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The Ionic Liquid Tolerant Evaluation Method Using Formazan Dye in *Escherichia Coli*

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Abstract

Ionic liquids (ILs) are used in a variety of applications. Although ILs are called "green solvents," they exhibit significant toxicity. The toxicity of ILs presumably manifests owing to their interaction with the cell membrane. Organic solvents bind to and penetrate the cell membrane and severely affect cell permeability. Therefore, there is a possibility that the tolerance to ILs could be enhanced by utilizing organic solvent tolerance mechanisms. In this article, we proposed a method for visually evaluating the toxicity of ILs to Escherichia coli using formazan dye. Furthermore, we examined whether the method could be used to evaluate the difference in ionic liquid susceptibility of E. coli transformed with an organic solvent tolerance gene marA. E. coli transformed with marA were found to be more tolerant to several ILs. It was suggested that the organic solvent tolerance mechanism effectively works for ILs.

Keywords: ***Ionic liquid tolerance; Organic solvent tolerance; Escherichia coli; Tetrazolium violet.***

1. Introduction

Ionic liquids (ILs), commonly termed as designer solvents, are organic molten salts comprising bulky asymmetrical cations and weakly coordinating anions. They have remarkable features that can be easily custom-made by a plethora of cations and anions. Owing to these tailor-made properties, ILs have been used in many applications including biotechnology [1], green chemistry [2], [3], and pharmaceuticals [4], [5]. Although ILs are called "green solvents," they exhibit significant toxicity. The ionic character of ILs makes many of them water soluble. Therefore, they easily migrate into the environment and affect the living biota, and there is a valid expectation that the toxicity of ILs should be determined before they are considered for any application [6]-[8]. In recent years, the cytotoxicity, eco-toxicity, and microbial toxicity of ILs have been extensively studied. Microorganisms have been utilized as indicators in toxicity studies owing to their short generation time, rapid growth, and environmental and industrial relevance [9].

The toxicity of ILs presumably manifests owing to their interaction with the cell membrane. It is suggested that ILs with long alkyl chains penetrate the lipid bilayer and disturb its structure [10]. Organic solvents bind and penetrate the cell

membrane and severely affect cell permeability. The current knowledge and understanding of solvent tolerance mechanisms is majorly obtained by studying various bacteria [11]. Several organic solvent tolerance mechanisms have been reported in *E. coli*; the most representative of which is induced by the overexpression of the transcription factor MarA [12]. MarA induces the expression of the AcrAB multidrug efflux pump [13] and promotes extracellular efflux of organic solvents.

In this article, we proposed a method for visually evaluating the toxicity of ILs to *E. coli* using formazan dye. Furthermore, we examined whether the method could be used to evaluate the difference in ionic liquid susceptibility of *E. coli* transformed with an organic solvent tolerance gene *marA*.

2. Materials and Methods

2.1 Bacterial strains

E. coli JM109 was purchased from Takara Bio Inc., Japan. The plasmids pCA24N and pCA24N-*marA* were incorporated into pCA24N and distributed by NBRP-*E.coli* at NIG. *E. coli* JM109 was transformed with pCA24N and pCA24N-*marA* to obtain *E. coli* JM109 pCA24N and *E. coli* JM109 pCA24N-*marA*, respectively.

2.2 Ionic liquids

The ILs are shown in Table 1. Each IL was abbreviated as shown in Table 1.

Table 1: ILs.

