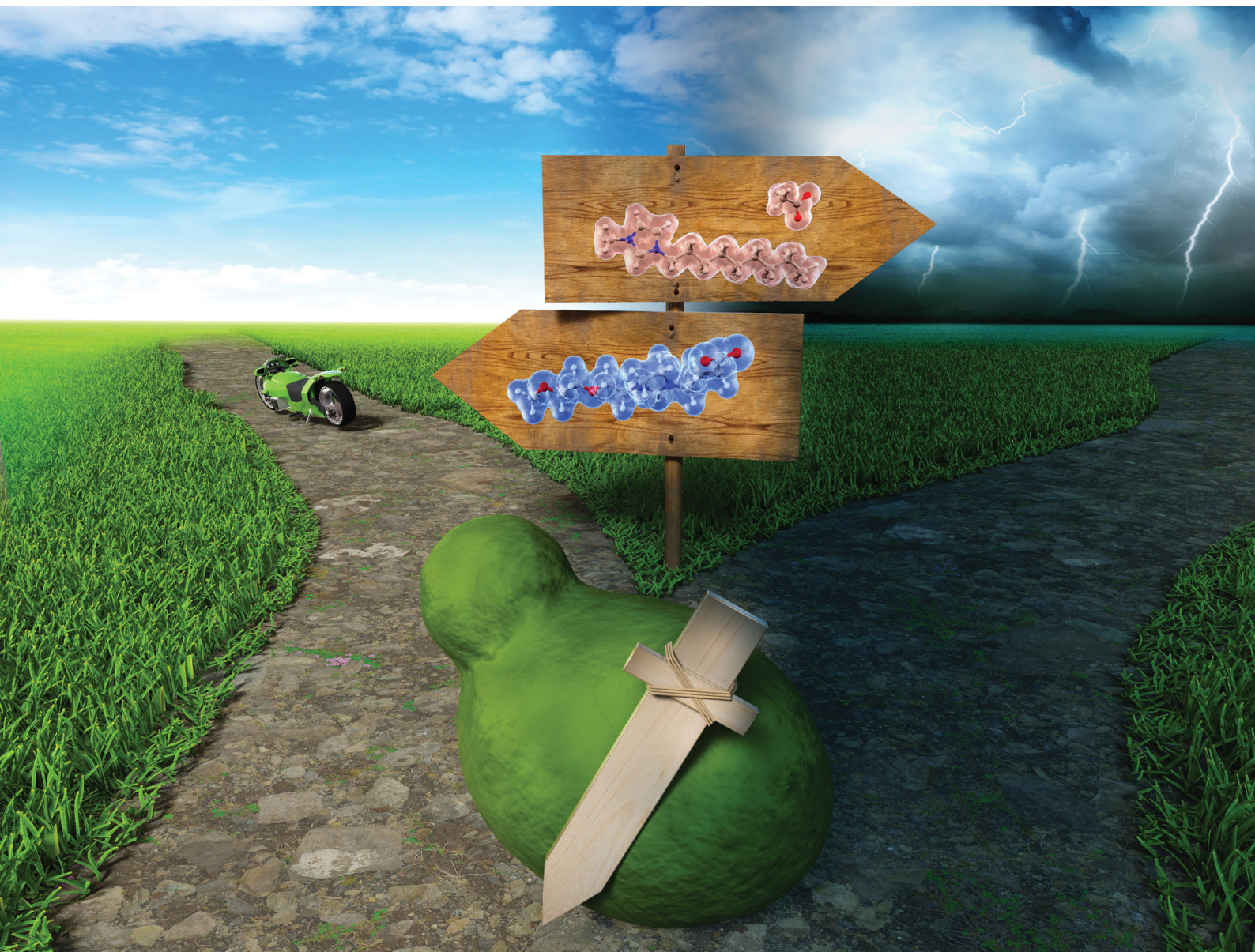


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**PERSPECTIVE**

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mini review



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## Bioethanol fermentation in the presence of ionic liquids: mini review

Kosuke Kuroda  <sup>ab</sup>

Ionic liquids are known as efficient pretreatment solvents for cellulosic biomass, but typical cellulose-dissolving ionic liquids are toxic to microorganisms, hindering the fermentation process for bioethanol production. Although the ionic liquids can be removed by washing after pretreatment, it negatively impacts the energy balance. Therefore, successive/simultaneous biomass pretreatment, hydrolysis, and fermentation processes with low-toxicity ionic liquids are desired. Herein, especially for the beginners and students, the toxicity of ionic liquids and their mechanisms by which the microorganisms are destroyed are simply discussed. Furthermore, two currently evolving solutions, low-toxicity ionic liquids and ionic liquid-resistant microorganisms for bioethanol production, are discussed. To the best of my knowledge, this is the first review specifically focusing on bioethanol fermentation in ionic liquid solutions, whereas there are many reports on cellulose dissolution.

