Biocatalytic anti-Prelog stereoselective reduction of 4′-methoxyacetophenone to (*R*)-1-(4-methoxyphenyl)ethanol with immobilized *Trigonopsis variabilis* AS2.1611 cells using an ionic liquid-containing medium†

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The biocatalytic anti-Prelog enantioselective reduction of 4′-methoxyacetophenone (MOAP) to (*R*)-1-(4-methoxyphenyl)ethanol {(*R*)-MOPE} using immobilized *Trigonopsis variabilis* AS2.1611 cells was, for the first time, successfully conducted in an ionic liquid (IL)-containing system. Of all the tested ILs, the best results were observed in the co-solvent system containing 1-(2′-hydroxyl)ethyl-3-methylimidazolium nitrate ($C_2OHMIM{\cdot}NO_3$), which showed the good biocompatibility with the cells and only a moderate increase in the cell membrane permeability. It was found that the optimal content of $C_2OHMIM{\cdot}NO_3$, buffer pH, reaction temperature, substrate concentration and shaking rate were 2.5% (v/v), 8.5, 30 °C, 15 mM and 200 r min^{-1}, respectively. Under these optimized conditions, the initial reaction rate, the maximum yield and the product e.e. were 7.1 μmol h^{-1}, 97.2% and >99%, respectively, which are much higher than the corresponding values previously reported. The presence of $C_2OHMIM{\cdot}NO_3$ in aqueous buffer allowed the cells to tolerate relatively high temperatures and substrate concentrations compared with those in aqueous buffer without IL. Additionally, the immobilized cells manifested excellent operational and storage stability. Hence, the whole-cell biocatalytic anti-Prelog reduction of MOAP to (*R*)-MOPE on a preparative scale in the presence of $C_2OHMIM{\cdot}NO_3$ appears to be a promising and competitive reaction.

Introduction

The asymmetric reduction of prochiral ketones is one of the most important and fundamental reactions for producing enantiomerically pure chiral alcohols, which are valuable chiral building blocks for the synthesis of many industrially important chemicals such as pharmaceuticals, agrochemicals, liquid crystals and flavors.[1–3] Enantiopure aromatic alcohols are especially widely used as key intermediates for the synthesis of chiral drugs.[4,5] Among them, (*S*)-1-(4-methoxyphenyl)ethanol {(*S*)-MOPE} can be utilized as a crucial synthon for the preparation of cycloalkyl[*b*]indoles for clinical treatment of general allergic response,[6,7] and (*R*)-1-(4-methoxyphenyl)ethanol {(*R*)-MOPE} can be employed for the synthesis of chiral 3-aryl-3-substituted propanoic acids with anti-inflammatatory activity.[8] Accordingly, there is an increasing need for a highly efficient synthesis of enantiopure chiral 1-(4-methoxyphenyl)-ethanol. Currently, enantiopure chiral alcohols can be prepared by chemical or biological approaches. Most traditional chemical strategies can ensure chiral purity only by utilizing expensive chiral feedstock chemicals under harsh reaction conditions and often bring environmental concerns. Therefore, the economic preparation of enantiomerically pure chiral alcohols *via* green biocatalytic routes has become a subject of great interest due to their high enantioselectivity, mild reaction conditions and low environmental pollution. Among various biological methods, biocatalytic asymmetric reduction of prochiral ketones catalyzed by whole cells or isolated dehydrogenases has become the preferred procedure for preparation of enatiopure chiral alcohols. Generally, whole microbial cells are preferred to isolated enzymes because they avoid the need for enzyme purification and coenzyme addition or the requirement for an additional system for coenzyme regeneration.

To date, there have been several reports on the biocatalytic Prelog stereoselective reduction of 4′-methoxyacetophenone (MOAP) to (*S*)-MOPE with plant cell cultures,[9,10] microbial cells[11–14] and ketoreductases[15] as the biocatalysts. However, the yields of (*S*)-MOPE were disappointingly low (≤62.3%) and most of the biocatalysts afforded relatively low product e.e. (≤77%) in aqueous systems, even under a relatively low substrate concentration (<10 mM), possibly resulting from the inhibition of the reaction by the substrate or product, or their toxicity to the cells. Recently, we have reported markedly improved Prelog stereoselective reduction of MOAP to (*S*)-MOPE catalyzed by

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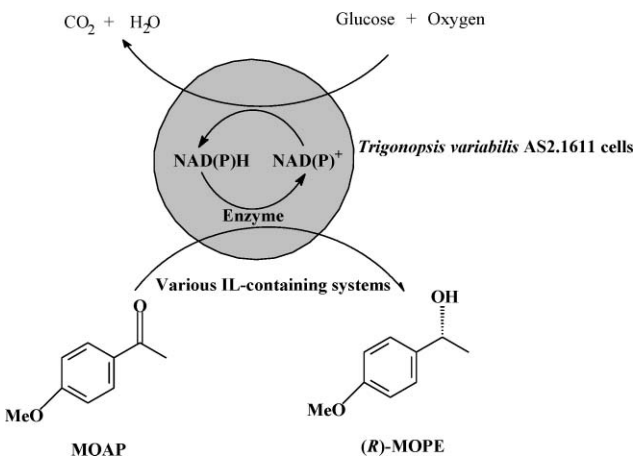
Rhodotorula sp. AS2.2241 cells in a biphasic system consisting of an aqueous phase and a water-immiscible ionic liquid (yield: 95.5%, product e.e. >99%).[16] However, to our knowledge, the biocatalytic anti-Prelog stereoselective reduction of MOAP to (*R*)-MOPE using microbial cells has so far remained largely unexplored, with only few published accounts[12,17,18] where the bioreduction of MOAP to (*R*)-MOPE was conducted in aqueous buffer systems with very low substrate concentration (2–3 mM) and a low yield of (*R*)-MOPE (< 40%), although the e.e. of the (*R*)-MOPE product was high (>99%).