IL	Abbreviation
1,3-Diallylimidazolium Bis(trifluoromethanesulfonyl)imide	Aaim TFSI
1-Allyl-3-butylimidazolium Tetrafluoroborate	Abim BF_4
1-Allyl-3-butylimidazolium Bis(trifluoromethanesulfonyl)imide	Abim TFSI
1-Allyl-3-methylimidazolium Bis(trifluoromethylsulfonyl)imide	Amim TFSI
1-Butyl-2,3-dimethylimidazolium Tetrafluoroborate	Bdmim BF_4
1-Butyl-3-methylimidazolium Bis(fluorosulfonyl)imide	Bmim FSI
1-Butyl-3-methylimidazolium Iodide	Bmim I
1-Butyl-3-methylimidazolium Bis(nonafluorobutanesulfonyl)imide	Bmim NFSI
1-Butyl-3-methylimidazolium Hexafluorophosphate	Bmim PF_6
1-Butyl-3-methylimidazolium Bis(trifluoromethanesulfonyl)imide	Bmim TFSI
1-Ethyl-2,3-dimethylimidazolium Bis(trifluoromethanesulfonyl)imide	Edmim TFSI
1-Ethyl-3-methylimidazolium Trifluoro(trifluoromethyl)borate	Emim CF_3BF_3
1-Ethyl-3-methylimidazolium Chloride	Emim Cl
1-Ethyl-3-methylimidazolium Diethylphosphate	Emim $dEPO_4$
1-Ethyl-3-methylimidazolium Bis(fluorosulfonyl)imide	Emim FSI
1-Ethyl-3-methylimidazolium Hydrogen sulfate	Emim HSO_4
1-Ethyl-3-methylimidazolium L-Lactate	Emim Lactate
1-Ethyl-3-methylimidazolium 2-(2-Methoxyethoxy)ethyl sulfate	Emim $MEeSO_4$
1-Ethyl-3-methylimidazolium Dicyanamide	Emim $N(CH)_2$
1-Ethyl-3-methylimidazolium Trifluoromethanesulfonate	Emim Tfo
1-Hexyl-3-methylimidazolium Tetrafluoroborate	Hmim BF_4
1-Hexyl-3-methylimidazolium Bromide	Hmim Br
1-Hexyl-3-methylimidazolium Chloride	Hmim Cl
1-Hexyl-3-methylimidazolium Bis(fluorosulfonyl)imide	Hmim FSI
1-Hexyl-3-methylimidazolium Hexafluorophosphate	Hmim PF_6
1-Hexyl-3-methylimidazolium Bis(trifluoromethylsulfonyl)imide	Hmim TFSI
1,3-Dimethylimidazolium Dimethyl phosphate	Mmim $dMPO_4$

1-Methyl-3-n-octylimidazolium Bromide	Moim Br
1-Methyl-3-octylimidazolium Chloride	Moim Cl
1-Methyl-3-octylimidazolium Hexafluorophospate	Moim PF_6
1-Methyl-3-octylimidazolium Bis(trifluoromethylsulfonyl)imide	Moim TFSI
1-Methyl-3-propylimidazolium Bis(trifluoromethylsulfonyl)imide	Mpim TFSI

2.3 Evaluating toxicity of ionic liquids

E. coli were grown aerobically in test tubes overnight at 37°C using LB medium containing 30 mg/L chloramphenicol. Approximately 100 µL of preculture was mixed with 10 mL of fresh LB medium containing 30 mg/L chloramphenicol and 100 mg/L tetrazolium violet. Previous reports [14], [15] have confirmed that the concentration of tetrazolium violet has no effect on growth. Approximately 300 µL of the culture solution was placed in a 96-well plate, and 3 µL of ionic liquid was placed in each well containing the culture solution. Even if the ionic liquid is insoluble in water, 1%v/v to the culture solution is excessive, and sufficient ionic liquid is present in the culture solution. Wells that did not contain ionic liquid were used as positive controls. After incubation for 24 h at 37°C, a picture of the 96-well plate was taken and the absorbance was measured at 540 nm using Multiskan MS (Thermo Fisher Scientific, USA).

3. Results and Discussion

Fig. 1 shows an image of a 96-well plate after 24 h of incubation. Table 2 shows the *E. coli* and ILs in each well. In the positive control, when *E. coli* proliferated, tetrazolium violet was oxidized to produce the violet formazan dye. Depending on the type of IL, the well containing the IL did not produce formazan dye when the growth was inhibited (Fig. 1).