### Lignocellulose as a resource of bioethanol

The global demand for energy is increasing, and concerns about climate change and the instability of oil resources have led to renewed interest in alternative energy sources to replace fossil fuels. As a result, many countries have been focusing on sustainable and renewable biofuels.<sup>1,2</sup> Currently, corn starch, sugarcane, and beet monosaccharides are directly converted to ethanol, but these edible sugars compete with food and feed. Therefore, lignocellulose, a major component of plant cell walls, is recognized as one of the most promising resources for ethanol production.<sup>3</sup> Specifically, cellulose and hemicellulose in lignocellulose are the resources of bioethanol. Although hemicellulose is relatively easy to convert, cellulose is not; it is one of the most chemically and physically robust and recalcitrant natural polymers.<sup>3,4</sup>

### Significance of bioethanol fermentation in the presence of ionic liquids

Typical ionic liquids, which are liquid salts below 100 °C,<sup>5–14</sup> are toxic to microorganisms.<sup>15–20</sup> Then, why is it necessary to

conduct microbial fermentation in the presence of ionic liquids? To figure this out, we first look at the overall picture of the bioethanol production process (Fig. 1).

Polysaccharides can be converted to ethanol by the following three processes: (1) Pretreatment with ionic liquids to improve enzyme accessibility of the polysaccharides; (2) enzymatic hydrolysis of the polysaccharides to sugars, such as glucose and xylose; (3) ethanol fermentation by microorganisms, such as yeasts.

Energy cost is one of the most important factors in the production of ethanol as a fuel. It is essential to reduce energy costs during production and thus maximize the energy obtained from ethanol. Although there are several cellulose solvents such as LiCl/*N,N*-dimethylacetamide and ethylene diamine/salt,<sup>21–23</sup> they do not efficiently dissolve cellulose at room temperature and ambient pressure. The high temperature and/or pressure are very energy intensive, hindering the practical industrialization of bioethanol.

On the other hand, ionic liquids, especially those containing carboxylate, dialkylphosphate, or alkylphosphonate anions, efficiently dissolve cellulose/biomass even at room temperature.<sup>24–31</sup>

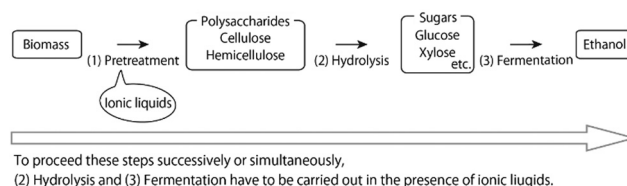


Fig. 1 Overview of the bioethanol production process and the significance of fermentation in the presence of ionic liquids.

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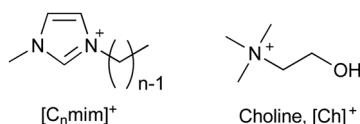
(Chloride-type ionic liquids are less effective in pretreatment.<sup>32,33</sup>) Therefore, ionic liquids are recognized as the most promising solvents for bioethanol production. Recently, ionic liquid-like onium salts with hydroxide anions were also reported as cellulose/biomass solvents working at room temperature.<sup>34–36</sup> Despite these advantages,<sup>5–13</sup> using ionic liquids alone is not sufficient for commercialization because ethanol has a low combustion energy density: around 60% of gasoline per volume. Further reduction of the energy cost is necessary and the processes (1)–(3) must be carried out in succession in the same reaction pot (called one-pot ethanol production), indicating that hydrolysis and fermentation must be carried out in the presence of ionic liquids used for pretreatment. However, typical cellulose-dissolving ionic liquids are toxic to microorganisms, which can hinder the fermentation process.<sup>15–20</sup> There are several good reviews on biomass pretreatment with ionic liquids<sup>6,10,28,37,38</sup> and hydrolysis in the presence of ionic liquids.<sup>39,40</sup> Comparatively, there is less focus on fermentation in the presence of ionic liquids, which is reviewed here as a mini-review.

