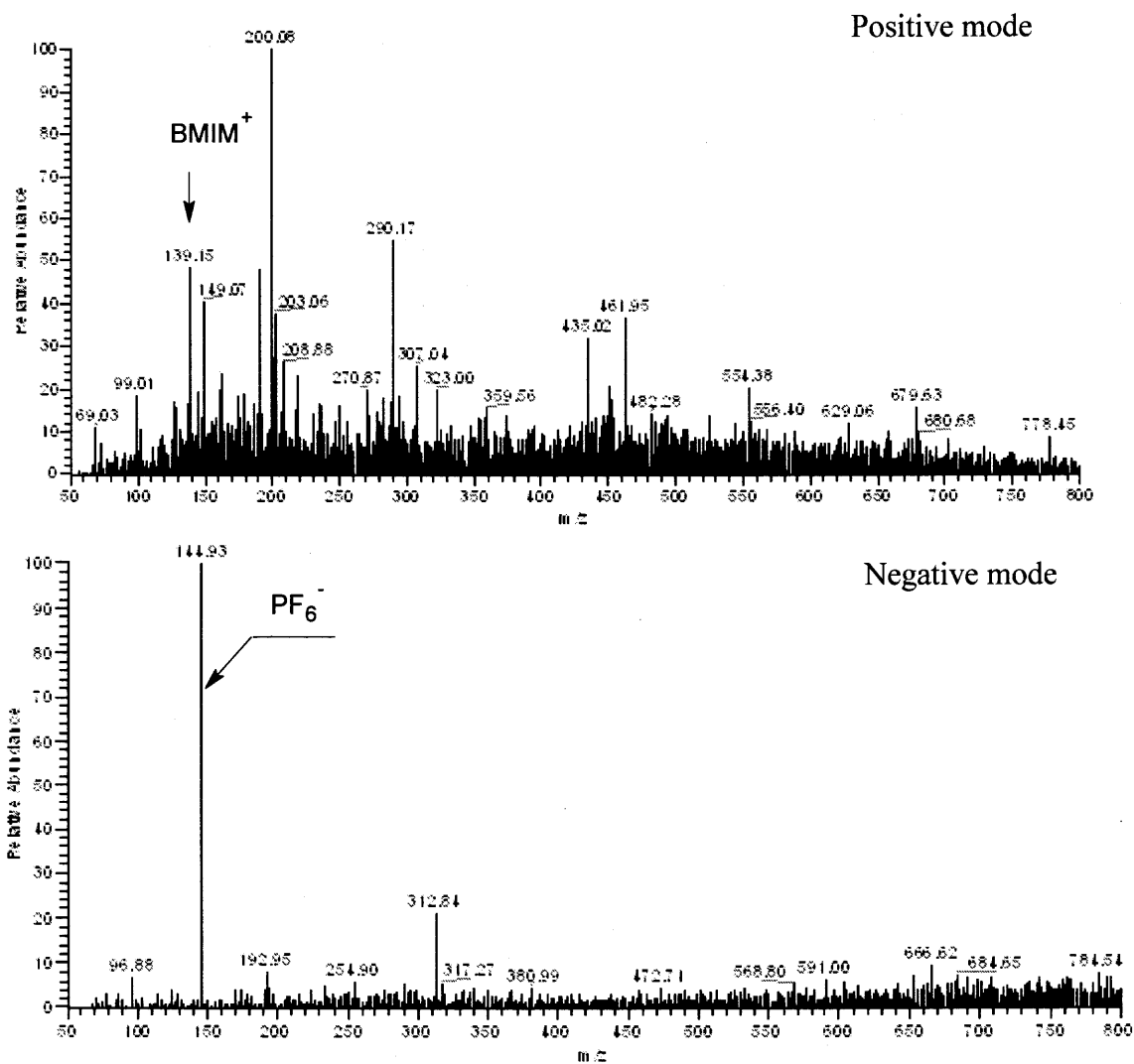


Figure 3.8 Mass spectroscopy of medium containing [BMIM][PF₆] (0.1%) after 48h incubation.

After a 48h incubation, the cation and anion remain the same. No degradation products were observed.

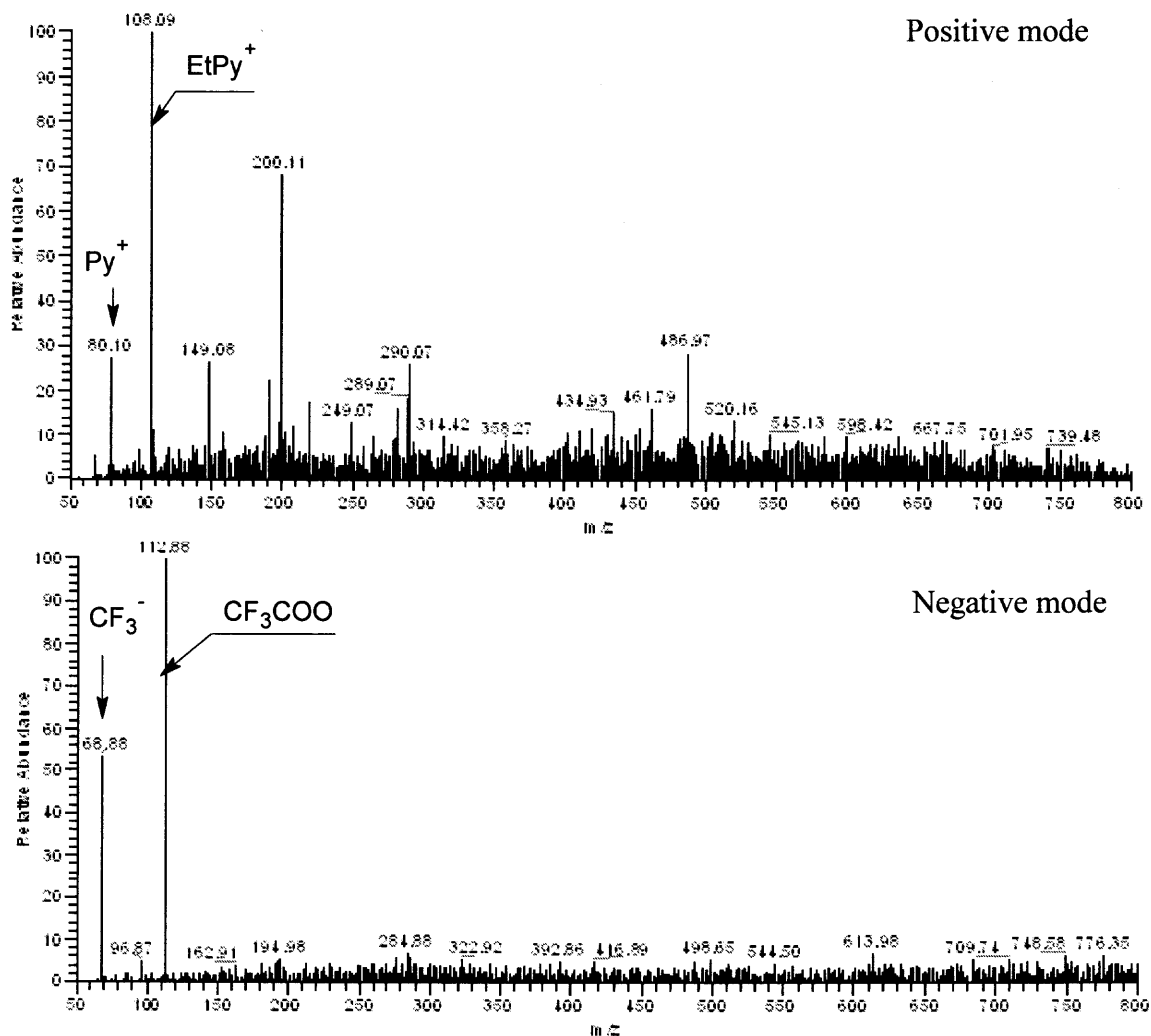


Figure 3.9 Mass spectrometry of medium containing [EtPy][CF₃COO] (0.1%) at 0h.

Under positive mode, the addition of 0.1% [EtPy][CF₃COO] gives EtPy⁺ peak at 108 (m/z), and Py⁺ fragment at 80 (m/z). Under negative mode, a peak at 113 (m/z) was due to the CF₃COO⁻ anion, and a peak at 69 (m/z) was due to the fragment of CF₃⁻.

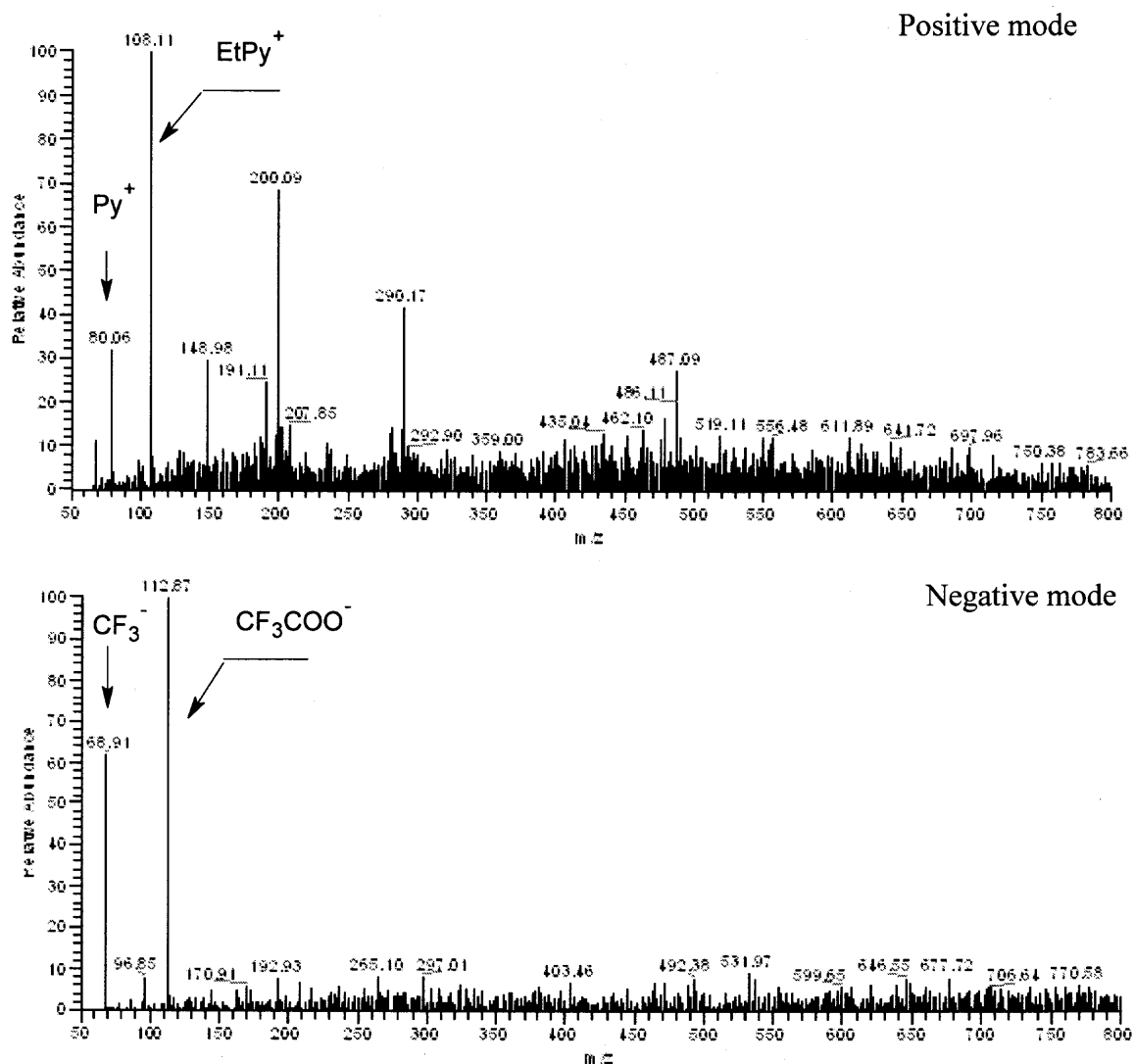


Figure 3.10 Mass spectroscopy of medium containing [EtPy][CF₃COO] (0.1%) after 48h incubation.

Both the anion and the cation were intact after 48h of incubation with *Clostridium*.

sp.

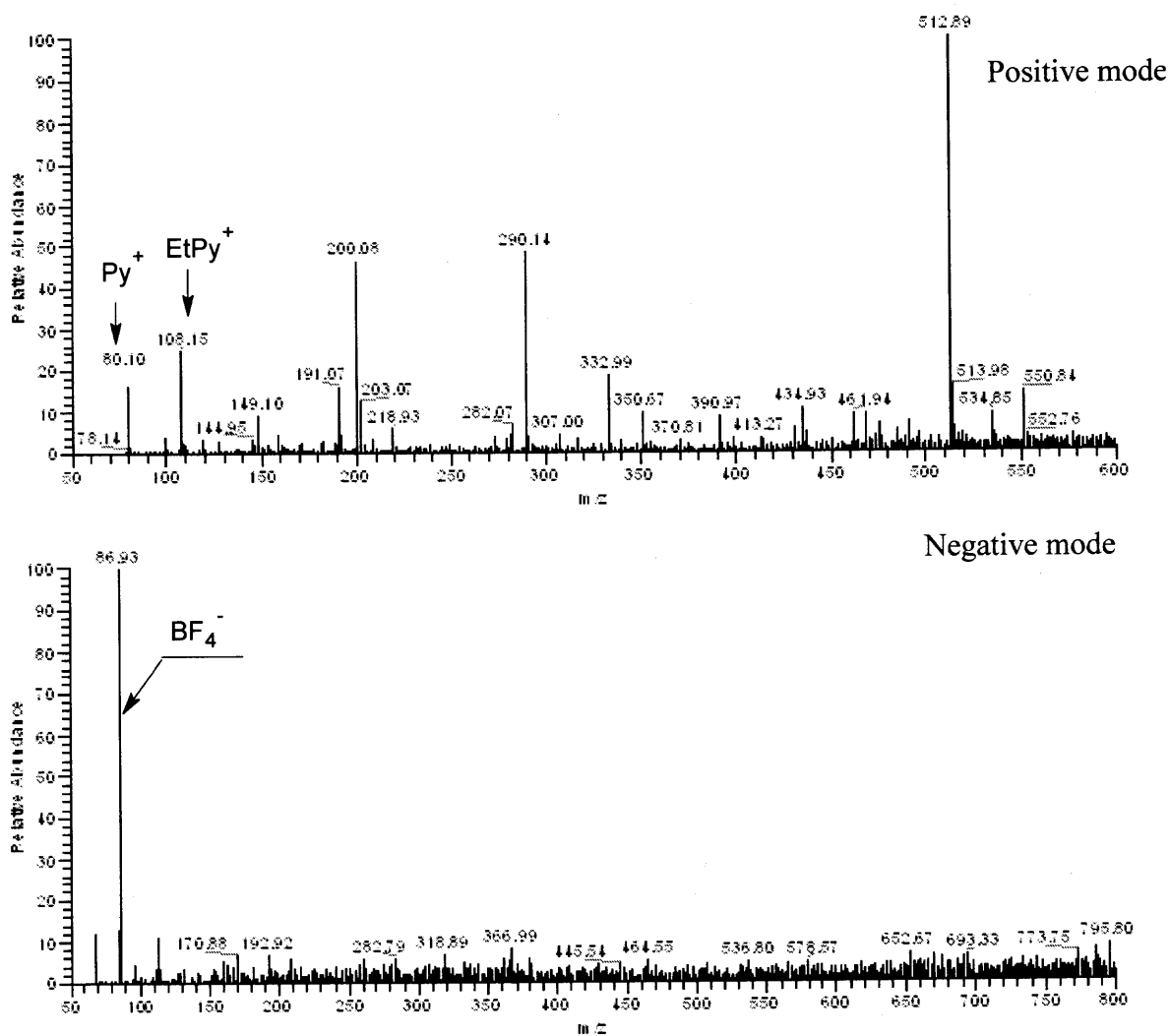


Figure 3.11 Mass spectrometry of medium containing [EtPy][BF₄] (0.1%) at 0h.

Except for the cation peak of 108 (m/z), there is new peak 512.89 (m/z) revealed in the MS under positive mode. This may be due to interference between the ionic liquid and the medium component thus forming a cluster in solution. BF₄ anion was detected under negative mode at 86.93 (m/z).

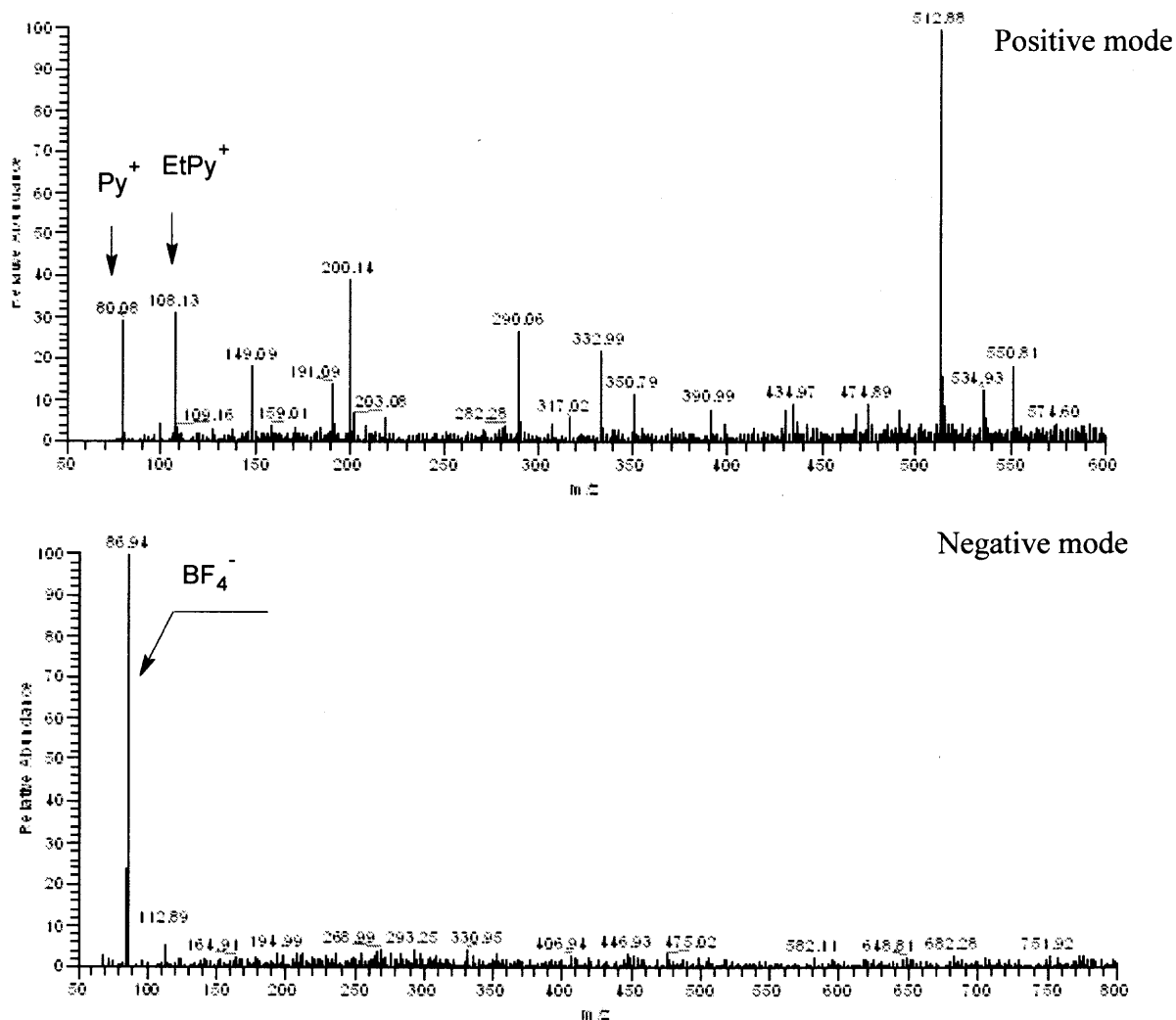


Figure 3.12 Mass spectrometry of medium containing [EtPy][BF₄] (0.1%) after 48h incubation.

After 48h of incubation with *Clostridium* sp., no degradation products of ionic liquids were observed.

3.4 Summary

All three ionic liquids affected the growth of *Clostridium* sp. as evidenced by the decrease in optical density and changes in pH and gas production. Among these ionic liquids, [BMIM][PF₆] and [EtPy][CF₃COO] showed higher toxicity than [EtPy][BF₄]. This observation is similar to Couling's study (2006), which showed that toxicity increased as the number of nitrogen atoms increased. The anion may also play an important role in the toxicity of the compounds studied here (i.e. CF₃COO⁻ and BF₄⁻). Moreover, the presences of positively charged atoms on the anion are predicted to increase toxicity slightly (Couling, et al., 2006). For example, monatomic anions such as bromide and chloride are predicted to be less toxic than larger anions containing a region of positive charge, such as bis(trifluoromethanesulfonyl)imide. Therefore, it is important to understand the relationship between structures and the biological effects of different types of cations and anions in ionic liquids.

CHAPTER 4

EFFECTS OF IONIC LIQUIDS ON URANIUM BIOSORPTION

4.1 Introduction

The biosorption of metal by various biological materials is through physico-chemical interactions or metabolically mediated pathways of uptake. Algae, bacteria, fungi and yeasts have shown to be potential metal biosorbents. The major advantages of biosorption include: low cost, high efficiency rate, minimization of sludge generation by chemical and biological treatment processes, no requirement for additional nutrients, and the regeneration potential for biosorbents and metal.

The mechanism of biosorption can be classified according to the following criteria: (1) biosorption can be metabolism-dependent or non- metabolism dependent; (2) depending on the location of metal, it can be extracellular accumulation/precipitation; cell surface sorption and/or intracellular accumulation. The non-metabolism dependent biosorption is mostly by physico-chemical interactions between metal and the functional group present on the microbial cell surface. The functional groups will include carboxyl, sulfate, phosphate hydroxyl and/or amino groups. The mechanism includes physical adsorption, ion exchange, and chemical sorption.

It is well known that microorganisms accumulate on uranium and play an important role in regulating the mobility of uranium in the environment. There have been extensive studies done on the accumulation of bacteria on U(VI) (Kelly, et al., 2002; Francis, et al., 2004; Gorman, et al., 2005). This accumulation depends on the chemical species extant at different conditions. For example, between pH 3 and 5, U(VI) exists

mostly as UO_2^{2+} ; while above pH 7, the most prevalent species is uranium carbonate. At a high pH, it may also precipitate as uranium hydroxide (Ohnuki, et al., 2005).

However, the effects of ligands on metal-ion sorption by microorganisms are still not clearly understood. They either increase or decrease the metal-ion sorption capabilities depending on the stability of the complex (Yoshida, et al., 2005), bioavailability of organic ligands (Sakamoto, et al., 2005), pH (Suzuki, et al., 2005), and even the competition of inorganic cations (Ozaki, et al., 2006).

Since we have been investigating the effects of ionic liquids on complex formation with uranyl ion and the growth of *Clostridium* sp., we are also interested in the sorption behavior of the U(VI) and U-ILs complex by *Clostridium* sp. Results from this study are reported here.

4.2 Materials and Methods

4.2.1 Preparation of Uranium and Ionic Liquids Mixture

Uranium nitrate 0.25 mM and 1% of ionic liquid were mixed in 50 ml of sterilized de-ionized water. The ionic strength of this mixture was adjusted to 20 mM by KCl. The solution was left overnight to reach equilibrium and then adjusted to pH 3.5 by adding HCl or KOH.

4.2.2 Biosorption Experiment

Cells for the adsorption experiments were grown by first inoculating them with 2ml of 18h old culture, and then incubating them for 18 hours at 26°C. The cells were harvested after they reached an early stationary growth phase (by centrifugation at 5000g, for

25min). After the measurement of the wet weight of the cell pellet, 5 ml of pH 3.5 sterilized 20 mM KCl solution were added to the centrifuge tube to re-suspend the cell. The cells were treated as follows: (i) cells + 0.25mM $\text{UO}_2(\text{NO}_3)_2$; (ii) cells + 0.25 mM U + 1% ILs. The samples were mixed and a 2 ml sample was withdrawn every 10 minutes for up to 30 minutes, after which samples were taken every 30 minutes for 2h. A control experiment (0.25 mM $\text{UO}_2(\text{NO}_3)_2$ alone) was also conducted 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.

4.2.3 Uranium Measurement

Each sample was filtered by a 0.45 μm syringe filter and diluted by filtered de-ionized water. After acidifying each with 2.5M sulfuric acid, samples were analyzed by KPA (Kinetic Phosphorescence Analysis, Chemchek inc.) for the uranium concentration in solution. The KPA's technology is based on the measurement of a sample's phosphorescence taken at selected time intervals to determine the precise concentration of the analyte.

4.2.4 Transmission Electro Microscopy (TEM) and Energy Dispersive X-ray Spectroscopy (EDS) Study

After a 2h incubation with uranium and ionic liquids, the solution was centrifuged at 5000g for 25min, and cells were collected. The solution was then washed two times with a 5 ml solution of 20 mM KCl (pH 3.5). The uranium in the washed solution was also analyzed by the KPA method. The cells were fixed by 2ml of 20mM KCl solution

containing 2.5% (V/V) glutaraldehyde (EM grade) for transmission electronic microscope analysis.

For TEM analysis, samples were washed twice with 0.05 M HEPES buffer (pH 6.8) and suspended overnight in 2% Glutaraldehyde. The samples were then washed three times with HEPES buffer. Each of these samples was enrobed in 2% Noble agar and dehydrated through a graded ethanol series, (25, 50, 75, 95 and 2 x 100% ethanol) for 15 minutes. The samples were then suspended in 50/50 ethanol/LRWhite resin for 1 hour, and then in pure LRWhite resin for 1h. Afterwards they were put into gelatin capsules containing pure LRWhite and polymerized at 60 °C for 1 hour. Sections (of approx 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 1K 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 (approx 200 nm diameters) on a Philips 400T at 100kV with an EDAX, Sapphire detector using the Phoenix software.

4.3 Results

4.3.1 Accumulation of Uranium by *Clostridium* sp.

Normally, the cell wall of Gram positive bacteria are comprised of approximately 25 layers of cross-linked polymers of peptidoglycan (Pg), which is made up of 40-50 repeating dimmers of N-acetylglucosamine and N-acetylmuramic acid rich in carboxylate groups. Teichoic and teichuronic acid are intermeshed in the Pg framework and contain phosphate groups and alanine (Madigan, et al., 2003). The cell walls of bacteria,

exopolymers, protein, and lipids contain functional groups of carboxyl, hydroxyl, amino acid, and phosphate, which are all capable of forming complexes with uranium (Fowle, et al. 2000). The biosorption of U by *Clostridium* was shown in Figure 4.3 below.

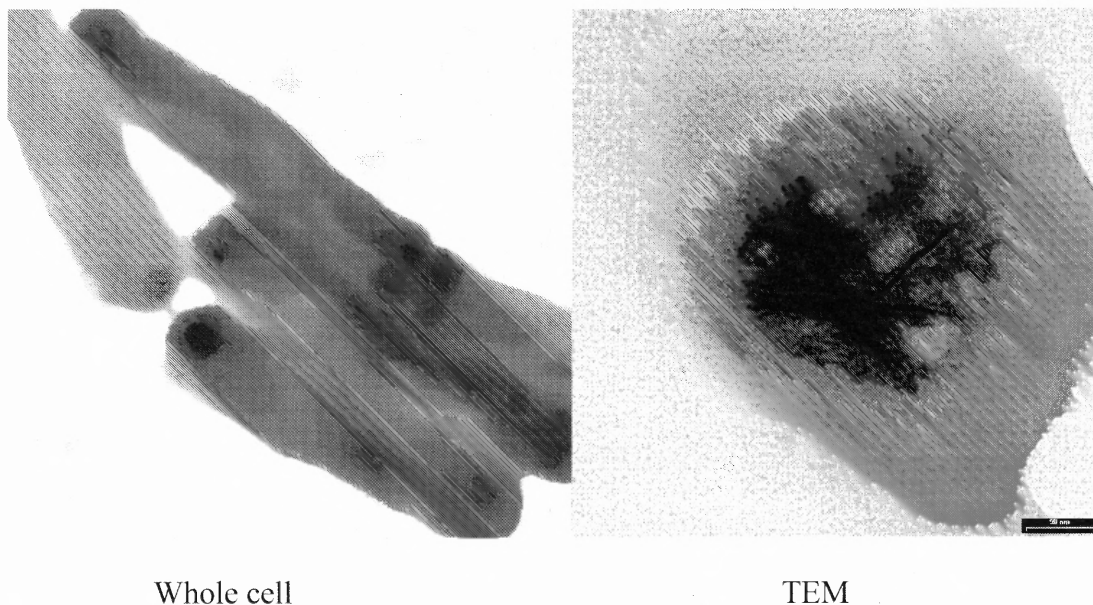


Figure 4.1 TEM of *Clostridium* sp. treated by uranium.

