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Tolerance of Immobilized Yeast Cells in Imidazolium-Based Ionic Liquids

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Summary

Ionic liquids (ILs) are considered as another 'green solvent', after the supercritical carbon dioxide. They are a promising reaction medium for biocatalysis process. The tolerance of active cells in hydrophobic imidazolium-based ILs (1-R-3-methylimidazolium hexafluorophosphate, [RMim][PF_6]) has been studied in this work. Calcium-alginate-entrapped baker's yeast has been chosen as the model of living cells. The results show that this kind of ILs possess a certain degree of biocompatibility. The tolerance of yeast cells to the ILs decreases with the increase of the R chain length of these ILs. The experiment indicated that 1-butyl-3-methylimidazolium hexafluorophosphate ([BMim][PF_6]) possessed excellent biocompatibility compared to the other imidazolium-based ILs. The moisture content in the ILs was the key factor that affected the tolerance. The activity retention of yeast cells pretreated with [BMim][PF_6] saturated with water and aqueous [BMim][PF_6] biphasic system was about 70 %, but it was only 50 % with the anhydrous [BMim][PF_6]. Although the yeast cells were pretreated with [BMim][PF_6] for 24 h, the activity retention was up to 45 %. The yeast cells had around 50 % activity after being pretreated 4 times with [BMim][PF_6]. This shows that the water immiscible ILs possess good biocompatibility, and they are suitable for application as the reaction medium catalyzed by living cells.

Key words: ionic liquid, biocompatibility, cell toxicity, yeast cell, biocatalysis

Introduction

Whole cell biocatalysis can be effectively used for the production of enantiopure compounds (*1,2*). It is applied especially in asymmetric reduction of prochiral ketones to produce the corresponding chiral alcohols as the key chiral building blocks for many enantiopure pharmaceuticals (*3*). Many asymmetric reduction reactions with excellent enantioselectivity are catalyzed by various microbes (*3–7*). However, the space-time yield does not meet the practicable demand (*8*). Toxicity of the substrate and product to living cells, and poor solubility of substrates are the main obstacles (*9,10*). Coupling the asymmetric reduction reaction with separation process is a promising technique to improve the reaction efficiency. The main idea is to introduce another phase as the substrate reservoir and *in situ* extracting agent for the reaction product (*11,12*). Macroporous adsorbing resins and organic solvents have been successfully applied as the second phase (*13,14*). Ionic liquids (ILs) should also be applied as the second phase (*15*). In previous reports the efficiency has not met the expectations (*16*), the main reason is perhaps the biocompatibility of ionic liquids with the living cell.

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Ionic liquids (ILs) are considered as another 'green solvent', after the supercritical carbon dioxide, due to their excellent properties, such as nonvolatile, thermal stability, designability, reusage and solvability to hydrophobic substrate as well as being environmentally friendly (*17*). That is why they have been recognized as an excellent and promising biocatalysis medium, as an alternative to conventional volatile chemical solvents (*18*). They were successfully applied as the reaction media for the reactions catalyzed by lipase and other enzymes (*19*). The biocompatibility of ILs with enzymes and proteins has been widely studied (*20*). Previous reports show that ILs, especially the imidazolium-based and pyridinium--based ones, possess good biocompatibility with enzymes and proteins (*20*). Unfortunately, there are very few reports about the biocompatibility of ILs with active cell, only Matsumoto *et al.* (*21*) and Ranke *et al.* (*22*) reported some simple experiments on it. Matsumoto *et al.* (*21*) used ILs as an extractant in the lactic acid fermentation process. The toxicity of ILs to lactic acid bacteria (LAB) was simply investigated by measuring the decrease of the ability of LAB affected by ILs to produce lactic acid. Ranke *et al.* (*22*) reported the ecotoxicity of ILs (methyl- and ethyl-imidazolium ionic liquids) using *Vibrio fischeri* and WST-1 cell as the model system. To our knowledge, the biocompatibility of ILs with living cell has not been reported in detail. It is important to understand the asymmetric reduction process catalyzed by living cells with ILs applied as substrate reservoir and product extractant *in situ*.