Dialkylimidazolium-based ILs are similar in structure to cationic surfactants, which may therefore be able to make the microbial cell membrane more permeable and change the intracellular concentration of substrate and product.[19–21] Therefore, the addition of environmentally compatible ILs instead of cationic surfactants into whole-cell reaction systems may lower product concentration within microbial cells and reduce the inhibition and toxic effects of the product, thus possibly enhancing the reaction efficiency of the bioreduction. Furthermore, in some cases, ILs can act as enzyme activators.[22–24]

Here we have examined the effect of several hydrophilic dialkylimidazolium-based ILs, in particular 1-(2′-hydroxyl)ethyl-3-methylimidazolium nitrate (C_2OHMIM·NO_3), on anti-Prelog stereoselective reduction of MOAP for the synthesis of (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells (Scheme 1), which are capable of effectively catalyzing anti-Prelog stereoselective reduction of a number of carbonyl compounds.[25–28] In this scheme, MOAP is reduced to enantiopure (*R*)-MOPE while converting NAD(P)H to NAD(P)$^+$, and glucose is simultaneously oxidized to CO_2, presumably driving the reduction reaction by regenerating NAD(P)H from NAD(P)$^+$.



Scheme 1 The biocatalytic anti-Prelog stereoselective reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells in IL-containing co-solvent systems.

Results and discussion

Effect of various ILs on the anti-Prelog stereoselective reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells

In order to take advantage of the environmental benefits of ILs[29–32] for the biocatalytic anti-Prelog stereoselective reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells and to explore the effects of changes in IL structure that are known to influence biotransformation in related systems,[33–35] we conducted this reaction in various dialkylimidazolium-based ILs containing co-solvent systems to determine the influence of the cations and anions of the ILs on the bioreduction.

Table 1 The biocatalytic stereoselective reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells in various media[a]

Entry	Medium	V_0/μmol h^{-1}	Yield[b] (%)	e.e.[c] (%)
1	Aqueous buffer	4.8	80.0	71
2	C_2OHMIM·NO_3-buffer	6.3	95.0	>99
3	C_2OHMIM·PF_6-buffer	5.3	30.5	60
4	C_2OHMIM·BF_4-buffer	3.2	4.5	5
5	C_2OHMIM·Cl-buffer	4.1	41.4	73
6	C_2OHMIM·TfO-buffer	5.0	34.6	60
7	C_2MIM·NO_3-buffer	4.6	71.5	87
8	C_4MIM·NO_3-buffer	4.2	39.2	84
9	C_4MMIM·NO_3-buffer	3.9	34.3	70
10	C_2MIM·BF_4-buffer	4.1	8.6	22
11	C_3MIM·BF_4-buffer	3.4	7.7	15
12	C_4MIM·BF_4-buffer	3.1	4.5	11
13	C_5MIM·BF_4-buffer	2.3	2.3	8
14	C_4MIM·Cl-buffer	6.0	18.9	33
15	C_4MIM·Br-buffer	5.3	24.6	12
16	C_5MIM·Br-buffer	4.9	17.1	18
17	C_6MIM·Br-buffer	4.4	9.8	25
18	C_7MIM·Br-buffer	2.6	6.8	28

[a] Reaction conditions: 10 mM MOAP, 2.5 ml of Tris-HCl buffer (50 mM, pH 8.5) containing 5.0% (v/v) ILs, 20% (w/v) glucose, 0.40 g ml^{-1} immobilized *Trigonopsis variabilis* AS2.1611 cells, 30 °C, 200 r min^{-1}. [b] The maximum yield. [c] The product e.e.

As evident from the data summarized in Table 1, the biocatalytic reduction of MOAP varied greatly with the use of different ILs, with respect to initial reaction rate, yield and product e.e. The addition of the five BF_4^--based ILs (C_2OHMIM·BF_4, C_2MIM·BF_4, C_3MIM·BF_4, C_4MIM·BF_4, C_5MIM·BF_4) into aqueous buffer system led to a clear drop in initial reaction rate, maximum yield and product e.e. (Table 1, entries 4, 10–13). In the case of the C_2OHMIM$^+$-based ILs (C_2OHMIM·NO_3, C_2OHMIM·PF_6, C_2OHMIM·BF_4, C_2OHMIM·Cl, C_2OHMIM·TfO), the anions greatly influenced the initial reaction rate, the product e.e. and the maximum yield of the bioreduction (Table 1, entries 2–6). For NO_3^- and Cl^--based ILs, when the cation in ILs changed from C_4MIM$^+$ to C_2OHMIM$^+$ (Table 1, entries 2,5,7,14), both the yield and the product e.e. were markedly enhanced. For C_nMIM·NO_3 (n = 2, 4) and C_nMIM·Br (n = 4–7), the initial reaction rate and the maximum yield clearly decreased with the elongation of the alkyl chain attached to the cation (*i.e.* increasing n value) (Table 1, entries 7–8, 15–18). Of the 17 ILs that were tested, the IL C_2OHMIM·NO_3 gave the fastest initial reaction rate, the highest yield and the highest product e.e., and consequently was considered as the most suitable IL for the bioreduction reaction.

The difference in the effects that the various ILs had on the biocatalytic reduction of MOAP to (*R*)-MOPE may be attributable to one or more of the following reasons: (1) the various ILs have different effects on the viability of *Trigonopsis variabilis*

AS2.1611 cells; (2) the various ILs influence the cell membrane permeability of *Trigonopsis variabilis* AS2.1611 differently; (3) ILs might enter into *Trigonopsis variabilis* AS2.1611 and then interact with the enzymes responsible for the bioreduction in the cells, affecting the catalytic activity and enantioselectivity of the cellular enzymes differently.

