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SHORT COMMUNICATION

Influence of Ionic Liquids on the Growth of *Escherichia coli*

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Abstract–Ionic liquids are compounds that composed only of ions and are liquid at room temperature. Thus, it is normally named room temperature ionic liquid (RTIL). In this study, the application of RTILs to the extractive fermentation of biomaterials was investigated as a substitute of organic solvents. The relative toxicity of the RTILs on the growth of *E. coli* was tested. The inhibition of cell growth in the presence of various ionic liquids was measured using solid and liquid culture, and EC_{50} of each RTILs was calculated. The number of viable and total cells was measured by the number of colonies and optical density, respectively. Effective concentrations of toxicity (EC_{50}) in these tested systems were similar with conventional solvents, such as acetone, acetonitrile, and ethanol. The viability of *E. coli* was affected by the polarity and ionic properties of ionic liquids. The resistance of the microorganisms against ionic liquids was different with the cations and anions composing ionic liquids. No general influence of the anionic compound of the ionic liquids was found on toxicity comparing with distinctive influence of cationic moiety.

Key words: Ionic Liquids, Cell Viability, *Escherichia coli*, Toxicity, Cation, Anion

INTRODUCTION

Recently, ionic liquids have gained considerable attention as alternative solvents in many biological and chemical reactions. Ionic liquids are compounds that composed only of ions and many of them are liquid at room temperature. Thus, it is generally named as room temperature ionic liquids. Structural variations of cations and anions could be permuted to make thousands of RTILs [Wilkes, 2004]. It is well known that the characteristics of ionic liquids are significantly changed by anions and cations. Generally, RTILs have melting point lower than 100 °C. Their low melting point is originated from the asymmetry of at least one of the ions and low intermolecular attractions [Ranke et al., 2004]. The size, charge, and charge distribution of the individual ions mainly affects the properties of an ionic liquid [Wilkes, 2004]. The advantages of RTILs as green solvents for chemical and biological processes have been extensively recognized due to their negligible vapor pressure [Ranke et al., 2004; Wilkes, 2004]. In the field of biotransformation, it has been reported that the use of RTILs improved the selectivity and yield in enzymatic reactions as an alternative to organic solvents [Lee et al., 2004]. Specifically, biotransformations using two-phase systems are promising application of RTILs in the field of biotechnology [Matsumoto et al., 2004]. The goal of such processes is to enhance the yield of bioconversion by continuously removing the water insoluble compounds from the aqueous reaction solution. Normally, microorganisms live, grow, divide, and work in aqueous media [Matsumoto et al., 2004; Ranke et al., 2004]. Bioconversion of hydrophobic molecules, such as steroid, aliphatic and aromatic hydrocarbon, has been carried out in two-phase systems [Lee et al., 2004]. The whole cells or enzymes originated from Gram negative bacteria were most frequently used in bioreaction systems [Peinado et al., 2002; Sardessai and Bhosle, 2002; Swatloski et al., 2004]. In a standard method, the toxicity of substances and commercial products are identified using bacteria as indicators [Peinado et al., 2002]. There are only a few results on the toxicity of RTILs, while a lot of researches have been performed on organic solvents. The appropriate selection of the ionic liquid for bioprocesses requires general knowledge on the toxicity of RTILs, including whole cell fermentation and enzymatic reaction system [Matsumoto et al., 2004; Ranke et al., 2004].

In this paper, we examined whether RTILs can replace conventional organic solvents by investigating their toxicity for the *in-situ* separation of the product during fermentation. *E. coli* has been used as a model microorganism in order to qualify a toxicity of various RTILs. Growth of *E. coli* in the presence of a series of RTILs is identical on both of solid media and in liquid media. Solid and liquid media were prepared on agar plate and glass tube, respectively. The present study with 13 different RTILs shows that *E. coli* cells were affected by wide range of RTILs from 20 mg/L to 50,000 mg/L.

MATERIALS AND METHODS

1. Room Temperature Ionic Liquids

All RTILs for toxicity test were purchased from the C-tri Co., Korea. 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([Emim][CF3SO3], m.w. 260.24), 1-methyl-3-octylimidazolium methylsulfate ([Omim][CH3SO4], m.w. 278.37), 1-phenylpropyl-3-methylimidazolium trifluoromethanesulfonate ([Pmim][CF3SO3], m.w. 350.36), 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF4], m.w. 197.97), 1-ethyl-3-methylimidazolium methylsulfate ([Emim][CH3SO4], m.w. 222.27), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF6] m.w. 284.18), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF4], m.w. 226.03), 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Bmim][(CF3SO2)2N], m.w. 419.37), 1-methyl-3-octylimidazolium hexaflu-

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orophosphate ([Omim][PF6], m.w. 340.29), 1-phenylpropyl-3-methylimidazolium hexafluoroantimonate ([Pmim][SbF6], m.w. 346.25), 1-hexyl-3-methylimidazolium hexafluorophosphate ([Hmim][PF6], m.w. 312.24), 1-hexyl-3-methylimidazolium hexafluoroantimonate ([Hmim][SbF6], m.w. 403.02), 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Hmim][(CF3SO2)2N], m.w. 447.42) were used.