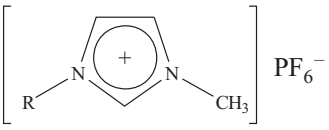
The biocompatibility of imidazolium-based ILs is studied in detail in the present work. Baker's yeast is chosen as the model of living cells, because it is the best model cell for these asymmetric reduction reactions (*3*). Imidazolium-based ILs, 1-R-3-methylimidazolium hexafluorophosphate ([RMim][PF_6]), are chosen as the research object, since they are water immiscible and considered to be the most promising ILs as the active cell biocatalysis medium (*15*,*16*,*19*). Also, this kind of ILs are tailored solvents, *i.e.* their properties can be tailored to fit the application *via* selection of the anion constituents or the R-chain (*17*).

Materials and Methods

Materials

1-butyl-, 1-pentyl, 1-hexyl- and 1-octyl-3-methylimidazolium hexafluorophosphate ([BMim][PF_6], [PMim][PF_6], [HMim][PF_6] and [OMim][PF_6], respectively) were used in the work. The methodology in the preparation of these ILs includes a metathesis of a bromide salt of the organic cation, [RMim]Br, with a sodium salt containing the desired anion, $NaPF_6$ (*23*). The structure of the ILs is shown in Scheme 1. All other chemicals were of analytical purity grade and commercially available.

The active dried baker's yeast was obtained from Meishan-Mauri Yeast Co., Ltd. (China) and it was activated to obtain the living cells as described in our previous work (*13*).



R=C_4H_9 for [BMim][PF_6]
R=C_5H_{11} for [PMim][PF_6]
R=C_6H_{13} for [HMim][PF_6]
R=C_8H_{17} for [OMim][PF_6]

Scheme 1. Imidazolium-based hexafluorophosphate ILs

Immobilization of yeast cell

Yeast cell was immobilized by the entrapped calcium alginate. The activated cell paste was resuspended in 25 mL of 20 mmol/L of Tris-HCl buffer (pH=7). The cell suspension was then mixed with 75 mL of 2 % (by mass per volume) sodium alginate solution prepared using the same buffer. The alginate/cell solution was extruded through a 19-gauge syringe needle, dripped into chilled 0.1 mol/L calcium chloride solution, and allowed to gel for 30 min at 4 °C. The hardened beads (3.3 mm average diameter) were then dried on a paper towel and stored at 4 °C in sealed vials. The cell mass of the alginate beads was typically 90 mg of dry cell mass per gram of beads.

Determination of the tolerance of yeast cells treated with ILs

The main experimental procedure was the following: the calcium-alginate-entrapped yeast cells were pretreated with the ILs for a certain period under the definite conditions described in the following paragraphs. Then, the activity of the yeast cells was measured in two ways. First, relative activity retention of the yeast cells was measured by glucose consumption velocity. Second, the ratio of living to dead cells was measured with an optical microscope and haemocytometer based on methylene blue dye staining. They will be described in detail in the following paragraphs.

Pretreatment of yeast cells with ILs

The pretreatment of yeast cells with ILs was carried out in 10-mL screw-capped vials placed in a thermostatic orbital shaker running at 75 rpm and 30 °C. Typically, a mass of 1.0 g of alginate beads (embedding yeast cells) and 5 mL of ILs were added into each vial. After 12 h, the alginate beads were taken out by filtration and washed with Tris-HCl buffer at 4 °C, after which the cell activity was determined. Each experiment was carried out three times in parallel, and the average results with the standard deviation were recorded.

