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ARTICLE

Using deep eutectic solvents to improve resolution of racemic 1-(4-methoxyphenyl)ethanol through *Acetobacter* sp. CCTCC M209061 cells-mediated asymmetric oxidation

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As novel low-viscosity and environmentally-friendly reaction media, deep eutectic solvents (DESs) have gained much attraction in biocatalysis. In this study, various DESs were prepared and their effects on the asymmetric oxidation of racemic 1-(4-methoxyphenyl)ethanol (MOPE) catalyzed by *Acetobacter* sp. CCTCC M209061 cells to give enantiopure (*S*)-MOPE were examined. The best results were obtained with the DES [ChCl][Gly] and its concentration exerted a significant influence on the reaction, with the optimal content being 10% (v/v). In [ChCl][Gly]-containing system, the substrate concentration was substantially increased(55 mmol/L *vs* 30 mmol/L) as compared with the [ChCl][Gly]-free aqueous system, while the residual substrate *e.e.* was kept as high as 99.9%. The good biocompatibility of [ChCl][Gly] with the cells and the improved cell membrane permeability in [ChCl][Gly]-containing system could partly account for the clearly enhanced reaction efficiency.

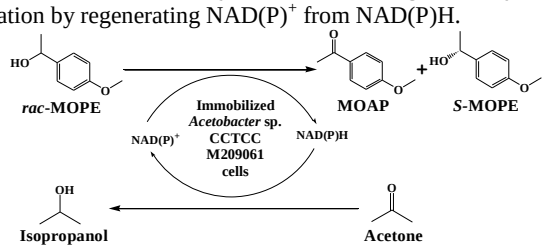
Introduction

Chiral alcohols are important building blocks for the synthesis of chiral pharmaceuticals, agrochemicals, flavors, fragrances and functional materials[1,2]. For instance, enantiopure (*S*)-1-(4-methoxyphenyl)ethanol {(*S*)-MOPE} is a key chiral building block for the synthesis of cycloalkyl [*b*] indoles used to treat general allergic responses[3, 4]. (*S*)-MOPE can be prepared through asymmetric resolution of racemic MOPE either chemically or biologically. For economic, environmental and social reasons, biological approaches have become a subject of great interest. Recently, whole-cells based biocatalyst has attracted much attention due to the unique advantages such as outstanding enantioselectivity, environmental friendliness and regeneration of cofactors *in situ*[5, 6]. In addition, immobilized cells are recyclable and reusable, thus their use greatly simplifies the process and lowers the cost of production.

To date, there have been a few published accounts on the resolution of racemic MOPE via biocatalytic routes[7,8]. For example, feruloyl esterase was used for the asymmetric transesterification of racemic MOPE with vinyl acetate and the relatively low product *e.e* (88%) and conversion of 18% were recorded. In addition, lipase was found to give high yield (99%) and product *e.e.*(99.9%) in the dynamic kinetic resolution of racemic MOPE, but with a poor substrate concentration (20 mmol/L) and a long reaction time (10 h). In our previous study, the asymmetric oxidation of MOPE catalyzed by immobilized *Acetobacter* sp. CCTCC M209061 cells was conducted successfully in an aqueous monophasic system with the *e.e.* of residual substrate (*S*)-MOPE being more than 98.0%. However, the substrate concentration was only 30 mmol/L, possibly due to the pronounced inhibitory and toxic effects of the substrate and the product at higher substrate loading.

On the other hand, deep eutectic solvents (DESs) have emerged as a good alternative to conventional ones with tailored properties, depressed toxicological traits, adequate biodegradability and acceptable cost, and a large number of promising application have already appeared in current literature[9-13]. Some DESs have been used as benign solvents for biocatalytic processes[14-17]. To our knowledge, only one work on whole-cell biocatalysis in DESs has been published so far, wherein enantioselective reduction of ketone was observed with baker's yeast as biocatalyst[18].

Herein, we for the first time report the use of various DESs as co-solvents to improve the performance of immobilized *Acetobacter* sp. CCTCC M209061 cells for asymmetric oxidation of MOPE, and the effects of these DESs on the asymmetric oxidation reactions with MOPE as an initial model substrate (Scheme 1). In the scheme, MOPE is oxidized to 4'-methoxyacetophenone(MOAP) to give enantiopure (*S*)-MOPE, while $NAD(P)^+$ is converted to NAD(P)H and the co-substrate, acetone, is simultaneously reduced, driving the asymmetric oxidation by regenerating $NAD(P)^+$ from NAD(P)H.



Scheme 1 The asymmetric oxidation of racemic MOPE catalyzed by immobilized *Acetobacter* sp. CCTCC M209061 cells

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Results and discussion

The comparison of the asymmetric oxidation of MOPE using immobilized and free *Acetobacter* sp. CCTCC M209061 cells showed that although immobilized cells afforded nearly the same maximum conversion (49.4% *vs* 49.7%) and residual substrate *e.e.* (99.0% vs 99.4%) as the free cells, the stability of immobilized cells was much better (Fig. S1-4 in ESI). Consequently, immobilized cells were used in this work.

