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Use of Ionic Liquid To Significantly Improve Asymmetric Reduction of Ethyl Acetoacetate Catalyzed by *Acetobacter sp.* CCTCC M209061 Cells

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Supporting Information

ABSTRACT: Biocatalytic reduction of ethyl acetoacetate (EAA) to ethyl (*R*)-3-hydroxybutyrate [(*R*)-EHB] with *Acetobacter sp.* CCTCC M209061 cells was successfully conducted in ionic liquid (IL)-based biphasic systems. Several water-immiscible ILs were used to construct biphasic systems. The best IL investigated was 1-butyl-3-methylimidazolium hexafluorophosphate (C_4mim·PF_6), which also had good biocompatibility. Several influential variables were examined. The optimum parameters were as follows: volume ratio of buffer to C_4mim·PF_6, 1/2 (v/v); substrate concentration, 55 mmol/L; buffer pH, 5.5; cosubstrate concentration, 80 mmol/L; reaction temperature, 35 °C; and shaking speed, 220 rpm. Under these optimal conditions, the initial reaction rate, the yield, and the product *e.e.* were 0.39 mmol/(L min), 90.8%, and >99%, respectively, which were much better than results reported previously. This efficient whole-cell biocatalytic process was feasible on a 450-mL preparative scale, and the immobilized cells showed excellent operational stability and could be reused for at least 10 batches.

1. INTRODUCTION

Enantiopure chiral alcohols have proven to be versatile intermediates for the synthesis of many chiral pharmaceuticals, agrochemicals, liquid crystals, and flavors.[1,2] Among them, enantiomerically pure ethyl 3-hydroxybutyrate (EHB) is a versatile intermediate for the synthesis of various biologically and structurally interesting compounds and pharmaceuticals.[3,4] For example, ethyl (*S*)-3-hydroxybutyrate [(*S*)-EHB] is used as a key chiral intermediate for the synthesis of lavandulol, sulcatol, and prenophorin,[5] whereas ethyl (*R*)-3-hydroxybutyrate [(*R*)-EHB] plays an important role in the synthesis of (+)-decarestrictine L.[5] Enantiopure chiral alcohols can be synthesized mainly by the asymmetric reduction of prochiral ketones using either chemical or biological methods. For economic, environmental, and social reasons, biocatalytic methods have recently gained much attention.[6−8] Whole cells rather than isolated enzymes are preferentially used to avoid enzyme purification and cofactor addition, as well as to avoid the requirement for an associated system for cofactor regeneration, since such reactions often require stoichiometric amounts of cofactors. In addition, using immobilized cells as biocatalysts can not only facilitate product separation, but also make cells recyclable and reusable, thus greatly simplifying the process and lowering the cost of production.

In our previous study, the asymmetric reduction of ethyl acetoacetate (EAA) to (*R*)-EHB catalyzed by immobilized *Acetobacter sp.* CCTCC M209061 cells was conducted successfully in a neat aqueous monophasic system with an optimum substrate concentration (35 mmol/L) and a faster initial reaction rate (0.43 mmol/(L min)). Also, the obtained yield (82.6%) and product *e.e.* (above 99.0%) were much better than those reported previously.[9] However, the substrate and product had pronounced inhibitory and toxic effects on the microbial cells in the aqueous monophasic system, thus resulting in relatively low reactant concentration and reaction efficiency.[10] In order to overcome these limitations, a biphasic system has been developed wherein an aqueous buffer contains the microbial cells and a water-immiscible organic phase acts as a reservoir for the substrate and product.[11−13] There have been several reports on using organic solvents for biocatalytic asymmetric reduction of EAA, but the products obtained mostly consisted of (*S*)-EHB. Most of the reported reaction systems generated a product *e.e.* above 94.8%, but with a relatively low product yield (≤87%). The only exception to these results was for the asymmetric reduction of EAA to (*S*)-EHB catalyzed by immobilized baker's yeast in glycerol (product *e.e.*, 99%; product yield, 99%), but this bioreduction required as long as 48 h.[14−17] It is well-known that, using conventional organic solvents in such processes can be problematic; in many cases, they are toxic to the microbial cells used and lead to poor operational stability. Also, they may be explosive and are usually environmentally harmful. Hydrophobic ionic liquids (ILs) are a promising new class of alternative green solvents that are obvious candidates for a great variety of biocatalytic transformations.[18−21] Many types of ILs have proven to be biocompatible with efficient biotransformations catalyzed by diverse microbial cells, including *Saccha-*