The TEM indicates whether or not there is accumulation of U by the cells, while the EDS spectra identify the elements associated with the cells. The whole amount of the sample exposed to uranium (VI) manifests a clear contrast without further staining.

Uranium is accumulated by *Clostridium* both extracellularly and intracellularly. The TEM analysis of our sample (Figure 4.1) shows uranium to be accumulated intracellularly as needle-like fibrils, having a size of around 50 nm. The EDS study of the cells (Figure 4.2) shows the presence of Oxygen(O), Nitrogen(N), Carbon(C), Phosphorus(P), Sulfur(S), Chlorine(Cl), and Potassium (K). Uranium was present both in the center and in the pole. The high copper (Cu) peak is from the EM grid used to support the specimen.

4.3.2 Accumulation of Uranium in the Presence of [BMIM][PF₆]

From the kinetic study (Figure 4.3) we find that the uranium absorption in 0.02M KCl reached the maximum in twenty minutes. Further accumulation was observed up to 2h. However, the uranium absorption in a KCl solution containing 1% of [BMIM][PF₆] reached the maximum in about 20 minutes, but the amount of absorption was 75% less than that without IL.

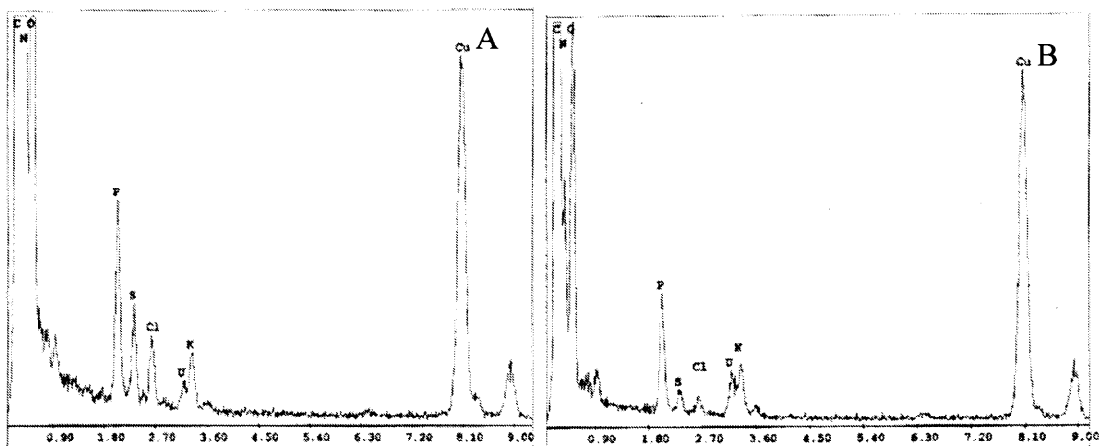


Figure 4.2 EDS of *Clostridium* sp. treated by $\text{UO}_2(\text{NO}_3)_2$: (A) Center; (B) Pole.

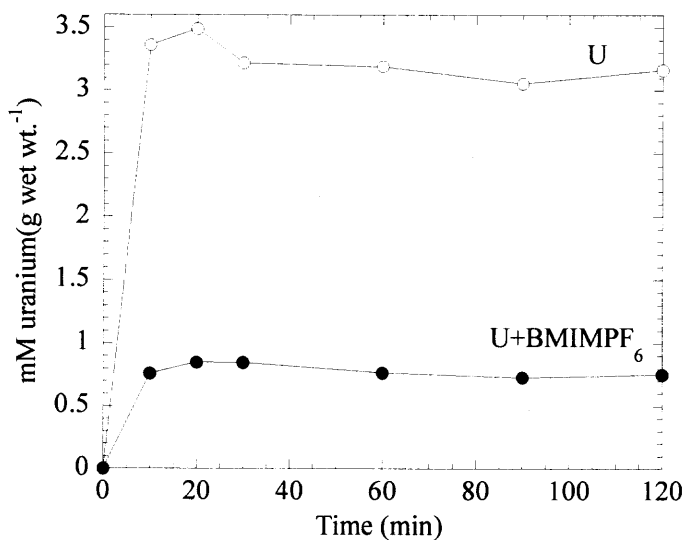
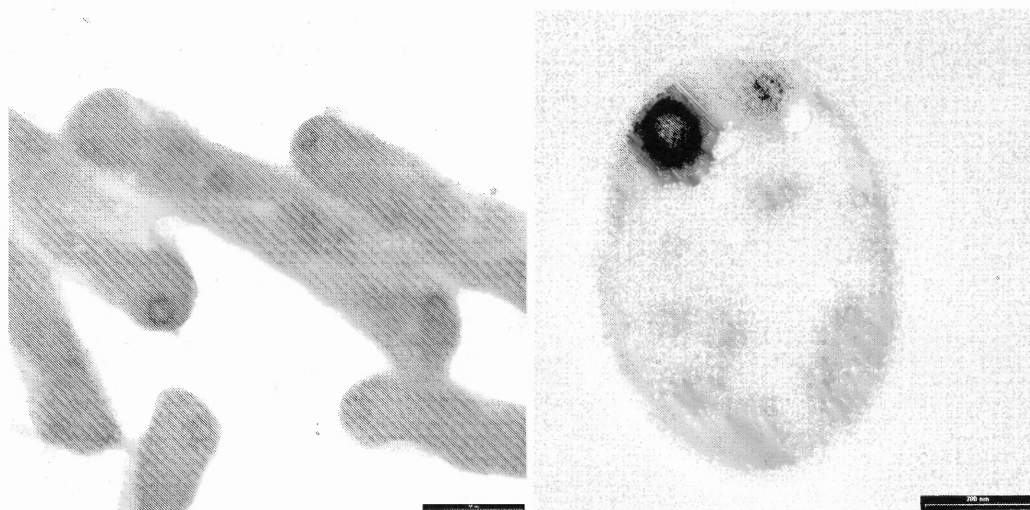


Figure 4.3 Kinetic study of U accumulation by *Clostridium* sp. in presence of 1% [BMIM][PF₆].



Whole Cell

TEM

Figure 4.4 TEM of *Clostridium* sp. exposed to uranium and 1% [BMIM][PF₆].

As the TEM (Figure 4.4) shows, most of the uranium accumulated extra-cellularly and coated on the surface of vesicles. EDS analysis (Figure 4.5) shows there is less oxygen, phosphorus, sulfur, potassium and uranium present in the center. Also less oxygen and phosphate were observed in the pole. Furthermore, the EDS analysis confirmed that less uranium accumulated inside the cell.

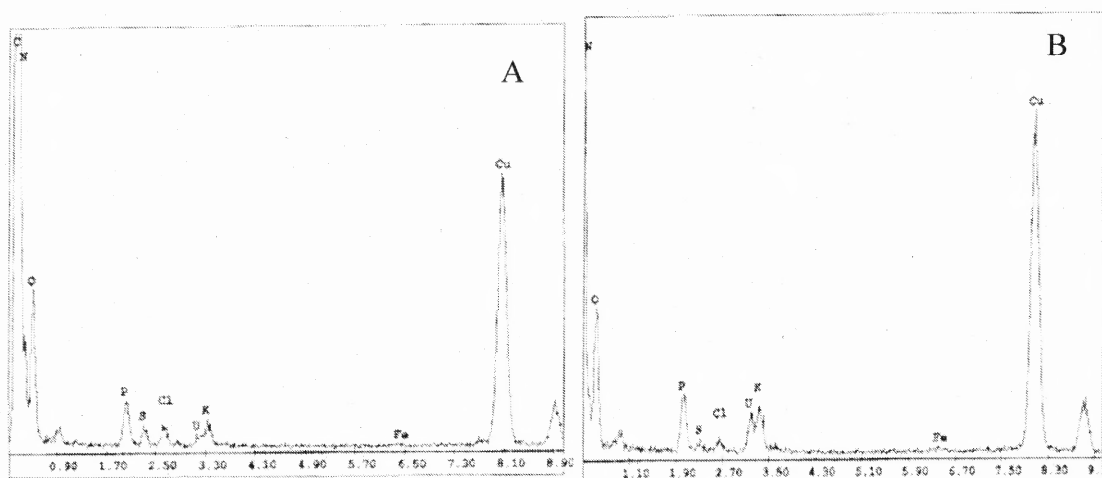


Figure 4.5 EDS of *Clostridium* sp. exposed to uranium and 1% [BMIM][PF₆] (A) Center; (B) Pole.

4.3.3 Accumulation of Uranium in the Presence of [EtPy][BF₄]

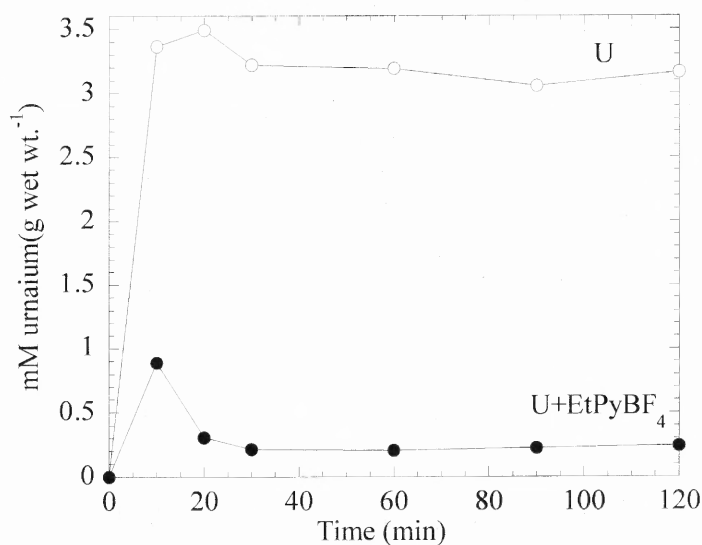
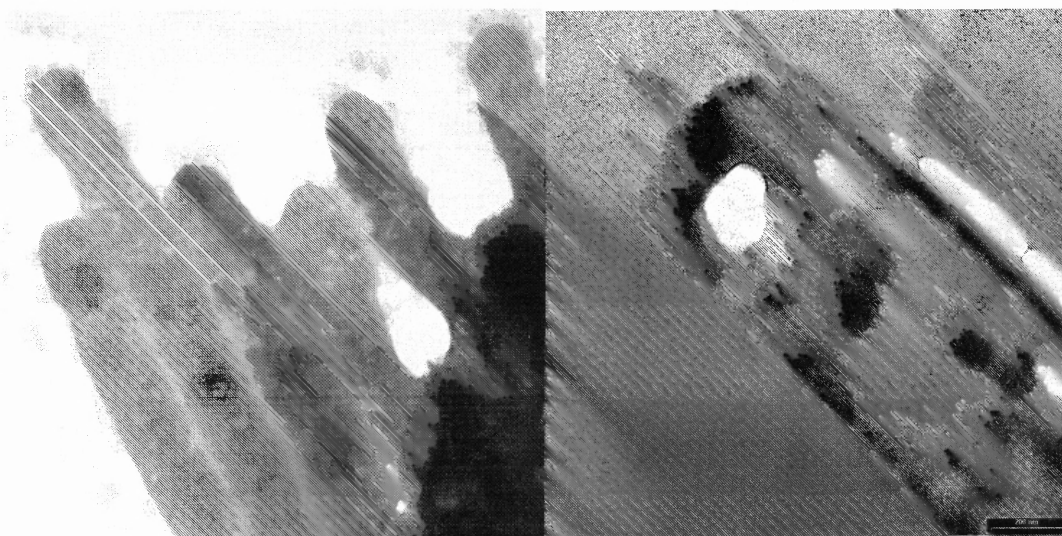


Figure 4.6 Kinetic study of U accumulation by *Clostridium* sp. in presence of 1% [EtPy][BF₄].

The kinetic study shows that absorption reaches the maximum in 10 minutes. It then decreased dramatically in the next thirty minutes. The absorption of uranium in the presence of [EtPy][BF₄] by *Clostridium* is almost 90% less than that of uranium alone.



Whole cell

TEM

Figure 4.7 TEM of *Clostridium* sp. exposed to uranium and 1% [EtPy][BF₄].

Only the extra-cellular accumulation of uranium was observed in solution containing 1% of [EtPy][BF₄] (Figure 4.7). However, many tiny pores were observed on the membrane, which could be due to the action of the [EtPy][BF₄]. Biological cells are surrounded by a cytoplasmic membrane, which works as a barrier between the cytoplasm and the extra-cellular environment. 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 (Konings, et al., 2002). Therefore, any effect on the integrity of the membrane will influence the ion transfer process significantly. As described by Bashford (1986), leakage is 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 progressively larger molecules, such as phosphorylated metabolites and (eventually) cytoplasmic proteins.

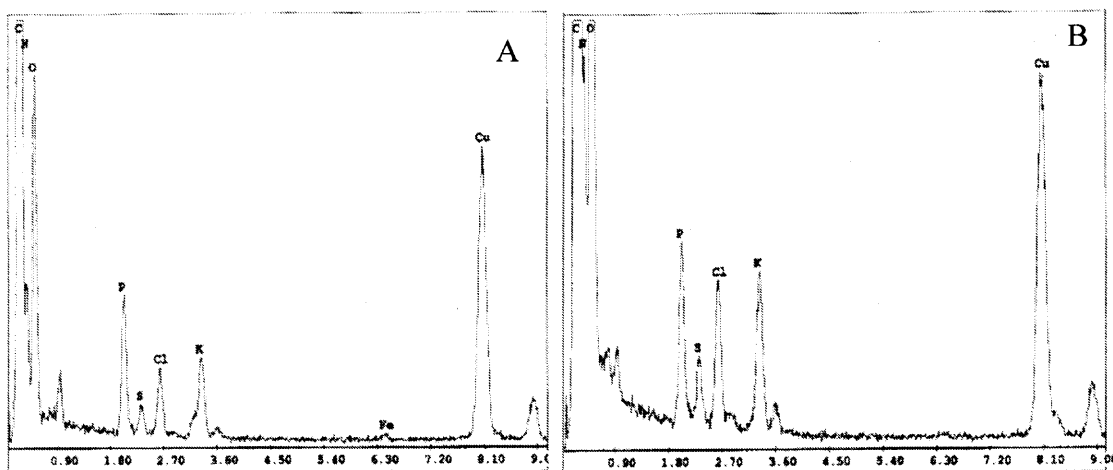


Figure 4.8 EDS of *Clostridium* sp. exposed to uranium and 1% [EtPy][BF₄] (A) Center; (B) Pole.

The EDS data (Figure 4.8) clearly demonstrates that there is no uranium accumulated in both the center and poles of the bacteria cell. As compared with the uranium alone treated bacterium, there was less of a presence of P, S and O in the center, but relatively a high presence of P, Cl, and K in the pole, which indicates the altering of the cellular chemistry and potential for an efflux of salt from the damaged membrane.

4.3.4 Accumulation of Uranium in the Presence of [EtPy][CF₃COO]

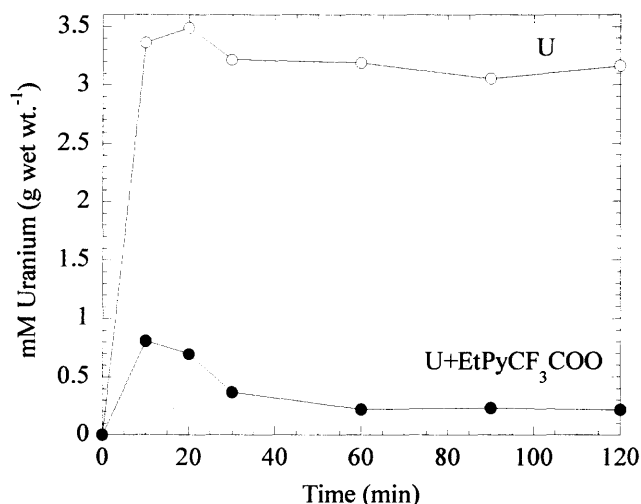


Figure 4.9 Kinetic study of U accumulation by *Clostridium* sp. in presence of 1% [EtPy][CF₃COO].

The absorption of uranium by *Clostridium* in a [EtPy][CF₃COO] solution shows the same behavior as [EtPy][BF₄]. Coated vesicles by uranium were clearly observed in the TEM (Figure 4.10). The structure of the cellular membrane was severely damaged, which also could be concluded from the EDS analysis (Figure 4.11). Both in the center and pole, less P and O were observed, which indicated the leakage of large molecular phosphorylated compounds. No uranium was detected in the pole and trace uranium was observed in the center. In particular, it should be noted that almost no K and/or Cl were

detected in either the center and/or the pole. A high intracellular K^+ concentration is necessary for the growth of living cells. The K leakage usually is an early indicator of membrane damage (Lambert, et al., 1973).

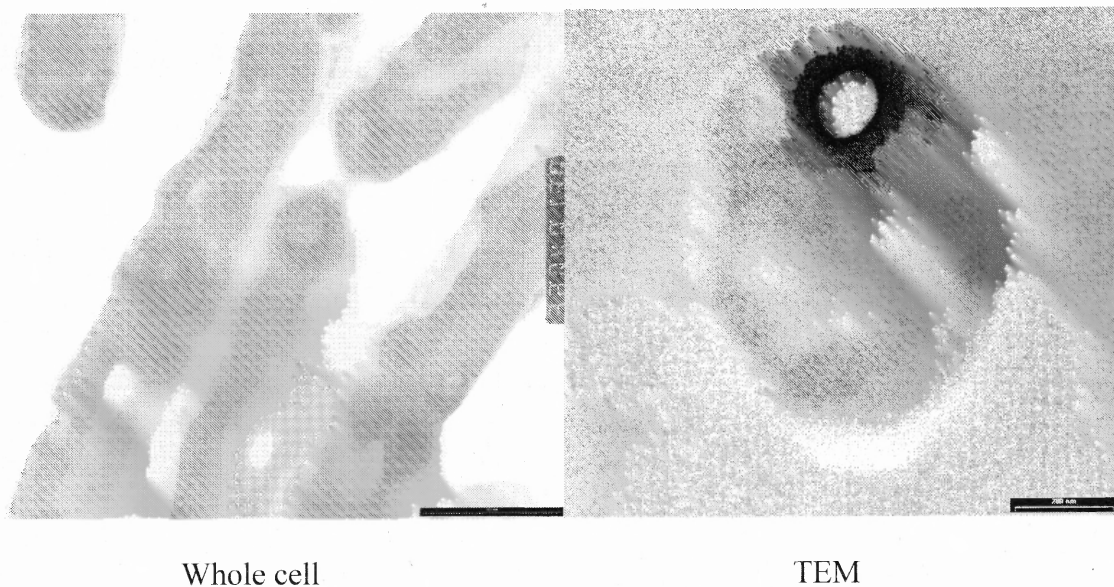


Figure 4.10 TEM of *Clostridium* sp. exposed to uranium and 1% [EtPy][CF₃COO].

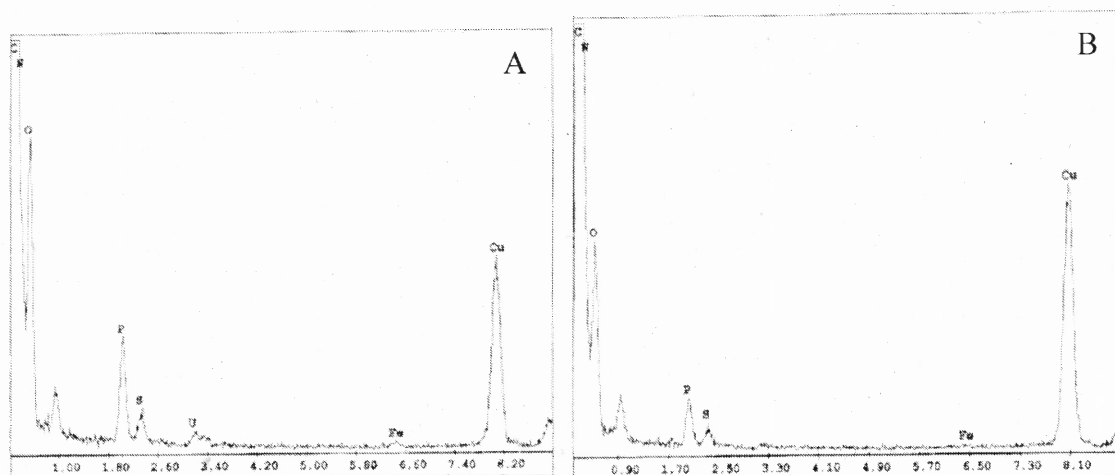


Figure 4.11 EDS of *Clostridium* sp. exposed to uranium and 1% [EtPy][CF₃COO] (A) Center; (B) Pole.

4.4 Discussion and Conclusions

The biosorption of uranium decreased in the presence of three ionic liquids. There are several reasons that may be responsible for this phenomenon. Firstly, when they combine with different ligands, the uranium complexes, U-BF₄, U-PF₆ and U-CF₃COO could show different affinities to cell surfaces. For example, U(VI) carbonate complex has a lower sorption ability on minerals and microorganisms comparing with U(VI)-phosphate (Francis, et al., 2000). Moreover, the absorption-ability is structure-related. As we know, biosorption is the competition equilibrium between the complex-ability with a cell surface and the stability of uranium complex in an aqueous solution. Stronger complexes show less absorption affinity. This study shows that the biosorption of uranium decreased in the order of U > U+[BMIMPF₆] (weak monodentate complex) > U+[EtPy][BF₄] (strong monodentate complex) and U+[EtPy][CF₃COO] (strong bidentate complex).

Secondly, the decrease of P element in the center and pole indicates the change of the cellular chemistry, which may also affect the sorption of uranium ions by decreasing the concentration of phosphate species able to complex with uranium. Fowle et al. (2000) postulated that absorption at low pH was caused by uranyl-binding with protonated phosphory groups on the cell wall, whereas the behavior at a higher pH was caused by uranyl binding with bacterial carboxyl groups.

Thirdly, ligand-gated channels typically have a high specificity for a specific ion species. When binding with different ligands, the ion channel may be blocked, thereby eliminating their transport inside the cell. When in a complex with different ionic liquids, the permeability of uranium may change.

Toxicity is another issue. As described by Coulding (2006), ionic liquids act as a surfactant and prove toxic to cells through interference with the cell membrane. Our results show that ILs affect the integrity of cell membrane structure. The toxicity of those three uranium and ionic liquids mixtures were in the order of $U+[EtPy][CF_3COO] > U+[EtPy][BF_4] > U+[BMIM][PF_6]$. For $U+[EtPy][BF_4]$ and $U+[EtPy][CF_3COO]$, cell membrane damages and an efflux of cellular solutes were observed. The damage of the membrane destroys the cell's function thus greatly influencing the biosorption process.

CHAPTER 5

EFFECTS OF IONIC LIQUIDS ON URANIUM BIOREDUCTION

5.1 Introduction

5.1.1 Bioremediation of Uranium

The contamination of sediment and groundwater by uranium has become a global problem. Various chemical and biological methods have been attempted to remediate such issues. Ion-exchange (Gu, et al. 2005), photodegradation (Dodge, et al. 2002), electrosorption (Xu, et al., 2000), biosorption (Sar, et al., 2004; Beyenal, et al., 2004) and chemical reduction (Jeon, et al., 2005) have been reported to remove uranium from water successfully. Among them, bioremediation is viewed to be a cost-effective and environmentally-friendly alternative for the clean up of uranium contamination.

The mechanisms involved in bioremediation include: 1) oxidation-reduction reaction; 2) changes in pH and Eh; 3) chelation or a form-specific complex with sequestering agents; 4) biosorption; 5) formation of stable mineral products; 6) biodegradation of a uranium-organic complex (Francis, et al., 1994, 1998).

Dissolved uranium in the oxidized form U(VI) is highly soluble whereas, the reduced form, U(IV), is highly insoluble in water. Several microorganisms have shown the ability to convert dissolved uranium to the insoluble form, U(IV). This results in a more concentrated extracellular precipitate, proven to be an effective means for bioremediation of soluble uranium in water. The bacteria studied so far include the Fe(III)-reducing *Geobacter* sp. and *Shewanella* sp. (Lloyd, et al., 2002), the Fe(III)- and sulfate-reducing *Desulfotomaculum* sp. (Pietzsch, et al., 1999), the sulfate-reducing

Desulfovibrio (Yong, et al., 2002), and the fermentative anaerobic *Clostridium* sp (Francis, et al., 1994).

5.1.2 Effect of Ligands on the Bioreduction

Many organic compounds can form stable complexes with uranium and thus increase their solubility and leaching capabilities. Chelating agents are often used in clean-up operations and separation processes. However, there is less information known about the rate and extent of the bioavailability of those organic compounds (with the exception of some widely used chelating agents, such as citric acid and carboxylic acid). Also, the bioavailability of those complexes varies with the functional groups involved in the complexation. In Robinson's study of acetate, oxalate, citrate and tiron (4,5-dihydroxy,1,3-benzenedisulfonic acid), the rate of the acetate-complexed uranium reduction was the fastest while that of the citrate-complexed uranium was the slowest (Robinson, et al., 1998). The choice among the various reducing bacteria for the uranium complex with organic ligands may produce different effects on the rate of bioreduction. For example, it was found that uranium complexed with monodentate ligands such as acetate, relative to polydentate ligands such as malonate, oxalate or citrate (Ganesh, et al., 1999) exhibited an increased rate of U(VI) reduction by *Desulfovibrio*; but when using *Shewanella* with a uranium complex with polydentate ligands, it reduced even faster (Ganesh, et al., 1997). It was found that the choice among various organic ligands could also impact uranium precipitation. The removal of uranium from waste water then becomes a problem.

The commonly formed inorganic complexes include: carbonate, sulfate, nitrate, and phosphate. The complexes affect the speciation of uranium in aqueous solution and its bioavailability. In a study by Markich (2002), U(VI) complexation with inorganic ligands (e.g., carbonate or phosphate) and humic substances (e.g., uranyl fulvate), has been suggested to reduce the bioavailability of U by reducing the activity of UO_2^{2+} and $\text{UO}_2(\text{OH})^+$. It is suggested that elevated NO_3^- and SO_4^{2-} concentrations can interfere with the bioreduction process by *Desulfovrio*. For example, Tucker et al. (1998) report a decrease in U(VI) removal-efficiency, in the presence of as little as $50 \text{ mg l}^{-1} \text{NO}_3^-$, which most probably is due to the toxicity of NO_2^- .

These studies clearly suggest that the chelating/complexation molecules affect uranium bioreduction. Therefore, we carefully studied the physic-chemical interactions between uranium and ILs, the effects of ILs on the growth of *Clostridium* sp., and the biosorption process under aerobic conditions. The uranium bioreduction under anaerobic conditions in presence of U-ILs complex is reported here.

5.2 Materials and Methods

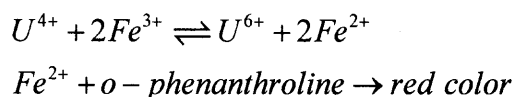
5.2.1 Comparison of Different Concentration of Ionic Liquid

A mixture of uranyl nitrate (final concentration 0.25 mM) with various concentrations of $[\text{EtPy}][\text{BF}_4]$ was prepared. Each mixture was added to an 18 h old growing culture (pH 3.6), and samples were taken every two or three hours in order to measure the totals of U and U(IV) in solution. IL was monitored by ESI-MS. Another set of growing culture was kept intact for 24 hours after this addition, then the U(IV) and total U in solution was

measured. After centrifugation at 5000 g for 20min, the precipitates were washed three times with N₂ purged, pre-reduced water, and then re-suspended in 10 ml of 5 mM citric acid overnight and in darkness. All operations were carried out under anaerobic conditions. The solutions were analyzed by the following method to determine U(IV) and total U in precipitate.

5.2.2 Detection Methods of U(IV) and Total Uranium

U(IV) detection is accordance with the reaction below. Ferrous complex was monitored by UV spectrometer at 510 nm.



Preparation of color development mixture Thirty-five milliliters of 1 mM FeCl₃ solution containing concentrated HCl (6.4 ml/liter mixture) was mixed with 7.5 ml of 10 mM o-phenanthroline and 7.5 ml of 1M acetate buffer (pH 5).

Sample measurement A filtered sample of 0.25 ml was added to the cell containing 0.25 ml filtered de-ionized water and 0.5ml color development mixture. It was allowed to stand in darkness for 3 hours, and then the absorbance was measured at 510 nm. Solutions containing 0.01, 0.025, 0.05 and 0.1 mM ferrous ion were used to prepare a standard curve. The culture without uranium was tested for both background ferrous ion in the medium and for that released by bacteria.

After dilution and acidifying with 2.5 M sulfuric acid, the total U in solution was measured by KPA. The difference between the total U and U(IV) yields the un-reduced U(VI).

5.2.3 Comparison of Different ILs Effects

Mix 75 μ l of 141 mM U(VI) with 1% [BMIM][PF₆] (48mM), or [EtPy][BF₄] (45mM), or [EtPy][CF₃COO](54mM) respectively. Keep the mixture in a dark glove box overnight, and then add it to the 18h growing culture. Follow the same procedure as above (5.2.1 and 5.2.2) to measure the U(IV) and total uranium concentrations in solution.