Effects of various DESs on the asymmetric oxidation of racemic MOPE catalyzed by immobilized *Acetobacter* sp. CCTCC M209061 cells

According to the studies on biocatalytic reactions in various DESs-containing systems, the catalytic performance exhibited by a biocatalyst was closely related to the hydrogen bond donor types of the DESs, and the effects of different DESs on biocatalytic reactions have been found to vary widely[9]. Therefore, it is of great significance to explore the effects of DESs with different hydrogen bond donors on the asymmetric oxidation of MOPE catalyzed by immobilized *Acetobacter* sp. CCTCC M209061 cells in various DES-containing systems (Table 1). It was noted that immobilized *Acetobacter* sp. CCTCC M209061 cells were capable of catalyzing the asymmetric oxidation of racemic MOPE in the presence of DESs with the hydrogen bond donors being glycerol, urea, ethylene glycol or acetamide. In general, the initial reaction rate (90.2 μmol/min), the conversion (49.4%) and the residual substrate *e.e*. (98.7%) obtained in [ChCl][Gly]-containing system were higher than those with the control under the same conditions. To better understand the effects of [ChCl][Gly] on the reaction, the oxidation reaction following the addition of [ChCl][Gly], choline chloride or glycerol was investigated. As depicted in Table 2, the initial reaction rate and the conversion in [ChCl][Gly]-containing system were higher than those when choline chloride or glycerol was added individually. The reason for this might be that the hydrogen bonding interactions between glycerol molecules and the chloride ion would increase the nucleophilicity of the substrate, MOPE, thus leading to a faster oxidation reaction rate [19].

As a result, [ChCl][Gly] was chosen as the co-solvent in the DESs-containing system for subsequent experiments.

Effect of several key variables on the asymmetric oxidation of MOPE catalyzed by immobilized *Acetobacter* sp. CCTCC M209061cells

To see the potential of [ChCl][Gly]-containing system for the reaction, several key variables were examined. As evident in Table 3, the initial reaction rate increased with increasing [ChCl][Gly] content up to 10% (v/v), and a further rise in [ChCl][Gly] content led to a clear fall in the initial reaction rate. Additionally, adding [ChCl][Gly] allowed a residual substrate *e.e.* of 99.9% due to higher maximum conversions.

To get deeper insight into the influence of [ChCl][Gly] content, we investigated its effects on buffer pH. Unexpectedly, with [ChCl][Gly] concentration increasing from 0 to 20%, the corresponding buffer pH varied little (from 6.47 to 6.52). Obviously, the effects of [ChCl][Gly] concentration on the reaction could not attributed to the changes in buffer pH.

Table 1 Effect of various DESs on the asymmetric oxidation of racemic MOPE catalyzed by immobilized *Acetobacter* sp. CCTCC M209061cells

Media	Initial reaction rate (μmol/min)	Reaction time (h)	Conversion[a] (%)	*e.e*[b] (%)
Aqueous buffer	80.0	11.0	47.5	91.4
[ChCl][MA]/buffer	29.6	22.5	5.0	53.6
[ChCl][Gly] /buffer	90.2	9.0	49.4	98.7
[ChCl][EG]/buffer	63.8	12.0	42.9	76.2
[ChCl][Acet]/buffer	48.2	13.0	39.8	67.1
[ChCl][U]/buffer	77.7	10.5	47.7	92.3
[ChCl][IM]/buffer	34.3	22.0	6.1	65.1
[Bu_4NBr][IM]/buffer	23.5	23.0	3.2	34.1

ChCl: Choline chloride, MA: Malonic acid, Gly: Glycerol, EG: Ethylene glycol, Acet: Acetamide, U: Urea, IM: Imidazole, Bu_4NBr: Tetrabutylammonium bromide.

Reaction conditions: 5.0 mL TEA-HCl buffer (100 mmol/L, pH 6.5) consisting of 20% (v/v) various DESs, 40 mmol/L MOPE, 0.3 g immobilized cells, 50 mmol/L acetone, 30 ℃, 200 rpm.

[a] The maximum conversion

[b] The residual substrate *e.e.*

Table 2 Effect of [ChCl][Gly] and its components on the asymmetric oxidation of racemic MOPE with immobilized *Acetobacter* sp. CCTCC M209061 cells

[ChCl][Gly] or its components[a]	Initial reaction rate (μmol/min)	Reaction time (h)	Conversion[b] (%)	*e.e.*[c] (%)
Control	101.6	9.0	49.3	98.2
ChCl	99.1	9.0	49.3	98.2
[ChCl][Gly]	125.3	7.0	52.0	99.9
Gly	100.8	8.0	48.6	95.5

Reaction conditions: 5.0 mL TEA-HCl buffer (100 mmol/L, pH 6.5) containing 10% (v/v) [ChCl][Gly] , 55mmol/L MOPE, 0.3 g immobilized cells, 50 mmol/L acetone, 30 ℃, 200 rpm.