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romyces cerevisiae, Escherichia coli, Geotrichum candidum, Candida parapsilosis CCTCC M203011, *Pichia membranaefaciens* Hansen ZJPH07, and *Lactobacillus kefir*.[4,22−27] To date, no report has been published on biocatalysis with immobilized *Acetobacter sp.* CCTCC M209061 cells in a two-phase system involving ILs.

In this study, we have for the first time used various water-immiscible ILs in a biphasic system to improve the biocatalytic asymmetric reduction of EAA to (*R*)-EHB, catalyzed by immobilized *Acetobacter sp.* CCTCC M209061 cells (Figure 1). We have also examined the effect of these ILs on the

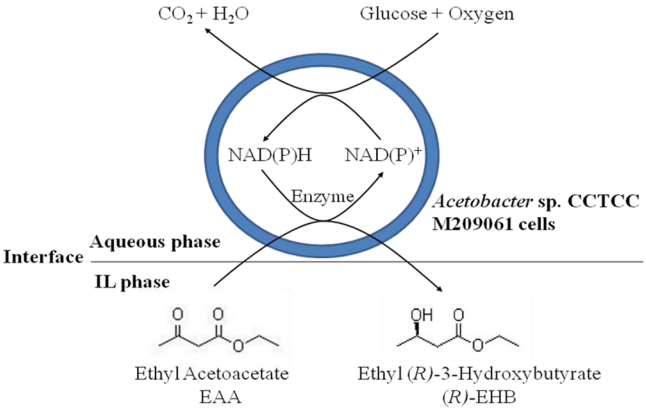

Figure 1. Biocatalytic anti-Prelog asymmetric reduction of EAA to (*R*)-EHB with immobilized *Acetobacter sp.* CCTCC M209061 cells in ionic liquid (IL)-containing biphasic systems.

biocatalytic reaction. In this process, EAA was reduced to enantiopure (*R*)-EHB, while NAD(P)H was converted to NAD(P)$^+$ and cosubstrate glucose was simultaneously oxidized to CO_2, presumably driving the reduction reaction by regenerating NAD(P)H from NAD(P)$^+$. In addition, this efficient biocatalytic process in the presence of ILs was tested on a preparative scale and shown to be effective and competitive with previous biocatalytic reactions.

2. EXPERIMENTAL SECTION

2.1. Biological and Chemical Materials. *Acetobacter sp.* CCTCC M209061 was isolated from Chinese kefir grains by our research group and conserved in our laboratory.[28]

Ethyl acetoacetate (99% purity, EAA) was purchased from Alfa Aesar (USA). Ethyl 3-hydroxybutyrate (98% purity, EHB), ethyl (*R*)-3-hydroxybutyrate {98% purity, (*R*)-EHB} and *n*-decane (>99% purity) were obtained from Sigma−Aldrich (USA). The nine ILs shown in Table S1 in the Supporting Information were purchased from Lanzhou Institute of Chemical Physics (China) and all had purities of >98%. All other chemicals were also from commercial sources and were of analytical grade.