2. Growth of *Escherichia coli* in the Presence of Room Temperature Ionic Liquids

E. coli (DE3)/pET-hIL6 that produces human interleukine-6 [Jung et al., 2004] was selected as a model recombinant microorganism. The Luria-Bertani (LB) medium containing 100 μg/mL ampicillin was used. Cultivation was performed at 37 °C, 200 rpm. The solid LB-agar plate containing 15 g/L of Bacto agar (Difco Lab, USA) was used for colony counting to measure the number of viable cells. The plates were incubated for 8 h at 37 °C. The toxicity of RTILs was measured in both of suspension and solid culture. Glass tube and 100-well plates were used for suspension culture. The series of medium that has different concentrations of RTILs were made in 100-well plates and optical density was measured by Bioscreen C (Oy Growth Curves AB Ltd, USA) micro-plate reader. The cell viability assays were carried out in the concentration range of ILs from 20 mg/L to 50,000 mg/L. Culture medium that has ionic liquids was prepared with ethanol to solvate immiscible ionic liquids. The final concentration of ethanol on the medium was 0.1, 0.2, and 0.5% (v/v), respectively. In these concentrations of ethanol has proven non-cytotoxic.

3. Cell Viability Assay

Viability of *E. coli* in glass tube culture was measured using a spectrophotometer (Spectronic Instruments Co., USA) at the wave length of 600 nm. Cell concentration was measured at 600 nm in the Bioscreen C micro-plate reader with UV/VIS detector. The measurement was duplicated on each plate containing blanks, controls, and samples. Each colonies in solid plate were counted after incubation for 8 h, 37 °C. The number of colonies formed on the solid culture plate was converted as colony forming units (CFUs). The viability of each culture was calculated by comparing the number of colonies in control culture in which no ionic liquids. The concentration of added RTILs which inhibits 50% of the growth of microorganisms comparing with the control culture was defined as EC_{50}, and it was calculated by regression method.

RESULTS AND DISCUSSION

1. Growth of *E. coli* in the Presence of Room Temperature Ionic Liquids

The toxicity of RTILs was measured by CFU in solid agar plates. The suspension of *E. coli* at stationary phase of the growth was plated on the agar plate which contains RTILs. The toxicity of RTILs on suspension culture of *E. coli* was measured in glass tubes and 100-well micro-plates. The results of the toxicity test are summarized in Table 1. In these methods, the cell solution at stationary phase of *E. coli* was inoculated in the media which has RTILs, thus it reflects the influence of RTILs on active microorganisms during early adaptation period of the cells to the new media. Normally, hydrophobic RTILs are water-immiscible, while hydrophilic RTILs are water-miscible. In this experiment, most frequently used hydrophobic, such as [Bmim][PF6] and [Omim][CH3SO4] and hydrophilic RTILs, such as [Emim][BF4] and [Emim][CH3SO4] were used. The hydrophobic and hydrophilic RTILs showed opposite results of toxicity in solid and suspension culture. The hydrophobic RTILs showed lower toxicity in solid culture, but hydrophilic RTILs presented lower toxicity in suspension culture, among tested RTILs.

The difference of diffusivity between hydrophilic and hydrophobic RTILs in solid and suspension culture is considered as a major factor affecting the influence of RTILs on microorganisms. The diffusivity of hydrophobic RTILs should be smaller than hydrophilic one in hydrophilic solid media, resulted in the lower possibility of contact between water-immiscible RTILs and microorganisms. However, formation of the aggregates of hydrophobic RTILs in hydrophilic solid media could disturb the contact with microorganisms. A few conditions should be considered for these results. Firstly, both of hydrophobic and hydrophilic RTILs would be uniformly distributed in suspension culture different from solid culture. Secondly, the actual number of viable microorganisms in suspension culture is smaller, because non-viable but not lysed cells are encountered in the concentrations. Consequently, the hydrophobic RTILs are supposed to have higher toxicity than hydrophilic one, because smaller amount of hydrophobic RTILs showed larger toxicity in suspension culture.

The viable ranges of *E. coli* were obtained with RTILs from 20 mg/L to 50,000 mg/L (Table 2). The toxicity of the various RTILs is widely spanned. The parameter, log P, is defined as the distribution coefficient of the given solvent in an equimolar mixture of octanol and water [Sardessai and Bhosle, 2002]. The larger polarity lowered the log P value of the solvent. The same RTILs showed similar trend of the parameters, log P and EC_{50}. The growth of *E. coli* was

Table 1. Influence of representative ionic liquids on the growth of *E. coli*. The concentration range of RTILs were identified in solid and suspension culture

		[Emim][BF4] (mg/L)	[Emim][CH3SO4] (mg/L)	[Omim][CH3SO4] (mg/L)	[Bmim][PF6] (mg/L)
Solid culture	Growth	<100	<100	<100	<1,000
	Low growth	500-5,000	100-200	200-10,000	5,000-50,000
	No growth	>10,000	>200	>10,000	>50,000
Suspension culture	Growth	<2,000	<800	<20	<1,500
	Low growth	4,000-40,000	1,000-2,000	50-200	2,000-5,000
	No growth	>50,000	>3,000	>300	>7,000

Growth: colony and optical density in the solid and suspension culture, respectively were same as control; Low growth: below the control; No growth: no cell, respectively.