## Microbial toxicity of ionic liquids

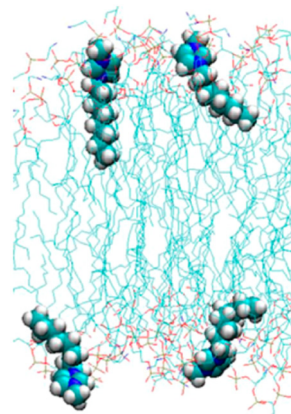
The toxicity of ionic liquids strongly depends on the alkyl chain length of the cation (Table 1).<sup>19,41–44</sup> Long alkyl chains of the cation (e.g., 1-methyl-3-octylimidazolium acetate: [C<sub>8</sub>mim]OAc; Fig. 2) enter the cell membrane and eventually destroy it (Fig. 3).<sup>41–44</sup> The mechanism of alkyl chain entry is as follows: (i) A positively charged cation comes into close contact with a negatively charged phosphate group of the cell membrane. (ii) The alkyl group of the cation interacts with the acyl group (long-alkyl chain) of the cell membrane by hydrophobic interactions and enters the cell membrane. On the other hand, short alkyl chains of the cation (e.g., [C<sub>2</sub>mim]OAc, the most well-known pretreatment solvent for biomass) are relatively less toxic than their long counterparts, but are still more toxic than common organic solvents.<sup>45,46</sup> For example, a commonly used indicator of toxicity, half maximal effective concentration (EC<sub>50</sub>), is defined as

**Table 1** The alkyl chain length of the cations and the minimum growth inhibition concentration of yeast (*Saccharomyces cerevisiae*). The minimum growth inhibitory concentration is the lowest concentration that prevents the growth; the higher this value, the lower the toxicity

|                        | Minimum inhibitory concentration (mM) |
|------------------------|---------------------------------------|
| [C <sub>2</sub> mim]Cl | 261.8                                 |
| [C <sub>4</sub> mim]Cl | 207.5                                 |
| [C <sub>6</sub> mim]Cl | 7.8                                   |
| [C <sub>8</sub> mim]Cl | 0.5                                   |



**Fig. 2** Ionic liquid cations presented in this review.



**Fig. 3** Cell membrane with inserted [C<sub>8</sub>mim]<sup>+</sup>. Adapted with permission.<sup>41</sup> Copyright 2014 American Chemical Society.

the concentration of ionic liquids at which the growth of microorganisms is halved compared to that without ionic liquids. The EC<sub>50</sub> values of [C<sub>2</sub>mim]OAc, dimethyl sulfoxide, and ethanol against *Escherichia coli* (*E. coli*) are 9, 91, and 17 g L<sup>−1</sup>, respectively.<sup>45</sup> The toxicity is assumed to be derived from the entry of cations into the cell *via* transporters on the cell membrane.<sup>47</sup> The cations alter the mitochondrial membrane potential, leading to apoptosis (cell death).

[C<sub>2</sub>mim]OAc has a lethal effect on the growth and fermentation of *Saccharomyces cerevisiae* (*S. cerevisiae*) and *E. coli* even at concentrations below 1%.<sup>20</sup> Comparatively, [C<sub>2</sub>mim]Cl is slightly less toxic, but still has a significant impact at concentrations of 1–2%.<sup>20</sup> Additionally, phosphate ionic liquids exhibit a similar toxicity to chloride ionic liquids.<sup>48,49</sup> Therefore, typical imidazolium-based ionic liquids are effective pretreatment solvents,<sup>50,51</sup> but are highly toxic and unsuitable for successive/simultaneous ethanol production processes.

## Countermeasures against ionic liquid toxicity

Various countermeasures have been proposed to counteract the toxicity of typical ionic liquids, which are broadly classified into three categories.

### 1. Increase the concentration of microorganisms

The simplest countermeasure is to increase the concentration of microorganisms. Generally, most microorganisms can resist some stresses including ionic liquids at high microbial concentrations. For example, it has been reported that concentrated *S. cerevisiae* can produce ethanol with little inhibition in a 3% [C<sub>2</sub>mim]OAc solution.<sup>49</sup> A similar trend has been shown with recombinant *E. coli* (*E. coli* KO11) that can ferment ethanol.<sup>48</sup>

Increasing the concentration of microorganisms also contributes to increasing the ethanol yield. When fermentation is initiated with a high microbial concentration, glucose is not used for microbial growth, thereby improving the ethanol yield.