2.2. Cultivation and Immobilization of *Acetobacter sp.* CCTCC M209061 Cells. *Acetobacter sp.* CCTCC M209061 was cultivated in a medium (pH 5.7) containing 8.26 g/L glucose, 2.50 g/L fructose, 83.92 g/L soy peptone, 0.088 g/L $MnSO_4 \cdot H_2O$, and 10% (v/v) inoculum at 30 °C and 80 rpm for 30 h. The harvested cells were immobilized using calcium alginate entrapment and then coated with chitosan, according to our previously described methods.[9] In brief, a homogeneous cell/sodium alginate suspension was first prepared and added dropwise by an injector to a gently stirred $CaCl_2$ solution (2%, w/v), where the calcium alginate beads were precipitated. The beads were then transferred to chitosan solution to form the membrane structure. The coated beads with chitosan were collected and stored at 4 °C for later use.[29,30]

2.3. General Procedure for Biocatalytic Asymmetric Reduction of EAA. In a typical experiment, a biphasic system (3.0 mL) consisted of a water-immiscible IL and triethanolamine (TEA)-HCl buffer (100 mmol/L), contained in a 10-mL Erlenmeyer flask capped with a septum. The alginate beads (0.45 g/mL) with a load of 10% (w/w) *Acetobacter sp.* CCTCC M209061 cells {based on cell wet mass (cwm)} were added, together with 80 mmol/L glucose. The reaction mixture was preincubated in an air-bath shaker at 180 rpm and an appropriate temperature for 15 min. Then, the reaction was initiated by adding EAA to the mixture. Aliquots (25 μL) were withdrawn at specified time intervals from the IL phase and the aqueous phase, respectively. The product and the residual substrate were extracted with acetic ether (50 μL) containing 15.7 mmol/L *n*-decane (internal standard) prior to gas chromatography (GC) analysis. Details about the used water-immiscible ILs, volume ratio of buffer to IL, substrate and cosubstrate concentrations, buffer pH, reaction temperature and shaking speed are specified for each case.

The initial reaction rate was calculated as

$$\text{Initial reaction rate (mmol/(L min))} = \frac{C_t}{t}$$

where C_t (mmol/L) is the concentration of the formed product EHB after reaction for t minutes, and t (min) is the initial reaction time of 10 min.

The yield was calculated as

$$\text{Yield (\%)} = \frac{M_t}{M_0} \times 100$$

where M_0 (mmol) is the theoretical molar mass of the formed product EHB based on the molar mass of the used substrate EAA, and M_t (mmol) is the determined molar mass of the released EHB after the reaction for t minutes.

2.4. Preparative Scale Biocatalytic Reduction of EAA in an IL-Based Biphasic System. The preparative scale biocatalytic reduction of EAA to (*R*)-EHB was performed by adding 202.5 g of immobilized *Acetobacter sp.* CCTCC M209061 cells and 55 mmol/L (3.2 g) of EAA to 450 mL of the biphasic system (volume ratio: 2/1) consisted of C_4mim·PF_6 and TEA-HCl buffer (100 mmol/L, pH 5.5) containing 80 mmol/L glucose at 220 rpm and 35 °C. The reaction was terminated when no substrate was detectable by GC analysis. The immobilized cells were removed by filtration, and the reaction mixture was extracted with acetic ether. The product *e.e.* and the isolated yield were determined by GC analysis.

2.5. Analytical Methods. The reaction mixtures were analyzed according to our previously reported GC analytical method.[9] The retention times for *n*-decane, EAA, and EHB were 4.9, 9.2, and 9.8 min, respectively. The product *e.e.* was determined after the derivatization of EHB with trifluoroacetic anhydride.[31] The retention times for (*R*)-EHB and (*S*)-EHB were 52.7 and 53.4 min, respectively.

The glucose concentration was determined by high-performance liquid chromatography (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

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rate, 0.5 mL/min; column temperature, 65 °C; and detector temperature, 50 °C. The retention time for glucose was 12.1 min.

The average error for this determination was <1.0%. All reported data are averages of experiments performed at least in duplicate.