Viability of *Trigonopsis variabilis* AS2.1611 cells in various IL-containing co-solvent systems

A number of reports[16,34,36–40] indicate that the biocompatibility of an IL must be established before it can be exploited as a reaction medium for a whole-cell biocatalytic process. Indeed, most ketone substrates and/or their alcohol reduction products display pronounced toxicity to cells. Therefore, cell viability of *Trigonopsis variabilis* AS2.1611 was measured before and after 24 h exposure to co-solvent systems consisting of buffer and 5.0% (v/v) of various ILs, in the absence and presence of substrate. As can be seen in Fig. 1, in the absence of substrate, the cell viability was lower in all the tested IL-containing systems than in aqueous buffer, indicating that the ILs were toxic to the cells to some extent. Among all the tested ILs, the IL $C_2OHMIM{\cdot}NO_3$ exhibited the highest biocompatibility with the cells as shown by the highest cell viability (90%) observed in the $C_2OHMIM{\cdot}NO_3$-based co-solvent system. Additionally, in the presence of 10 mM MOAP, the cell viability after reaction for 24 h decreased clearly in all the reaction systems as compared with that in the absence of substrate, suggesting that the substrate and product had substantial toxicity to the cells.

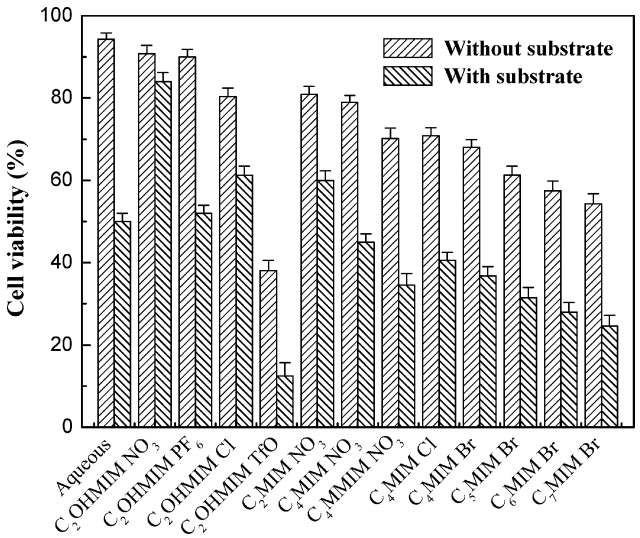


Fig. 1 Cell viability of *Trigonopsis variabilis* AS2.1611 after 24 h exposure to co-solvent systems comprising 5.0% (v/v) of various ILs and Tris-HCl buffer (50 mM, pH 8.5), with and without substrate (10 mM MOAP).

The cell viability in various IL-containing co-solvent systems varied greatly with the type of ILs. In the absence of substrate, the cells lost about 17% and 100% of their catalytic activity after 1 h and 24 h exposure, respectively, to the co-solvent systems involving BF_4^--based ILs ($C_2OHMIM{\cdot}BF_4$, $C_2MIM{\cdot}BF_4$, $C_3MIM{\cdot}BF_4$, $C_4MIM{\cdot}BF_4$, $C_5MIM{\cdot}BF_4$). This suggested that these BF_4^--based ILs had very low biocompatibility with, and substantial toxicity to, the cells. In addition, the cells lost their catalytic activity quickly in the BF_4^--based IL systems in the presence of substrate, which could explain the poor biotransformation results observed in these reaction media (Table 1). In the case of C_2OHMIM^+-based ILs ($C_2OHMIM{\cdot}NO_3$, $C_2OHMIM{\cdot}PF_6$, $C_2OHMIM{\cdot}Cl$, $C_2OHMIM{\cdot}TfO$), the cell viability in the presence of substrate varied greatly with the difference of anion types in the ILs (Table 1), suggesting that the anion types in ILs affect the reaction significantly. For Cl^- and NO_3^--based ILs, when the cation of IL changed from C_4MIM^+ to C_2OHMIM^+, the cell viability in the presence of substrate was markedly increased, which was in good accordance with the observation that the maximum yield and product e.e. were enhanced in a co-solvent system containing $C_2OHMIM{\cdot}Cl$ or especially $C_2OHMIM{\cdot}NO_3$, compared to $C_4MIM{\cdot}Cl$ or $C_4MIM{\cdot}NO_3$ (Table 1). Also, the cell viability in the co-solvent systems with substrate clearly declined with the elongation of the alkyl chain attached to the cation in $C_nMIM{\cdot}NO_3$ ($n = 2$, 4) or $C_nMIM{\cdot}Br$ ($n = 4$–7), which correlates with the observed decrease in initial reaction rate and maximum yield with increasing length of the alkyl chain in the IL cation. Of all the reaction systems tested (including the aqueous buffer system used as the control), the highest cell viability (83%) in the presence of substrate was observed in the $C_2OHMIM{\cdot}NO_3$-based co-solvent system. This coincides with the fastest initial reaction rate, the highest yield and product e.e., which were also achieved in the $C_2OHMIM{\cdot}NO_3$-based system (Table 1). Furthermore, the fall in cell viability caused by adding the substrate was only 7% in the $C_2OHMIM{\cdot}NO_3$-containing co-solvent system, which was much less than the fall in viability (around 45%) when the substrate is added to the aqueous control system.

Effect of various ILs on cell membrane permeability of *Trigonopsis variabilis* AS2.1611 cells

It is possible that ILs permeabilise the cell membrane by interacting with the hydrophobic tail regions of the membrane phospholipids. We further propose that membrane permeability changes might also be possible because of ionic interactions between the ILs and the charged headgroups of the lipid moieties. Either or both of these mechanisms may enable the dialkylimidazolium-based ILs used in this work to increase the permeability of the cell membrane,[19–21] and might thus allow the substrate and product to pass more quickly in and out of the cells, and hence accelerate the bioreduction reaction. On the other hand, the increase in the cell membrane permeability caused by the ILs could have a negative effect on the bioreduction because the damage to the membrane may lower cell viability and the availability of reducing equivalents for the reaction.