5.2.4 UV-Vis Spectroscopy Analysis of U-citrate Extract

After extraction with citric acid, the absorption spectra of uranium citrate were determined from 190 to 1100 nm using Hewlett Packard 8453 diode array scanning UV-Vis spectrophotometers. Similarly, the precipitate of U(VI) in a pure culture without bacteria was also collected and extracted with citric acid for comparison with U(IV).

5.2.5 XANES and EXAFS Analysis

After bioreduction for 24 hours, the precipitates were collected by centrifugation. Samples were stored in darkness in an anaerobic glove box. Both XANES and EXAFS analyses of the precipitate were performed as described previously in chapter 2 (2.2.6).

5.3 Results and Discussion

5.3.1 Effect of Various Concentrations of [EtPy][BF₄] on U Bioreduction

In a medium containing only uranium, the U(VI) precipitated from the solution (due to the interference with medium components), and in 6 hours almost all the uranium precipitated out. The concentration of U(IV) is comparatively very low (0.005 mM) in

the solution. In two hours, it reached its highest concentration (0.015 mM) then precipitated.

The disappearance of U(VI) in the solution containing uranium alone is about 0.047 mM/h. In the presence of various concentrations of [EtPy][BF₄], the rates of decrease of U(VI) concentration in solution are 0.047 mM/h, 0.068 mM/h, and 0.06 mM/h respectively. The increased concentration of U(IV) in solution is in the order of 0.006 mM/h, 0.005 mM/h, 0.014 mM/h, and 0.011 mM/h. In the presence of IL, the rates of decrease of U(VI) in solution and the increase of U(IV) in solution are relatively higher than the rates in the medium containing uranium alone. Moreover, the rates accelerated with the increase of the IL concentration.

In the presence of different contents of [EtPy][BF₄], U(IV) could maintain a high level of concentration. After 10 hours, however, it would slowly start to precipitate. The U(IV) concentration in solution increased with the increase of the [EtPy][BF₄] concentration. Upon the addition of 1% [EtPy][BF₄], the U(IV) concentration in solution is almost 6 times higher than it is in a pure culture after 24h of bioreduction (Figure 5.1).

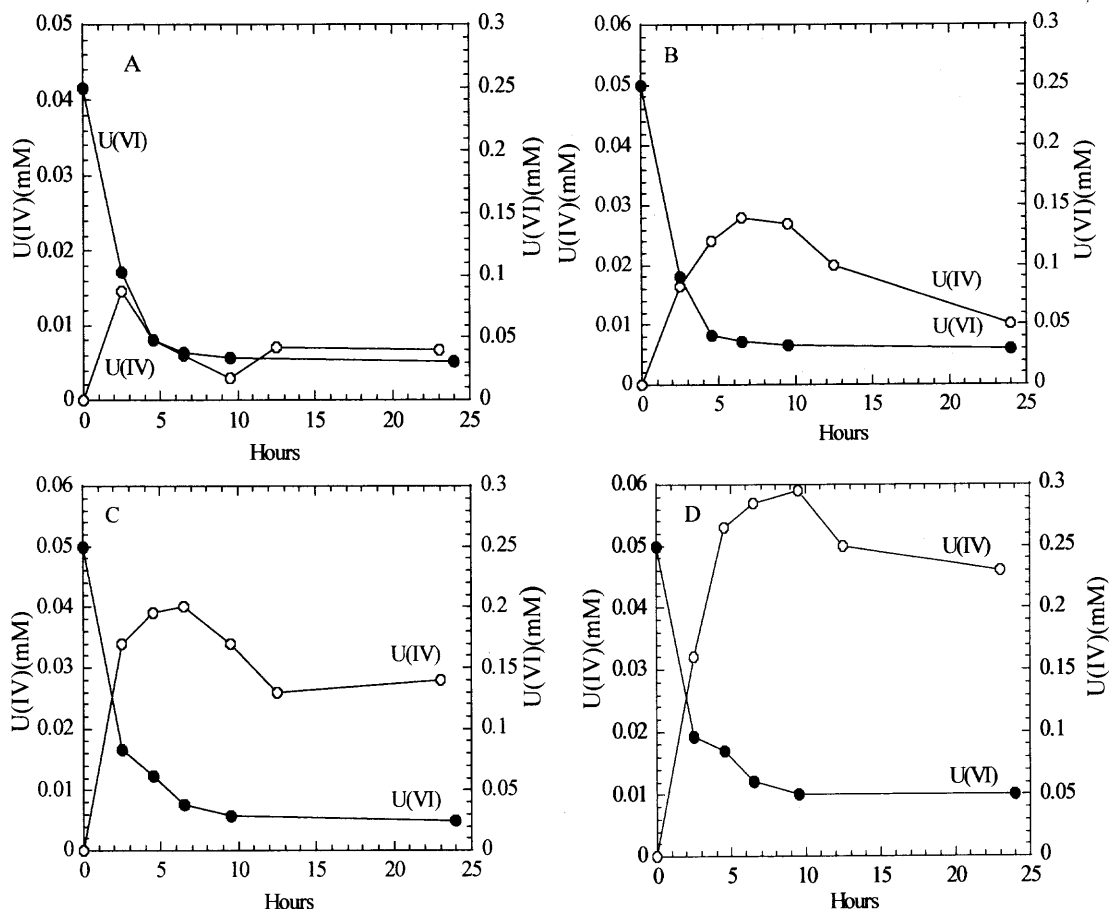


Figure 5.1 U bioreduction by *Clostridium* sp. in the presence of (A) 0% (B) 0.1% (C) 0.5% (D) 1% of [EtPy][BF₄].

5.3.2 The Mass Balance of U in a Medium after Bioreduction in the Presence of Various Concentrations of [EtPy][BF₄]

Figure 5.2 gives the mass balance of uranium after bioreduction. We find that by increasing the [EtPy][BF₄] concentration, the total uranium concentration in solution is increased from 16.9% (U nitrate) to 20.9% (U:0.1%[EtPy][BF₄]), 30.2% (U:0.5%[EtPy][BF₄]), and 40.8% (U:1%[EtPy][BF₄]) respectively. We also find that the U(IV) concentration in the solution increased from 1.7% (U nitrate), 3.8% (U:0.1%[EtPy][BF₄]), 16.1% (U:0.5%[EtPy][BF₄]), to 19.8% (U:1%[EtPy][BF₄]). The

uranium(VI) precipitates were decreased in the order of 37.9%, 15.8%, 4%, and 2.4% respectively.

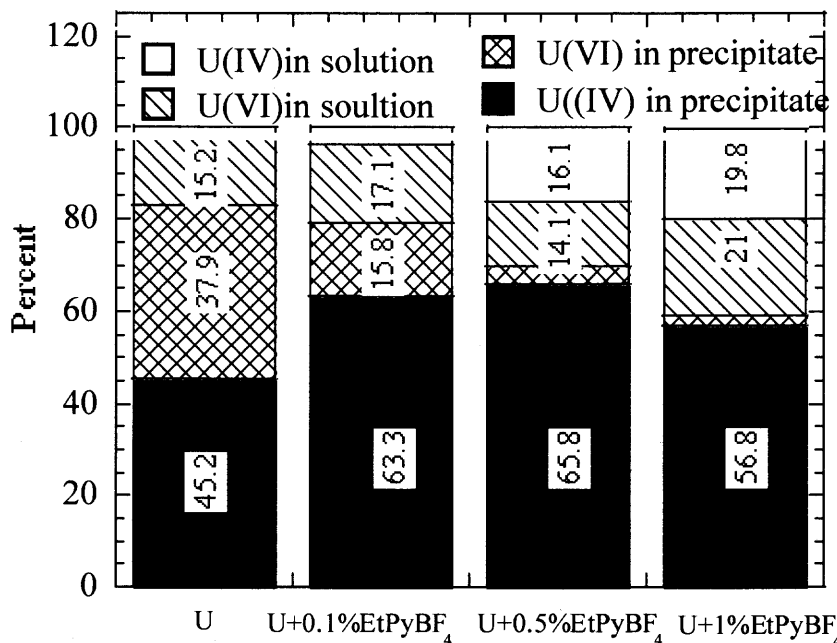


Figure 5.2 Mass balance of U after reduction.

These results indicated that the increased solubility of uranium in solution was due to the complexation between U(VI) and [EtPy][BF₄], as we previously described. Equilibrium constants for a complex are usually stated for reactions and written according to the following complex formula:



$$K \text{ (equilibrium constant)} = \frac{[\text{complex}]}{[\text{ligand}]^n \times [\text{central metal ion}]^m} \quad (2)$$

Although we are unable to calculate the K value, due to a lack of information about ionic liquid dissociation, we could infer that the increase of the [EtPy][BF₄] concentration shifts the balance to form more U(VI)-BF₄ complex. However, when the [EtPy][BF₄] concentration increased, the U(IV) species, which usually precipitated out

early, also maintained a high concentration in solution. We further observed that the medium containing 29 mM (0.5%) and 58 mM (1%) of [EtPy][BF₄] remained cloudy after 24h of incubation, but the medium containing only uranium became clear with a green precipitate in just 5h. We suggest that U(IV)-BF₄ complex also forms in the solution and that an increase in the [EtPy][BF₄] concentration facilitates the formation of the U(IV) complex. There was a similar observation reported by Ganesh et al. (1999) and Francis et al. (2002). They found that some organic ligands are able to form soluble complexes with microbial reduced uranium and thereby prevent the uranium's precipitation. Most multidentate organic ligands, such as oxalate and citrate, can retard the reduction rate by forming a complex with U(VI), and can limit its ability for microbial reduction. Furthermore, they can form soluble complexes with reduced U(IV) and prevent the precipitation of the uranium.

In the complexation of uranium with a weak coordination ligand, there are many influences on the stability of chelates. For example, (1) the concentration and redox status of the metal ion, (2) the concentration and structure of the chelate, (3) the nature of the metal and donor atoms etc. (Chao, et al., 1998). Metal ions may compete with hydrogen ions (in this study it is EtPy⁺ cation) for the available donor atoms. Therefore, simultaneous equilibrium exists between the formation of the chelates and the dissociation of the chelating agents. In aqueous systems, water is a competing ligand. It dissociates into hydrogen and hydroxyl ions, and can also form a competing uranium hydroxide. Due to these varying factors, we were able to observe the instability of the weak complex between U(IV) and [EtPy][BF₄]. These observations are noted in this study.

During the first 24 hours, we observed high levels of U(IV) in the solution, however, over the course of 5 days the U(IV) slowly precipitated out. Reasonably, the aggregation properties of ionic liquids in water (Dietz, et al., 2003; Miskolcym, et al., 2004), and/or the strong electrostatic interactions between ionic compounds could also be in effect. These weak complexes may not, however, prove to be an encumbrance for future separation processes.

As we know, a high ratio of sulfate/nitrate will decrease the uranium's reduction rate, because of its potential/tendency to thermodynamically favor the reduction of these anions over that of uranium reduced by the bacteria, *Shewanella*, *Geobacter* and/or *Desulfotomaculum*) (Ganesh, et al. 1999). With the exception of [EtPy][BF₄], despite the fact that they have reached a high ratio (around 180:1), the rate of reduction was not impacted with *Clostridium* sp. In contrast, an increase of [EtPy][BF₄] concentration enhanced the uranium reduction. With the addition of 1% [EtPy][BF₄], almost all of the precipitates yield as uranium (IV), and the reduction ratios reach 77% (as compared to a 46% conversion in a uranium nitrate solution).

As a result of hydrolysis and condensation reactions with the medium components, U(VI) underwent precipitation due to the formation of a colloidal precipitates. As we know, because of the transport mechanism, soluble actinides and metal are more likely to be bioavailable to microorganisms than precipitated or mineral-absorbed actinides and/or metals (Ruggiero, et al., 2005). The study by Francis et al. shows that the amount of uranium is reduced only by the growing and resting cells and not by the heat-killed cells nor by the cell free spent medium (Francis, et al., 1994). So it is the living bacteria, which play an important role in the bioreduction of uranium. Moreover, in the presence of

[EtPy][BF₄], biosorption decreased and both the U(VI) and U(IV) solubility increased, thus allowing more U(VI) to reach the active site of the bacteria thereby enhancing bioreduction (Figure 5.3).

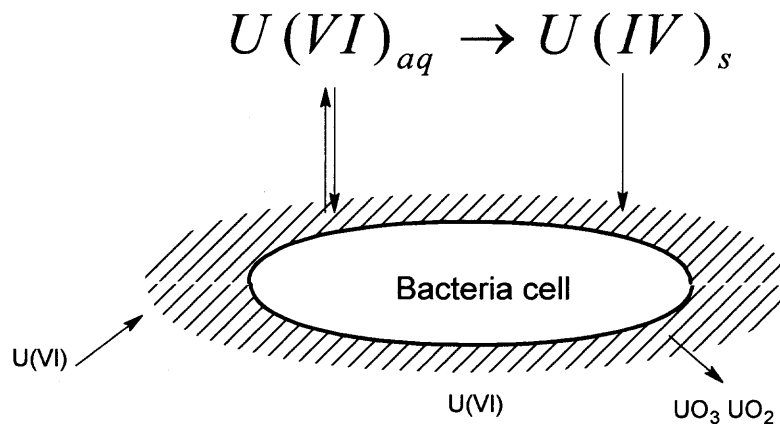


Figure 5.3 Enhanced U reduction by decrease of biosorption.

5.3.3 Determination of Uranium Accumulated in Bacterial Cell during Reduction

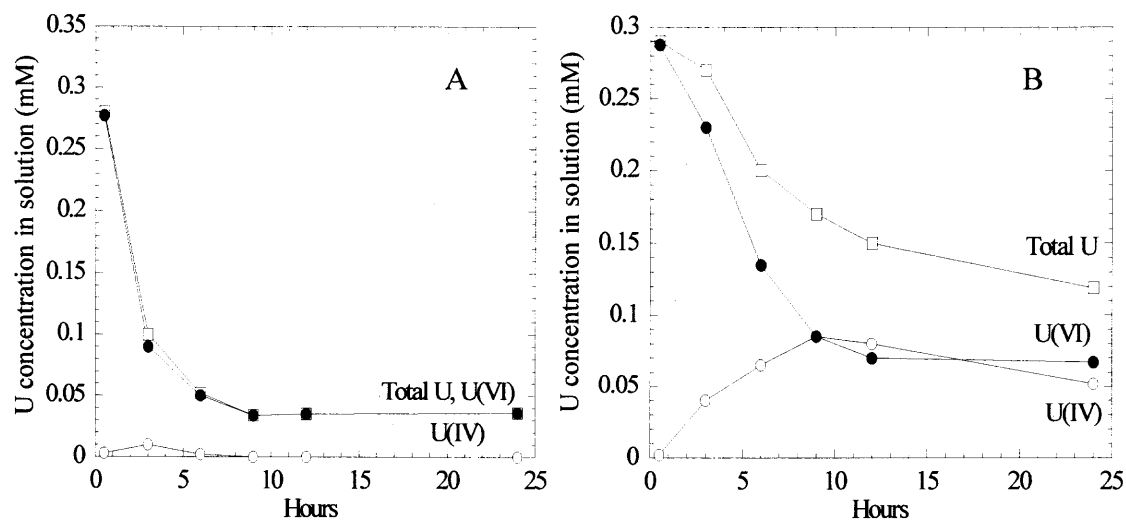


Figure 5.4 Kinetic study of uranium concentration in solution during bioreduction exposed to: (A) uranium; (B) uranium + 1% [EtPy][BF₄].

In order to confirm the amount of accumulation of uranium in a cell, culture samples incubating with uranium were removed at 0h, 6h and 24 h. As shown in Figure 5.4, in the presence of [EtPy][BF₄], the total uranium and U(IV) concentrations in the solution increased. At 6h, the total of the U are 4 times higher, while U(IV) concentrations are more than 20 times higher in the presence of an ionic liquid than with uranium alone. More than 30% of the U(VI) has been converted to U(IV) by *Clostridium* sp. in the presence of [EtPy][BF₄] (Figure 5.5).

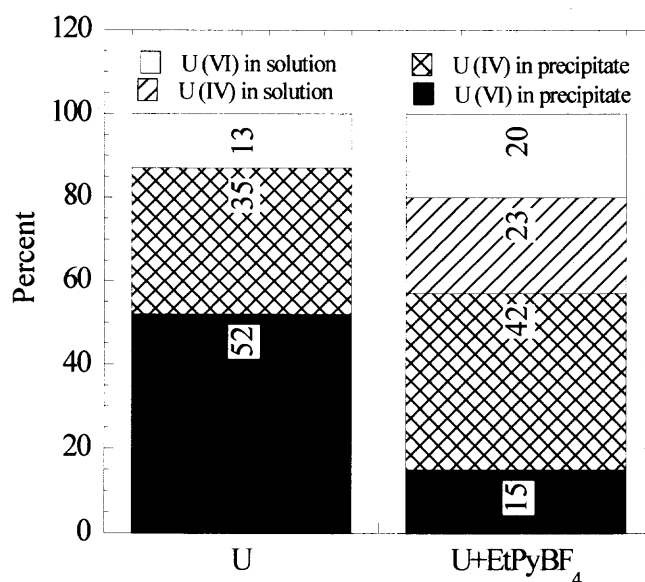


Figure 5.5 Mass balance of U after 24h incubation.

The bacterial morphology maintained the same rod shape during 24h of incubation. Figure 5.6 (B) shows that *Clostridium* sp. produces a large amount of extracellular polymeric substance (EPS). The major components of the hydrolyzed EPS are polysaccharides (Sutherland, et al., 1973). EPS also contains small amounts of phosphate, and nucleic acid that contributes phosphate. The phosphate group is a good candidate for extracellular accumulation of uranium (Merroun, et al., 2003; Bonthron, et al., 2000). The slime has several functions such as cell-to-cell adhesion and the adhesion

to the substrate facilitating bacteria growth. The slime may also assist in aiding gliding-motility, in preventing dehydration, and in protecting against phagocytosis and toxins (Dworkin, 1993).

Using Figure 5.7A to illustrate, imagine that the white spots in the thin section are polyglucoside (glycogen) granules. *Clostridium* can accumulate polyglucose which is surrounded by a single-layered membrane. The granules first appear in the cells of the early log-phase cultures and become more numerous as the culture ages, reaching their maximum at the outset of sporulation (Shively, 1974). EDS analysis (Figure 5.7 B) shows there to be Carbon (C), Oxygen (O), Phosphorus (P), Sulfur (S), Chlorine (Cl), and Potassium (K).

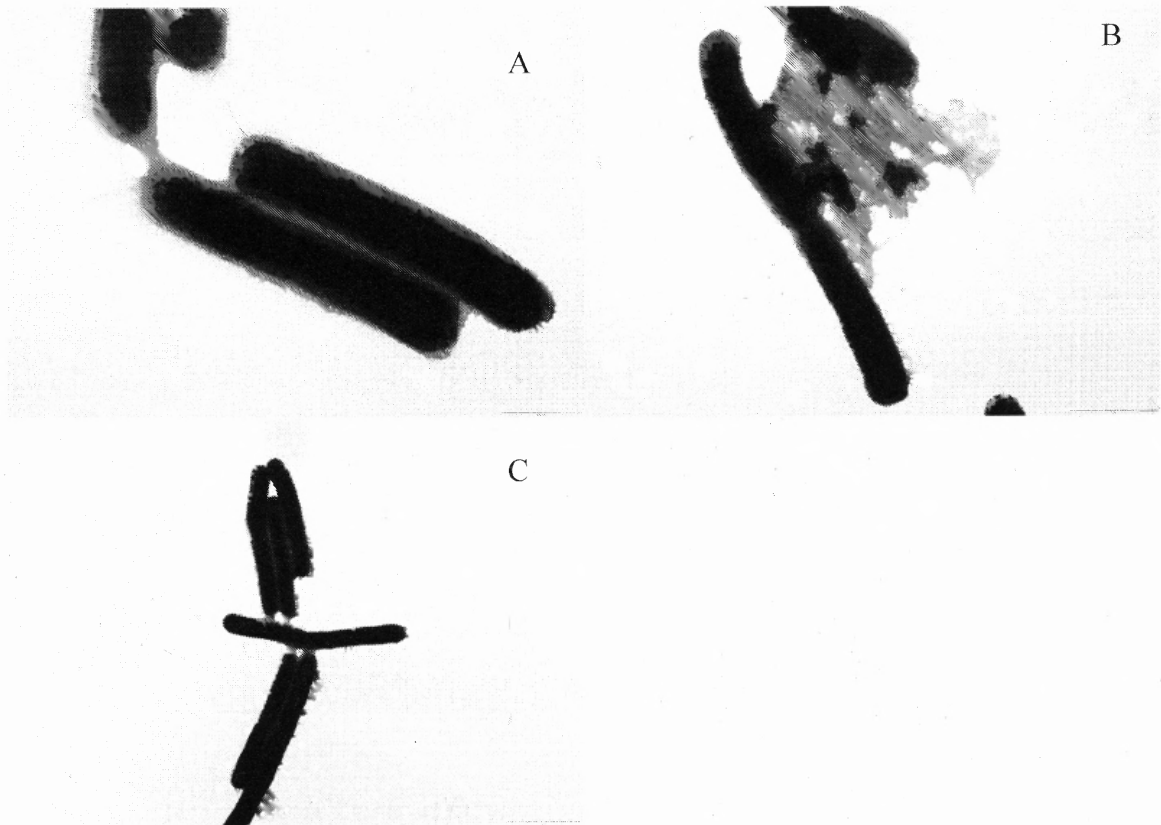


Figure 5.6 Whole cell of *Clostridium* sp. alone at (A) 0h; (B) 6h; (C) 24 h.(uranium came from the staining during TEM sample preparation)

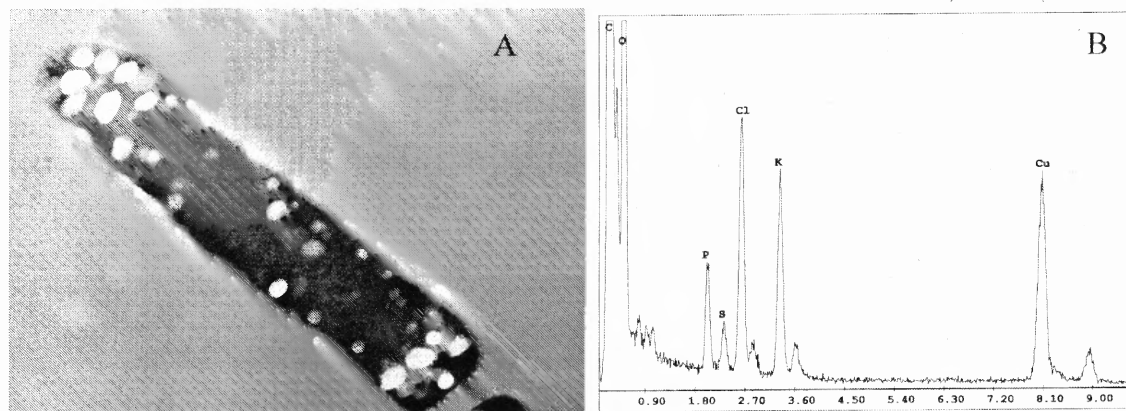


Figure 5.7 (A) Thin section image of *Clostridium* sp. alone (24h); (B) EDS analysis (24h).

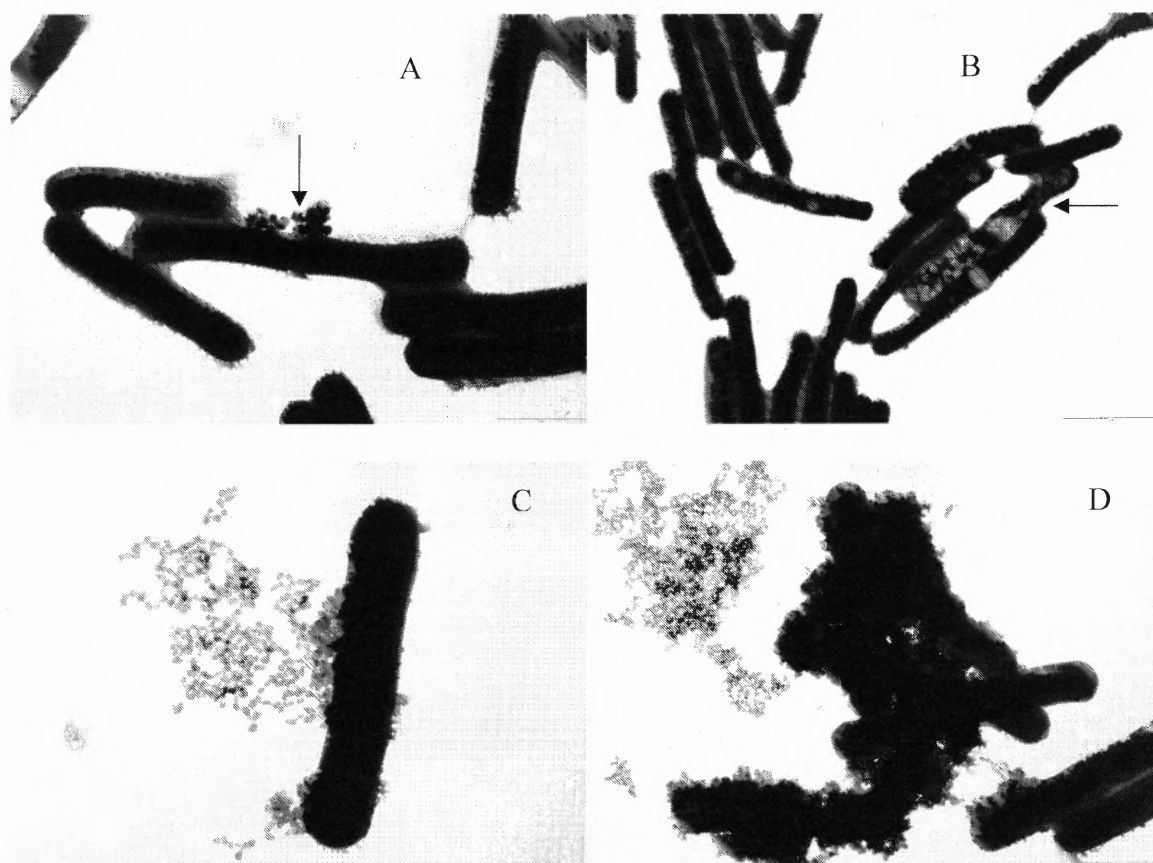


Figure 5.8 Whole cell of *Clostridium* sp. in the presence of uranium: (A and B) 0h; (C) 6h; (D) 24h.

Although we cannot identify the oxidation status of the uranium through EDS analysis only, we can still observe from the Figure 5.8 that uranium accumulated extracellularly upon its interaction with a bacterium cell (Figure 5.8 A). EPS containing a large phosphate group serves as a net that leads to uranium accumulation primarily around the cells (Figure 5.8 B and C). After 24h of incubation, the cell surfaces were covered with precipitated uranium (Figure 5.8 D).

The thin section clearly shows the precipitate of uranium granules associating with the cell wall (Figure 5.9 A). The penetration of uranium into the cytoplasm of bacteria was also observed as shown by the arrow mark. The intracellular uranium deposits appear to be randomly distributed. The amount of U deposited in the EPS and in the cell wall is higher than that deposited inside the cell. The extracellular association of uranium with the surface of a bacterial cell is due to a physical and/or chemical interaction, such as absorption, ion exchange, or complexation, (which is not directly dependant on metabolism) whereas the intracellular accumulation of uranium is conducted via passive transport mechanisms.

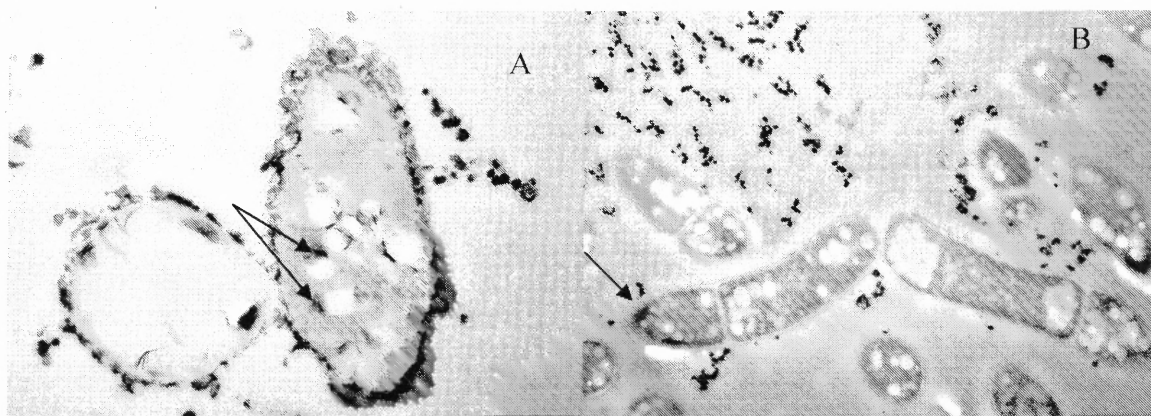


Figure 5.9 (A and B) Thin section of *Clostridium* sp. exposed to uranium after 24h incubation.