[a] ChCl or Gly was added according to the mass fraction in [ChCl][Gly]

[b] The maximum conversion

[c] The residual substrate *e.e.*

A great effect of buffer pH on the reaction in the [ChCl][Gly]-containing system was observed(Fig. S5). When buffer pH increased from 4.0 to 6.5, the reaction sped up (83 μmol/min vs 107.9 μmol/min), and the maximum conversion increased(39.0% vs 50.5%) with the residual substrate *e.e.* rising from 63.9% to 99.9%. Further increases in buffer pH, however, led to a remarkable drop in initial reaction rate, the maximum conversion and the residual substrate *e.e*. Thus, pH 6.5 was regarded as the optimal buffer pH.

Increasing the reaction temperature from 20 ℃ to 30 ℃ caused increased initial reaction rate (79.8 μmol/min *vs* 109.1 μmol/min), maximum conversion (44.4% vs 52.8%) and residual substrate *e.e* (97.6% *vs* 99.9%)(Fig. S6). However, the initial reaction rate (109.1μmol/min *vs* 88.5 μmol/min), the maximum conversion(52.8% *vs* 46.5%) and the residual substrate *e.e*(99.9% *vs* 85.2%) decreased with increase in reaction temperature from 30 ℃ to 45 ℃. So the most suitable reaction temperature was 30 ℃.

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Table 3 Effect of [ChCl][Gly] content on the asymmetric oxidation of racemic MOPE with immobilized Acetobacter sp. CCTCC M209061 cells

[ChCl][Gly] content	Initial reaction rate (μmol/min)	Reaction time (h)	Conversiona (%)	e.e.b (%)
0	89.0	10.0	48.0	93.5
5%	94.1	9.0	50.0	99.9
8%	97.1	8.0	50.5	99.9
10%	102.3	7.0	51.7	99.9
12%	98.0	8.0	50.3	99.9
15%	94.2	8.5	49.8	99.2
20%	90.3	9.0	49.3	98.3

Reaction conditions: 5.0 mL TEA-HCl buffer (100 mmol/L, pH 6.5) containing various concentrations of [ChCl][Gly] , 40 mmol/L MOPE, 0.3g immobilized cells, 50mmol/L acetone, 30 ℃, 200 rpm, incubation time7h.

[a] The maximum conversion

[b] The residual substrate *e.e.*

Raising the concentration of co-substrate acetone from 40.0 mmol/L to 50.0 mmol/L gave rise to increased initial reaction rate (109.1 μmol/min vs 126.5 μmol/min), maximum conversion (47.5% vs 52.0%)(Fig. S7) and residual substrate *e.e.* (97.0% vs 99.9%). However, further rise in acetone concentration from 50.0 mmol/L to 65.0 mmol/L led to declined initial reaction rate(126.5 μmol/min vs 101.2 μmol/min), maximum conversion(52.0% vs 48.9%) and residual substrate *e.e.* (99.9% vs 95.7%). Therefore, the optimal acetone concentration was considered to be 50.0 mmol/L.

The initial reaction rate increased rapidly from 100.5 μmol/min to 126.0 μmol/min when the shaking rate was increased from 160 rpm to 220 rpm, suggesting that mass transfer was the rate-limiting step (Fig. S8). However, a further increase in the shaking rate had little effect on the initial reaction rate, maximum conversion, and residual substrate *e.e.*, indicating that 220 rpm was the optimal shaking rate.

As shown in Table 4, the initial reaction rate increased with increasing substrate concentration up to 55 mmol/L, while the maximum conversion and the residual substrate *e.e.* showed no clear variation. However, further increases in substrate concentration from 55 mmol/L to 65 mmol/L led to a clear drop in the initial reaction rate, the maximum conversion and residual substrate *e.e.*, possibly due to inhibitory and toxic effects of the substrate and the product. It is worth noting that the optimal substrate concentration in the [ChCl][Gly]-containing system was much higher than that in the aqueous monophasic system (55 mmol/L vs 30 mmol/L) and this will doubtlessly improve the reaction efficiency substantially.

In order to understand why [ChCl][Gly]-containing system could allow a higher substrate loading, we examined the biocompatibility of various DESs with *Acetobacter* sp.CCTCCM209061 cells and the effects of DESs on the cells' membrane integrity.