An increase in medium OD_{260} and OD_{280} values after removal of the cells was used to show the release of intracellular components (we presume primarily nucleic acids and proteins) into the medium during 24 h incubation with each IL (at 5% v/v). These increases in OD can therefore be taken as a measure of the effect of the IL on the permeability of the cell membrane.[41] As can be seen from the data listed in Table 2, addition of various ILs in aqueous buffer appeared to increase the cell membrane permeability of *Trigonopsis variabilis* AS2.1611 cells, because

Table 2 Effect of various ILs on the cell membrane permeability of *Trigonopsis variabilis* AS2.1611 cells

Medium[a]	Net OD_{260}[b]	Net OD_{280}[b]
Aqueous buffer	0.019	0.030
C_2OHMIM·NO_3-buffer	0.052	0.070
C_2OHMIM·PF_6-buffer	0.090	0.128
C_2OHMIM·BF_4-buffer	0.119	0.269
C_2OHMIM·Cl-buffer	0.102	0.178
C_2OHMIM·TfO-buffer	0.161	0.384
C_2MIM·NO_3-buffer	0.088	0.129
C_4MIM·NO_3-buffer	0.093	0.168
C_4MMIM·NO_3-buffer	0.079	0.158
C_2MIM·BF_4-buffer	0.083	0.148
C_3MIM·BF_4-buffer	0.089	0.178
C_4MIM·BF_4-buffer	0.146	0.204
C_5MIM·BF_4-buffer	0.186	0.265
C_4MIM·Cl-buffer	0.061	0.115
C_4MIM·Br-buffer	0.072	0.140
C_5MIM·Br-buffer	0.079	0.172
C_6MIM·Br-buffer	0.091	0.190
C_7MIM·Br-buffer	0.100	0.222

[a] The IL content in various co-solvent media is 5.0% (v/v). [b] OD_{260} and OD_{280} values given here show the increase in OD_{260} and OD_{280} of the medium from 0 h to 24 h, and thus indicate the cellular contents released during that period, which were taken as a measure of cell membrane permeability.

the OD_{260} and OD_{280} values were clearly higher in IL-containing systems than in the aqueous control system. The BF_4^--based ILs gave relatively high OD_{260} and OD_{280} values, implying that these ILs greatly increase the cell membrane permeability and thus effectively reduce the product inhibition. However, in the co-solvent systems involving these BF_4^--based ILs, the immobilized *Trigonopsis variabilis* AS2.1611 cells displayed a very low viability (Fig. 1) and a very poor catalytic efficiency in the biocatalytic reduction of MOAP to (*R*)-MOPE (Table 1). One possible explanation of these data is that the BF_4^--based ILs damage the cell membrane too seriously for the cells to maintain their catalytic activity. Similarly, the cell membrane permeability was increased with the elongation of the alkyl chain attached to the cation in C_nMIM·NO_3 ($n = 2, 4$) or C_nMIM·Br ($n = 4$–7), while the cell viability in the presence of substrate was clearly reduced (Fig. 1) together with the catalytic efficiency of the cells (Table 1). Of all the ILs used, the lowest OD_{260} and OD_{280} values were observed in the C_2OHMIM·NO_3-containing system with the best biotransformation results and the highest cell viability, strongly suggesting that substantial increase in membrane permeability has a net negative effect on the reaction, although stimulation of the reaction by moderate membrane permeabilisation remains a possibility.

Effect of key variables on the biocatalytic reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells

We investigated the effect of the amount of IL in the co-solvent system containing C_2OHMIM·NO_3, which gave the best performance in the above reduction experiments, since our previous studies have demonstrated that the amount of IL in co-solvent systems markedly affects the activity, enantioselectivity and stability of enzymes and microbial cells.[39,42] As shown in Fig. 2, an increase in C_2OHMIM·NO_3 content up to 2.5% (v/v)

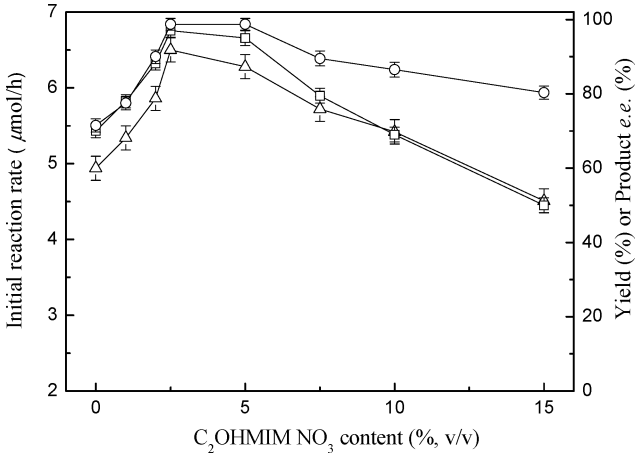


Fig. 2 Effect of C_2OHMIM·NO_3 content on the biocatalytic reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells. Reaction conditions: 10 mM MOAP, 2.5 ml of Tris-HCl buffer (50 mM, pH 8.5) with various C_2OHMIM·NO_3 contents, 20% (w/v) glucose, 0.40 g ml^{-1} immobilized cells, 30 °C, 200 r min^{-1}. Symbols: (○) product e.e.; (□) maximum yield; (△) initial reaction rate.

brought about a clear increase in the initial reaction rate, the product e.e. and the yield, while further increase in IL content up to 5.0% (v/v) led to a slight decline in the yield and the initial reaction rate. The product e.e. was consistently above 99% over a C_2OHMIM·NO_3 concentration range from 2.5% (v/v) to 5.0% (v/v). When the C_2OHMIM·NO_3 content in the co-solvent system exceeded 5.0% (v/v), the initial reaction rate, the product e.e. and the yield decreased rapidly with increasing IL content. Compared to the reaction conducted in neat aqueous buffer system, the reaction was accelerated by addition of small amounts (≤10%, v/v) of C_2OHMIM·NO_3, but became significantly slower when the C_2OHMIM·NO_3 content was above 12.5% (v/v). Higher C_2OHMIM·NO_3 content (15.0%, v/v) resulted in poor initial reaction rate, product e.e. and yield. This is possibly because a high concentration of IL caused not only a high ionic strength of the reaction medium that might inactivate the cells or the enzymes present in the cells, but also a high viscosity of the reaction mixture, which can limit the diffusion of substrates and products in and out of the immobilized cells.