The EPS structure was not seen in Figure 5.9 B; only the uranium granule was observed around the cell. That is because water is the most abundant constituent of EPS, and also because it is difficult to keep it intact for ultrastructural study due to the structure's collapse during the dehydration process. The deposit of uranium inside the cell was confirmed by EDS analysis.

Uranium concentration increased with time in both the cytoplasm (Figure 5.10 A and C) and in the slime (Figure 5.10 B and D). An extremely high concentration of U in the slime indicates that most of the uranium accumulated outside the cytoplasm. Although no quantitative microanalyses were carried out, it can be observed that higher peaks of P correspond to higher peaks of U. The presence of P in the cell wall and in the cell interior is common because of its presence in phospholipids, nucleic acids, polyphosphates, etc. Although just how the U cation diffuses into the cell is still unknown, it is notable that whenever this diffusion appeared in all parts of the cell, U was accompanied by P. It is assumed that the phosphate structure may be more favorable for bacteria for the entrance of U. Similar phenomena were found in the bioaccumulation of La (Bayer, et al., 1991).

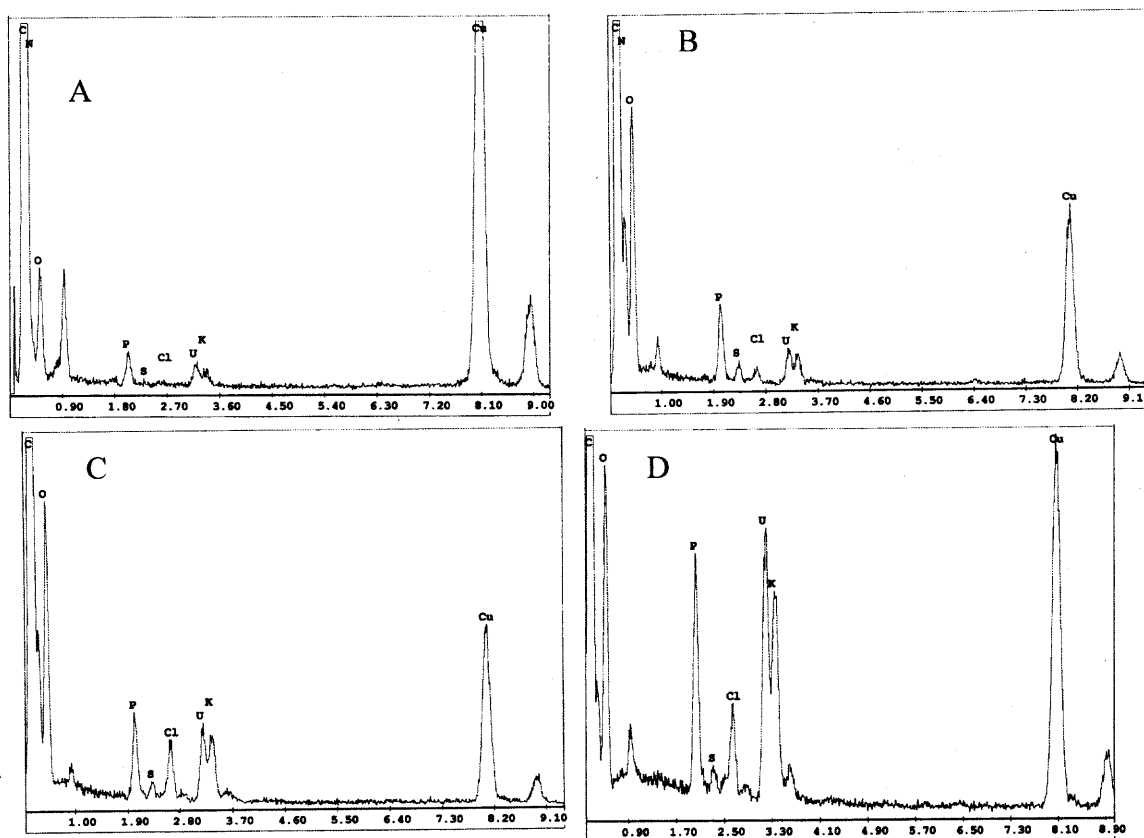


Figure 5.10 EDS of *Clostridium* exposed to Uranium (A) in cell (0h); (B) slime (0h); (C) in cell (24h); (D) slime (24h).

EPS was also observed in presence of the ionic liquid [EtPy][BF₄] as shown in Figure 5.11 A. After 6h of incubation, uranium precipitation was found outside the cytoplasm (Figure 5.11 B). According to the kinetic study (Figure 5.4), less uranium precipitated from the solution and deposited outside of cytoplasm of the bacteria. This is confirmed by following EDS analysis. Damage to the membrane was also observed here.

In Figure 5.11 C a clear contrast of cell surface indicates the structure change of the cytoplasm membrane, which was more pronounced after 24h of incubation (Figure 5.11 D).

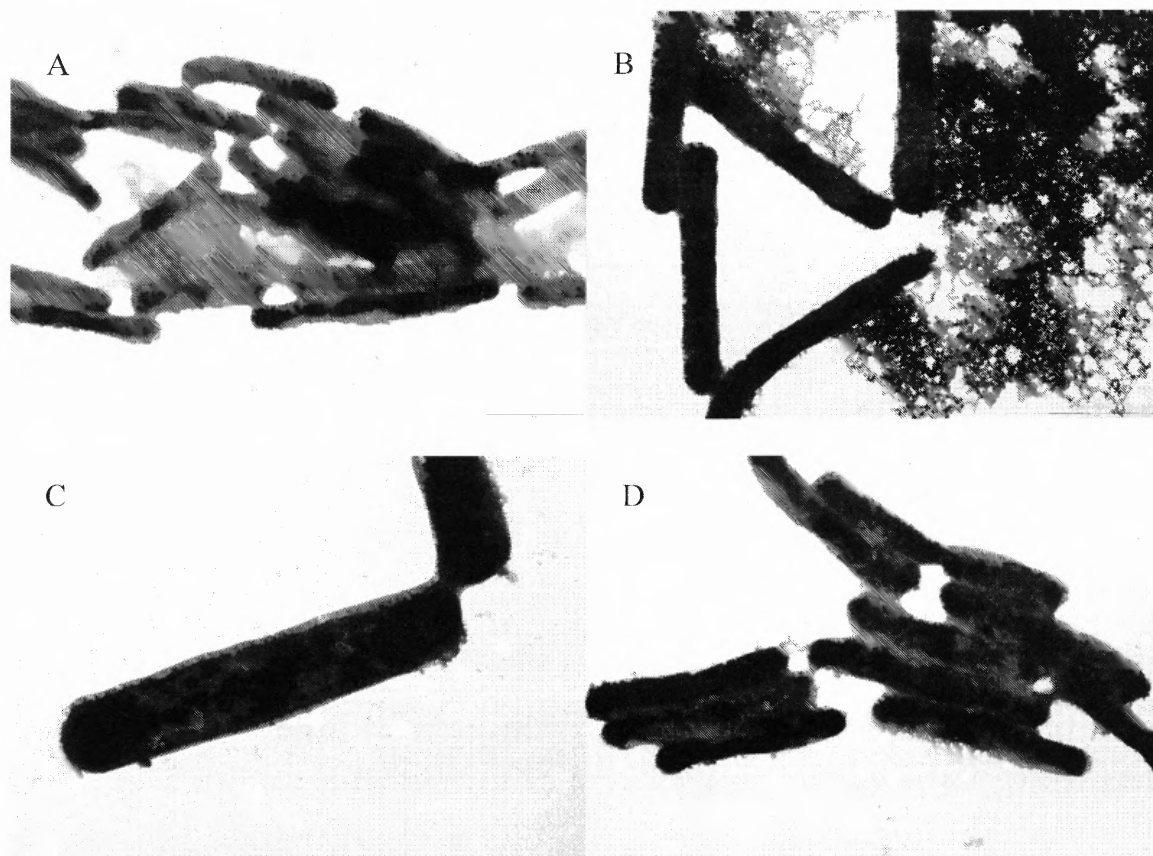


Figure 5.11 Whole cell of *Clostridium* sp. exposed to Uranium and 1% [EtPy][BF₄]: (A) 0h; (B and C) 6h; (D) 24h.

The accumulation of surfactant on the membrane may cause a change in the membrane's function by alternating the lipid order, orientation, and fluidity, or dissolving the membrane lipids (Xia, et al., 2000). Based on the toxicity study by Pretti (Pretti, et al., 2006), ionic liquids show a similar toxicity to surfactants. IL could increase the membrane permeability and thereby alter the physical properties of the lipid bi-layer and enhance its permeability for an external ion. The appearance of fluorine (F) and sodium (Na) at 6h and 24h in the EDS analysis (Figure 5.12) indicates the transport of external ions inside cell. A relative high potassium (K) and Chlorine (Cl) concentration in the cell were observed after 6 and 24h incubations with ionic liquids, which may also due to the increased permeability of the cytoplasm membrane.

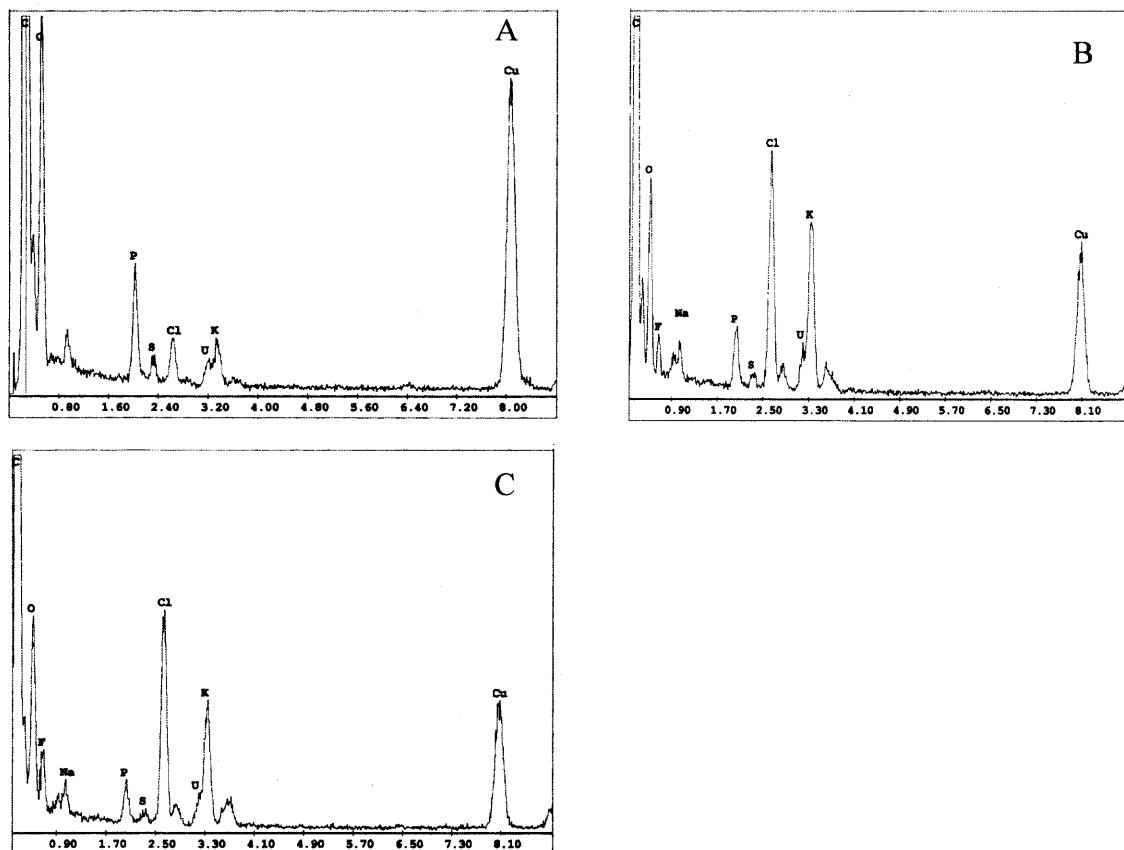


Figure 5.12 EDS (in cell) of *Clostridium* sp. exposed to uranium and [EtPy][BF₄]: (A) 0h; (B) 6h; (C) 24h.

The sequential onset of a permeability change could result in an increase in the metabolic potentiality of the cells (Bashford, et al., 1986). Although the metabolism of U(VI) bioreduction is still not completely established, the electron transfer chain that traverses the periplasm and terminates at the cytoplasmic membrane, periplasm, or outer membrane, has been assumed to play an important role in the reduction process (Lloyd, et al., 2002). Our results indicate that most uranium was precipitated outside of the cell, but some of the U(VI) was able to traverse the outer membrane and penetrate the periplasm (wherein it was reduced and precipitated). Similar results were noted in Gorby's (1992) and Lovely's (1992) studies. Also, an enhanced permeability may influence the

bioreduction process. However, many other factors may also affect the reduction, such as the transport limitations, structural incompatibilities, transport mechanisms, or the involvement of different enzyme systems.

Similar to the uranium treated bacteria, uranium not only accumulated in the periplasmic space, but also transported into the bacteria's cytoplasm in U:[EtPy][BF₄] system (Figure 5.13). However, the uranium concentration inside cell almost maintained stability during the 24h incubation. The enhanced membrane permeability seems only to influence the mobility of the small molecular ion, and shows little effect on the deposit of heavy metal uranium.

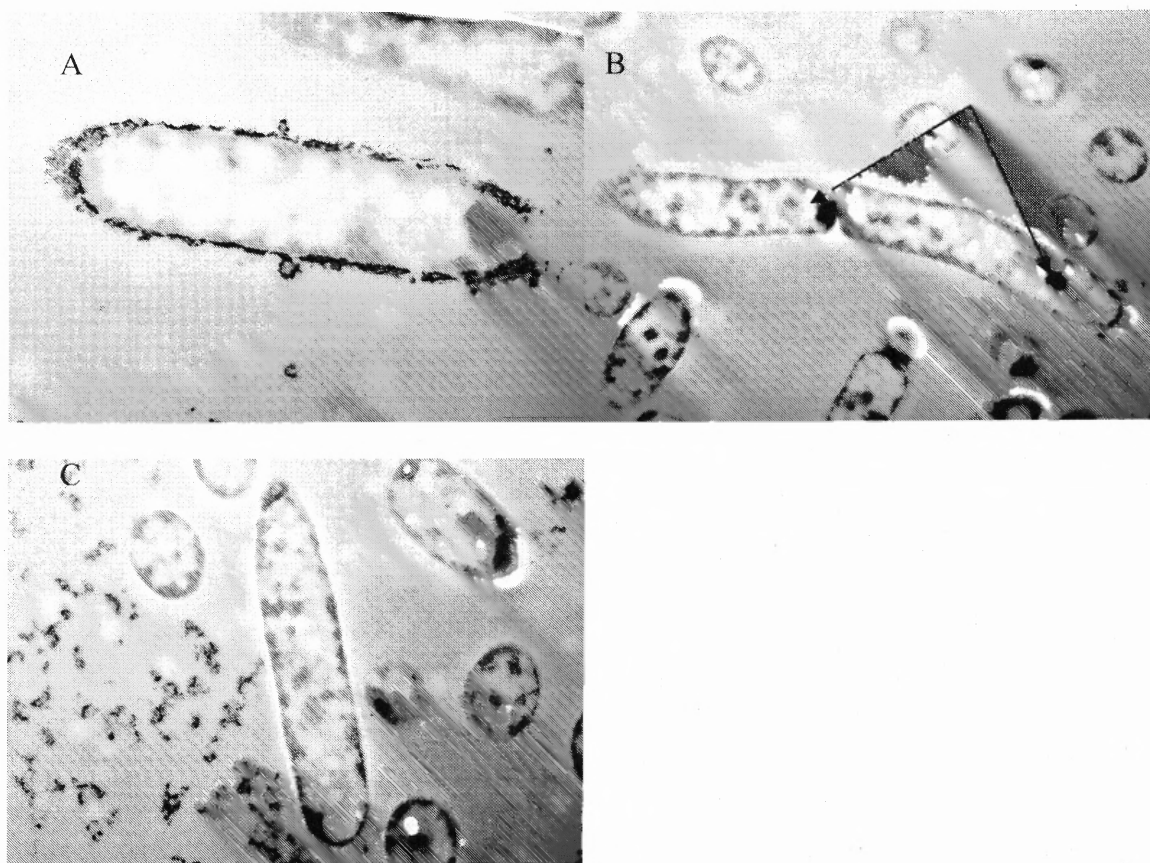


Figure 5.13 Thin section image of *Clostridium* sp. exposed to U and [EtPy][BF₄]: (A and B) 6h; (C) 24h.

5.3.4 Effects of Different Ionic Liquids on Bioreduction

Bioreduction of U(VI) by the anaerobic bacteria *Clostridium* sp. was affected by the presence of ionic liquids. The rate of reduction of U(VI) to U(IV) in the presence of [BMIM][PF₆] and [EtPy][BF₄] was similar to the reduction of uranyl nitrate. However, there were differences in the rate of precipitation from the solution of reduced uranium. The precipitation rate of bioreduced U(IV) from solution appears to be related to the nature of a complex formation with the ILs.

In contrast, the [EtPy][CF₃COO] and U(VI) complexes increase their solubility and stability in medium, and after 3 days, no precipitate was observed. However, almost no reduction was detected in the solution either. Although all three of these ionic liquids could form a complex with uranium, not all of the complexes could be bioavailable.

As we know, bidentate complex formed between U(VI) and [EtPy][CF₃COO]. Normally the stability constants of the multidentate complexes are from one to several orders of magnitude greater than those of monodentate complexes, thus making them resistant to a microorganism attack. Toxicity may be another factor. We find in the TEM analysis that U:[EtPy][CF₃COO] shows dramatic damage in the cellular membrane and affects the bacteria's function. Similar anion effects were suggested by Yang et al. (2005). They demonstrated that usually BF₄⁻ and PF₆⁻ benefit enzyme activity, but CF₃COO⁻ is not conducive to bioactivity.

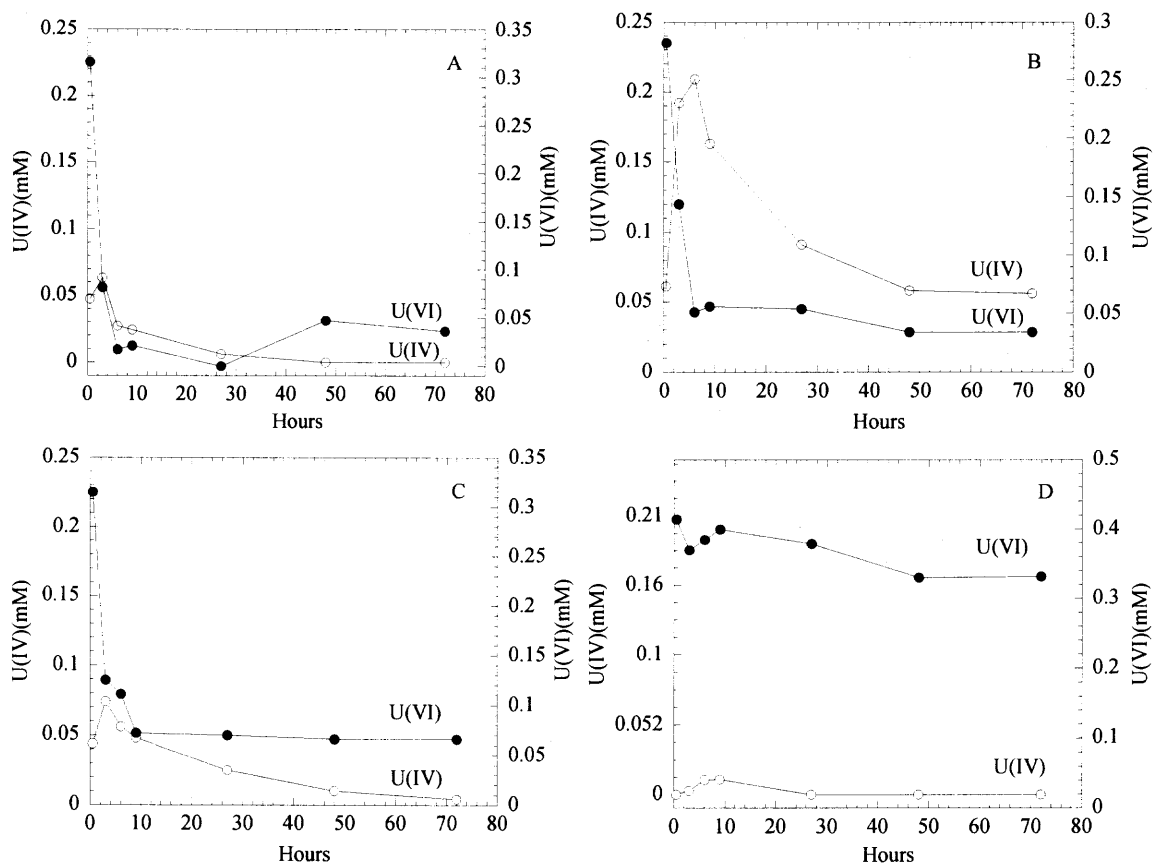


Figure 5.14 U bioreduction in presence of various ILs: (A) uranium; (B) [EtPy][BF₄]; (C) [BMIMPF₆]; (D) [EtPy][CF₃COO].

5.3.5 Mass Balance of U in Medium after Bioreduction

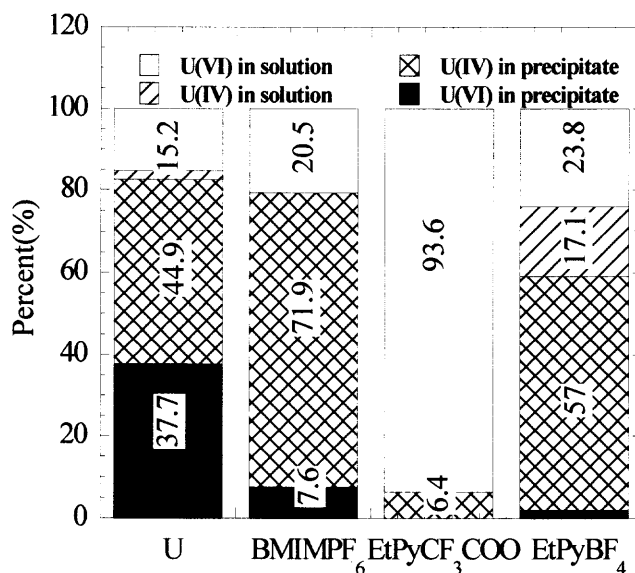


Figure 5.15 Mass balance of U in medium containing different ionic liquids.

Additions of different ionic liquids show various effects on bioreduction. [EtPy][CF₃COO] forms a bidentate complex with uranium(VI), and retains all uranium in solution. However, almost no reduction occurs. (The extremely low concentration of U(IV) in the precipitate may be due to the U's contact with the bacterial surface.) The addition of [BMIM][PF₆] and [EtPy][BF₄] increase the reduction. The reduction ratio was in order of U+[EtPy][BF₄](74.1%) > U+[BMIM][PF₆](71.9%) > U (47.1%) > U+[EtPy][CF₃COO](6.4%). Around 40% of the total uranium was maintained in solution when in the presence of the U-BF₄ complex. 20.5% and 23.8% of U(VI) were detected in solution in a U-[BMIM][PF₆] and U-[EtPy][BF₄] mixture. However, those two ionic liquids show different complex-ability to U(IV). With the addition of [EtPy][BF₄], the solubility of U(IV) in solution was greatly increased (17.1% U(IV) was maintained in solution after 72h incubation). But this phenomenon is not observed in the solution containing [BMIM][PF₆].

5.3.6 Comparison of UV-Vis Spectroscopy of U Before and After Bioreduction Extracted by 5mM Citric Acid

In comparing those two pictures (Figure 5.16), we can see that before the bacteria reduction, the maximum absorbance is at 435nm, an absorbance characteristic of U(VI). After bioreduction, however, the absorbance shifts to 555 and 665nm, clearly demonstrating the conversion of U(VI) to the reduced U(IV). This information is also confirmed in the XANFS and EXAFS analyses below.

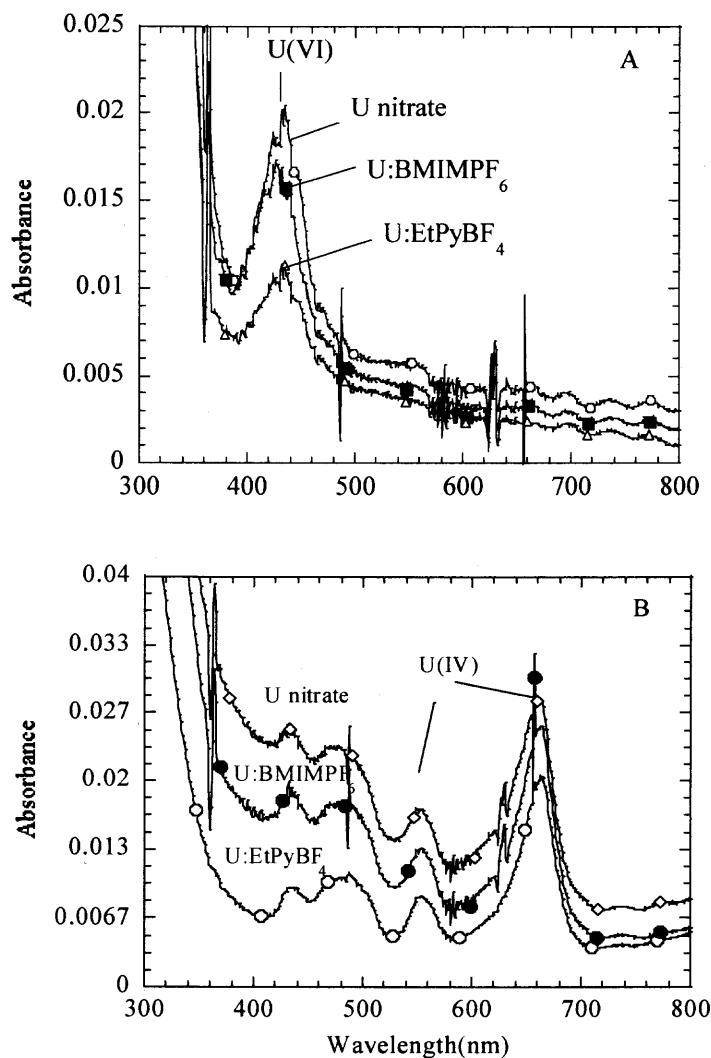


Figure 5.16 UV-Vis spectroscopy of precipitates extracted by citric acid (A) before bioreduction; (B) after bioreduction.

5.3.7 XANES and EXAFS Analysis

XANES spectra (Figure 5.17) show that after reduction, the energy changes from 17175 to a low energy of 17170 and 17169 eV. The complex with [EtPy][BF₄] and [BMIM][PF₆] makes the energy shift a little bit.

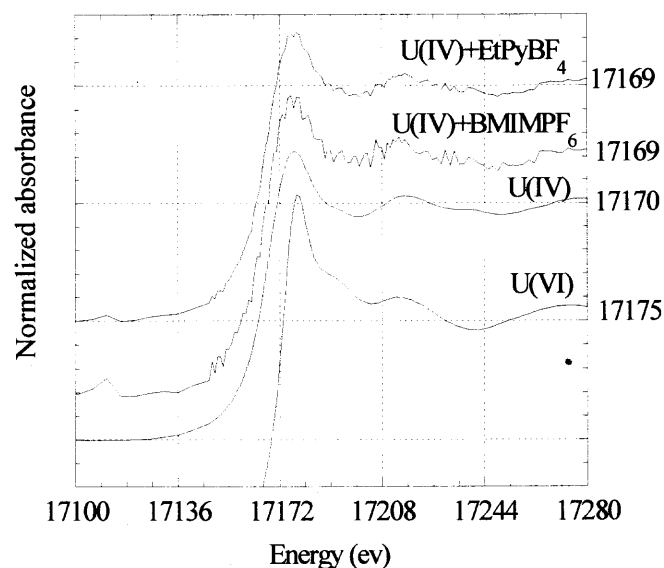


Figure 5.17 Normalized XANES spectra.

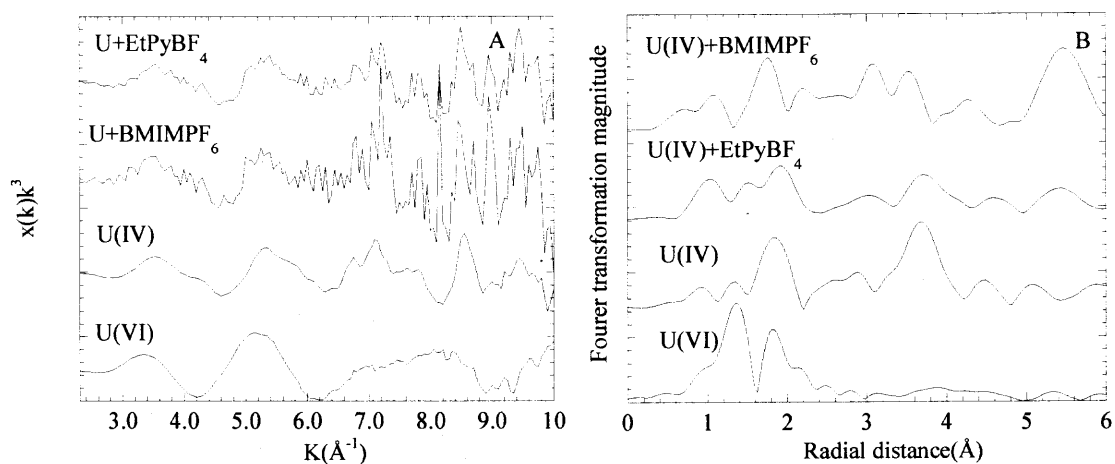


Figure 5.18 EXAFS analysis of bio-reduced biomass.