## 2. Use of ionic liquid-tolerant microorganisms

**2-(1) Search for ionic liquid-tolerant microorganisms.** *Yarrowia lipolytica* (*Y. lipolytica*), a type of yeast, is highly tolerant to ionic liquids.<sup>52</sup> For example, *Y. lipolytica* is stress tolerant by nature and has the ability to adapt to a wide range of pH (2–11) and high salt concentration such as 12% NaCl. Therefore, it can grow and ferment even in 10% [C<sub>2</sub>mim]OAc. Although it is not capable of producing ethanol, it can produce useful substances such as  $\alpha$ -ketoglutaric acid and eicosapentaenoic acid. Among yeasts capable of ethanol fermentation, *Kazachstania telluris* and *Wickerhamomyces anomalus* exhibit high ionic liquid tolerance;<sup>53</sup> however, it is not as high as that of *Y. lipolytica*.

**2-(2) Incubation in ionic liquids to confer ionic liquid tolerance.** Walker *et al.* succeeded in improving the ionic liquid tolerance of *Y. lipolytica*.<sup>54</sup> They started culturing *Y. lipolytica* in 5% [C<sub>2</sub>mim]OAc and gradually increased the concentration of [C<sub>2</sub>mim]OAc to obtain a *Y. lipolytica* strain that could grow in an 18% [C<sub>2</sub>mim]OAc solution. This strain showed an increase in the phospholipid content and changes in its composition, as well as an increase in sterol content after incubation. In particular, it has been suggested that the decrease in cell membrane fluidity associated with the increase in sterol content is important.

**2-(3) Gene modification to confer ionic liquid tolerance.** To confer ionic liquid tolerance to microorganisms *via* genetic modification, it is necessary to identify factors that are important for ionic liquid tolerance. Ionic liquids affect the mitochondria in *S. cerevisiae* because of the cation uptake transporters.<sup>55</sup> Therefore, attempts have been made to knock out its related gene, *ptk2*, to avoid the uptake of ionic liquids. The *ptk2* knockout greatly improved the resistance to [C<sub>2</sub>mim]OAc and [C<sub>2</sub>mim]Cl.<sup>55</sup> Furthermore, *E. coli* with improved ionic liquid resistance by genetic recombination has been reported.<sup>56</sup>

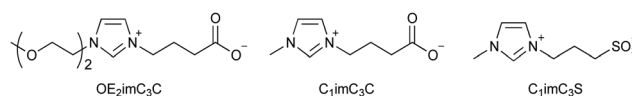
Other mechanisms of ionic liquid resistance have been investigated in *Enterobacter lignolyticus*.<sup>55</sup> These include: (1) decreased intracellular permeability of ionic liquids due to changes in the phospholipid composition and reduction of porin (a passive transporter that penetrates through the plasma membrane), (2) extracellular efflux of ionic liquids by multidrug efflux pumps, and (3) accumulation of sugars and amino acids to confer osmotolerance. It can be expected that ionic liquid tolerance will be conferred by using these mechanisms.

## 3. Use of low toxicity ionic liquids

Although [C<sub>2</sub>mim]<sup>+</sup>-based ionic liquids effectively delignify and reduce cellulose crystallinity in biomass,<sup>10,57,58</sup> they are basically highly toxic. Therefore, low-toxicity ionic liquids are being developed. One strategy for developing low-toxicity ionic liquids is to use bio-derived ions such as choline acetate ([Ch]OAc). As expected, [Ch]OAc has been reported to be less toxic than [C<sub>2</sub>mim]OAc.<sup>59</sup> Although its pretreatment capacity is somewhat less than that of [C<sub>2</sub>mim]OAc, it is reported to be adequate.<sup>60–62</sup> Pretreatment with [Ch]OAc also occurs through the mechanisms of cellulose crystallinity reduction and delignification. However, despite its low toxicity toward microbial growth, it