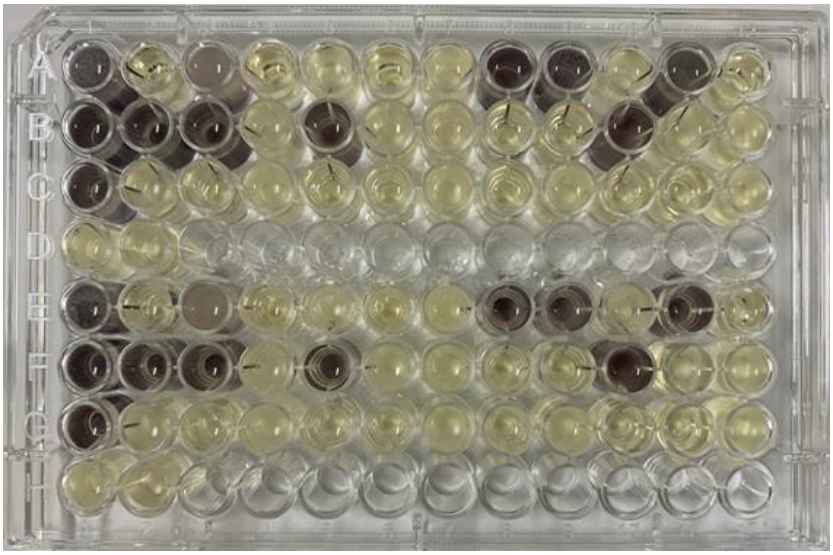

Fig. 1. The image of a 96-well plate after 24 h incubation.

It was confirmed that formazan dye was not produced only by adding the IL to the LB medium containing tetrazolium violet and chloramphenicol (data not shown). On the other hand, it was observed that the wells in which the formazan dye was produced were reproducibly produced in the three experiments. ILs with long alkyl chains may penetrate the lipid bilayer and disturb its structure [10]. ILs with short alkyl chains (Mmim, Emim, Bmim) were filled in the wells

where the formation of formazan dye was confirmed, suggesting that there is a correlation between the length of the alkyl chains and toxicity. However, formazan dye was not generated in the wells containing the ILs that produce Emim and Bmim cations; therefore, the influence of anions and other factors was considered. Cellular enzymes are inactive in ILs that possess BF_4, PF_6, and TFSI anions but are active in ILs that contain Cl, NO_3, CF_3SO_3, CF_3COO, or CH_3COO anions [16]. The anions in the IL filled in the wells where the formazan dye was produced were relatively small in size and often did not inhibit the activity of cellular enzymes. Comparing *E. coli* pCA24N (row A-D) and E. coli pCA24N-*marA* (row E-H), the presence or absence of formazan dye production and the difference in the amount of formazan dye produced was observed in some wells. Table 3 shows the difference in absorbance at 540 nm between 0 and 24 h-incubation in each well (mean of three experiments). The wells in which formazan dye was formed had an absorbance of >0.5, and it was confirmed that there is also repeatability (Table 4). *E. coli* pCA24N-*marA* had a higher absorbance than *E. coli* pCA24N and had an absorbance of ≥0.1 in five wells, except for the positive control (Table 5). The difference in the well containing Amim TFSI or Hmim PF_6 was not observed visually (Fig. 1), but the difference in absorbance was clear. Especially, Hmim PF_6 has a long cation alkyl chain, and the anion PF_6 inactivates cellular enzymes; therefore, it is generally considered to be highly toxic. Therefore, it was suggested that the overexpression of the AcrAB multidrug efflux pump caused by the high expression of the *marA* gene showed tolerance to the IL by causing intracellular IL efflux. EC_{50} and minimum inhibitory concentration (MIC) are usually used to evaluate the toxicity of compounds. In the case of bacteria, the survival is confirmed by proliferation (increased turbidity or colony formation). This method utilized that tetrazolium violet was oxidized with the survival of *E. coli* to produce formazan dye. The production of formazan dye visually revealed the survival of *E. coli*. Further, by measuring the absorbance of the formazan dye, the degree of tolerance to the IL could be evaluated. As a result, it was possible to show the possibility that IL tolerance could be improved by *marA*, which is an organic solvent tolerance gene. Moreover, since this method did not require complicated experimental procedures and advanced equipment, it could be a very powerful tool for screening tolerance to ILs.

Table 2: The E. Coli and ILs Filled in Each Well.