5.4 Summary

Ionic liquids, [EtPy][BF₄], [BMIM][PF₆], [EtPy][CF₃COO], can form different complexes with uranium and exhibit various effects on the uranium bioreduction process. Unlike with other organic ligands where the complex formed retards the reduction rate, in the presence of [BMIM][PF₆] and [EtPy][BF₄], the bioreduction is increased from 47% to 71.9% and 74.1% respectively. Most particularly, with the addition of 1% [EtPy][BF₄], a

high concentration of reduced U(IV) could be maintained in a medium. These results indicate that ionic liquids could be useful as efficient chelates that could convert U(VI) to U(IV) through biocatalysis, thus separating the uranium from an aqueous solution. Although the strong complex between U and [EtPy][CF₃COO] increases solubility of uranium in solution dramatically, it does not render it available for biotransformation. The result demonstrates the potential application of [EtPy][CF₃COO] as an efficient chelate and extractant in uranium separation.

In summary, our preliminary results show a promising future for the separating of target metals/radionuclides by tuning the properties of ionic liquids.

CHAPTER 6

BIODEGRADATION OF IONIC LIQUIDS

6.1 Introduction

Although ionic liquids are considered green solvents, their accidental or intentional release into the environment is a major concern. The persistence and fate of ionic liquids in the environment are not fully understood. There are only a few papers published about the biodegradation of imidazolium-based ionic liquids (Gathergood, et al., 2004; Boethling, et al., 1996).

The biodegradation of pyridine and pyridine derivative compounds has been studied extensively (Liu, et al., 1998; Robert, et al., 2002; Berry, et al., 1987). Metabolism of alky pyridines usually follows three initial steps: (i) reduction of the aromatic ring (Rhee et al., 1997; Watson, et al., 1975), (ii) oxidation of the aromatic ring (Feng, et al., 1994; Kaiser, et al., 1993), and (iii) oxidation of the alky group (Korosteleva, et al., 1981). Heterocyclic compounds can be degraded under both aerobic and anaerobic conditions. For the most part, aerobic biodegradation involves the general hydroxylation steps followed by the dioxygenolytic cleavage of the hetero-aromatic ring (Fetzner, 1998). Under anaerobic conditions, metabolism of pyridine is initiated either by ring reduction or ring hydroxylation.

Most of this work is focused on pyridinium-based ionic liquids. These ionic liquids belong to heterocyclic aromatic compounds. Heterocyclic aromatic compounds exist naturally from biological systems as electron carriers, nucleotides, and energy storage molecules. Also synthetic heterocyclic aromatic compounds are widely used in

industry as solvents, dyes, pharmaceuticals and pesticides (Kaiser, et al., 1996). Because of their heterocyclic structure, these compounds are more soluble in water than other homocyclic analogs and can be transported to ground water more easily.

Pyridinium salts are an important class of cationic surfactants and they are different from pyridine compounds. They contain a long alky chain attached to the positive charged nitrogen atom. Thousands of pyridinium salts can be synthesized with different substituents and anions. Because of the formal positive charge on the nitrogen atom, those salts have many unique properties such as antimicrobial, anti-electrostatic, surface activity and adsorption onto negatively charged solids (Pernak, et al., 2004; Pernak, 2001). As a functionalized surfactant, they can aggregate and exhibit chemical and biochemical reactivity simultaneously. It is also observed that in pyridinium salts, the electro-density distribution around the heterocyclic nucleus may differ significantly from that of ring-substituted pyridines. This is due to the fact that the distribution of positive charge severely hinders electrophilic substitutions in the deactivated pyridine ring.

6.2 Degradation of ILs by *Pseudomonas fluorescence*

According to previous research (Mohan, et al., 2003; Kim, et al., 2006) *Pseudomonas* sp. is one of the more effective bacterium able to degrade pyridine compounds. *Pseudomonas fluorescence* is a common, nonpathogenic bacterium that exists in soil, in water, and on plant surfaces. It produces a soluble, greenish fluorescent pigment, particularly under conditions of low iron availability. It has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources. They have been known to biodegrade many xenobiotics

(Bencheikh, et al, 2003; Kao, et al., 2005; Pazarlioğlu, et al., 2005; Francis, et al., 1978). In this study we investigated the degradation of ILs by a pure culture of *Pseudomonas fluorescens*.

6.2.1 Materials and Methods

6.2.1.1 *Pseudomonas* Culture *Pseudomonas fluorescens* (ATCC No.55241) was grown in the following manner (per liter): citric acid 2g, NH₄Cl 1g, KH₂PO₄ 1g, K₂HPO₄ 1g, NaCl 4g, MgSO₄ 0.2g. After adding different amounts of IL, the pH was adjusted to 6.5 and then the specimen was autoclaved. Two milliliters of an 18h-old culture of *P. fluorescens* grown in the same medium was used to inoculate 50 ml of the medium containing varying amounts of ILs. Duplicate samples of each treatment were incubated in darkness on a rotary shaker at 26°C. Periodically a 5ml sample was removed, filtered through a 0.22 µm filter and analyzed for pH, for citric acid by HPLC using a Bio-Rad HPX-87H column with 0.003M sulfuric acid as a mobile phase, and a UV-Vis detector at 210nm. IL in the medium before and after microbial growth was analyzed by LC-MS (Advantage LCQ ESI-MS).

6.2.1.2 Gram Stain To determine the effect of ILs on bacterial cell morphology, Gram staining was performed. That process, briefly, manifested as follows: we placed the slides on a staining rack and flooded the thin, air-dried, heat fixed smear with the crystal violet staining reagent for 1min. Then wash the smear in a gentle and indirect stream of tap water for 2s. And then smear was flooded with an iodine mordant for 1min. Again we washed the smear as before for 2s, and blotted the film dry with absorbent paper. The

smear was then flooded with 95 % ethanol for 30s with agitation, and the film was blotted dry with absorbent paper. We then flooded the smear with the safranin counterstain for 10s; washed it with a gentle and indirect stream of tap water until no color appeared in the effluent. The film was then blotted dry with absorbent paper. Pictures of the bacterial cells were taken by NIKON photomicrography under 100 magnification.

6.2.2 Results and Discussion

6.2.2.1 Effects of Different Ionic Liquids on the Growth of *Pseudomonas fluorescens*

The effects of ILs, [EtPy][BF₄], [EtPy][CF₃COO] and [BMIM][PF₆], on the growth of bacteria were investigated.

[EtPy][BF₄] 1, 5, or 10 % (v/v) was added to the growth medium at the onset of the experiment. Growth was observed only in the presence of 1 % of [EtPy][BF₄] (Figure 6.1 A). Little growth was observed in the medium containing 0.5 % of [BMIM][PF₆] (Figure 6.1 B). No growth was observed in the medium containing [EtPy][CF₃COO] (Figure 6.1 C).

The properties of ionic liquids may affect the growth of bacteria dramatically. Although [EtPy][BF₄] and [EtPy][CF₃COO] have the same cation, the different anion may play an important role in the toxicity. As described by Garcia et al. (2005) biological membranes are essentially non-polar interfaced; the toxicity is caused by a disruption of the integral membranes by a hydrophobic/ionic adsorption at the cell membrane-water interface. Hydrophobic molecules have a greater ability to accumulate at the interface, so higher toxicity would be expected with increasing hydrophobicity. The hydrophobic

[BMIM][PF₆] showed high inhibitory effects in this study. Based on our knowledge from studies conducted with diverse natural or laboratory microbial cultures, we know that trifluoroacetic acid is expected to be generally resistant to microbial degradation (<http://www.afeas.org/newtfa.html>).

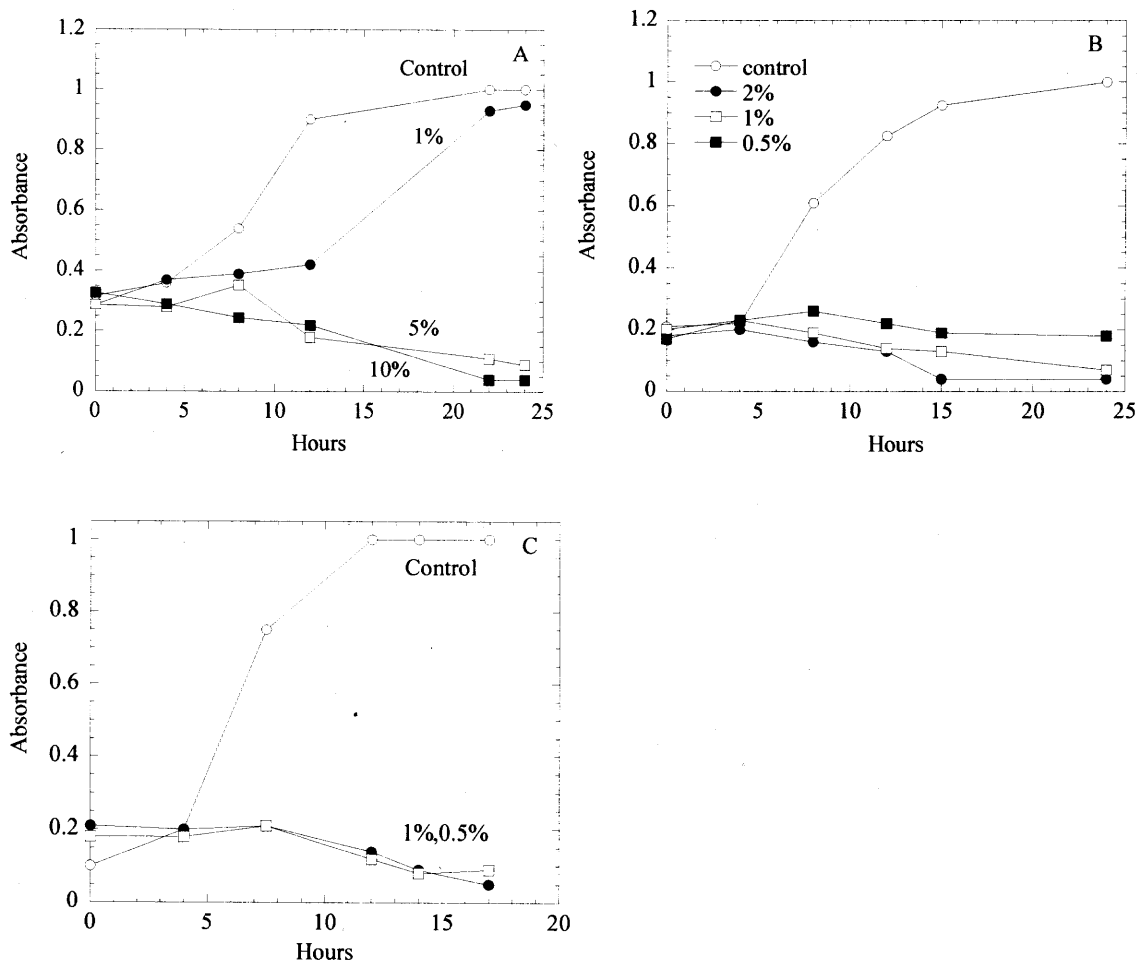


Figure 6.1 Effects of ILs on the growth of *P. fluorescens* (A) [EtPy][BF₄]; (B) [BMIM][PF₆]; (C) [EtPy][CF₃COO].

6.2.2.2 Effects of Different Concentrations of [EtPy][BF₄] To determine the effect of [EtPy][BF₄] concentration on growth of the bacterium, 0.05 %, 0.75 %, and 1.0 % of [EtPy][BF₄] were added to the growth medium and the optical density, pH, degradation of citric acid, and change of bacterial morphology were monitored.

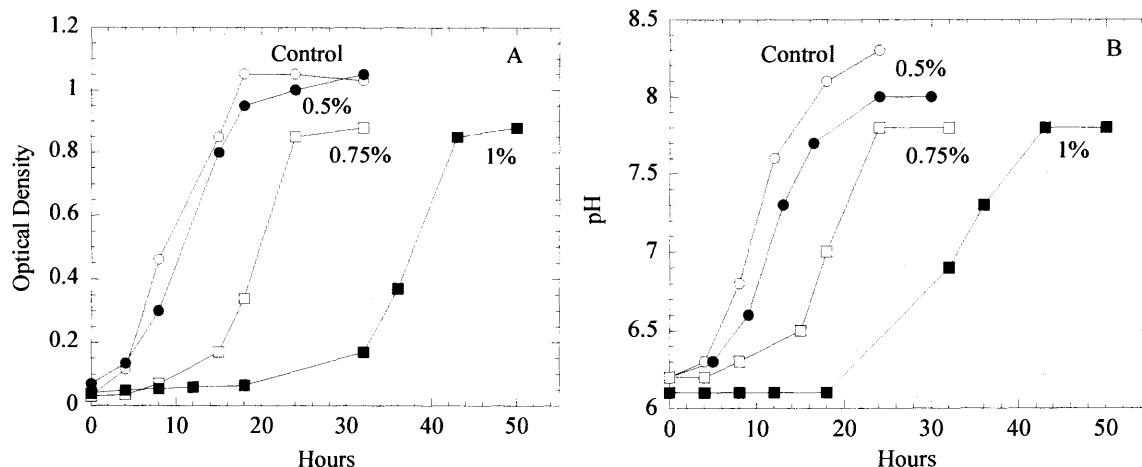


Figure 6.2 Effects of different concentration of [EtPy][BF₄] on change of (A) optical density; (B) pH.

Bacterial growth was retarded with increasing concentrations of [EtPy][BF₄] (Figure 6.2 A). The lag time increased with an increase of the ionic liquid concentration. There was about 18 h lag time in the medium containing 1% [EtPy][BF₄]. When bacteria experience various stresses, such as temperature, pH, salt concentration, low nutrition, the presence of toxic chemicals, a lag phase may appear for bacteria to accommodate the new environment. However, the lag phase can be eliminated by inoculating the medium with a growing culture derived from a medium containing [EtPy][BF₄] (Figure 6.3).

In a control sample containing no [EtPy][BF₄], the pH changed from 6.2 to 8.3 after incubation because the citric acid was consumed as a carbon source. However, in a medium containing different concentrations of ionic liquids, less citric acid was consumed by the bacterium, resulting in a pH relatively lower after incubation, as compared to the pH in the control. For the medium containing 1% [EtPy][BF₄], the pH only reached 7.7 after incubation.

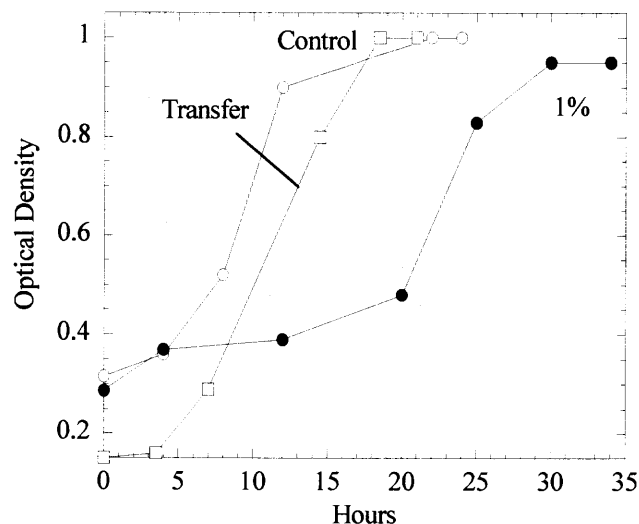


Figure 6.3 Growth of *Pseudomonas* in presence of [EtPy][BF₄].

We also tried to decrease the citric acid concentration, and explored the use of ionic liquids as a carbon source for the bacterium. For this purpose, media containing 0 % and 50 % citric acid were used to incubate the bacteria while maintaining the same concentration of [EtPy][BF₄] (1%). No growth was observed. This indicates that the bacterium cannot use [EtPy][BF₄] as sole carbon source, and that the [EtPy][BF₄] can be co-metabolized only in the presence of another carbon source such as citric acid.

The HPLC analysis of citric acid degradation showed that the rate of consumption of citric acid in a IL treated culture is almost half that of a culture containing no [EtPy][BF₄], as shown in Figure 6.4. In the presence of ILs, the bacterium's consumption of citric slows down.

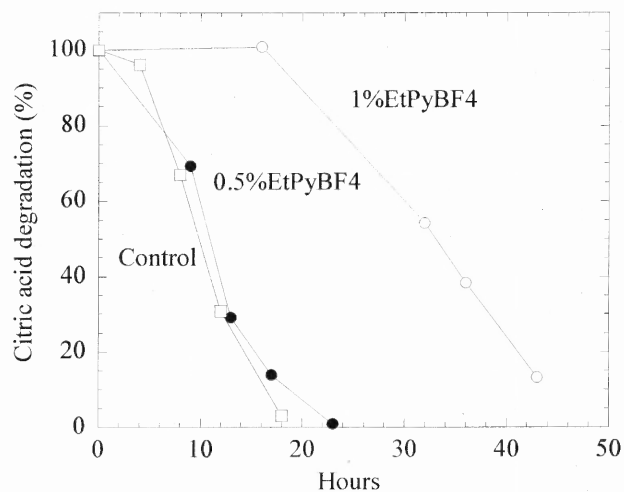
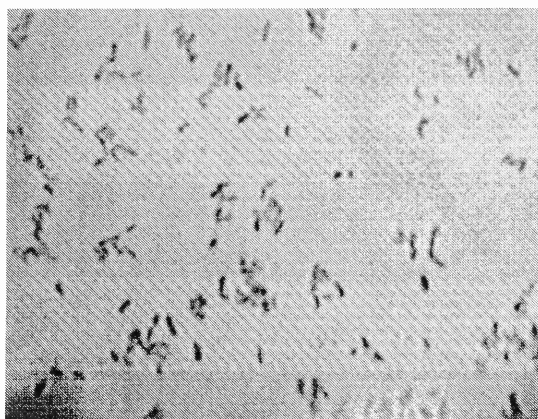


Figure 6.4 Effect of $[\text{EtPy}][\text{BF}_4]$ on citric acid degradation.

In the presence of $[\text{EtPy}][\text{BF}_4]$, the morphology of the bacteria changed from an original shape of a small dot to a long wide rod. The length of the bacteria is about $0.5\mu\text{m}$. After IL treatment, however, the length of bacteria increased to $0.8\mu\text{m}$ to $1.1\mu\text{m}$ (as shown in Figure 6.5). The cause for this phenomenon is not known.



P. fluorescens without IL $[\text{EtPy}][\text{BF}_4]$



P. fluorescens with IL $[\text{EtPy}][\text{BF}_4]$

Figure 6.5 Comparisons of *P. fluorescens* cell growth in the absence and presence of $[\text{EtPy}][\text{BF}_4]$.

6.2.2.3 LC-MS analysis

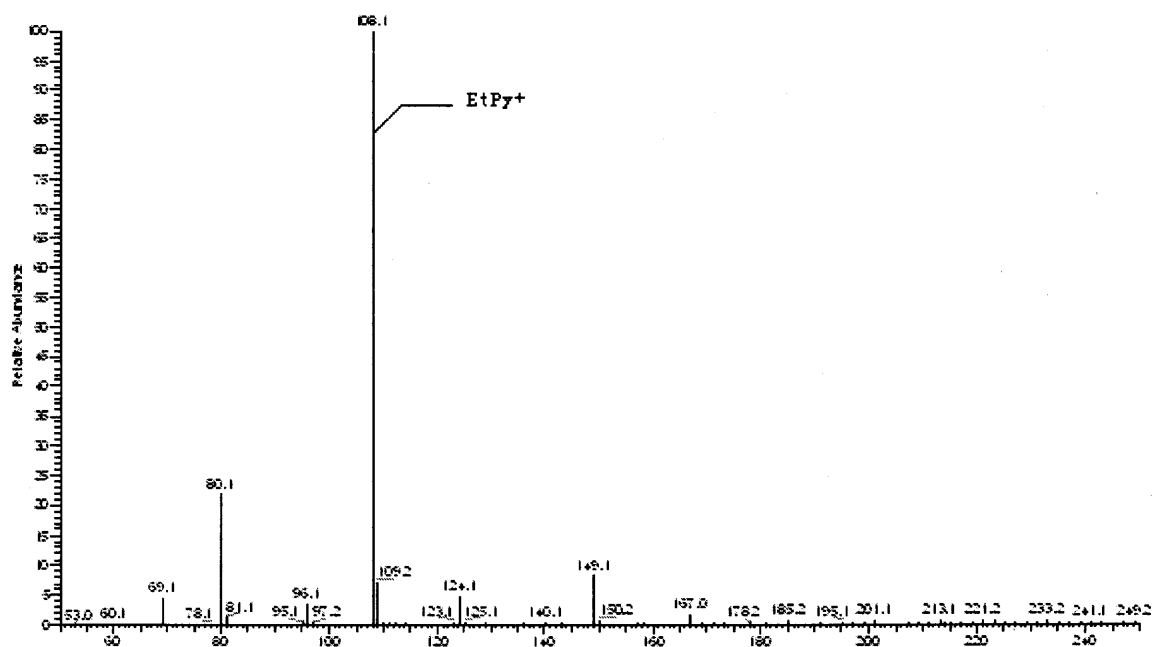


Figure 6.6 MS of mixture before biodegradation of [EtPy][BF₄] at positive mode.

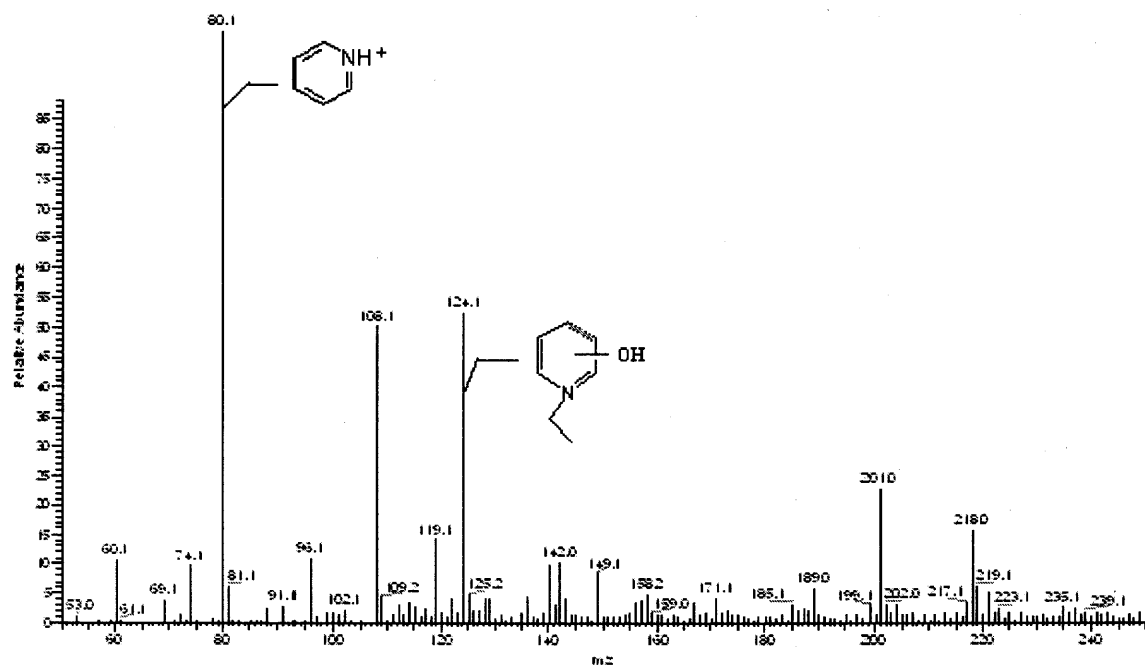


Figure 6.7 MS of mixture after 24 h biodegradation of a culture sample containing [EtPy][BF₄] at positive mode.

In the presence of bacterial activity, a 124 (m/z) peak manifests, which could be due to the nucleophilic addition. As suggested by Wright et al. (1971) nucleophilic substitutions are promoted by the electron distribution in pyridine (particularly in pyridinium nuclei). Moreover, the enzymatic hydroxylation of pyridine compounds frequently occurs by the incorporation of the O atom from water. Similar properties were also found in ethyl-pyridine biodegradation products (Bunting, et al., 1987; Feng et al., 1994).

The apparent increase of the peak 80 (m/z) and decrease of the peak 108 (m/z) can be attributed to the dealkylation process during bacteria incubation. Since the long alky chain increases the IL's hydrophobicity, dealkylation may reduce the IL's potential of retarding in the environment. Pyridinium can be reduced to pyridine, which can be completely metabolized by soil bacteria and converted to low molecular organic acid and ammonia (Watson et al., 1974; 1975; Sim, et al., 1985).

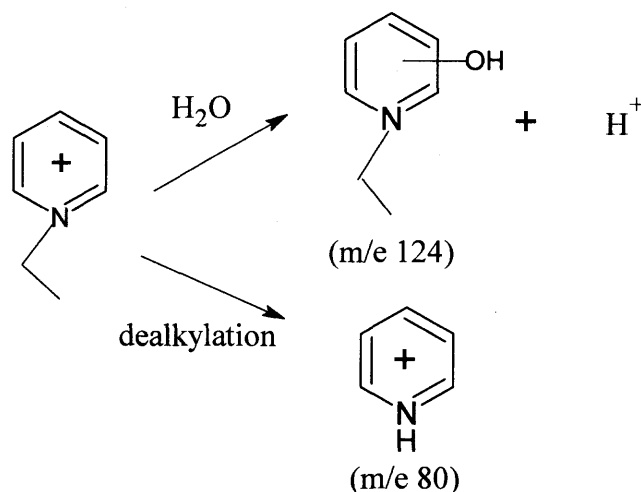
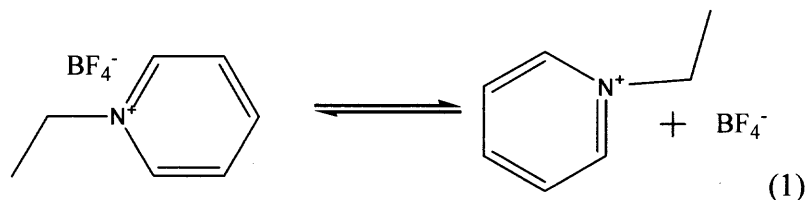


Figure 6.8 Nucleophilic addition and dealkylation of N-ethylpyridinium.

In a control medium without bacteria, a decrease of pH was also observed (from 7.8 to 6.7 in 4 days). This may be attributed to the hydrolysis of the anion BF_4^- . The equation is shown below:



The degradation of fluorinated acid has been reported by Menon et al. (1973). The release of hydrogen fluoride from ionic liquids containing the fluorinate anion also was investigated by Swatloski et al. (2003). This anion degradation process causes pH drift in aqueous solution and affects the biocatalysis process dramatically. The pyridinium cation maintained its integrity during the pH change. Thus, the degradation of the cation was caused solely by bacterial activity.

6.2.3 Summary

This study shows that the ionic liquid $[\text{EtPy}][\text{BF}_4]$ was not metabolized as the sole carbon source by a pure culture of *P. fluorescens*. But in the presence of an easily metabolized carbon source, such as citric acid, the IL partially degrades through cometabolism. Hydrolysis, dealkylation, and nucleophilic addition reactions were the three major processes. Hydroxyl-N-ethylpyridine and the pyridinium cation are the major cation degradation products. HBF_3OH and HF are suggested as anion degradation products. Also the presence of IL causes a morphological change in the bacteria *P. fluorescens*,

and decreases the rate of citric acid degradation. Different ILs show different inhibitory effects on the growth of bacteria.

6.3 Degradation of ILs by a Soil Enrichment Culture

In this research, I investigated (1) the ability of a soil enrichment culture to degrade [EtPy][BF₄] as a sole carbon source; (2) the character of the degradation products; (3) the degradation rate of ILs in the presence of uranium.

6.3.1 Materials and Methods

6.3.1.1 Source, Enrichment and Maintenance of the IL-utilizing Bacteria A sandy soil sample was obtained from a garden near the Brookhaven National Laboratory (Long Island, NY). Ten grams of soil were added to 250 ml flasks containing 100 ml of a mineral salt medium (MSM). Then [EtPy][BF₄] 10 μ l was added; it provided the sole carbon and nitrogen growth source.