Table 2. Comparison of toxicity (EC_{50}) and polarity (log P) of ionic liquids and organic solvents

Solvents	EC_{50} (mg/L)	log P
[Bmim][(CF3SO3)2N]	150±50	0.33[a]
[Hmim][(CF3SO3)2N]	150±50	0.65[a]
[Bmim][PF6]	4,000±500	−2.39[a]
[Hmim][PF6]	550±500	−1.86[a]
[Omim][PF6]	150±50	−1.33[a]
[Emim][BF4]	35,000±5,000	
[Bmim][BF4]	9,000±500	−2.44±0.23[d]
[Emim][CH3SO4]	2,500±500	-
[Omim][CH3SO4]	1,500±500	-
[Emim][CF3SO3]	20,000±10,000	-
[Pmim][CF3SO3]	1,000	-
[Pmim][SbF6]	3,000±2,000	-
[Hmim][SbF6]	550±500	-
Acetone	10,977.1[b]	−0.23[c]
Acetonitrile	8,734.3[b]	−0.33[c]
Ethanol	16,384.0[b]	−0.31[e]
Chloroform	1,285.9[b]	2.0[c]
Chlorohexane	>128[b]	3.2[c]
1-Chlorophenol	42.7[b]	-
Ethylbenzene	49.8[b]	-
Dimethyl sulfoxide (DMSO)	62,883.3[b]	-

Source; [a]Lee and Lee, 2004, [b]Peinado et al., 2002, [c]Hazarika et al., 2002, [d]Ulbert et al., 2004, [e]Willauer et al., 2002.

inhibited with increased RTILs in the medium. Most RTILs offered similar toxicity with frequently used organic solvents such as ethanol, acetone, acetonitrile and DMSO [Peiando et al., 2002].

2. Influence of Ions in Room Temperature Ionic Liquids on Viability of *E. coli*

The influence of the cations and anions on toxicity was surveyed. The influence of the anions on toxicity was tested by comparing the influence of [BF4], [PF6], [CF3SO3], [CH3SO4], [(CF3SO3)2N], and [SbF6] (Table 2). In this experiment, there was no significant influence of the anions, although it has been asserted by previous reports that modifications of the anion lead remarkable changes of chemical and physical properties of RTILs [Wilkes, 2004]. However, [BF4] gave lowest toxicity among tested anions of RTILs. The toxicity of [Bmim][BF4] is lower than [Bmim][PF6]. This could be due to the lower toxicity of [BF4] compared with other anions. An anion, [PF6], has larger number of fluoride than [BF4] which is well known toxicant to the microorganisms. However, combined influence of cation should be considered even there was no effect found in this research, because enormous compositions of anions and cations are possible. The toxicity was even higher than other ionic liquids for anion of [(CF3SO3)2N].

The influence of cations to toxicity was measured (Table 2). All cations of RTILs showed higher toxicity with increased length of n-alkyl chain. Thus, increased hydrophobicity is believed to enhance the toxicity of RTILs, because longer alkyl chains ascend the hydrophobicity of RTILs. These results show good agreement with previous report on toxicity study [Ranke et al., 2004], and are supported by previous reports suggesting the hydrophobicity governs the uptake rate of RTILs into the cells [Rajagopal, 1996; Sardessai and Bhosle, 2002]. [Emim] has lower toxicity than other cations of RTILs. However, various cations and anions showed no notable trends of influence on the toxicity to *E. coli*. More experiments using various RTILs are necessary to identify their toxicity. Further investigations would be focused on the RTILs composed of same anions or cations and various counter ions.

CONCLUSIONS

Most of the RTILs were less toxic than the organic solvents on *E. coli*. Cations of RTILs such as [Emim] showed lower toxicity than other cations. [BF4] presented lowest toxicity among tested anions of RTILs. Consequently, [Emim][BF4] seems one of the most favorable RTIL for bioprocesses because it exhibits lowest toxicity. No general influence of the anions of the RTILs could be found on toxicity. The RTILs with the longest alkyl chain showed highest toxicity. Therefore, from the point of view of sustainability, further research would be closely accompanied by ecotoxicological test [Jastorff et al., 2002]. However, DMSO showed smaller toxicity than tested ionic liquids as favorable solvent to microorganisms. Several RTILs could be used on *in-situ* production and separation bioprocesses with whole cell as a biocatalyst. In spite of the toxicity to the biomolecules, ionic liquids are promising molecule as green solvents for at least general extraction system. Further investigations are needed using more RTILs to identify their influence on biomolecules.

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