The initial reaction rate clearly increased with increasing substrate concentration up to 15 mM, and the product e.e. and the maximum yield showed no significant variation, but further increase in substrate concentration led to a decrease in the initial reaction rate, the yield and the product e.e. (Fig. 3). Obviously, the optimum substrate concentration for the bioreduction conducted in the C_2OHMIM·NO_3-buffer co-solvent system was shown to be 15 mM, which is much higher than that in the neat aqueous system (5 mM).

In other reaction systems, it is well known that buffer pH plays a crucial role in a biocatalytic reaction, where it influences not only the selectivity, activity and stability of the enzymes involved in the reaction, but also the regeneration of the coenzyme present in the microbial cells, which in turn can affect the reaction rate. Fig. 4 illustrates the significant effect of buffer pH of the C_2OHMIM·NO_3-buffer co-solvent system on the bioreduction

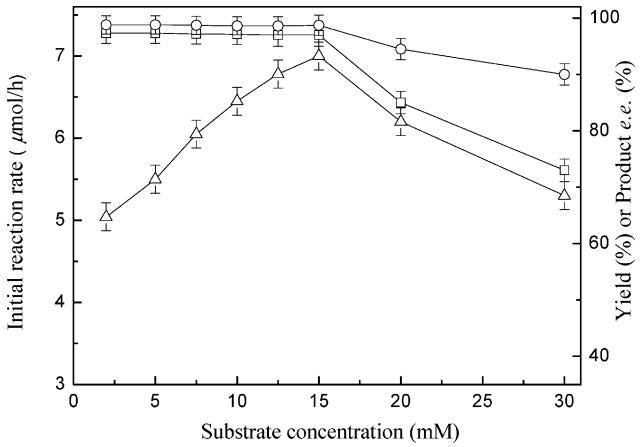


Fig. 3 Effect of substrate concentration on the biocatalytic reduction of MOAP with immobilized *Trigonopsis variabilis* AS2.1611 cells in the $C_2OHMIM{\cdot}NO_3$-buffer co-solvent system. Reaction conditions: various MOAP concentration, 2.5 ml of Tris-HCl buffer (50 mM, pH 8.5) containing 2.5% (v/v) $C_2OHMIM{\cdot}NO_3$, 20% (w/v) glucose, 0.40 g ml^{-1} immobilized cells, 30 °C, 200 r min^{-1}. Symbols: (○) product e.e.; (□) maximum yield; (△) initial reaction rate.

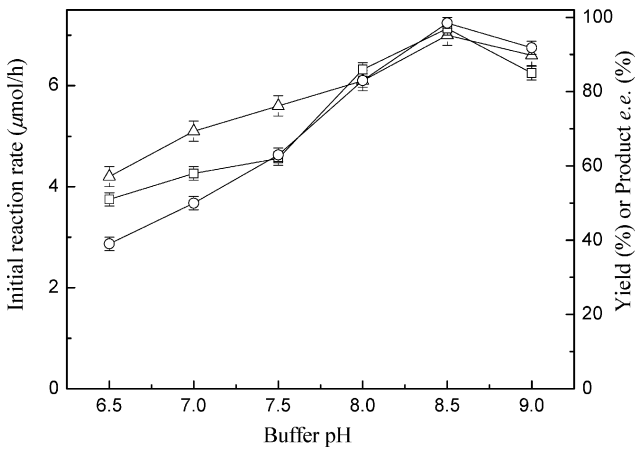


Fig. 4 Effect of buffer pH on the biocatalytic reduction of MOAP with immobilized *Trigonopsis variabilis* AS2.1611 cells in the $C_2OHMIM{\cdot}NO_3$-buffer co-solvent system. Reaction conditions: 15 mM MOAP, 2.5 ml of Tris-HCl buffer (50 mM, various pHs) containing 2.5% (v/v) $C_2OHMIM{\cdot}NO_3$, 20% (w/v) glucose, 0.40 g ml^{-1} immobilized cells, 30 °C, 200 r min^{-1}. Symbols: (○) product e.e.; (□) maximum yield; (△) initial reaction rate.

of MOAP with immobilized *Trigonopsis variabilis* AS2.1611. The reaction accelerated with increasing buffer pH from pH 6.0 to 8.5, and both the yield and the product e.e. increased markedly. The cells lost their activity dramatically above the optimal pH of 8.5, as shown by the poor initial reaction rate, product e.e. and maximum yield.

As can be seen in Fig. 5, the bioreduction proceeded rapidly with increasing reaction temperature up to 35 °C, and a further rise in temperature led to a sharp drop in the initial reaction rate, which could be explained by the partial inactivation of the cells. The maximum yield and the product e.e. clearly decreased when the temperature was above the optimum temperature of 30 °C, possibly resulting from the inactivation of the cells after being incubated for a long time at a higher temperature.