Measuring the relative activity retention by glucose consumption velocity

The method is based on the glucose consumption velocity (*24*). The procedure of the determination of relative activity retention of yeast cell pretreated with ILs was the following: after pretreatment with ILs, the alginate beads were added into 5 mL of Tris-HCl buffer containing 2.0 g/L of glucose. The amount of consumed

glucose was determined in the first 30 min. Glucose concentration was determined with DNS method (*25*). The relative activity retention of yeast cells was expressed as follows:

$$\text{Relative activity retention} = \frac{n_i}{n_b} \times 100 \qquad /1/$$

where n_i is the amount of glucose consumed after 30 min by the yeast cells pretreated with Ils, and n_b is the amount of glucose consumed after 30 min by the yeast cells pretreated in a buffer (blank).

Measuring the ratio of living cells

The living and dead cells were identified, and the ratio of living cells was calculated. The activity of cells pretreated with ILs can also be expressed as the ratio of living cells. The method to identify the dead and living cells is based on methylene blue dye staining, which reveals the presence of cells with ruptured walls (*26*). The dye causes the dead cells to appear dark blue, while the living cells remain colourless. For this method, 0.1 g of alginate beads pretreated with ILs were dissolved in 2 mL of sodium citrate solution (20 mmol/L of Tris, 20 mmol/L of Na_3PO_4, and 100 mmol/L of citric acid, pH=7.0) for 1 h. Next, 0.2 mL of the dissolved alginate/cell solution were mixed with 0.8 mL of methylene blue dye solution (containing in g per 100 mL of distilled water: methylene blue 0.025, NaCl 0.9, KCl 0.042, $CaCl_2$ 0.048, $NaHCO_3$ 0.02, and glucose 1). After 10 min, the suspended mixture was added into the haemocytometer. The living and dead cells were then able to be distinguished with an optical microscope, and the ratio of living cells to total cells was recorded.

Results and Discussion

Effect of R-chain length of the imidazolium-based ILs on yeast cell activity

The tolerance of yeast cells to imidazolium-based ILs with different R-chain length was investigated. The *n*-alkyl chain length of these ILs varied from 4 to 8 carbon atoms, which were saturated with water. The activity of calcium-alginate-entrapped yeast cells preincubated with ILs is shown in Fig. 1. The relative activity retention based on glucose consumption velocity and the ratio of living cells are both given in Fig. 1.

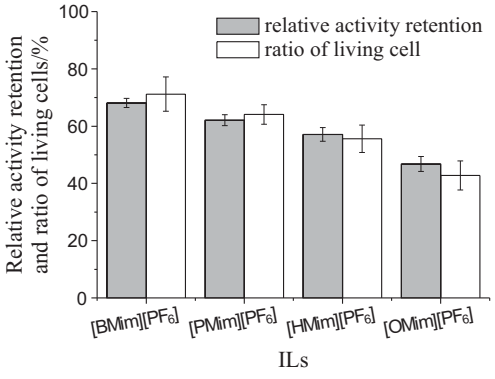


Fig. 1. Biocompatibility of imidazolium-based ILs with different R-chain length with yeast cells

The results clearly show that the water immiscible imidazolium-based ILs are biocompatible with yeast cells. The activity of yeast cells decreases with the increase of R-chain. [BMim][PF_6] possessed the best biocompatibility compared to the other ILs. The relative activity retention of yeast cells pretreated with [BMim][PF_6] for 12 h reached up to 70 %. The results are consistent with the previously published work about Gram-positive *Lactobacillus kefir* in biphasic aqueous-ionic liquid system (*21*). Also, Ranke *et al.* (*22*) found the ecotoxicity increasing with lengthening of the *n*-alkyl chain using *Vibrio fischeri* and the mammalian cell lines as the assay system in their biological study of imidazolium-based ILs. However, these results are contrary to the effect of the alkyl chain length on the activity and stability of enzymes in imidazolium-based ILs (*20,27,28*). It has been reported that the enzyme stability increased along with the alkyl chain length increase. The reason for the inactivation of enzymes in ILs is mainly that the enzyme is dissolved in the Ils, while the effect of ILs on living cells is more complicated.