Biocompatibility of various DESs with *Acetobacter* sp.CCTCCM209061 cells

Despite being regarded as green solvents, DESs have been found to be toxic to microorganisms[14]. Thereby, it is necessary to assess the biocompatibility of the DESs used with *Acetobacter* sp.CCTCC M209061 with the sugar metabolic activity retention (MAR) of the microbial cells as a criterion [20]. As shown in Figure 1, the MAR value of the cells in the presence of substrate was lower than that of cells in the absence of substrate, suggesting that MOPE was toxic to the cells. In addition, the cells in the aqueous monophasic system showed higher MAR value than those in all tested DESs-containing system without substrate, implying that the examined DESs were toxic to the cells to some extent

Table 4Effect of substrate concentration on the asymmetric oxidation of racemic MOPE with immobilized *Acetobacter* sp. CCTCC M209061 cells

Substrate concentration (mmol/L)	Initial reaction rate (μmol/min)	Reaction time (h)	Conversion[a] (%)	*e.e.*[b] (%)
40.0	109.1	6.0	52.8	99.9
45.0	118.2	6.5	52.2	99.9
50.0	126.5	7.0	51.6	99.9
55.0	126.7	7.5	51.5	99.9
60.0	107.0	9.0	49.2	97.8
65.0	93.1	10.0	48.3	94.5

Reaction conditions: 5.0 mL TEA-HCl buffer (100 mmol/L, pH 6.5) containing 10% (v/v) [ChCl][Gly], various concentrations of MOPE, 0.3 g immobilized cells, 50mmol/L acetone, 30 ℃, 200 rpm.

[a] The maximum conversion

[b] The residual substrate *e.e.*

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The MAR value varied greatly in different DESs-containing systems, of which [ChCl][Gly] exhibited the best biocompatibility with the cells and the cells displayed the highest MAR value of 94.5%. In the case of ChCl-based DESs, the variation of MAR value in the presence of substrate with different hydrogen bond donors in the DESs (Fig. 1) could well account for the significant influence of these hydrogen bond donors in DESs on the reaction (Table 1). In addition, the MAR value of the cells with $[Bu_4NBr][IM]$ was inferior to that of [ChCl][IM] irrespective of the presence of substrates,. For all the tested DESs, the MAR values of the cells when the hydrogen bond donors were glycerol, urea, and ethylene glycol were higher than those when the hydrogen bond donors were carboxylic acid and imidazole, which could explain the catalytic efficiency shown in Table 1.

It has been speculated that the good biocompatibility of the DESs with glycerol, urea or ethylene glycol with microbial cells stem from the OH-bridge protic microenvironment formed by their hydroxyl groups[21]. More attention, however, should be paid to the underlying mechanisms and relevant studies are now underway in our laboratory. The highest MAR value achieved with the cells in [ChCl][Gly]-based co-solvent system, agreed with the observation in Table 1. It is interesting to note that the MAR value of the cells with substrate was only 3% lower than that without substrate in the [ChCl][Gly]-containing system, while the corresponding value was around 25% in aqueous buffer, demonstrating that the addition of [ChCl][Gly] to the reaction system substantially lowered the toxic effect of the substrate and the product to the cells, which contributes to the higher substrate loading.

Effect of various DESs on cell membrane integrity

The enhanced catalytic efficiency in [ChCl][Gly]-containing system might result partly from the improved cell membrane permeability, which could accelerate the mass transfer, thus leading to a higher initial rate, reduced toxic and inhibitory effects of the product and limited reverse reaction. On the other hand, cell death may be along with the damage to cell membrane and thus lower the availability of reaction equivalents for the reaction[15, 22]. Hence, it is of great interest to investigate the influence of various DESs on the cell membrane integrity.

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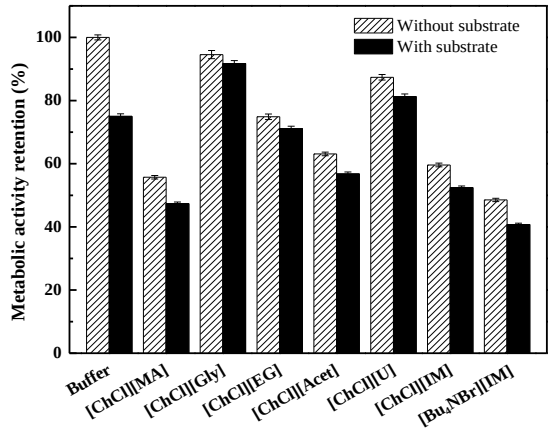

Fig. 1 Metabolic activity retention of immobilized *Acetobacter* sp. CCTCC M209061 cells after 24 h exposure to co-solvent systems consisting of 10% various DESs and TEA-HCl buffer (100 mmol/L, pH 6.5), with and without substrate (40 mmol/L MOPE)

Flow cytometry (FCM) with propidium iodide (PI) as cell fluorescein dye[23] was adopted as a simple and accurate method of measuring the membrane integrity of *Acetobacter* sp. CCTCC M209061 cells incubated with various DESs (10%, v/v)-containing systems for 24 h.