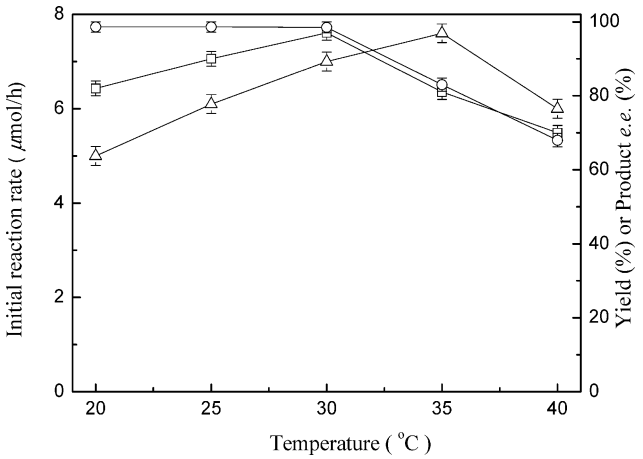


Fig. 5 Effect of reaction temperature on the biocatalytic reduction of MOAP with immobilized *Trigonopsis variabilis* AS2.1611 cells in the $C_2OHMIM{\cdot}NO_3$-buffer co-solvent system. Reaction conditions: 15 mM MOAP, 2.5 ml of Tris-HCl buffer (50 mM, pH 8.5) containing 2.5% (v/v) $C_2OHMIM{\cdot}NO_3$, 20% (w/v) glucose, 0.40 g ml^{-1} immobilized cells, various temperatures, 200 r min^{-1}. Symbols: (○) product e.e.; (□) maximum yield; (△) initial reaction rate.

Owing to its high viscosity, addition of an IL into a reaction system can limit the mass transfer of the substrate and the product to and from the microbial cells. Therefore, the effect of shaking rate on the bioreduction reaction was investigated using the $C_2OHMIM{\cdot}NO_3$-containing co-solvent system. It was found that the reaction speeded up with increasing shaking rate, indicating that the mass transfer was the rate-limiting step. The optimal shaking rate was found to be 200 r min^{-1}, above which little change in the initial rate and the maximum product yield were observed with a further rise in shaking rate. Throughout these experiments, the product e.e. was above 99%.

Under the optimum conditions described above, the efficiency of the biocatalytic anti-Prelog stereoselective reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells was substantially enhanced in the $C_2OHMIM{\cdot}NO_3$-based co-solvent system compared to the neat aqueous buffer system in terms of optimal substrate concentration (15.0 mmol L^{-1} *vs.* 5.0 mmol L^{-1}), initial reaction rate (7.1 μmol h^{-1} *vs.* 4.7 μmol h^{-1}), maximum yield (97.2% *vs.* 86.4%) and product e.e. (99.6% *vs.* 87.0%).

In order better to understand the effect of the various influential parameters on the bioreduction reaction, their effect on the viability of the cells was investigated. These experiments were done in the $C_2OHMIM{\cdot}NO_3$ (2.5%, v/v)-based co-solvent system and in the aqueous system without IL. The cell viability of *Trigonopsis variabilis* AS2.1611 was determined after 24 h exposure to both media at different buffer pHs, temperatures and substrate concentrations. As can be seen in Fig. 6a, the buffer pH inactivation profile of the cells in the $C_2OHMIM{\cdot}NO_3$-based co-solvent system was similar to that observed in the aqueous system without IL. The cells were more stable in relatively alkaline environments than in acidic ones, as indicated by the remarkably higher cell viability with buffer pH of above 7.0. When buffer pH was 8.5, which has been shown to be the optimal pH for the bioreduction, the cells had the highest viability in either the $C_2OHMIM{\cdot}NO_3$-based co-solvent system or the

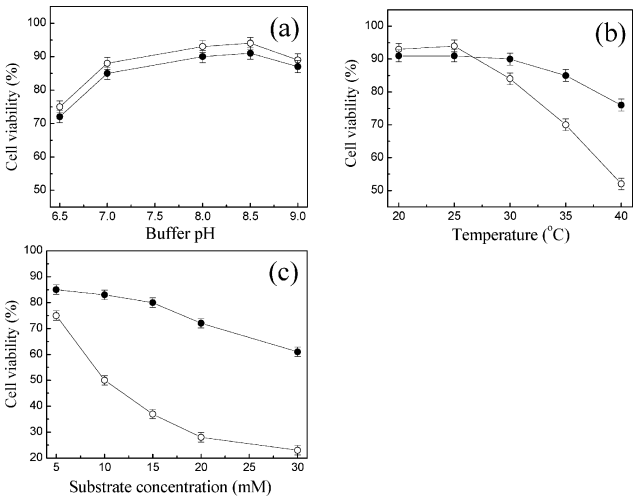


Fig. 6 Comparison of effect of several crucial variables on cell viability of *Trigonopsis variabilis* AS2.1611 after 24 h exposure to the aqueous buffer without IL (○) and the $C_2OHMIM{\cdot}NO_3$ (2.5%, v/v)-based co-solvent system (●). (A): effect of buffer pH; (b): effect of temperature; (c): effect of substrate concentration.

aqueous buffer without IL. In comparison with the aqueous buffer without IL, the $C_2OHMIM{\cdot}NO_3$-based co-solvent system gave slightly diminished cell viability within the tested range of buffer pH, indicating a small amount of toxicity of the IL to the cells (Fig. 1). Fig. 6b illustrates the significant effect of various temperatures on the cell viability. The cell viability clearly decreased in the non-IL aqueous buffer when temperature was above 25 °C, but started to reduce in the $C_2OHMIM{\cdot}NO_3$-containing buffer when temperature was above 30 °C. The culture retained only about 50% of its viability after being incubated in the non-IL aqueous buffer for 24 h, but still retained around 80% of viability in the presence of $C_2OHMIM{\cdot}NO_3$, showing that the IL could increase cell resistance to high temperature. As shown in Fig. 6c, a less clear drop in cell viability with increasing substrate concentration was observed in the $C_2OHMIM{\cdot}NO_3$-containing buffer, in comparison to the non-IL aqueous buffer. When substrate concentration was 30 mM, the cells had about 21% of viability in the non-IL aqueous buffer and 64% of viability in the $C_2OHMIM{\cdot}NO_3$-containing buffer, respectively, indicating that the relatively high concentration of substrate could cause pronounced inactivation of the cells in aqueous buffer without IL. However, the presence of $C_2OHMIM{\cdot}NO_3$ in aqueous buffer could allow the cells to tolerate a relatively higher concentration of substrate compared with that in aqueous buffer alone.