The flasks were placed on a rotary shaker (200 rpm) at 25°C in darkness. Deionized sterilized water was added weekly to make up for the loss of water due to evaporation. The concentration of N-ethyl pyridinium cation was monitored by UV-Vis spectroscope at 259 nm. Once the pyridinium cation has disappeared, 2 ml of supernatant was transferred to the 50 ml of fresh MSM containing 10 μ l [EtPy][BF₄]. Cultures were maintained through weekly transfers of 2 ml of turbid culture to 50 ml of fresh MSM containing 10 μ l of [EtPy][BF₄].

6.3.1.2 Mineral Salt Medium Mineral salts medium (MSM) contained the following ingredients as described by Houghton et al. (1972): K_2HPO_4 , 1g/liter of water; KCl, 0.25g/liter of water; $MgSO_4 \cdot 7H_2O$, 0.25g/l of water; trace element solution, 1ml; pH was adjust to 7.5.

The trace element solution contained (per liter): $FeSO_4 \cdot 7H_2O$, 40mg; $MnSO_4 \cdot 4H_2O$, 40mg; $ZnSO_4 \cdot 7H_2O$, 20mg; $CuSO_4 \cdot 5H_2O$, 5mg; $CoCl_2 \cdot 7H_2O$, 4mg; $Na_2MoO_4 \cdot 2H_2O$, 5mg; $CaCl_2 \cdot 6H_2O$, 0.5mg; NaCl, 1g. Several drops of concentrated HCl were added to the solution to prevent precipitation.

6.3.1.3 Growth of Bacteria At periodic intervals, 5 ml of the bacterial culture sample were withdrawn and analyzed for the growth of bacteria by measuring the optical density (O.D.) at 600 nm. The culture medium was filtered through a $0.45\mu m$ syringe filter. Changes in pH were determined by pH electrode and the concentration of N-ethylpyridinium cation was measured at 259 nm by UV-Vis spectroscope. Degradation products were analyzed by HPLC-ESI/MS (Bio-Rad organic acid analysis column, 300 mm X 7.8 mm, flow rate 0.7 ml/min, 210 nm) with 0.003 mM sulfuric acid as the mobile phase. Uninoculated MSM containing same amount of $[EtPy][BF_4]$ was used as a control to eliminate non-bioactive interference.

6.3.1.4 Effects of Different Ionic Liquids Ten microliters of the different ionic liquids, $[EtPy][BF_4]$, $[EtPy][CF_3COO]$ and $[BMIM][BF_4]$ were added to a MSM respectively as the sole carbon source. The cultures were inoculated with 2 ml of an early log phase

growing medium. Optical density, pH change, and LC-MS analyses were measured as described above.

6.3.1.5 Effects of Uranium on Biodegradation Uranium (0.25 mM) was mixed with 1.2 mM (10 μ l) of [EtPy][BF₄] and stabilized overnight. This U-IL complex was added to MSM (pH was adjusted to 6 by 0.01M NaOH) and inoculated with 2 ml early log phase growing medium. Growth of bacteria was monitored as above. Uranium concentrations in solution were measured by KPA after filtration. U-IL complex in uninoculated medium (pH 6) was also monitored as control.

6.3.2 Results and Discussion

6.3.2.1 Degradation of [EtPy][BF₄] The enrichment culture of bacteria from soil was able to metabolize [EtPy][BF₄] as the sole carbon and nitrogen source. The medium's optical density only reached 0.25 after 24 h of incubation as a result of the low content of carbon source (1.2mM). According to the UV-Vis spectroscopy, N-ethylpyridium metabolized completely in about 28 hours, and no other peaks were detected by UV (Figure 6.10). During incubation, the pH of the medium dropped from 6.65 to 6.55 (about 0.1 units) as compared to the control, which indicates that acid was produced during the degradation of [EtPy][BF₄].

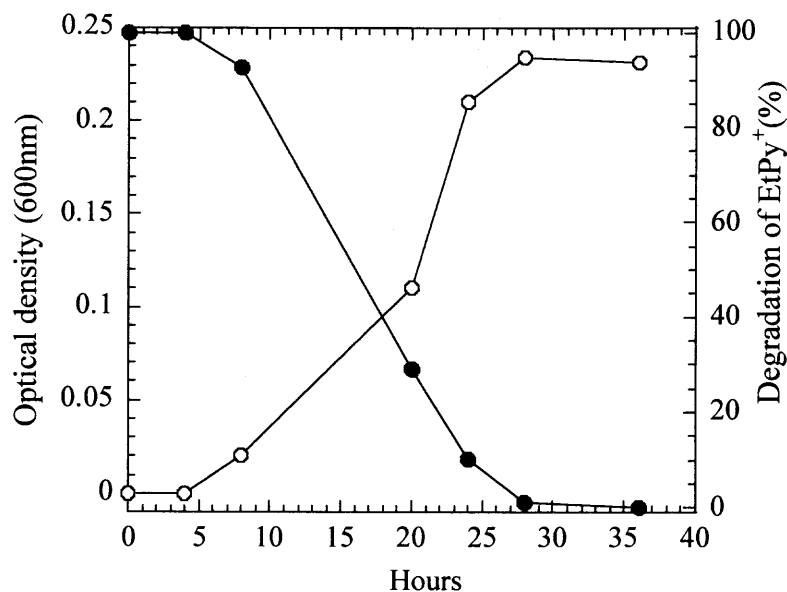


Figure 6.9 Degradation of 1.2mM [EtPy][BF₄] by enrichment culture.

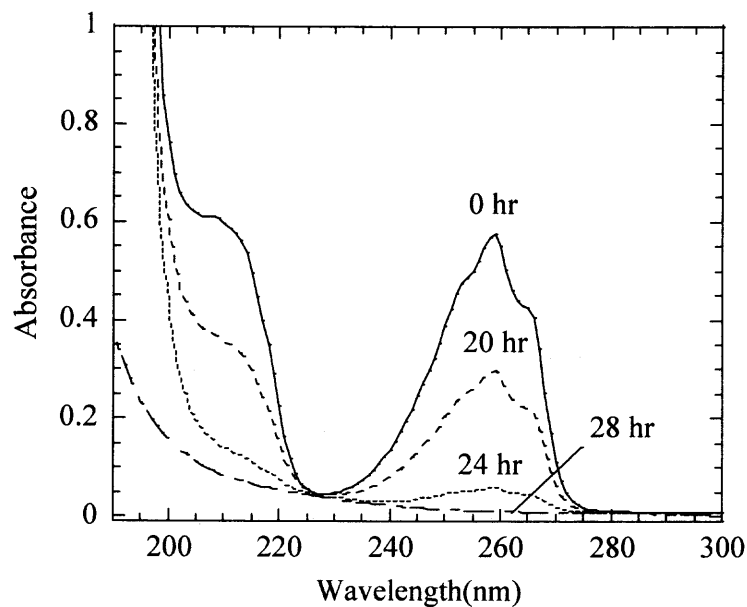


Figure 6.10 UV-Vis spectroscopy of EtPy⁺ during incubation.

The optical density reached around 0.8 when 4.8mM [EtPy][BF₄] were provided as the sole carbon sources. No inhibitory effects were observed (Figure 6.11). The decrease in the pH during incubation was also observed as shown in Table 6.1.

In the presence of other carbon sources, such as yeast extract and glucose, no ethylpyridinium degradation was detected.

Table 6.1 pH Changes of the medium during incubation

| Addition of [EtPy][BF ₄] | Before incubation | After incubation |
|--------------------------------------|-------------------|------------------|
| 1.2mM | 6.65 | 6.55 |
| 2.4mM | 6.50 | 6.45 |
| 3.6mM | 6.43 | 6.32 |
| 4.8mM | 6.38 | 6.28 |

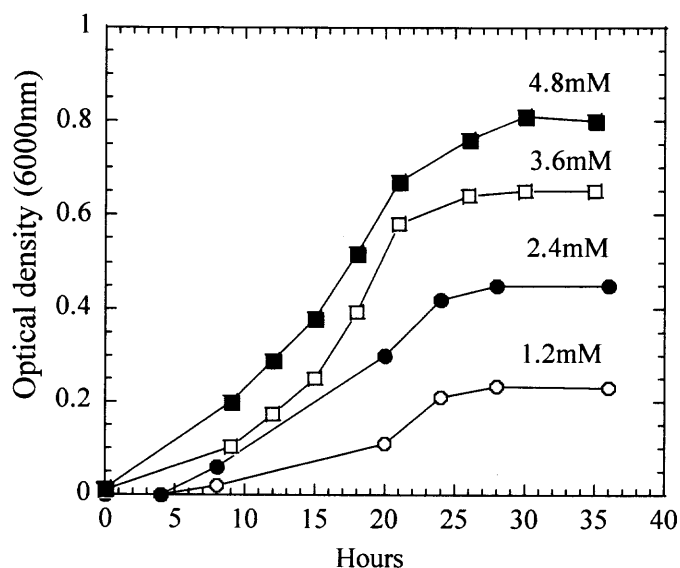


Figure 6.11 Biodegradation of [EtPy][BF₄] at different concentrations.

6.3.2.2 Degradation of Different Ionic Liquids The bacterium metabolized [EtPy][CF₃COO] and produced the same cation degradation products as [EtPy][BF₄]. However, for the imidazolium-based ionic liquid, [BMIM][PF₆] was not metabolized by the enrichment culture (Figure 6.12).

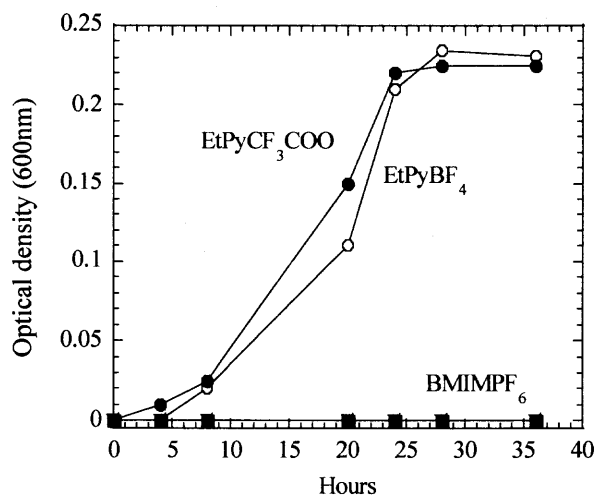


Figure 6.12 Degradation of different ionic liquids (10 μ l) by enriched culture.

6.3.2.3 Metabolites Analysis and Proposed Degradation Pathway HPLC analysis showed that there are two major peaks at the retention time of 24.4 minutes (A) and 35.5 minutes (B), which accumulated in the medium as intermediate degradation products within the first 25 hours. After reaching the highest concentration product A quickly disappeared in 4 hours, whereas the product B disappeared more slowly over the course of one week.

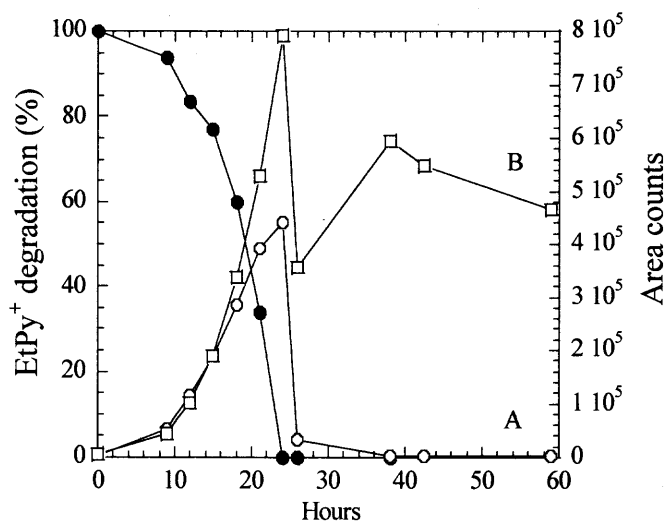


Figure 6.13 Metabolites during incubation by HPLC analysis.

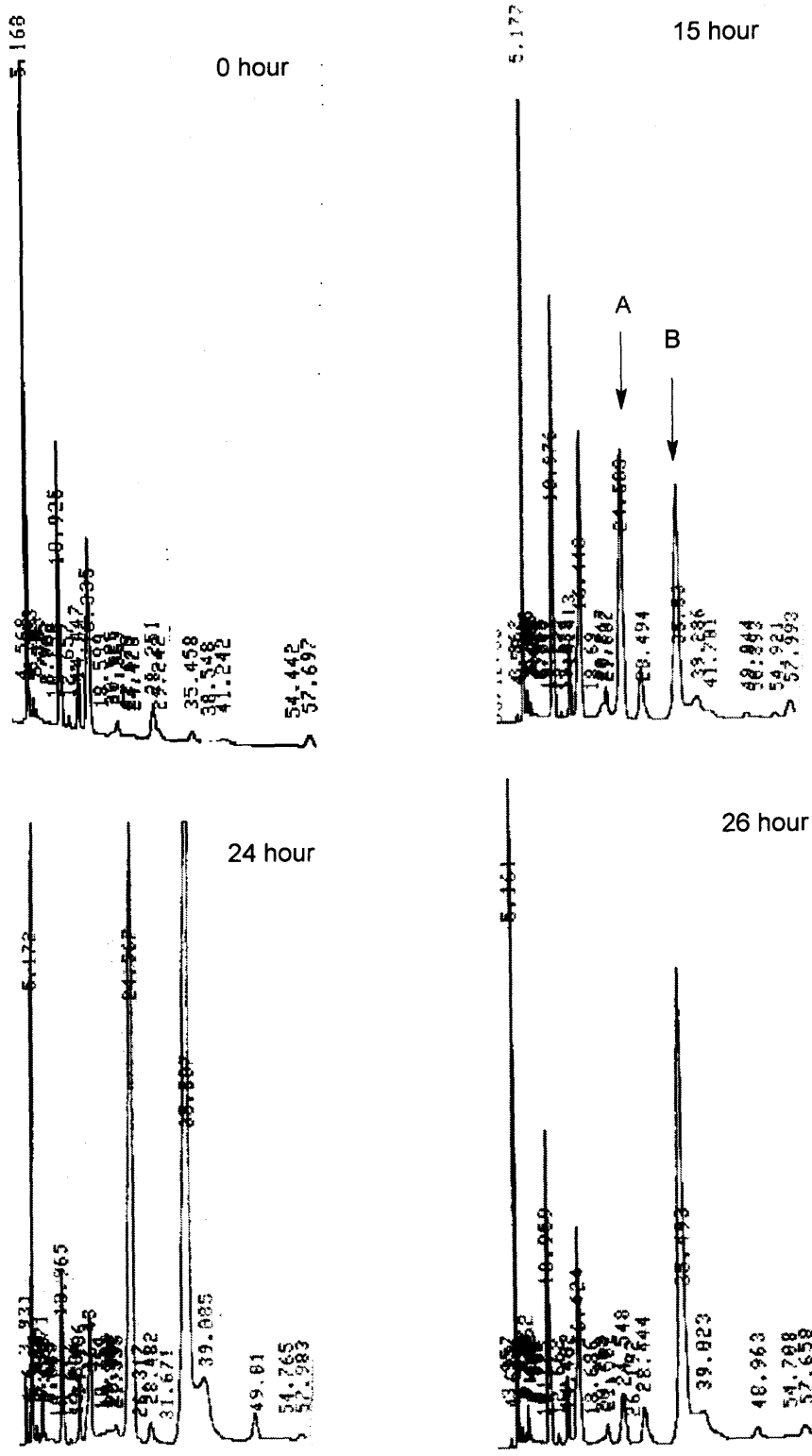


Figure 6.14 HPLC monitoring of metabolites from 0h to 26h.

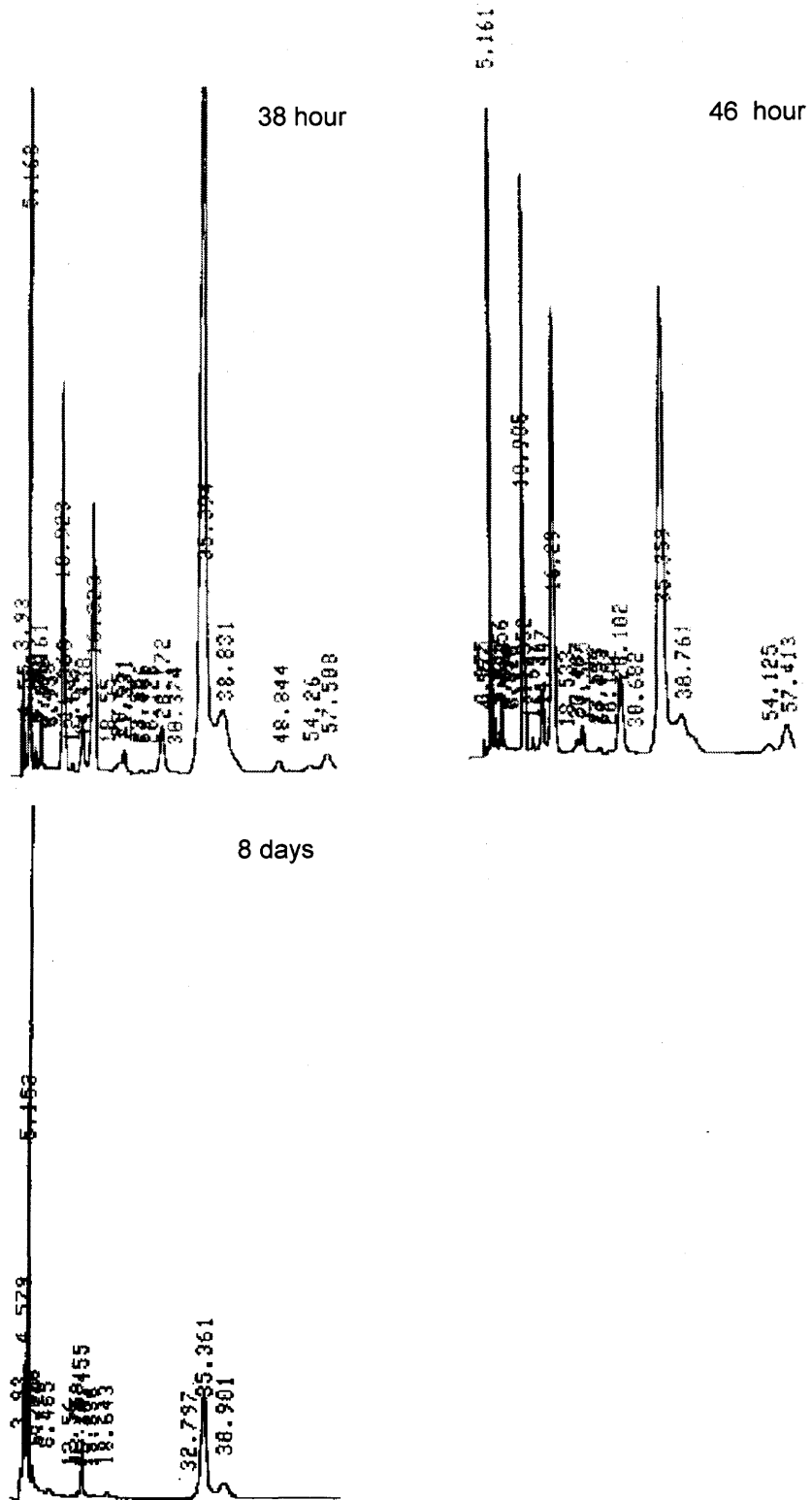


Figure 6.15 HPLC monitoring of metabolites from 38 h to 8 days.

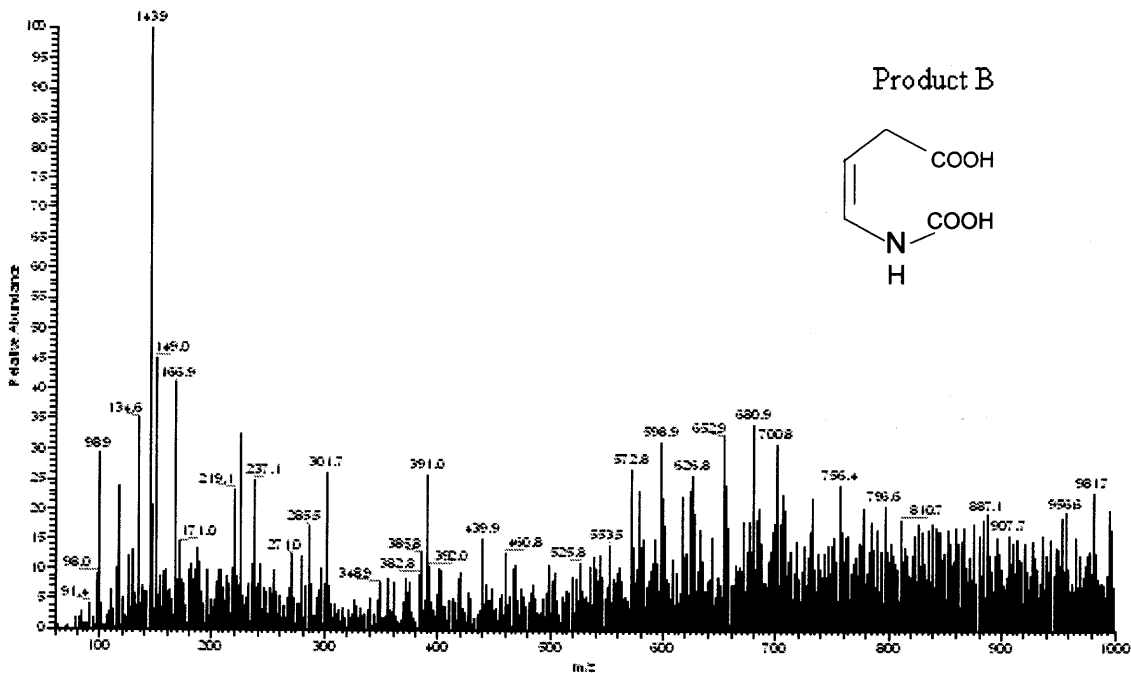
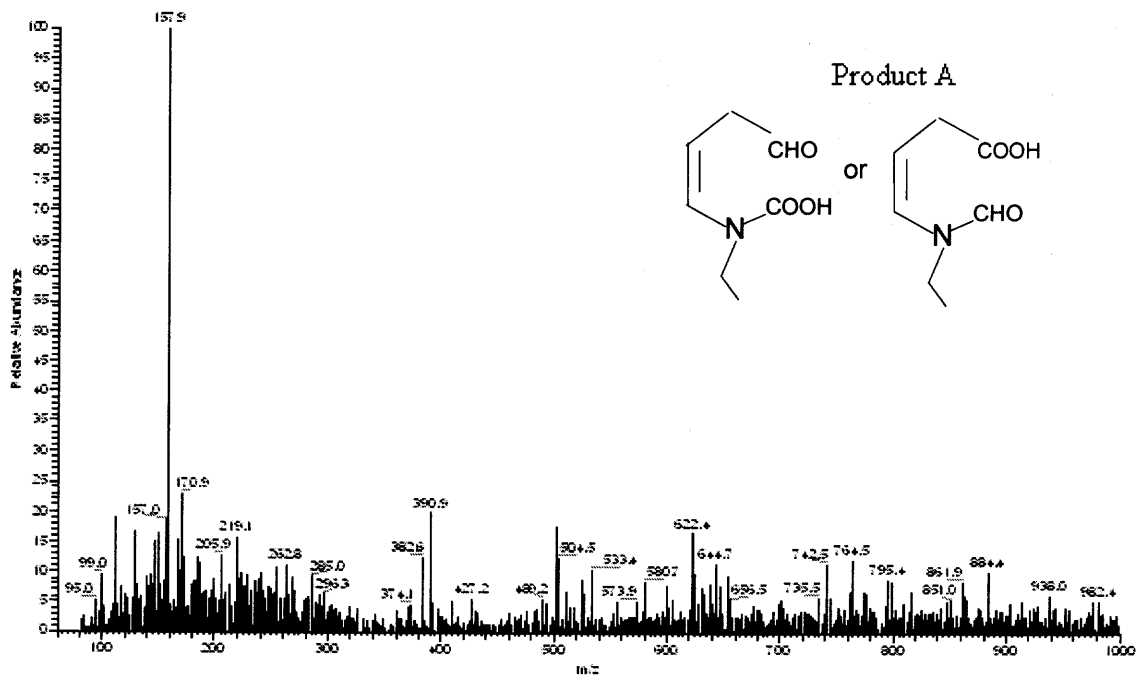


Figure 6.16 LC-ESI/MS analysis of products A and B.

LC-ESI/MS analysis clearly demonstrates the molecular weight of product A and B are 157.9 and 143.9 respectively. These products can be metabolites as result of C2-C3 ring opening. The proposed degradation pathway is shown below (Figure 6.17).

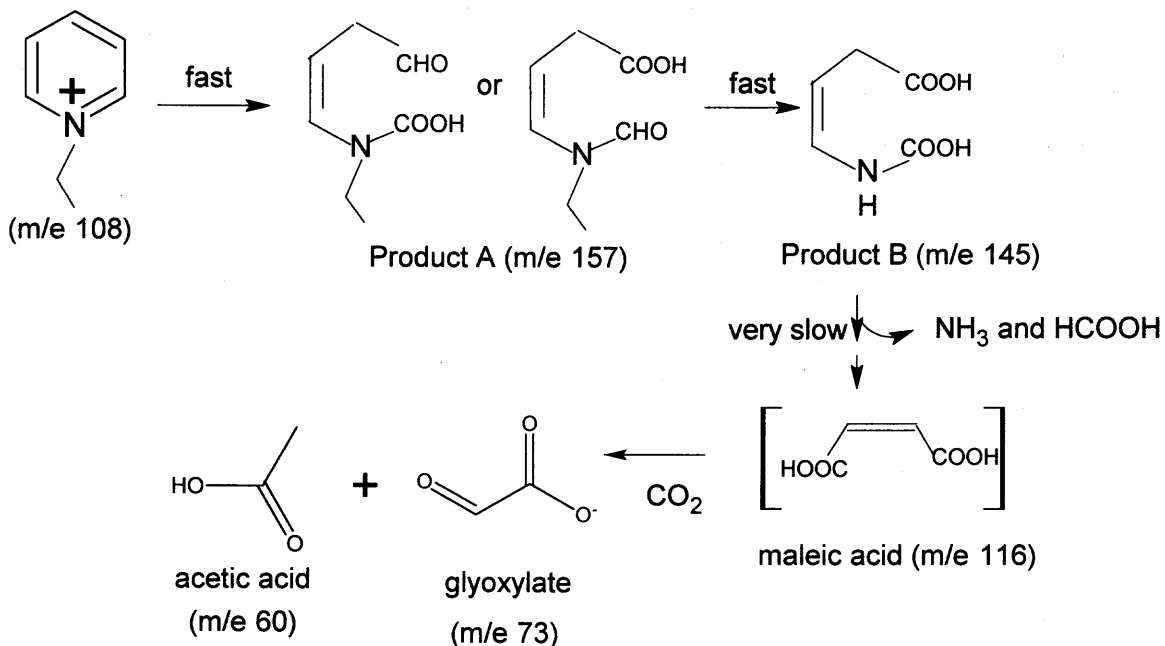


Figure 6.17 Proposed degradation pathway.

The ring opening step is fast since the absorbance of the ring disappeared in just 24 hours. The intermediate product A was converted to product B as evidenced by the increase of the peak density of B between 24 hours and 40 hours. The slow biodegradation of product B may be the result of the inhibition or the lack of nutrients. We tried to identify the final products, however due to the low molecular weights and volatile properties, no major peak was detected by LC-MS between 5 and 20 minutes. However, we found a 73 (m/z) peak and 60 (m/z) peak when the sample was injected to the MS directly without any separation (Figure 6.18). Especially at the negative mode, the acetic acid peak was prevalent (Figure 6.19), which confirmed our hypothesis that

acetic acid and glyoxylate can result as final products. The glyoxylate can be metabolized by the glyoxylate pathway (Kornberg, 1966).

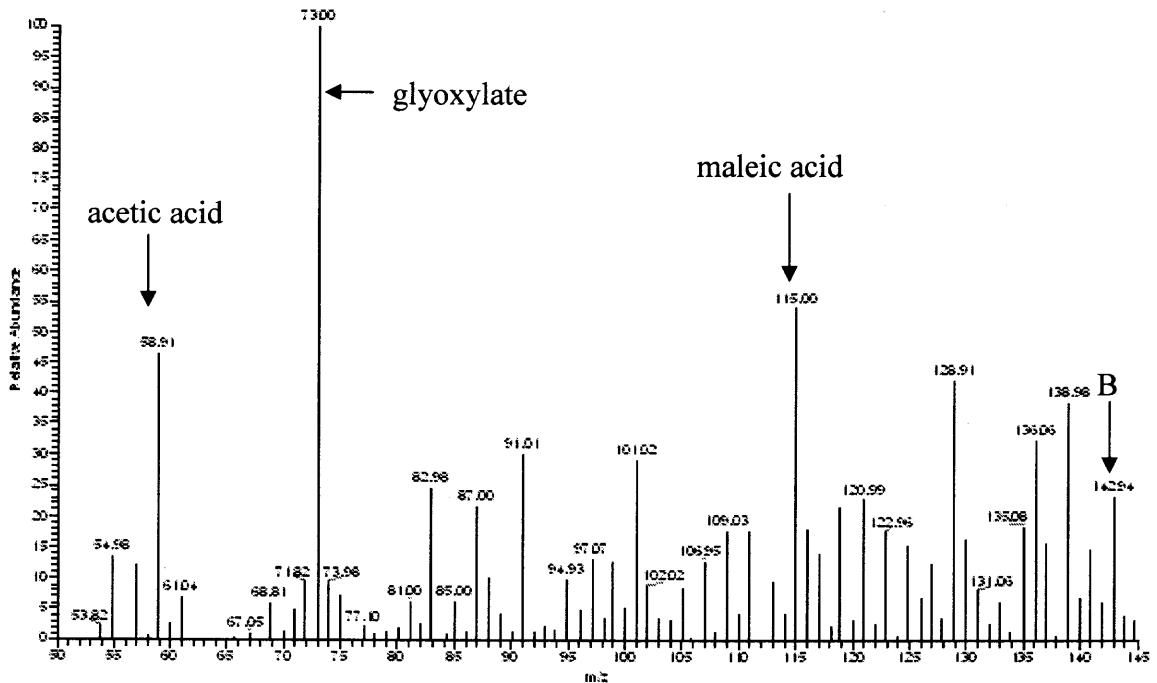


Figure 6.18 Mass spectroscopy of degradation products (after 35 hours) at positive mode.