It can be seen in Fig. 1 that relative activity retention based on glucose consumption velocity is consistent with the ratio of living cells. This indicates that the determination of the cell activity based on glucose consumption velocity is efficient, which is why only the relative activity retention was given in the following figures. It is evident that the use of [BMim][PF_6] as the reaction medium for active cell biocatalysis process is preferable, because of the good biocompatibility and the low ecotoxicity (*22*).

To further understand the effect of alkyl chain length on the biocompatibility of the ILs with yeast cells, the change of the activity of yeast cells pretreated with the intermediates of these ILs, *i.e.* 1-alkyl-3-methylimidazolium bromide, was also investigated. The results are shown in Fig. 2.

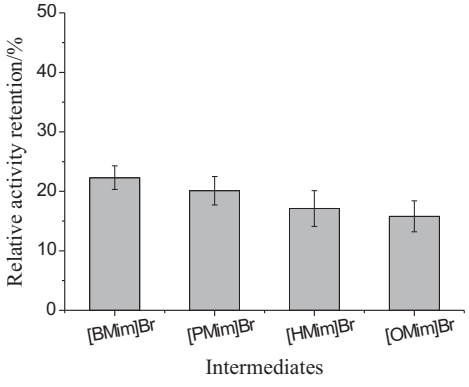


Fig. 2. Biocompatibility of the intermediates with yeast cells

Comparing Fig. 2 to Fig. 1, it is evident that the change in the trend of yeast cell activity in relation to the alkyl chain length is similar to that of the ILs. However, the relative activity retention of yeast cells pretreated with these intermediates is obviously smaller than of those pretreated with the corresponding ILs, although they are also ILs, but their biocompatibility is observably different from the corresponding hexafluoro-

phosphate ILs. There are two reasons for the difference. The first reason is that bromide is toxic to active cells, and the second is that the intermediates are water miscible, which results in the yeast cell dehydrating to be inactive. The second reason may be the main cause. The previous report also shows that only hydrophobic or water immiscible ILs can be applied as the reaction medium for active cell biocatalysis process (*29*).

Comparison of the cell activity between immobilized and free cells

Two kinds of cells, free and immobilized, with entrapped calcium alginate, were used to investigate the effect of the type of cell on the activity retention of yeast cell in imidazolium-based ILs. Fig. 3 shows the experiment results.

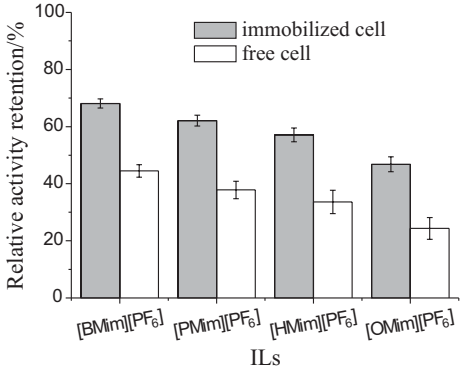


Fig. 3. Effect of the type of cell on the tolerance of yeast cell in ILs

It is evident that the relative activity retention of yeast cell entrapped in calcium-alginate bead is remarkably higher than that of free cell. Alginate, which is a kind of hydrogel, is quite hydrophilic and it protects the cell quite well against hydrophobic IL molecules because they have problems passing the barrier. This indicates that immobilized cell is preferable for the biocatalysis process in IL medium. The result is similar to that in organic solvent in a previous report (*24*).

Effect of moisture content of IL on the activity of yeast cells

In biocatalysis in nonaqueous media, moisture content is the key factor of the reaction process, since it influences the biocatalyst activity. Perhaps the moisture content also influences the activity of yeast cell in IL. The effect was investigated using anhydrous IL, biphasic aqueous-ionic liquid medium and water-saturated IL. Results are shown in Fig. 4.