Preparative scale biocatalytic reduction of MOAP to (*R*)-MOPE in $C_2OHMIM{\cdot}NO_3$-based co-solvent system

To show the applicability of the bioreduction on a larger scale, we also carried out the reaction on a 250 ml preparative scale under the optimal conditions described above. The reaction was monitored by GC analysis and the product was extracted from the reaction mixture with isopropyl ether upon the termination of the reaction. Although slightly lower than that obtained on the 2.5 ml scale, the isolated yield (95.7%) was much higher than that reported previously[12,18] and the product e.e. was very high (>99%). Furthermore, no emulsification of the $C_2OHMIM{\cdot}NO_3$-based co-solvent system was observed, so the product could be separated readily by simple extraction. No by-products accumulated in the co-solvent system, and consequently the $C_2OHMIM{\cdot}NO_3$-based co-solvent system may be easily recycled, reducing the overall cost of the biocatalytic process. Additionally, the immobilized *Trigonopsis variabilis* AS2.1611 cells still maintained above 85.0% of their original catalytic activity after being used repeatedly for 12 batches, showing the good operational stability of the immobilized cells under the optimized reaction conditions. From a practical viewpoint, it is also important to investigate the storage stability of the immobilized cells. The fresh immobilized cells were suspended in an aqueous solution of 20% (w/v) glucose, 0.9% (w/v) NaCl and 0.05% (w/v) $CaCl_2$ for a specified time, followed by the measurement of their remaining activity. After being stored for 7 days, the immobilized cells still retained more than 91.0% of their initial catalytic activity, clearly showing the excellent storage stability under the storage conditions.

Conclusion

The synthesis of (*R*)-MOPE can be successfully conducted with high yield and excellent product e.e. by means of the biocatalytic anti-Prelog stereoselective reduction of MOAP using immobilized *Trigonopsis variabilis* AS2.1611 cells in IL-containing systems. Various ILs exerted significant but different influences on the bioreduction. Among all the tested ILs, $C_2OHMIM{\cdot}NO_3$ can substantially boost the reaction efficiency of the bioreduction, possibly due to the markedly enhanced cell viability and a possible moderate increase in cell membrane permeability by the IL. Also, the presence of the IL in aqueous buffer could allow the cells to tolerate relatively higher temperature and substrate concentration compared with those in aqueous buffer without IL. Furthermore, the results described here clearly show that the whole-cell biocatalytic process in the presence of $C_2OHMIM{\cdot}NO_3$ is feasible up to a 250 ml scale. If further scale-up is possible, the reaction may be attractive for large-scale industrial application.

Experimental

Biological and chemical materials

The yeast strain, *Trigonopsis variabilis* AS2.1611, was obtained from China General Microbiological Culture Collection Center (CGMCC, China).

4′-Methoxyacetophenone (MOAP, >97% purity) and *n*-nonane (>99% purity) were purchased from Aldrich-Fluka (USA). (*R*)-1-(4-Methoxyphenyl)ethanol {(*R*)-MOPE} and (*S*)-1-(4-methoxyphenyl)ethanol {(*S*)-MOPE) were from Alfa Aesar (China) and were both of over 98% purity.

The eight ionic liquids (ILs), 1-(2′-hydroxyl)ethyl-3-methylimidazolium nitrate ($C_2OHMIM{\cdot}NO_3$), 1-(2′-hydroxyl)ethyl-3-methylimidazolium hexafluorophosphate ($C_2OHMIM{\cdot}PF_6$), 1-(2′-hydroxyl)ethyl-3-methylimidazolium tetrafluoroborate

(C_2OHMIM·BF_4), 1-(2′-hydroxyl)ethyl-3-methylimidazolium chloride (C_2OHMIM·Cl), 1-(2′-hydroxyl)ethyl-3-methylimidazolium trifluoromethanesulfonate (C_2OHMIM·TfO), 1-ethyl-3-methylimidazolium nitrate (C_2MIM·NO_3), 1-butyl-3-methylimidazolium nitrate (C_4MIM·NO_3) and 1-butyl-2, 3-dimethylimidazolium nitrate (C_4MMIM·NO_3), were purchased from Lanzhou Institute of Chemical Physics (China) and were all of over 99% purity. 1-Ethyl-3-methylimidazolium tetrafluoroborate (C_2MIM·BF_4) and 1-butyl-3-methylimidazolium tetrafluoroborate (C_4MIM·BF_4), were from Aldrich-Fluka (USA) and were both of over 97% purity. The other seven ILs, 1-propyl-3-methylimidazolium tetrafluoroborate (C_3MIM·BF_4), 1-pentyl-3-methylimidazolium tetrafluoroborate (C_5MIM·BF_4), 1-butyl-3-methylimidazolium chloride (C_4MIM·Cl), 1-butyl-3-methylimidazolium bromide (C_4MIM·Br), 1-pentyl-3-methylimidazolium bromide (C_5MIM·Br), 1-hexyl-3-methylimidazolium bromide (C_6MIM·Br) and 1-heptyl-3-methylimidazolium bromide (C_7MIM·Br) were kindly donated by Dr Xue-Hui Li (Department of Chemical Engineering, South China University of Technology, China) and were all of above 96% purity.

All other chemicals were obtained from commercial sources and were of analytical grade.

Cultivation of *Trigonopsis variabilis* AS2.1611 cells

The yeast strain, *Trigonopsis variabilis* AS2.1611, was cultivated in medium containing 2.0% (w/v) glucose, 1.0% (w/v) yeast extract, 2.0% (w/v) peptone, 0.3% (w/v) $NaNO_3$, 0.05% (w/v) K_2HPO_4, 0.05% (w/v) NaCl and 0.05% (w/v) $MgSO_4·7H_2O$. The medium was autoclaved at 121 °C for 20 min. A preculture was prepared by inoculation of 100 ml of the complex medium with fresh cells from an agar plate culture. Incubation was carried out in a 300 ml Erlenmeyer shaking-flask at 150 rpm and 30 °C. After 20 h incubation *Trigonopsis variabilis* AS2.1611 cells in the exponential growth phase were collected by centrifugation (1150 × *g*, 15 min), washed twice with distilled water, and separated from the aqueous medium by centrifugation to give a cell wet mass (cwm) of around 5–6 g per 300 ml batch. The ratio 'cell wet mass'/'cell dry mass' of about 4.0 was determined by lyophilization of samples of wet cells. The wet cells were immobilized on calcium alginate as described below.