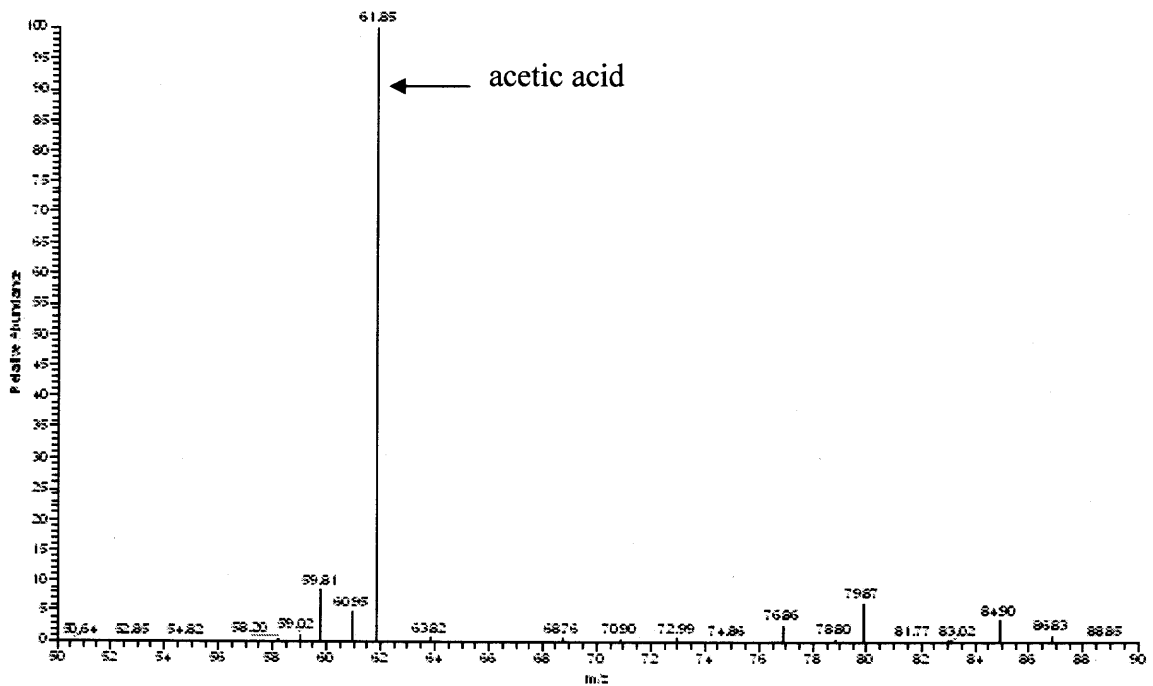


Figure 6.19 Mass spectroscopy of degradation products (after 35 hours) at negative mode.

Products A and B were further identified by LC-ESI/MS/MS analysis as shown in Figure 6.20 and 6.21.

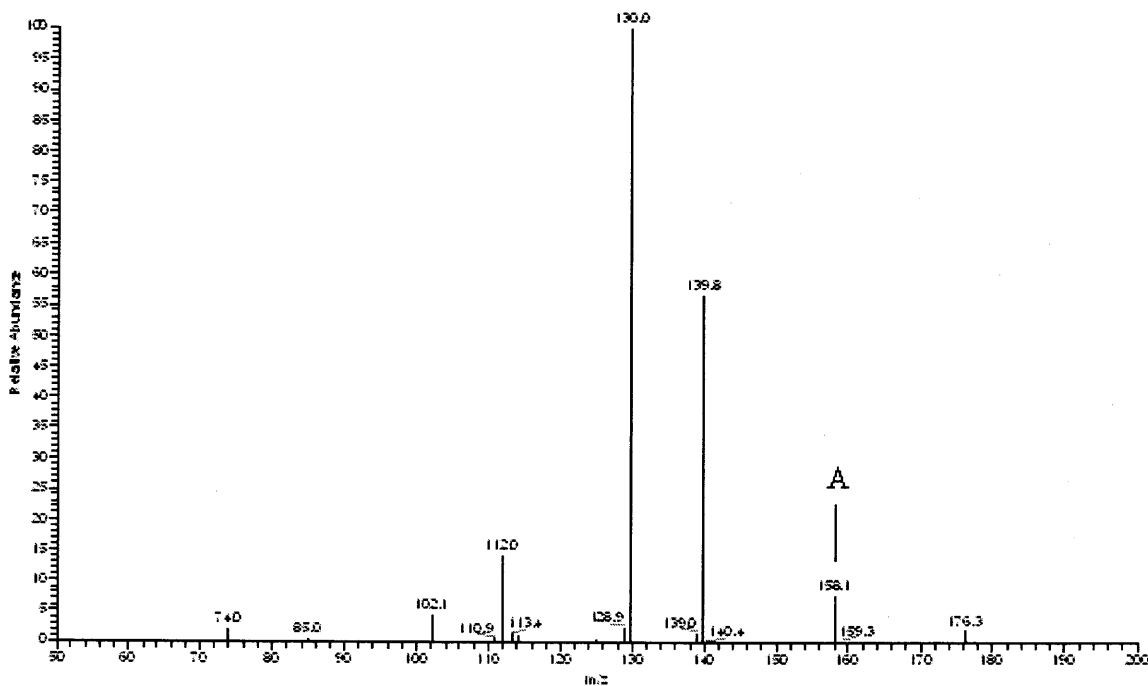


Figure 6.20 LC-ESI/MS/MS analysis of product A at 40% collision energy.

The fragment of 139.8 (m/z) could result from the loss of $-OH$ (m/z 17) group from the carboxyl group. The fragment of 130.0 (m/z) is the molecule loss of the $-CHO$ group (m/z 29). The fragment of 112 (m/z) is the molecule loss of the $-COOH$ group (m/z 45).

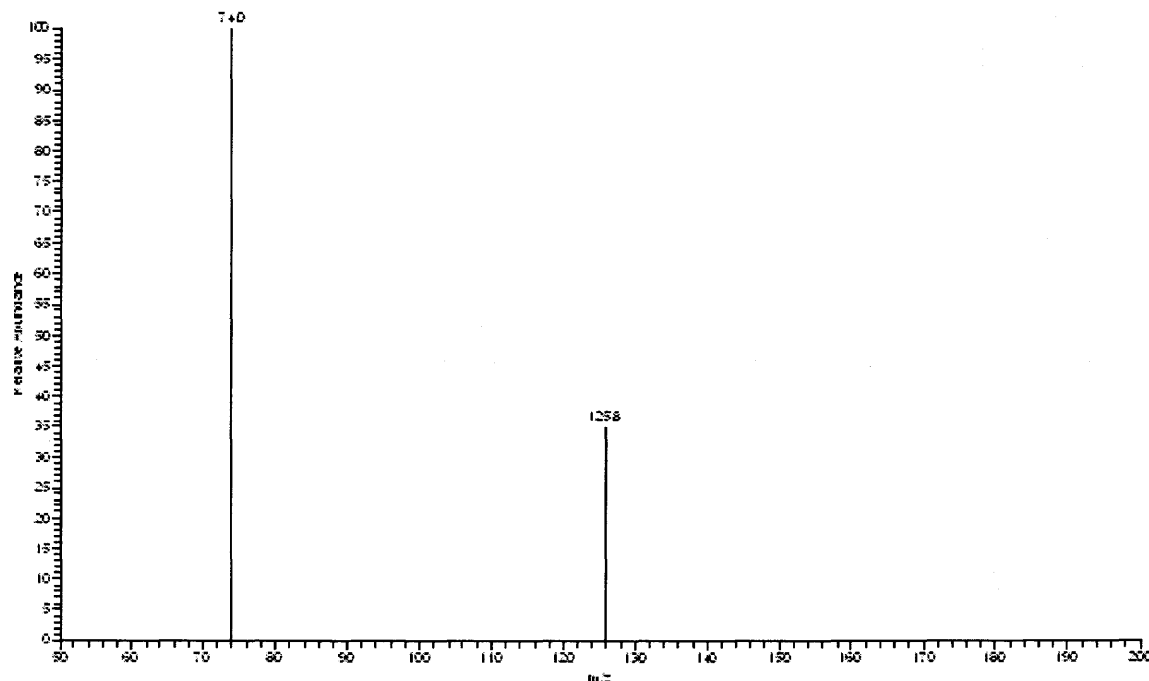


Figure 6.21 LC-ESI/MS/MS analysis of product B at 40% collision energy.

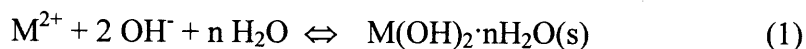
Product B showed fewer fragments. The fragment of 125.8 (m/z) could result from the loss of an $-OH$ group (m/z 17). The fragment of 74 (m/z) may be due to the cleavage between the double bonds ($-CH_2-NHCOOH$).

Similar degradation pathways have been suggested by Wright and Cain (1971). They investigated the transformation of 4-carboxy-1-methylpyridinium chloride by *Achromobacter* Stain D, which could use this compound as its sole carbon and nitrogen source. Succinic acid, formic acid, methylamine, and carbon dioxide are identified as end products. The radioisotopic experiments in their study indicate that the ring cleavage occurs between carbon 2 and 3 of the heterocyclic ring by multi-enzyme reactions. The N-formyl group was hydrolyzed to formic acid, while γ -(N-formyl-N-methylamino)vinylacetaldehyde was hydrolyzed to the corresponding acid. The

following hydrolysis processes lead to the formation of succinic acid semialdehyde and methylamine. A similar pyridine ring cleavage process was also found in the biotransformation of pyridine by *Bacillus* strain 4 (Watson, et al., 1975).

6.3.2.4 Effects of Uranium on [EtPy][BF₄] Biodegradation The biodegradation of a uranium complex varied with the type of microorganism used. It also varied the type of complex that formed between the metal and the organic ligand. Biodegradation of uranium-citrate complex was investigated by Francis, et al. (1994; 2002). It was shown that biodegradation of metal-citrates should result either in the bio-precipitation of release ions as water insoluble hydroxides, oxides, carbonates, salts or in biosorption by the biomass. However, the complex formed with [EtPy][BF₄] differs from the others, where uranium formed a complex with the anion and the cation could be used as carbon source by the bacteria. The effect of uranium on the biodegradation of ionic liquids is not known.

Since bacterium can only grow and function near a neutral pH in a buffered environment, the increased pH affects the stability of U-BF₄ dramatically. Previous studies performed under acidic conditions (around pH 3), [EtPy][BF₄] showed complexation with uranium. As we can see from Figure 6.22, because of the high pH of the medium, most uranium was precipitated out at the beginning when it mixed with the MSM. That is due to the fact that in an aqueous solution, metal ions may form insoluble forms of metal hydroxides with OH⁻:



$$K_{sp} = [M^{2+}][OH^{-}]^2, \text{ and } K_w = [H^{+}][OH^{-}] \quad (2)$$

$$pM = 2 pH + \log (K_w^2/K_{sp}) \quad (3)$$

From this equation we can see that as the chelate becomes more stable it can maintain under a higher pH. We also can see that as its stability increases, it necessitates a higher pH to precipitate metal hydroxide. On the other hand, the effect of pH on chelation can be utilized to liberate metals from chelates that have already participated in another stage of a process, so that the metal, chelate, or both can be recovered separately. Although we cannot calculate the speciation of a uranium complex due to a lack information about ionic liquids dissociation, and/or the stability constants of chelates, we are still able to perceive that at this pH, $U-BF_4$ is replaced by an insoluble uranium hydroxide hydrate, and precipitates a yellow cake-like material (Hausen, 1998).

In the presence of bacteria, the slowly increased concentration of uranium may be due to the formation of the degradation products, N-ethyl-4-(carbosy-amino)but-3-enoic acid semialdehyde (product A), and 4-(carbosy-amino)but-3-enoic acid (product B). These organic acids contain strong carboxyl function groups and can form complexes with uranium, thereby increasing their solubility. Less than 10% of uranium was maintained in solution after 3 days of incubation.

However, the remaining uranium still affects the degradation rate of pyridinium dramatically (as shown below in Figure 6.23). The degradation rate decreased more than two times, and following 3 days of incubation there is still 30% of $EtPy^+$ remaining in the solution. Product A and B accumulated in the solution but no further degradation was observed. This may be due to the toxicity of uranium. As reported by Leduc et al. (1997) uranium is twelve times more toxic than copper and nickel to *Thiobacillus ferrooxidans*.

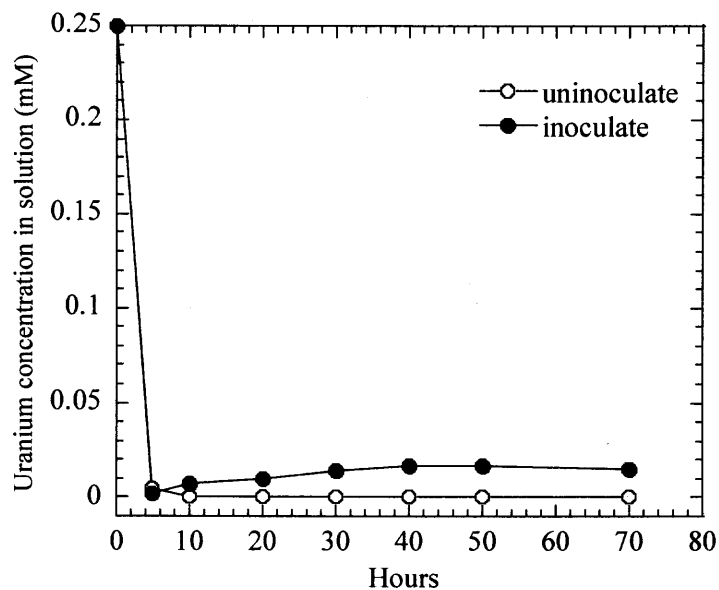


Figure 6.22 Uranium concentrations in solution during incubation.

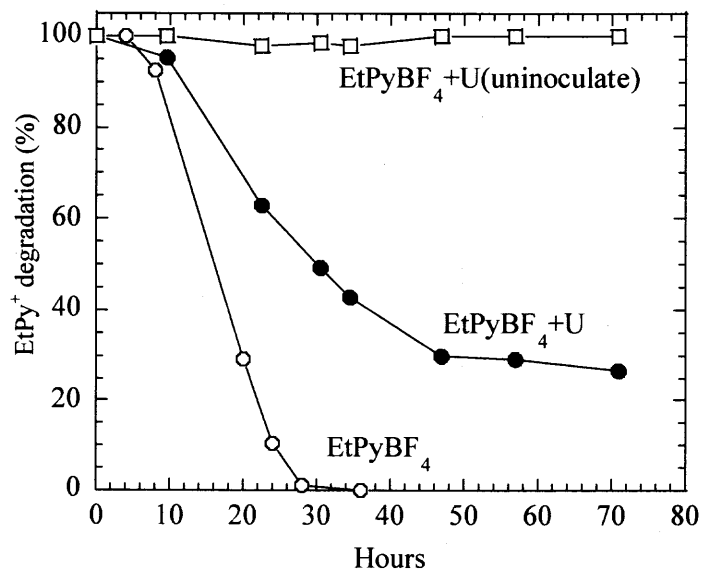


Figure 6.23 Effects of uranium on biodegradation of [EtPy][BF₄].

6.3.3 Summary

N-ethyl pyridinium-based ionic liquids were biodegraded by an enrichment culture of soil bacteria. The ring cleavage between C2-C3 was proposed as a degradation pathway during incubation. The intermediate products identified by LC-ESI/MS/MS were N-ethyl-(4-(carbosy-amino)but-3-enoic acid semialdehyde and (4-(carbosy-amino)but-3-enoic acid. The final products (acetic acid and glyoxylate) are confirmed by MS. In the presence of uranium, the degradation rate of [EtPy][BF₄] slowed.

This the first report showing the complete biodegradation of a N-substitute of pyridinium compound, and thus provides fundamental information concerning the assessment of the environmental risks posed by those “environmental friendly” ionic liquids.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Conclusions

1. The interactions between uranium and three ionic liquids, N-ethylpyridinium tetrafluoroborate ([EtPy][BF₄]), N-ethylpyridiniumtrifluoroacetate ([EtPy][CF₃COO]), 1-butyl-3-methylimidazoliumhexafluorophosphate ([BMIM][PF₆]), were identified by various analytical methods, potentiometric titration, UV-Vis spectroscopy, LC-MS, XANES (X-ray near edge spectroscopy) and EXAFS (extended X-ray absorption fine structure analysis). Monodentates were formed between uranyl and BF₄⁻, PF₆⁻. A bidentate complex was formed between uranyl ion and CF₃COO⁻. During complexation, the change of UV absorbance and acid release were found only in the mixture of U and [EtPy][CF₃COO].
2. All of these three ionic liquids show inhibitory effects on the growth of the anaerobic bacteria, *Clostridium* sp. Compared with [EtPy][BF₄], [EtPy][CF₃COO] and [BMIM][PF₆] show a higher toxicity, due to a decrease in the optical density, a change of pH, and the production of gas. Therefore, a living culture was selected for the experiments in order to ensure the best performance.
3. Biosorptions of uranium decreased in the presence of ionic liquids.
4. Ionic liquids have a significant effect on the rate and the extent of uranium reduction. The monodentate complexes U-BF₄ and U-PF₆ enhanced the reduction rate and extent, while the strong bidentate complex of U-CF₃COO was not

bioavailable. [EtPy][BF₄] and [BMIMPF₆] show different affinities toward U(IV). High U(IV) concentration in solution was only observed in a medium containing [EtPy][BF₄], which may be due to the complex formed between U(IV) and BF₄⁻. The reduced U(IV) in precipitate was confirmed by UV-Vis spectroscopy, XANES and EXAFS analyses.

5. The biodegradation of [EtPy][BF₄] was investigated by testing a pure culture *Pseudomonas fluorescens* and a mixed enrichment culture isolated from soil. N-ethylpyridinium was co-metabolized by *Pseudomonas fluorescens*, and produced hydroxyl-N-ethylpyridine and pyridinium cation. The cleavage of C2-C3 to a pyridium ring was suggested as the N-ethylpyridinium degradation pathway for enriched soil bacterium. The dominant intermediate products, N-ethyl-4-(carboxyamino)but-3-enoic acid and semialdehyde, were identified by LC-ESI/MS/MS. Finally, the N-ethylpyridinium cation is converted to a low molecular acetic acid and glyoxylate and the rate of biodegradation retards in the presence of uranium contamination.

7.2 Recommendations

1. If ionic liquids are chosen to be the solvents for an actinide extraction, the interactions that occur between ionic liquids with actinide should be considered. The complexation of IL with the actinide may result in the loss of the cationic component from IL. On the other hand, functionalized ionic liquids could be designed to aim toward the target metal/radionuclide. By tuning the properties of

ionic liquids, one augments their potential to achieve recoverability, effectiveness and selectivity.

2. Imidazolium-based ionic liquids are recalcitrant. Since they have been widely used in most ionic liquids, their potential environmental risk now becomes a major concern. Issues of toxicity, degradability, and mechanism should be explored through further investigation.

REFERENCES

- Abbott, A.P., Boothby, D., Capper, G., Davies, D., & Rasheed, R. (2004). Deep eutectic solvents formed between choline chloride and carboxylic acid: versatile alternatives to ionic liquids. Journal of American Chemical Society, *126*, 9142-9147.
- Abbott, A.G., Capper, D., Davies, D., Rasheed, R., & Shikotra, P. (2005). Selective extraction of metals from mixed oxide matrices using choline-based ionic liquids. Inorganic Chemistry, *44*, 6497-6499.
- Allen, P.G., Bucher, J.J., Clark, D.L., Edelstein, N.M., Ekberg, S.A., Gohdes, J.W., Hudson, E.A., Kaltsoyannis, N., Lukens, W.W., Neu, M.P., Palmer, P.D., Reich, T., Shuh, D. K., Tait, C.D., & Zwick, B.D. (1995). Multinuclear NMR, Raman, EXAFS, and X-ray diffraction studies of uranyl carbonate complexes in near-neutral aqueous solution: X-ray structure of $[\text{C}(\text{NH}_2)_3]_6[(\text{UO}_2)_3(\text{CO}_3)_6] \cdot 6.5\text{H}_2\text{O}$. Inorganic chemistry, *34*, 4797-4807.
- Allen, P.G., Bucher, J.J., Shuh, D.K., Edelstein, N.M., & Reich, T. (1997). Investigation of aquo and chloro complexes of UO_2^{2+} , NpO^{2+} , Np^{4+} , and Pu^{3+} by X-ray absorption fine structure spectroscopy. Inorganic Chemistry, *36*, 4676-4683.
- Allen, P.G., Shuh, D.K., Bucher, J.J., Edelstein, N.M., Reich, T., Denecke, M.A., & Nitsche, H. (1996). EXAFS determination of uranium structures; the uranyl ion complexed with tartaric, citric, and malic acids. Inorganic Chemistry, *35*, 784-787.
- Anastas, P.T., & Warner, J.C. (1998). Green Chemistry: Theory and Practice, Oxford University Press, p.30.
- Anderson, C.J., Deakin, M.R., Choppin, G.R., D'Olieslager, W., & Heerman, L. (1991). Spectroscopy and Electrochemistry of U(IV)/U(III) in basic aluminum chloride-1-ethyl-3-methylimidazolium chloride. Inorganic Chemistry, *30*, 4013-4016.
- Anderson, J.L., Ding, J., Welton, T., & Armstrong, D.W. (2002). Characterizing ionic liquids on the basis of multiple solvation interaction. Journal of American Chemical Society, *124*, 14247-14254.
- Anthony, J.L., Maginn, E.J., & Brennecke, J.F. (2001). 'Solution thermodynamics of imidazolium-based ionic liquids and water.' Journal of Physical Chemistry B, *105*, 10942-10949.
- Antonio, M.R., Soderholm, L., Williams, C.W., Blaudau, J.P., & Bursten, B.E. (2001). Neptunium redox speciation. Radiochimica Acta, *89*, 17-25.

- Baranyai, K.J., Deacon, G.B., MacFarlane, D.R., Pringle, J.M., & Scott, J.L. (2004). Thermal degradation of ionic liquids at elevated temperatures. Australian Journal of Chemistry, 57(2), 145-147.
- Bashford, C.L., Alder, G.M., Menestrina, G., Micklem, K.J., Murphy, J.J., Pasternak, C.A. (1986). Membrane damage by hemolytic viruses, toxins, complement, and other cytotoxic agents. The Journal of Biological Chemistry, 261(20), 9300-9308.
- Bayer, M.E., & Bayer, M.H. (1991). Lanthanide accumulation in the periplasmic space of *Escherichia coli*. Journal of Bacteriology, 173, 141-149.
- Bencheikh, L.R.; Bargar, J.R.; & Leckie, J.O. (2003). Fate of uranyl in a quaternary system composed of uranyl, citrate, goethite, and *Pseudomonas fluorescens*. Environmental Science and Technology, 37(16), 3555-3559.
- Bernot, R.J., Brueseke, M.A., Evans-White, M.A., & Lamberti, G.A. (2005). Acute and chronic toxicity of imidazolium-based ionic liquids on *DAPHNIA MAGNA*. Environmental Toxicology and Chemistry, 24(1), 87-92.
- Bernot, R.J., Kennedy, E.E. & Lamberti, G.A. (2005). Effects of ionic liquids on the survival, movement, and feeding behavior of the freshwater snail, *Physa acuta*. Environmental Toxicology and Chemistry, 24(7), 1759-1765.
- Berry, D.F., Francis, A.J., & Bollag, J.M. (1987). Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiological Reviews, 51(1), 43-59.
- Beyenal, H., Sani, R.K., Peyton, B.M., Dohnalkova, A.C., Amonette, J.E., & Lewandowski, Z. (2004) Uranium immobilization by sulfate-reducing biofilms. Environmental Science and Technology, 38(7), 2067-2074.
- Boethling, R.S. (1996). A.C.S. Symposium Serials, 640, 156.
- Bon throne, K.M., Quarmby, J., Hewitt, C.J., Allan, V.J.M., Paterson-Beedle, M., Kennedy, J.F., & Macaskie, L.E. (2000). The effect of the growth medium on the composition and metal binding behavior of the extracellular polymeric material of a metal accumulating *Citrobacter* sp. Environmental Technology, 21, 123-134.
- Bonhôte, P., Dias, A.P., Papageorgiou, N., Kalyanasundaram, K., & Gratzel, M. (1996). Hydrophobic, highly conductive ambient temperature ionic liquids. Inorganic Chemistry, 35, 1168-1178.
- Bunting, J.W. (1987). pK_R^+ values for pyridinium cations. Tetrahedron, 43(19), 4277-4286.

- Carda, B.S., Berthod, A., & Armstrong, D.W. (2003). Solvent properties of the 1-butyl-3-methylimidazolium hexafluorophosphate ionic liquid. Analytical and Bioanalytical Chemistry, *375*, 191-199.
- Chao, J.C., Hong, A., Okey, R.W., & Peters, R.W. (1998). Selection of chelating agents for remediation of radionuclide contaminated soil. Proceeding of the 1998 Conference on Hazardous Waste Research, 142-160.
- Chiappe, C., Leandri, E., Lucchesi, S., Pieraccini, D., Hammock, B.D., & Morisseau, C. (2004). Biocatalysis in ionic liquids: the stereoconvergent hydrolysis of trans- β -methylstyrene oxide catalyzed by soluble epoxide hydrolase. Journal of Molecular catalysis B: Enzymatic, *27*, 243-248.
- Corman, L.D.J., & Fein, J.B. (2004). Experiment study of the adsorption an ionic liquid onto bacterial and mineral surface. Environmental Science & Technology, *38*, 2491-2495.
- Couling, D.J., Bernot, R.J., Docherty, K.M., Dixon, J.K., & Maginn, E.J. (2006). Assessing the factors responsible for ionic liquid toxicity to aquatic organism via quantitative structure property relationship modeling. Green Chemistry, *8*, 82-90.
- Dietz, M.L., & Stepinski, D.C. (2005). A ternary mechanism for the facilitated transfer of metal ions into room-temperature ionic liquids (RTILs): implications for the "greenness" of RTILs as extraction solvents. Green Chemistry, *7*, 747-750.
- Dietz, M.L., Dzielawa, J.A., Jensen, M.P., & Firestone, M.A. (2003). Conventional aspects of unconventional solvents: Room temperature ionic liquids as ion-exchangers and ionic surfactants. American Chemical Society Meeting Serials, *41*, 527-543.
- Diniz, V., & Volesky, B. (2005). Effects of counterions on lanthanum biosorption by *Sargassum polycystum*. Water Research, *39*, 2229-2236.
- Docherty, K.M., & Kulpa, C.F.J. (2005). Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. Green Chemistry, *7*, 185-189.
- Dodge, C.J., & Francis, A.J. (2002) Photodegradation of a ternary Iron (III)-Uranium (VI)-Citric Acid complex. Environmental Science and Technology, *36*(9), 2094-2100.
- Dodge, C.J., & Francis, A.J. (2003) Structural characterization of ternary Fe (III)-U(VI)-citrate complex. Radiochim Acta, *91*, 525-532.
- Dodge, C.J., Francis A.J., Gillow, J.B., Halada G.P., Eng, C., & Clayton, C.R. (2002). Association of uranium with ion oxides typically formed on corroding steel surface. Environmental Science and Technology, *36*(16), 3504-3511.