**Table 2** Half maximal effective concentration (EC<sub>50</sub>) of zwitterions, an ionic liquid, organic solvents against *E. coli* KO11 and relative ethanol concentration in 0.5 mol L<sup>−1</sup> solutions.<sup>45,48</sup>

|              |                                    | EC <sub>50</sub><br>(g L <sup>−1</sup> ) | Relative ethanol<br>concentration<br>(%) | Pretreatment<br>ability |
|--------------|------------------------------------|------------------------------------------|------------------------------------------|-------------------------|
| Zwitterion   | OE <sub>2</sub> imC <sub>3</sub> C | 158                                      | 96                                       | Yes                     |
|              | C <sub>1</sub> imC <sub>3</sub> C  | 141                                      | 100                                      | Yes                     |
|              | C <sub>1</sub> imC <sub>3</sub> S  | > 200                                    | 104                                      | No                      |
| Ionic liquid | [Ch]OAc                            | 70                                       | 15                                       | Yes                     |
|              | Dimethyl<br>sulfoxide              | 91                                       | —                                        | No                      |
|              | Ethanol                            | 17                                       | —                                        | No                      |
| Control      | —                                  | —                                        | 100                                      | —                       |



**Fig. 4** Structures of zwitterions described in this review.

is highly toxic to fermentation, resulting in low ethanol yields (Table 2).<sup>48</sup>

Ionic liquids with choline cations and amino acid anions have also been reported.<sup>60,63–66</sup> These ionic liquids are capable of pretreating biomass, with choline lysinate being particularly effective. The primary pretreatment mechanism of choline lysinate is delignification, but it can also slightly reduce cellulose crystallinity.

Artificial ions can also exhibit low toxicity. Zwitterionic liquids (*e.g.*, OE<sub>2</sub>imC<sub>3</sub>C; Fig. 4), in which the anion and cation are covalently bonded, have been reported to be much less toxic than the corresponding ionic liquids.<sup>45,48,67,68</sup> The greatest advantage of zwitterionization is that zwitterionic liquids exhibit low toxicity without loss of their functionality, because the constituent cations or anions are the same as the original ionic liquids. OE<sub>2</sub>imC<sub>3</sub>C, which combines an imidazolium cation with a carboxylic acid anion, can not only perform delignification and reduce cellulose crystallinity, but also dissolution of cellulose.<sup>45,48,68–70</sup> In addition, microbial fermentation is possible even in OE<sub>2</sub>imC<sub>3</sub>C solutions of more than 50 wt%, showing its extremely low toxicity.<sup>48</sup>

## Feasibility of continuous ethanol production

One-pot ethanol production from plant biomass is at the edge of realization through the various innovations described above. In particular, the Joint BioEnergy Institute and its affiliated research institutes in the United States are vigorously pursuing research toward practical application, including protic ionic liquids and deep eutectic solvents.<sup>66,71–75</sup> They have already been continuously converting tens of kilograms of woody biomass into ethanol with relatively high yields.<sup>72</sup> The one-pot ethanol production process also reduces the capital cost and wastewater treatment cost. They then estimate the



production cost of ethanol to be approximately \$5.3 per gallon, which can be lowered to \$1.8 per gallon with further improvements.<sup>72</sup> The price is similar or a little bit higher than the case produced by the other pretreatment methods,<sup>76</sup> although precise comparison of the values from different literature is difficult. Reducing the price of ethanol to match that of gasoline will facilitate the practical application of bioethanol.

## Conclusions

The practical application of cellulosic bioethanol is an extremely difficult task. It is evidenced by the fact that it has not yet been economically industrialized on a large scale, despite knowing that “cell walls are made of glucose units” for 100 years. However, as mentioned in the previous section, the possibility of practical application is gradually emerging. In Europe, a venture company, Lixea, has been established, and its practical application is expected in the future.

On the other hand, bioethanol production includes chemical, enzymatic, and microbial processes, requiring interdisciplinary insights and techniques such as those from biochemistry, genetics, organic chemistry, and chemical engineering. Agriculture is also important considering the cultivation of biomass. Thus, new ideas and innovation are urgently required from areas other than of ionic liquids and biomass pretreatment.

While this paper mainly focuses on ethanol production by yeast, a wide variety of fermentation products can be obtained from glucose and other sugars. For example, limonene<sup>56</sup> and isopentenol<sup>66</sup> were produced by *E. coli*, which can be easily genetically modified. The technologies described in this review are expected to expand into many other areas, not limited to bioethanol production.

## Conflicts of interest

There are no conflicts to declare.

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