Immobilization of *Trigonopsis variabilis* AS2.1611 cells

A homogenous cell/sodium alginate suspension was prepared at 25 °C by adding 10 g of a suspension containing fresh cells (5 g wet cells in 5 g water) into 30 ml of a homogeneous aqueous sodium alginate solution (2%, w/v), which was prepared by dissolving sodium alginate in deionized water, heating and stirring vigorously. The suspension was added dropwise by an injector into a gently stirred $CaCl_2$ solution (2%, w/v), where the calcium alginate pearls with a load of 15.6% (w/w) of *Trigonopsis variabilis* AS2.1611 cells (based on cwm) precipitated. The pinhole size of injector and the drop rate were adjusted in such a way that the diameter of the pearls was about 1 mm. The pearls were kept in the $CaCl_2$ solution (2%, w/v) for another 1 h at 25 °C and collected by filtration, washed with water and re-suspended in an aqueous solution of 20% (w/v) glucose, 0.9% (w/v) NaCl and 0.05% (w/v) $CaCl_2$ at 4 °C for 24 h. They were stored at 4 °C for subsequent use.

General procedure for the asymmetric reduction of MOAP to (*R*)-MOPE with immobilized *Trigonopsis variabilis* AS2.1611 cells in IL-containing systems

In a typical experiment, 2.5 ml of different IL-containing co-solvent systems or aqueous Tris-HCl buffer (50 mM) monophasic system containing a predetermined amount of MOAP, 20% (w/v) glucose and 0.05% (w/v) $CaCl_2$ was pre-incubated in a 10 ml Erlenmeyer flask capped with a septum for 30 min at the appropriate temperature and shaking rate, and the bioreduction was initiated by adding 0.40 g ml^{-1} immobilized *Trigonopsis variabilis* AS2.1611 cells to the reaction system. Aliquots (10 μl) were withdrawn at specified time intervals from the co-solvent system, and then the product and the residual substrate were extracted with isopropyl ether (30 μl) containing 5.6 mM *n*-nonane (as an internal standard) prior to GC analysis. Details about IL content, substrate concentrate, buffer pH, reaction temperature and shaking rate are specified in each case.

The preparative scale biocatalytic reduction of MOAP to (*R*)-MOPE was carried out by adding 40 g of immobilized *Trigonopsis variabilis* AS2.1611 cells and 3.75 mmol of MOAP to 250 ml of the co-solvent system of 2.5% (v/v) C_2OHMIM·NO_3 and Tris-HCl buffer (50 mM, pH 8.5) containing 20% (w/v) glucose at 30 °C and 200 r min^{-1}. The reaction was terminated when no substrate was detectable by GC analysis. The immobilized cells were removed by filtration, and the products were extracted from the reaction mixture with isopropyl ether. The product e.e. and the isolated yield were determined by GC analysis.

Cell viability assay

The viability of *Trigonopsis variabilis* AS2.1611 cells was assayed after cells were incubated for 24 h in various IL (5%, v/v)–Tris-HCl buffer (50 mM, pH 8.5) co-solvent systems containing 20% (w/v) glucose, or in aqueous Tris-HCl buffer (50 mM, pH 8.5) system containing 20% (w/v) glucose in the absence of substrate and in the presence of substrate (10 mM MOAP), respectively. The beads of immobilized *Trigonopsis variabilis* AS2.1611 cells were withdrawn from the reaction systems and then added to 0.1 M trisodium citrate to dissolve the beads. The microbial cell suspension was diluted and dyed with 0.1% Methylene Blue for 5 min. Micrographs were taken and analyzed for blue dead cells and colourless viable ones.

Cell membrane permeability assay

In a typical experiment, 20 ml of different IL-containing co-solvent mixtures or aqueous Tris-HCl buffer (50 mM) containing 0.32 g ml^{-1} immobilized *Trigonopsis variabilis* AS2.1611 cells were incubated in a 100 ml Erlenmeyer flask capped with a septum at 30 °C and 180 r min^{-1}. The cell-free supernatants (2 ml) containing ILs and released intracellular components were withdrawn from the co-solvent system at 0 h (controls with ILs) and 24 h and diluted for measurement of OD. The OD_{260} and OD_{280} of the samples, were determined by using an ultraviolet spectrophotometer (Hitachi UV-3010). The OD_{260} and OD_{280} values at 24 h were corrected for the absorbance of the ILs

by subtracting the corresponding 0 h absorbance values, hence these corrected values for the 24 h samples are a measure of the release of intracellular components (primarily nucleic acids and proteins) into the medium during the incubation with the ILs.

GC analysis

The reaction mixtures were assayed by a Shimadzu GC 2010 model with a flame ionization detector and a chiral column (20% permethylated β-cyclodextrin 30 m × 0.25 mm × 0.25 μm) from Hewlett Packard (USA). The split ratio was 100 : 1. The injector and the detector were both kept at 250 °C. The carrier gas was nitrogen at 3.0 mL min^{-1}. The column temperature was held at 140 °C for 10 min and was raised to 145 °C at a rate of 1 °C min^{-1}, and then kept constant for 4 min. The retention times for *n*-nonane, MOAP, (*R*)-MOPE and (*S*)-MOPE were 2.00, 17.00, 17.70 and 18.20 min, respectively. The initial reaction rate, the maximum yield and the product e.e. were all calculated according to the GC data. All reported data were averages of experiments conducted at least in duplicate and the average error for the results was less than 1.0%.

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