As can be seen in Fig. 2, the cell membrane integrity decreased significantly, indicating that the examined DESs damaged and permeated the cell membrane. The different types of DESs exerted distinctly different effects on the cell membrane integrity and thus on the cell membrane permeability. The cell membrane integrity as well as the cell membrane permeability was clearly dependent on the different hydrogen bond donors. With DESs in which the hydrogen bond donors were carboxylic acid and imidazole, the cell membrane was relatively less integrated, indicating that these DESs seriously damaged the cell membrane, leading to cell death. As a result, in the presence of these DESs, the microbial cells displayed a very low activity (Table 1) and MAR (Fig. 1). For DESs in which the hydrogen bond donors was glycerol, urea, or ethylene glycol, the cell membrane integrity decreased slightly. Of all the DESs assessed, the highest cell membrane integrity was recorded with [ChCl][Gly], which correlated with the best performance of the cells in the [ChCl][Gly]-containing system. Obviously, only a moderate increase in cell membrane permeability could enhance the reaction efficiency.

Preparative scale biotransformation in the [ChCl][Gly]-containing system

To further show the feasibility of applying [ChCl][Gly]-containing system to practical use, the reaction was conducted on a 500 mL preparative scale under the optimal reaction conditions {55 mmol/L MOPE, [ChCl][Gly]-containing system (10% [ChCl][Gly], 100 mmol/L, pH 6.5), 50 mmol/L acetone, 0.3 g/mL immobilized cells, 30 ℃, and 220 rpm}. The reaction process was monitored by GC analysis, and the product was extracted from the reaction mixture with isopropyl ether upon the exhaustion of (*R*)-MOPE. The residual substrate *e.e.* was above 99.9% after reacting for 7h when the conversion was 51.5%. Therefore, the whole-cell biocatalytic resolution of MOPE in the [ChCl][Gly]-containing system is though promising.

Experimental

Biological and chemical materials

Acetobacter sp. CCTCC M209061 was isolated from Chinese kefir grains by our research group and conserved in our laboratory[24]. MOPE (98% purity) was purchased from Alfa Aesar (USA). 4´-methoxyacetophenone (99%) and n-tetradecane (>99%) were purchased from TCI (Japan). All other chemicals were from commercial sources and were of analytical grade.

Cultivation and immobilization of *Acetobacter* sp. CCTCC M209061cells

Acetobacter sp. CCTCC M209061 was cultivated according to our previous described methods[25]. The medium was sterilized by autoclaving at 121 ℃ for 20 min. After 30 h incubation, the cells were in the late exponential growth phase and were harvested by centrifugation (6010 ×g, 10 min, 4 ℃) to give a biomass of 6.11 ± 0.17 g/L (wet weight). The wet cells were immobilized as described below before use in the asymmetric oxidation. A homogenous cell/chitosan suspension was prepared at 25 ℃ by adding 6 g of fresh cell suspension (3 g wet cells in 3 mL water) into 47 mL of a homogeneous aqueous chitosan solution (3%, w/v), which was prepared by dissolving chitosan in acetate buffer (pH 4.2), heating and ultrasonic processing (20 KHz, 30 min)
The suspension was added dropwise from a syringe to a cross-linking solution, which was mixed by 4% (w/v) glyoxal solution with an equal volume of 3% (w/v) tetrasodium pyrophosphate solution (pH 8.0). The gel beads were hardened for 30 min at room temperature. The beads were then transferred to 0.05% glutaraldehyde for reinforcement treatment[26, 27]. The coated beads were collected by filtration and washed with sterilized water to remove the residual solution. The beads had a load of 15% (w/w) of *Acetobacter* sp. CCTCC M209061 cells (based on cell wet mass). The resulting beads were stored in triethanolamine (TEA)-HCl buffer (100 mmol/L, pH 6.5) at 4 ℃ for later use.

Preparation of DESs

The preparation of DESs involved the reaction of choline chloride (1 mol) with different hydrogen bond donors (2 mol) at 100 ℃ in a flask with stirring for 2 h until a clear solution was obtained which was used for the reactions without purification. This method gave DESs with 100% atom economy as it completely formed a eutectic mixture with no by-product formation. Another DES was similarly prepared by combining tetrabutyl ammonium bromide (3 mol) with imidazole (7 mol) under the same conditions[18] .

General procedure for asymmetric oxidation of MOPE (oxidation activity assay)

In a typical experiment, a system (5.0 mL) consisted of DESs and TEA-HCl buffer (100 mmol/L), added to a 10-mL Erlenmeyer flask capped with a septum. Predetermined free or immobilized *Acetobacter* sp. CCTCC M209061 cells were added as well as 50 mmol/L acetone. The reaction mixture was pre-incubated at an appropriate temperature and shaking rare for 15 min. The reaction was then initiated by adding MOPE (precalculated concentration) to the reaction system. Aliquots (50 μL) were withdrawn at specified time intervals, and then the product and the residual substrate were extracted with isopropyl ether (50 μL) containing 5.04 mmol/L n-tetradecane (as an internal standard) prior to GC analysis. Details of the used DESs, content of DESs and substrate are specified in each case.