The results show that there is obvious effect of moisture content on the relative activity retention of the yeast cells. Relative activity retention of the yeast cells pretreated with anhydrous IL is the smallest, just about 50 %. However, when pretreated with biphasic aqueous IL and water-saturated IL, it can be around 70 %. This indicates that a certain amount of water is necessary when IL is applied as the reaction medium, catalyzed by active cells.

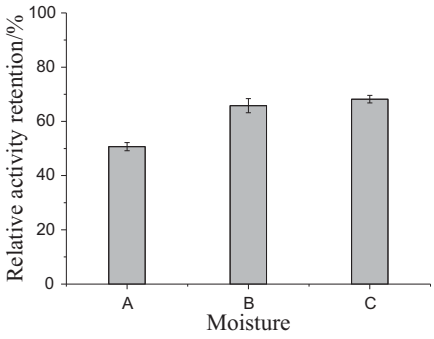


Fig. 4. Effect of moisture content on the activity of yeast cell in [BMim][PF_6]
A: anhydrous [BMim][PF_6], B: biphasic aqueous [BMim][PF_6], C: water-saturated [BMim][PF_6]

Effect of pretreatment period on the activity of yeast cell

The effect of pretreatment period on the tolerance of yeast cell was investigated. The pretreatment time was increased from 6 to 54 h in water-saturated ILs. Results are shown in Fig. 5.

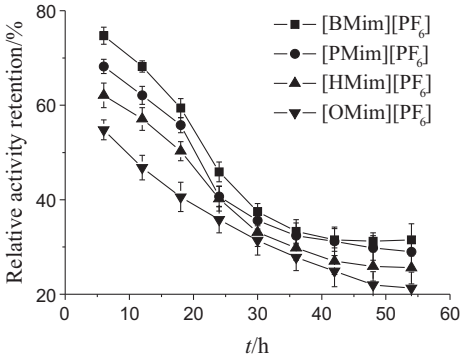


Fig. 5. Effect of pretreatment period on the activity of yeast cell in ILs

Relative activity retention decreases with the increase of contact time of yeast cell to ILs. However, the downtrend is gentle when the contact time is longer than 20 h. Although the yeast cells were pretreated with [BMim][PF_6] for more than 30 h, relative activity retention was able to reach 37.5 %.

Effect of pretreatment cycles on the activity of yeast cell

The effect of pretreatment with ILs on the activity of the yeast cells was investigated. Results are given in Fig. 6.

Experiments indicate that the immobilized yeast cells can be repeatedly used in ILs. Although pretreated four times with [BMim][PF_6], the relative activity retention can remain around 45 %.

Conclusions

The tolerance of yeast cells to imidazolium-based hexafluorophosphate ILs was investigated in this work. Experiments showed that the water immiscible ILs possess a certain degree of biocompatibility with active

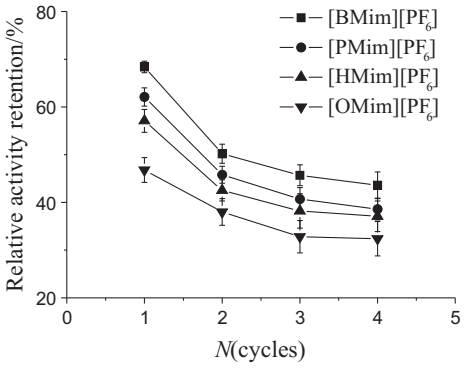


Fig. 6. Effect of pretreatment with ILs on the activity of yeast cell

cells. Relative activity retention of yeast cells decreases along with the increase of the length of alkyl chain of the ILs, and it is the largest in [BMim][PF_6], up to 70 %. Moisture content is one of the key factors that influence the yeast cell activity retention in ILs. Preferable cell activity can be kept in water-saturated IL. The results show that the ILs possess good biocompatibility and can be applied as the biocatalysis reaction medium.

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