- Dworking, M. (1993). Cell surfaces and appendages. II. American Society for Microbiology, Washington DC, USA, pp.63-83.
- Dzyuba, S.V., & Bartsch, R.A. (2001). New room-temperature ionic liquids with C₂-symmetrical imidazolium cations. Chemical Communication, 16, 1466-1467.
- Earle, M.J., & Seddon, K.R. (2000). Ionic liquids: Green solvents for the future. Pure and Applied Chemistry, 72, 1391-1398.
- Ezdihar, H.A., Abraham, M., Kini, V., Al-Ghafri, M., & Abushaban, A. (2002). Cytotoxicity of the ionic liquid, 1-N-butyl-3-methyl imidazolium chloride. Research Communications in Pharmacology and Toxicology, 7(1 & 2), 23-31.
- Feng, Y.C., Kaiser, J.P., Minard, R.D., & Bollag, J.M. (1994). Microbial transformation of ethylpyridines. Biodegradation, 5, 121-128.
- Fetzner, S. (1998). Bacterial degradation of pyridine, indole, quinoline and their derivatives under different redox conditions. Applied Microbiology and Biotechnology, 49, 237-250.
- Forsyth, S.A., Pringle, J.M., & Macfarlane, D.R. (2004). Ionic liquids-an overview. Australian Journal of Chemistry, 57, 113-119.
- Fowle, D.A., Fein, J.B., & Martin, A.M. (2000). Experimental study of uranyl adsorption onto *Bacillus subtilis*. Environmental Science and Technology, 34, 3737-3741.
- Francis, A.J., Dodge, C.J., Halada, G.P., & Clayton, C.R. (1994). XPS and XANES studies of uranium reduction by *Clostridium sp.* Environmental Science & Technology, 25, 636-639.
- Francis, A.J. (1994). Microbial transformations of radioactive wastes and environmental restoration through bioremediation. Journal of Alloys and Compounds, 213-214, 226-231.
- Francis, A.J. (1998). Biotransformation of uranium and other actinides in radioactive wastes. Journal of Alloys and Compounds, 271-273, 78-84 .
- Francis, A.J. (1999). Bioremediation of radionuclide and toxic metal contaminated soils and wastes. Bioremediation of Contaminated Soils, Agronomy Monograph, No. 37, p239-271.
- Francis, A.J., & Dodge, C.J. (1998). Remediation of soils and wastes contaminated with uranium and toxic metals. Environmental Science and Technology, 32, 3993-3998.

- Francis, A.J., Dodge C.J., Lu, F., Halada, G., & Clayton, C.R. (1994). XPS and XANES studies of uranium reduction by *Clostridium* sp. Environmental Science and Technology, 28, 636-639.
- Francis, A.J., Dodge, C.J., Gillow, J.B., & Papenguth, H.W. (2000). Biotransformation of uranium compounds in high ionic strength brine by a halophilic bacterium under denitrifying condition. Environmental Science and Technology, 34(11), 2311-2317.
- Francis, A.J., Gillow, J.B., Dodge, C.J., Dunn, M., Mantione, K., Strietelmeier, B.A., Pansoy-Hjelvik, M. E., & Papenguth, H. W. (1998). Role of bacteria as biocolloids in the transports of actinides from a deep understanding radioactive waste repository. Radiochimica Acta, 82, 347-354.
- Francis, A.J., Gillow, J.B., Dodge, C.J., Harris, R., Beveridge, T.J., & Papenguth, H.W. (2004). Uranium association with halophilic and non-halophilic bacteria and archaea. Radiochimica Acta, 92, 481-488.
- Francis, A.J., Joshi-tope, G.A., Dodge, C.J., & Gillow, J.B. (2002). Biotransformation of uranium and transition metal citrate complexes by Clostridia. Journal of Nuclear Science and Technology, supplement 3, 935-938.
- Gaillard, C., Azzi, A.E., Billard, I., Bolvin, H., & Hennig, C. (2005). Uranyl complexation in fluorinated acids (HF, HBF₄, HPF₆, HTf₂N): a combined experimental and theoretical study. Inorganic Chemistry, 44, 852-86.
- Ganesh, R., Robinson, K.G., Reed, G.D., & Sayler, G.S. (1997). Reduction of hexavalent uranium from organic complexes by sulfate and iron-reduction bacteria. Applied and Environmental Microbiology, 63, 4385-4391.
- Ganesh, R., Robinson, K.G., Chu, L., Kucsmas, D., & Reed, G.D. (1999). Reductive precipitation of uranium by *Desulfovibrio desulfuricans*: evaluation of co-contamination effects and selective removal. Water Research, 33(16), 3447-3458.
- Garcia, M.T., Gathergood, N., & Scammells, P.J. (2005). Biodegradable ionic liquids Part II. Effect of the anion and toxicology. Green Chemistry, 7, 9-14.
- Gathergood, N., Garcia, M.T., & Scammells, P.J. (2004). Biodegradable ionic liquids: part I. Concept, preliminary targets and evaluation. Green chemistry, 6, 166-175.
- Gianfreda, L., & Rao, M.A. (2004). Potential of extra cellular enzymes in remediation of polluted soils: a review. Enzyme and Microbial Technology, 35, 339-354.
- Giridhar, P., Venkatesan, K.A., Srinivasan, T.G., & Vasudeva Rao, P.R. (2004). Comparison of diluents characteristics of imidazolium hexafluorophosphate ionic

- liquid with n-dodecane. Journal of Nuclear and Radiochemical Science, 5(2), 17-20.
- Giridhar, P., Venkatesan, K.A., Srinivasan, T.G., & Vasudeva Rao, P.R. (2004). Effects of alky group in 1-alkyl-3-methylimidazolium hexafluorophosphate ionic liquids on the extraction of uranium by tri-n-butylphosphate diluted with ionic liquids. Journal of Nuclear and Radiochemical Sciences, 5(2), 21-26.
- Glen, H., Vadim, V.M., Chery, B., & Yuri, L.K. (2002). Oxidative enzymes possess catalytic activity in system with ionic liquids. Biotechnology Letters, 24, 2083-2087.
- Gorman, L.D., Elias, P.E., & Fein, J.B. (2005). Adsorption of Aqueous Uranyl Complexes onto Bacillus Subtilis Cells. Environmental Science & Technology, 39(13), 4906-4912.
- Gorby, Y.A., & Lovely, D.R. (1992). Enzymatic uranium precipitation. Environmental Science and Technology, 26, 205-207.
- Grabinska, S.E., & Kalka, J. (2003). An assessment of the toxicity of pyridinium chlorides and their biodegradation intermediates. Environmental International, 28, 687-690.
- Gu, B., Ku, Y.K., & Brown, G.M. (2005). Sorption and desorption of perchlorate and U (VI) by strong-Base Anion-Exchange Resins. Environmental Science and Technology, 39(3), 901-907.
- Gubicza, L., Nemestothy, N., Frater, T., & Belafi-Bako, K. (2003). Enzymatic esterification in ionic liquids integrated with pre-evaporation for water removal. Green Chemistry, 5(2), 236-239.
- Hardacre, C. (2004). Application of EXAFS to molten salts and ionic liquid technology. Annual Review of Materials Research, 35, 29-50.
- Hausen, D.M. (1998). Characterizing and classifying uranium yellow cakes: a background. Energy Resources, December, 45-48.
- Hirayama, N., Deguchi, M., Kawasumi, H., & Honjo, T. (2005). Use of 1-alkyl-3-methylimidazolium hexafluorophosphate room temperature ionic liquids as chelate extraction solvent with 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione. Talanta, 65, 255-260.
- Holbrey, J.D., Reichert, W.M., Nieuwenhoyzen, M., Sheppard, O., Hardacre, C., & Rogers, R.D. (2003). Liquid clathrate formation in ionic liquid–aromatic mixtures. Chemical Communication, 4, 476-477.

- Hopkins, T.A., Berg, J.M., Costa, D.A., Smith, W.H., & Dewey, H.J. (2001). Spectroscopy of $\text{UO}_2\text{Cl}_4^{2-}$ in basic aluminum chloride- 1-ethyl-3-methylimidazolium chloride. Inorganic Chemistry, 40, 1820-1825.
- Houghton, C., & Cain, R.B. (1972). Microbial metabolism of the pyridine ring. Biochemistry Journal, 130, 879-893.
- Huang, J.F., Chen, P.Y., Sun, W., & Wang, S.P. (2001). NMR evidence of hydrogen bonding in 1-ethyl-3-methylimidazolium tetrafluoroborate room temperature ionic liquids. Spectroscopy Letters, 34(5), 591-603.
- Husum, T.L., Jorgensen, C.T., Christensen, M.W., & Kirk, O. (2001). Enzyme catalyzed synthesis in ambient temperature ionic liquids. Biocatalysis and Biotransformation, 19(4), 331-338.
- Jastorff, B., Stoermann, R., Ranke, J., Moelter, K., Stock, F., Oberheitmann, B., Hoffmann, W., Hoffmann, J., Nuechter, M., Ondruschka, B., & Filser, J. (2003). How hazardous are ionic liquids? Structure-activity relationships and biological testing as important elements for sustainability evaluation. Green Chemistry, 5(2), 136-142.
- Jensen, M.P., Neufeind, J., Beitz, J.V., Skanthakumar, S., & Soderholm, L. (2003). Mechanisms of metal ion transfer into room-temperature ionic liquids: the role of anion exchange. Journal of American Society, 125(50), 15466-73.
- Jeon, B.H., Dempsey, B.A., Burgos, W.D., Barnett, M.O., & Roden, E.E. (2005) Chemical reduction of U(VI) by Fe(II) at the solid-water interface using natural and synthetic Fe(III) oxides. Environmental Science and Technology, 39(15), 5642-5649.
- Jiang, J., Rao, L.F., Bernardo, P., Zanonato P.L., & Bismondo, A. (2002). Complexation of uranium (VI) with acetate at variable temperature. Journal of American Chemical Society, 1832-1838.
- Kaar, J.L., Jesionowski, A.M., Berberich, J.A., Moulton, R., & Russell, A.J. (2003). Impact of ionic liquid physical properties on lipase activity and stability. Journal of American Chemical Society, 125, 4125-31.
- Kaftzik, N., Kroutil, W., Faber, K., & Kragl, U. (2004). Mandelate racemase activity in ionic liquids: scopes and limitations. Journal of Molecular Catalysis A: chemical, 214, 107-112.
- Kaftzik, N., Neumann, S., Kula, M.R., & Kragl, U. (2003). Enzymatic condensation reactions in ionic liquids. ACS Symposium Series, 856 (Ionic Liquids as Green Solvents), 206-211.

- Kaftzik, N., Wasserscheid, P., & Kragl, U. (2002). Use of ionic liquids to increase the yield and enzyme stability in the galactosidase catalyzed synthesis of N-acetyllactosamine. Organic Process Research & Development, 6(4), 553-557.
- Kaiser, J.P., Feng, Y., & Bollag, J.M. (1993) Transformation of 3-and 4- picoline under sulfate reducing conditions. Applied Environmental Microbiology, 59, 701-705.
- Kaiser, J.P., Feng, Y. C., & Bollag, J.M. (1996). Microbial metabolism of pyridine, quinoline, acridine, and their derivatives under aerobic and anaerobic conditions. Microbiological Reviews, 60(3), 483-498.
- Kao, C.M., Liu, J.K., Chen, Y.L., Chai, C.T., & Chen, S.C. (2005). Factors affecting the biodegradation of PCP by *Pseudomonas Mendocina* NSYSU. Journal of Hazardous Materials, 124(1-3),68-73.
- Kelly, S.D., Kemner, K.M., Fein, J.B., Fowle, D.A., Boyanov, M.I., Bunker, B.A., & Yee, N. (2002). X-ray absorption fine structure determination of pH-dependent U-bacterial cell wall interactions. Geochimica et Cosmochimica Acta, 66(22), 3855-3871.
- Keyser, H.H., & Munns, D.N. (1979). Tolerance of rhizobia to acidity, aluminum, and phosphate. Soil Science American Journal, 43, 519-523.
- Kim, K.W, Song, B., Choi, M.Y., & Kim, M.J. (2001). Biocatalysis in ionic liquids: marked enhanced enantioselectivity of lipase. Organic Letter, 3, 1507-1509.
- Kim, M.K., Singleton, I., Yin, C.R., Quan, Z.X., Lee, M., & Lee, X.T. (2006). Influence of phenol on the biodegradation of pyridine by freely suspended and immobilized *Pseudomonas putida* MK1. Letters in Applied Microbiology, 42(5), 495-505
- Konings, W.N., Albers, S.V., Konig, S., & Driessen, J.M. (2002). The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Antonie Van Leeuwenhoek, 81, 61-72.
- Kornberg, H.L. (1966). The role and control of the glyoxylate cycle in *Escherichia coli*. Biochemical Journal, 99, 1-11.
- Korosteleva, L.A., Kost, A.N., Vorob'eva, L.I., Modyanova, L.V., Terentev, P.B., & Kulikov, N. S. (1981). Microbiological degradation of pyridine and 3-methylpyridine. Applied Biochemistry and Microbiology, 17, 276-283.
- Kragl, U., Kaftzik, N., Schofer, S.H., Eckstein, M., Wasserscheid, P., & Hilgers, C. (2001). Enzyme catalysis in the presence of ionic liquids. Chimica Oggi, 19(7/8), 22-24.

- Kragl, U., Eckstein, M., & Kaftzik, N. (2002). Enzyme catalysis in ionic liquids. Current Opinion in Biotechnology, 13, 565-571.
- Kragl, U., Kaftzik, N., Schoefer, S., & Wasserscheid, P. (2002). Enzymatic catalysis in the presence of ionic liquids. European Patent Apply, 15 pp.
- Kargl, U., Eckstein, M., & Kaftzik, N. (2003). Biocatalytic reactions in ionic liquids. Ionic Liquids as Green Solvents: Progress and Prospects, ACS symposium serials, 856, 336-347.
- Lambert, P.A., Hammond, S.M. (1973). Potassium fluxes, first indications of membrane damage in micro-organisms. Biochemical and Biophysical Research Communications, 54, 796-799.
- Laszlo, J.A., & Compton, D.L. (2001). Chymotrypsin catalysis in imidazolium-based ionic liquids. Biotechnology and Bioengineering, 75(2), 181-186.
- Laszlo, J.A., & Compton, D.L. (2002). Chymotrypsin-catalyzed transesterification in ionic liquids and ionic liquid/supercritical carbon dioxide. ACS Symposium Series, 818(Ionic Liquids), 387-398.
- Laszlo, J.A., & Compton, D.L. (2002). Comparison of peroxidase activity of hemin, cytochrome c and microperoxidase-11 in molecular solvents and imidazolium based ionic liquids. Journal of Molecular Catalysis B: Enzymatic, 18, 109-120.
- Latala, A., Stepnowski, P., Nedzi, M., & Mroziak, W. (2005). Marine toxicity assessment of imidazolium ionic liquid: Acute effects on the Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana*. Aquatic Toxicology, 73, 91-98.
- Leduc, L.G., Ferroni, G.D., & Trevors, J.T. (1997). Resistance to heavy metals in different strains of *thiobacillus ferrooxidans*. World Journal of Microbiology & Biotechnology, 13, 453-455.
- Lee, J.K., & Mahn-Joo, Kim. (2002). Ionic liquid-coated enzyme for biocatalysis in organic solvent. Journal of Organic Chemistry, 67, 6845-6847.
- Liu, S.M., Wu, C.H., & Huang, H. J. (1998) Toxicity and anaerobic biodegradability of pyridine and its derivatives under sulfidogenic conditions. Chemosphere, 36(10), 2345-2357.
- Lloyd, J.R., Chesnes, J., Glasauer, S., Bunker, D.J., Livens, F.R., & Lovley, D.R. (2002). Reduction of actinides and fission products by Fe(III)-reducing bacteria. Geomicrobiology Journal, 19(1), 103-120.

- Lozano, P., De Diego, T., Guegan, J.P., Vaultier, M., & Iborra, J.L. (2001). Stabilization of chymotrypsin by ionic liquids in trans-esterification reactions. Biotechnology and Bioengineering, 75(5), 563-569.
- Lozano, P., De Diego, T., Carrie, D., Vaultier, M., & Iborra, J.L. (2003). Enzymatic ester synthesis in ionic liquids. Journal of Molecular Catalysis B: Enzymatic, 21(1-2), 9-13.
- Lozano, P., De Diego, T., Gmouh, S., Vaultier, M., & Iborra, J.L. (2004). Criteria to design green enzymatic processes in ionic liquid/supercritical carbon dioxide systems. Biotechnology Progress, 20(3), 661-669.
- Lovely, D.R., & Philips, E.J.P. (1992). Bioremediation of uranium contamination with enzymatic uranium reduction. Environmental Science and Technology, 26, 2228-2234.
- Madeira, L.R., Sorgedraeger, M.J., Carrea, G., Rantwijk, F.V., Secuundo, F., & Sheldon, R. A.(2004). Dissolution of *Candida antarctica* lipase B in ionic liquids: effects on structure and activity. Green Chemistry, 6, 483-487.
- Madigan, M., Martinko, J., & Parker, J. (2003). Brock Biology of Microorganisms (10th edition), Prentice Hall, Upper Saddle River, p 74.
- Magnuson, D.K., Bodley, J.W., Evans, D.F. (1984). The activity and stability of alkaline phosphatase in solution of water and the fused salt, ethyl ammonium nitrate. Journal of Solution Chemistry, 13(8), 583-587.
- Malhotra, S.V., & Zhao, H. (2005). Enantioseparation of the esters of α -N-acetyl amino acids by lipase in ionic liquids. Chirality, 17(suppl.), S240-S242.
- Manahan, S.E. (2004). Green Chemistry—Fundamentals of Sustainable Chemical Science and Technology, Chechar Research Inc. publisher. p8.
- Markich, S.J. (2002). Uranium speciation and bioavailability in aquatic systems: an overview. Scientific World Journal, 15, 707-729.
- Marsh, K.N., Boxall, J.A., & Lichtenthaler, R. (2004). Room temperature ionic liquids and their mixtures- a review. Fluid Phase Equilibria, 219, 93-98.
- Matsumoto, M., Mochiduki, K., Fukunishi, K., & Kondo, K. (2004). Extraction of organic acids using imidazolium-based ionic liquids and their toxicity to *Lactobacillus rhamnosus*. Separation and Purification Technology, 40(1), 97-101.
- Menon, M.P. (1973). Kinetics of the formation of trace amount of tetrafluoroboric acid. Journal of Inorganic Nuclear Chemistry, 35, 4183-4191.

- Merroun, M.L., Chekroun, K.B., Arias, J.M., & González-Muñoz, M. T. (2003). Lanthanum fixation by *Myxococcus xanthus*: cellular location and extracellular polysaccharide observation. Chemosphere, *52*, 113-120.
- Miskolczy, Z., Sebök-Nagy, K., Biczók, L., & Göktürk, S. (2004). Aggregation and micelle formation of ionic liquids in aqueous solution. Chemical Physics Letters, *400*, 296-300.
- Miyako, E., Maruyama, T., Kamiya, N., & Goto, M. (2003). Enzyme-facilitated enantioselective transport of (S)-ibuprofen through a supported liquid membrane based on ionic liquids. Chemical Communications, *23*, 2926-2927.
- Mohan, S.V., Sistla, S., Guru, R.K., Prasad, K.K., Kumar, C.S., Ramakrishna, S.V., & Sarma, P.N. (2003). Microbial degradation of pyridine using *Pseudomonas* sp. and isolation of plasmid responsible for degradation. Waste Management, *23*(2), 167-171.
- Nara, S.J., Mohile, S.S., Harjani, J.R., Naik, P.U., & Salunkhe, M.M. (2004). Influence of ionic liquids on the rates and regioselectivity of lipase-mediated biotransformations on 3,4,6-tri-O-acetyl-D-glucal. Journal of Molecular Catalysis B: Enzymatic, *28*(1), 39-43.
- Noel, M., Lozano, P., Vaultier, M., & Iborra, J.L. (2004). Kinetic resolution of rac-2-pentanol catalyzed by *Candida antarctica* lipase B in the ionic liquid, 1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide. Biotechnology Letters, *26*(4), 301-306.
- Oazki, T., Suzuki, Y., Nankawa, T., Yoshida, T., Ohnuk, T., Kimura, T., & Francis, A.J. (2006). Interactions of rare earth elements with bacteria and organic ligands. Journal of Alloys and Compounds, *408-412*, 1334-1338.
- Ohnuki, T., Yoshida, T., Ozaki, T., Samadfam, M., Kozai, N., Yubata, K., Mitsugashira, Y., Kasama, T., & Francis, A.J. (2005). Interaction of uranium and bacteria and kaolin clay. Chemical Geology, *220*, 237-243.
- Okrasa, K., Guibe-Jampel, E., & Therisod, M. (2003). Ionic liquids as a new medium for oxidase-peroxidase-catalyzed sulfoxidation. Tetrahedron: Asymmetry, *14*, 2487-2490.
- Oldham, W.J., Costa, D.A., & Smith, W.H. (2002). Development of room temperature ionic liquids for applications in actinide chemistry. ACS symposium Series, *818*, 188-198.
- Orpin, C.G., Knight, M., & Evans, W.C. (1971). The bacteria oxidation of N-methylisonicotinate. Biochemistry Journal, *122*, 58.

- Orpin, C.G., Knight, M., & Evans, W.C. (1972). The bacteria oxidation of N-methylisonicotinate, a photolytic product of paraquat. Biochemistry Journal, 127, 833-844.
- Overview of AFEAS research activity on TFA, Retrieved September 28, 2005 from the World Wide Web: <http://www.afeas.org/newtfa.html>.
- Park, S., & Kazlauskas, R.J. (2001). Improved preparation and use of room temperature ionic liquids in lipase-catalyzed enantio- and regioselective acylations. Journal of Organic Chemistry, 66, 8395-8401.
- Pazarlioglu, N.K., & Telefoncu, A. (2005). Biodegradation of phenol by *Pseudomonas putida* immobilized on activated pumice particles, Process Biochemistry, 40(5), 1807-1814.
- Pernak J., Sobaszekiewicz, K., Mirska, I. (2003). Antimicrobial activities of ionic liquids. Green Chemistry, 5, 52-56.
- Pernak, J. & Branicka, M. (2004). Synthesis and aqueous ozonation of some pyridinium salts with alkoxyethyl and alkylthiomethyl hydrophobic groups. Industrial Engineering and Chemical Research, 43, 1966-1974.
- Pernak, J., Rogoza, J., & Mirska, I. (2001) Synthesis and antimicrobial activities of new pyridinium and benzimidazolium chlorides. European Journal of Medicinal Chemistry, 36, 313-320.
- Pernak, J., Sobaszekiewicz, K., & Foksowicz-Flaczyk, J. (2004). Ionic liquids with symmetrical dialkoxyethyl substituted imidazolium cations. Chemical European Journal, 10, 3479-3485.
- Pietsch, K., Hard, B.C., & Babel, W. (1999). A *Desulfovibrio* sp. capable of growing by reducing U(VI). Journal of Basic Microbiology, 39(5-6), 365-372.
- Pretti, C., Chiappe, C., Pieraccini, D., Gregori, M., Abramo, F., Monni, G., & Intorre, L. (2006). Acute toxicity of ionic liquids to the zebrafish (*Danio rerio*). Green Chemistry, 8, 238-240.
- Pruender, H., Amidjojo, M., Kragl, U. & Weuster-Botz, D. (2004). Efficient whole-cell biotransformation in a biphasic ionic liquid/water system. Angewandte Chemie International Edition, 43, 4529-4531.
- Ranke, J., Moelter, K., Stock, F., Bottin-Weber, U., Poczobutt, J., Hoffmann, J., Ondruschka, B., Filser, J., & Jastorff, B. (2004). Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicology and Environmental Safety, 58(3), 396-404.

- Rantwijk V.F., Lau, R.M., & Sheldon, R.A. (2003). Biocatalytic transformation in ionic liquids. Trends of Biotechnology, 21(3), 131-138.
- Rao, T.P., Metilda, P., & Gladis, J.M. (2006). Preconcentration techniques for uranium(VI) and thorium(IV) prior to analytical determination-an overview. Talanta, 68, 1047-1064.
- Rhee, S.K., Lee, G.M., Yoon, J.H., Park, Y.H., Bae, H.S., & Lee, S.T. (1997). Anaerobic and aerobic degradation of pyridine by a newly isolated denitrifying bacterium. Applied Environmental Microbiology, 63, 2578-2585.
- Roberts, N.J., Seago, A., Carey, J.S., Freer, R., Preston, C., & Lye, G.J. (2004). Lipase catalyzed resolution of the Lotrafiban intermediate 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester in ionic liquids: comparison to the industrial t-butanol process. Green Chemistry, 6(9), 475-482.
- Roberts, T.R., Dyson, J.S., & Lane, M.C.G. (2002). Deactivation of the biological activity of paraquat in the soil environment: a review of long-term environmental fate. Journal of Agriculture Food Chemistry, 50, 3623-3631.
- Robinson, K.G., Ganes, R., & Reed, G.D. (1998). Impact of organic ligands on uranium removal during anaerobic biological treatment. Water Science and Technology, 37(8), 73-80.
- Ruggiero, C.E., Boukhalfa, H., Forsythe, J.H., Lack, J.G., Hersman, L.E., & Neu, M.P. (2005). Actinide and metal toxicity to prospective bioremediation bacteria. Environmental Microbiology, 7(1), 88-97.
- Sakamoto, F., Ohnuki, T., Kozai, N., Wakai, E., Fuji, T., Iefuji, H., & Francis, A.J. (2005). Effects of uranium (VI) on the growth of yeast and influence of metabolism of yeast on adsorption of U(VI). Journal of Nuclear and Radiochemical Science, 6(1), 99-101.
- Sar, P., Kazy, S.K., & Souza, S.F. (2004). Radionuclide remediation using a bacteria biosorbent. International Biodeterioration & Biodegradation, 54, 193-202.
- Sheng, D., Shin, Y.S., Toth, L. M., & Barnes, C.E. (1997). Comparative UV-Vis studies of uranyl chloride complex in two basic ambient-temperature melt systems: the observation of spectral and thermodynamic variations induced via hydrogen bonding. Inorganic Chemistry, 36, 4900-4902.
- Shively, J.M. (1974). Inclusion bodies of prokaryotes. Annual Reviews of Microbiology, 28, 167-188.
- Sim, G.K., & Sommers, L.E. (1985). Degradation of pyridine derivatives in soil. Journal of Environmental Quality, 14, 580-584.

- Spear, J.R., Figueroa, L.A., & Honeyman, B.D. (1999). Modeling the removal of uranium U(VI) from aqueous solutions in the presence of sulfate reducing bacteria. Environmental Science & Technology, 35(15), 2667-2675.
- Stepinski, D.C., Jensen, M.P., Dzielawa, J.A., & Dietz, M.L. (2005). Synergistic effects in the facilitated transfer of metal ions into room-temperature ionic liquids. Green Chemistry, 7, 151-158.
- Stepnowski, P. (2005). Preliminary assessment of the sorption of some alky imidazolium cations as used in ionic liquids to soils and sediments. Australian Journal of Chemistry, 58, 170-173.
- Stepnowski, P., & Zaleska, A. (2004). Comparison of different advanced oxidation processes for the degradation of room temperature ionic liquids. Journal of Photochemistry and Photobiology A: chemistry, 170, 45-50.
- Stepnowski, P., Skladanowski, A.C, Ludwiczak, A., & Iacyszynska, E. (2004). Evaluating the cytotoxicity of ionic liquids using human cell line Hela. Human & Experimental Toxicology, 23, 513-517.
- Stock, F., Hoffmann, J., Ranke, J., Stoermann, R., Ondruschka, B., & Jastorff, B. (2004). Effects of ionic liquids on the acetylcholinesterase - a structure-activity relationship consideration. Green Chemistry, 6(6), 286-290.
- Sutherland, I.W., Thomson, S. (1975). Comparison of polysaccharides produced by *Mycococcus* strains. Journal of General Microbiology, 89, 124-132.
- Suzuki, Y., Kelly, S.D., Kemner, K. M., Banfield, J.F. (2003). Microbial population stimulated for hexavalent uranium reduction in uranium mine sediment. Applied Environmental Microbiology, 69, 1337-1346.
- Suzuki, Y., Nankawa, T., Yoshida, T., Ozaki, T., Ohnuki, T., Francis, A.J., Tsushima, S., Enokida, Y., & Yamamoto, I. (2005). Sorption of Eu(III) on *Pseudomonas* fluorescence in the presence of citric acid. Journal of Nuclear and Radiochemical Science, 6(1), 91-93.
- Swatloski, R.P., Holbrey J.D., & Rogers, R.D. (2003). Ionic liquids are not always green: hydrolysis of 1-butyl-3-methylimidazolium hexafluorophosphate. Green Chemistry, 5, 361-363.
- Tucker, M.D., Barton, L.L., & Thomson, B.M. (1998). Removal of U and Mo from water by immobilized *Desulfovibrio desulfuricans* in column reactors. Biotechnology and Bioengineering, 60, 88-96.

- Turner, M.B., Spear, S.K., Huddleston, J.G., Holbrey, J.D., & Rogers, R.D. (2003). Ionic liquid salt-induced inactivation and unfolding of cellulase from *Trichoderma reesei*. Green Chemistry, 5(4), 443-447.
- Vallet, V., Wahlgren, U., Schimmelpfennig, B., Moll, H., Szabo, Z., & Grenthe, I., (2003). Structure and bonding in solution of dioxouranium(VI) oxalate complexes: isomers and intramolecular ligand exchange. Inorganic Chemistry, 42(6), 1982-1992.