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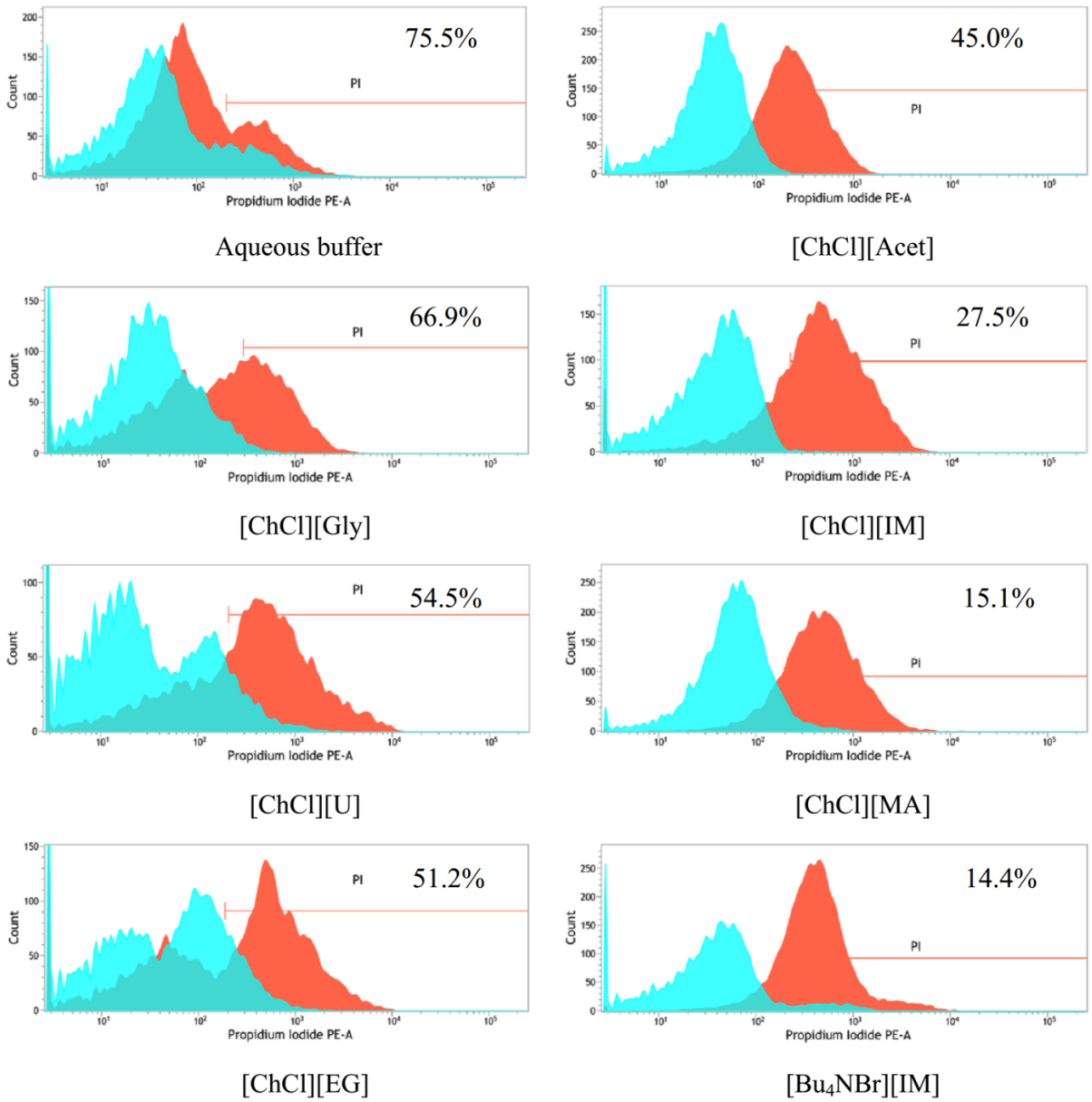

Fig. 2 Membrane integrity of *Acetobacter* sp. CCTCC M209061 cells measured by FCM with PI as cell fluorescein dye after being incubated in various DES (10%, v/v)-containing co-solvent systems for 24h

Stability of biocatalysts

Thermal stability

Wet-free cells (2.5 g) or immobilized beads (16.7 g, with a load of approximately 2.5 g cwm of *Acetobacter* sp. CCTCC M209061 cells) in 60 mL TEA-HCl buffer (100 mmol/L, pH 6.5) were incubated at 30, 40, and 50 ℃. Samples (0.3 g wet-free cells or 2.0 g immobilized beads) were withdrawn at specified time intervals and cooled to 30 ℃. The residual activity of biocatalysts was determined using the activity test described above.