		1	2	3	4	5	6	7	8	9	10	11	12
A	*E. coli* pCA24N	positive control	Bmim I	Bmim NFSI		Bmim TFSI	Emim HSO_4	Bmim PF_6	Emim $N(CH)_2$	Emim $MEeSO_4$	Emim CF_3BF_3	Emim $dEPO_4$	
B		Emim Lactate	Emim Tfo	Emim Cl	Mpim TFSI	Mmim $dMPO_4$	Abim TFSI	Moim PF_6	Abim BF_4	Moim Br		Moim TFSI	Edmim TFSI
C		Mmim $dMPO_4$	Aaim TFSI	Bdmim BF_4	Amim TFSI	Moim Cl	Hmim Cl	Hmim TFSI	Hmim BF_4	Bmim FSI	Hmim Cl	Hmim Br	Hmim FSI
D		Emim FSI	Hmim PF_4										
E	*E. coli* pCA24N-*marA*	positive control	Bmim I	Bmim NFSI		Bmim TFSI	Emim HSO_4	Bmim PF_6	Emim $N(CH)_2$	Emim $MEeSO_4$	Emim CF_3BF_3	Emim $dEPO_4$	
F		Emim Lactate	Emim Tfo	Emim Cl	Mpim TFSI	Mmim $dMPO_4$	Abim TFSI	Moim PF_6	Abim BF_4	Moim Br		Moim TFSI	Edmim TFSI
G		Mmim $dMPO_4$	Aaim TFSI	Bdmim BF_4	Amim TFSI	Moim Cl	Hmim Cl	Hmim TFSI	Hmim BF_4	Bmim FSI	Hmim Cl	Hmim Br	Hmim FSI
H		Emim FSI	Hmim PF_4										

Table 3: The Difference in Absorbance at 540 nm Between 0 and 24 h-Incubation in Each Well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.859	-0.003	0.875		-0.127	0.024	0.027	1.186	1.375	-0.029	1.370	
B	0.839	0.768	1.249	0.036	0.806	-0.564	0.059	-0.032	-0.009		-0.289	-0.241
C	0.829	-0.337	0.165	0.157	-0.001	-0.003	-0.443	-0.107	-0.111	-0.003	-0.003	-0.157
D	0.234	-0.044										
E	1.830	-0.002	1.158		-0.128	0.014	0.100	0.868	1.322	0.034	1.052	
F	0.599	0.736	0.953	-0.104	1.035	-0.184	0.094	-0.089	-0.006		-0.197	0.002
G	0.514	-0.132	-0.061	0.277	-0.002	0.000	-0.279	-0.043	-0.001	-0.004	-0.003	-0.320
H	0.116	0.289										

Table 4: Average and Standard Deviation of the Absorbance of the Wells in Which the Formazan Dye was Produced.

IL	*E. coli* pCA24N		*E. coli* pCA24N-marA	
	avg.	SD	avg.	SD
Bmim NFSI	0.875	0.185	1.158	0.268
Emim $N(CH)_2$	1.186	0.078	0.868	0.046
Emim $MEeSO_4$	1.375	0.050	1.322	0.236
Emim $dEPO_4$	1.370	0.095	1.052	0.031
Emim Lactate	0.839	0.088	0.599	0.111
Emim Tfo	0.768	0.074	0.736	0.193
Emim Cl	1.249	0.100	0.953	0.044
Mmim $dMPO_4$	0.806	0.102	1.035	0.081
Mmim $dMPO_4$	0.829	0.269	0.514	0.134

Table 5: *E. coli* pCA24N-*marA* had a Higher Absorbance Than *E. coli* pCA24N and had an Absorbance of ≥0.1.

IL	*E. coli* pCA24N		*E. coli* pCA24N-marA	
	avg.	SD	avg.	SD
Bmim NFSI	0.875	0.185	1.158	0.268
Bmim PF_6	0.027	0.024	0.100	0.040
Mmim $dMPO_4$	0.806	0.102	1.035	0.081
Amim TFSI	0.157	0.038	0.277	0.046
Hmim PF_6	-0.044	0.042	0.289	0.190

4. Conclusions

In this article, we proposed a method for visual evaluation of toxicity ILs to *E. coli* using formazan dye. ILs with short alkyl chains (Mmim, Emim, Bmim) were added in the wells in which formazan dye was formed, and we suggest a correlation between the length of the alkyl chains and toxicity. The anions in the IL filled in the wells where the formazan dye was produced were relatively small in size and often did not inhibit the activity of cellular enzymes. *E. coli* transformed with *marA* were found to be more tolerant to several ILs. It was tolerant to Hmim PF_6, which was composed of long alkyl chain cations and anions that inactivate cellular enzymes. It was suggested that the organic solvent tolerance mechanism works effectively for ILs.

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