Storage stability

Wet-free cells (2.5 g) or immobilized beads (16.7 g, with a load of approximately 2.5 g cwm of *Acetobacter* sp. CCTCC M209061 cells) were incubated in TEA-HCl buffer (100 mmol/L, pH 6.5) and stored at 4°C. Samples (0.3 g wet-free cells or 2.0 g immobilized beads) were withdrawn at specified time intervals and the residual activity was determined.

Operational stability

In order to text the operational stability of the cells, the re-use of immobilized and free *Acetobacter* sp. CCTCC M209061 cells was investigated in the aqueous monophasic system. Initially, an aliquot of the immobilized cells (0.3 g/mL) or free cells (0.045

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g/mL) was added to the TEA-HCl buffer system (100 mmol/L, pH 6.5) containing 50 mmol/L acetone and 30 mmol/L MOPE. The reactions were then carried out at 30 ℃ and 200 rpm and were repeated over 10 batches (12 h per batch) without changing the biocatalysts. Between batches, immobilized cells were filtered from the reaction mixture, washed three times with distilled water, and added to a fresh batch of reaction medium. Free cells were recycled by centrifugation after each batch (6010 ×g, 10 min, 4 ℃). Other conditions were the same as those used with immobilized cells. The activity of the cells, the conversion and the residual substrate *e.e.* were assayed in each batch. The relative activity of the cells employed for the first batch was defined as 100%.

Preparative scale biocatalytic resolution of MOPE in the [ChCl][Gly]-based system

The preparative scale biocatalytic resolution of MOPE was performed by adding 150.0 g immobilized *Acetobacter* sp. CCTCC M209061 cells and 55 mmol/L MOPE to 500 mL of the mixing system consisting of 50 mL [ChCl][Gly] and 450 mL TEA-HCl buffer (100 mmol/L, pH 6.5) containing 50 mmol/L acetone at 220 rpm and 30 ℃. The reaction was terminated when no (*R*)-MOPE was detected by GC analysis. The immobilized cells were removed by filtration, and the reaction mixture was extracted with acetic ether. The residual substrate *e.e.* and the conversion were determined by GC analysis.

Cell metabolic activity retention measurement

The metabolic activity retention (%, MAR) of immobilized *Acetobacter* sp. CCTCC M209061 cells was defined as the ratio of the amount of glucose consumed by the immobilized cells pretreated in various media to that by the immobilized cells pretreated in aqueous buffer (as the control)[20]. The MAR value of immobilized *Acetobacter* sp. CCTCC M209061 cells was assayed after 24 h exposure to various systems consisting of various DESs (10%, v/v)/TEA-HCl buffer (100 mmol/L, pH 6.5) or in a TEA-HCl buffer (100 mmol/L, pH 6.5) monophasic system in the presence and absence of substrate (40 mmol/L MOPE, based on the volume of the entire system), respectively, in a rotary incubator set at 30 ℃ and 200 rpm. After separation from the reaction medium and washed three times with fresh water, the beads of immobilized cells were transferred to glucose solution (10 mL, 1.0 g/L), and then incubated at 30 ℃ and 200 rpm for 4 h. The glucose concentration in the medium was then determined by HPLC.

Cell membrane integrity assay

In a typical experiment, 5 mL of a DES (10%, v/v)-containing system or aqueous TEA-HCl buffer (100 mmol/L, pH 6.5) containing 0.05 g/mL *Acetobacter* sp. CCTCC M209061 cells were incubated for 24 h in a 10 mL Erlenmeyer flask capped with a septum at 30 ℃ and 180 rpm.

For measurement of cell membrane integrity, the free cells were harvested and added to sterile normal saline to wash the cells. The cell suspension was diluted to 10^6 cfu/mL and dyed with propidium iodide (final concentration 50 μg/mL) at 4 ℃ in the dark, and then subjected to cell membrane integrity measurement using flow cytometry (FCM). The FCM assay was conducted with a BD FACSVerse flow cytometer and the FCM data were analyzed using BD FACSuite software.

Analytic methods

The reaction mixtures were analyzed using a Shimadzu GC 2010 model with a flame ionization detector and an Agilent chiral column (CP-ChiraSil-DEX CB 25 m×0.25 mm×0.25 μm) (USA). The injector and the detector were both kept at 250 ℃. The column temperature was held constant at 150 ℃ for 7 min. The carrier gas was nitrogen and its flow rate in the column was 1.5 mL/min. The retention times for *n*-tetradecane, MOAP, (*R*)-MOPE and (*S*) -MOPE were 3.3 min, 4.4 min, 5.4 min and 5.6 min, respectively.

The glucose concentration was determined by HPLC (515 pump and 2410 differential refraction detector, Waters Corp., USA), using an Aminex HPX-87H column (7.8 mm × 300 mm) under the following conditions: mobile phase, 5.0 mmol/L H_2SO_4; flow rate, 0.5 mL/min; column temperature, 65 ℃; and detector temperature, 50 ℃. The retention time for glucose was 12.1 min.
The average error for this determination was less than 1.0%. All reported data averages of the experiments were performed at least twice.

Conclusions

The DES [ChCl][Gly] could greatly enhance the efficiency of resolution of racemic MOPE using immobilized *Acetobacter* sp. CCTCC M209061 cells and improve the stability of the biocatalyst owing to the excellent solvent property of [ChCl][Gly] for MOPE and its benign biocompatibility with *Acetobacter* sp. CCTCC M209061 cells. Furthermore, the *Acetobacter* sp. CCTCC M209061 cells-catalyzed process with co-solvent [ChCl][Gly] is promising for industrial production of enantiopure (*S*)-MOPE. Further developments of the applications of DES-containing system in biocatalysis process are awaited with great interest.

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1. R. N. Patel, *Enzyme Microb. Technol.*, 2002, **31**, 804-826.
2. D. Gamenara and P. Dominguez de Maria, *Biotechnol. Adv.*, 2009, **27**, 278-285.
3. M. C. Hillier, J. i. Desrosiers, J. F. Marcoux and E. J. J. Grabowski, *Org. Lett.*, 2004, **6**, 573-576.
4. M. C. Hillier, J. F. Marcoux, D. Zhao, E. J. J. Grabowski, A. E. McKeown and R. D. Tillyer, *J. Org. Chem.*, 2005, **70**, 8385-8394.
5. R. Wohlgemuth, *Curr. Opin. Biotechnol.*, 2010, **21**, 713-724.
6. Y. Ni and J. H. Xu, *J. Mol. Catal. B: Enzym.*, 2002, **18**, 233-241.
7. N. S. Hatzakis and I. Smonou, *Bioorg. Chem.*, 2005, **33**, 325-337.
8. M. Egi, K. Sugiyama, M. Saneto, R. Hanada, K. Kato and S. Akai, *Angew. Chem. Int. Ed. Engl.*, 2013, **52**, 3742-3746.

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9. Q. H. Zhang, K. De Oliveira Vigier, S. Royer and F. Jerome, *Chem. Soc. Rev.*, 2012, **41**, 7108-7146.
10. K. Shahbaz, F. S. Mjalli, M. A. Hashim and I. M. AlNashef, *Energy Fuel.*, 2011, **25**, 2671-2678.
11. M. Francisco, A. van den Bruinhorst and M. C. Kroon, *Green Chem.*, 2012, **14**, 2153-2157.
12. D. Lloyd, T. Vainikka, L. Murtomäki, K. Kontturi and E. Ahlberg, *Electrochim. Acta*, 2011, **56**, 4942-4948.
13. M. Sharma, C. Mukesh, D. Mondal and K. Prasad, *Rsc Adv.*, 2013, **3**, 18149-18155.
14. J. T. Gorke, F. Srienc and R. J. Kazlauskas, *Chem. Commun.*, 2008, 1235-1237.
15. H. Zhao, G. A. Baker and S. Holmes, *Org. Biomol. Chem.*, 2011, **9**, 1908-1916.
16. H. Zhao, G. A. Baker and S. Holmes, *J. Mol. Catal. B: Enzym.*, 2011, **72**, 163-167.
17. D. Lindberg, M. de la Fuente Revenga and M. Widersten, *J. Biotechnol.*, 2010, **147**, 169-171.
18. Z. Maugeri and P. Domínguez de María, *ChemCatChem*, 2014, **6**, 1535-1537.
19. B. Singh, H. Lobo and G. Shankarling, *Catal. Lett.*, 2011, **141**, 178-182.
20. S. A. Gangu, L. R. Weatherley and A. M. Scurto, *Curr. Org. Chem.*, 2009, **13**, 1242-1258.
21. G. de Gonzalo, I. Lavandera, K. Durchschein, D. Wurm, K. Faber and W. Kroutil, *Tetrahedron: Asymmetr.*, 2007, **18**, 2541-2546.
22. M. Hayyan, M. A. Hashim, A. Hayyan, M. A. Al-Saadi, I. M. AlNashef, M. E. S. Mirghani and O. K. Saheed, *Chemosphere*, 2013, **90**, 2193-2195.
23. C. J. Bunthof and T. Abee, *Appl. Environ. Microbiol.*, 2002, **68**, 2934-2942.
24. Z. J. Xiao, M. H. Zong and W. Y. Lou, *Bioresour. Technol.*, 2009, **100**, 5560-5565.
25. X. H. Chen, W. Y. Lou, M. H. Zong and T. J. Smith, *BMC Biotechnol.*, 2011, **11**, 110.
26. M. Y. Chang and R. S. Juang, *Enzyme Microb. Technol.*, 2005, **36**, 75-82.
27. Ş. A. Çetinus, H. N. Öztop and D. Saraydın, *Enzyme Microb. Technol.*, 2007, **41**, 447-454.

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