2.6. Cell Metabolic Activity Retention Measurement. The metabolic activity retention (%, MAR) of immobilized *Acetobacter sp.* CCTCC M209061 cells was defined as the ratio of the consumed glucose amount by the immobilized cells pretreated in various media to that by the immobilized cells pretreated in aqueous buffer (as the control).[32,33] The MAR of immobilized *Acetobacter sp.* CCTCCM209061 cells was assayed after 24 h exposure to various biphasic systems consisting of hydrophobic organic solvents and TEA-HCl buffer (100 mmol/L, pH 5.5) (organic solvents/buffer volume ratio, 1/1), water-immiscible ILs and TEA-HCl buffer (100 mmol/L, pH 5.5) (IL/buffer volume ratio, 1/1) or in a TEA-HCl buffer (100 mmol/L, pH 5.5) monophasic system in the presence and absence of substrate (35 mmol/L EAA, based on the volume of the entire biphasic system), respectively, in a rotary incubator set at 30 °C and 180 rpm. After separated 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 °C and 180 rpm for 4 h. The glucose concentration in the medium was then determined by HPLC.

2.7. Determination of Partition Coefficients. Partition coefficients ($K_{IL/aq}$ and $K_{org/aq}$) were determined by dissolving 25, 35, or 45 mmol/L EAA or EHB, as appropriate, in each IL/buffer or organic solvent/buffer biphasic systems (volume ratio of two phases: 1/1) and shaking (220 rpm) for 24 h at 35 °C. The concentrations of EAA or EHB in the IL or organic phase and the aqueous phase were then analyzed by GC. The concentration of EAA or EHB in each phase varied linearly with the total amount of each chemical added to the two-phase system. The slopes then were calculated and used for the quantification of the partition coefficients of EAA and EHB between the IL or organic phase and the aqueous phase.

2.8. Operational Stability of Immobilized *Acetobacter sp.* CCTCC M209061 Cells. In order to assess the operational stability of immobilized *Acetobacter sp.* CCTCC M209061 cells, the reuse of the cell-loaded alginate-chitosan beads was investigated in the *n*-hexane/buffer biphasic system, the C_4mim·PF_6/buffer biphasic system, and the aqueous monophasic system, respectively. Initially, aliquots of the cells were added into separate screw-capped vials, each containing 3.0 mL of the appropriate medium {*n*-hexane/TEA-HCl buffer (100 mmol/L, pH 5.5) biphasic system (volume ratio = 2/1), C_4mim·PF_6/TEA-HCl buffer (100 mmol/L, pH 5.5) biphasic system (volume ratio = 2/1), or aqueous TEA-HCl buffer system (100 mmol/L, pH 5.5)}, together with the optimal amount of EAA and glucose for the reduction conducted in the above-mentioned media. Then, the reactions were performed at 35 °C and were repeated over 10 batches (reaction time: 4 h per batch) without changing the immobilized cells. Between batches, the immobilized cells were filtered off from the reaction mixture, washed twice with fresh water, and added to a fresh batch of reaction medium. The catalytic activity of the cells was assayed in each batch according to the above-described general procedure for asymmetric reduction of EAA (see section 2.3). The activity of the cells was defined as the amount of the cells required to catalyze the formation of 1 μmol product EHB per minute under the above-mentioned conditions. The relative activity of the cells employed for the first batch was defined as 100%.

3. RESULTS AND DISCUSSION

3.1. Effects of Various Water-Immiscible ILs on the Biocatalytic Asymmetric Reduction of EAA to (*R*)-EHB with Immobilized *Acetobacter sp.* CCTCC M209061 Cells. There have been many reports on the biocatalytic reduction of ketones using microbial cells in various IL-containing reaction systems, where the catalytic performance exhibited by the biocatalysts was closely related to the cation and anion types of the ILs; the effects of ILs on biocatalytic reactions have been found to vary widely.[26,27,34−37] Therefore, a comparative study of the effects of ILs with different combinations of cations and anions on the bioreduction of EAA with immobilized *Acetobacter sp.* CCTCC M209061 cells was carried out in various water-immiscible IL-based biphasic systems (see Table 1). Nine water-imiscible ILs were chosen, to allow exploration

Table 1. Effect of Various Water-Immiscible ILs or Organic Solvents on the Biocatalytic Asymmetric Reduction of EAA to (*R*)-EHB with Immobilized *Acetobacter sp.* CCTCC M209061 Cells[a]

medium	initial reaction rate ($\times 10^{-1}$ mmol/(L min))	yield (%)	product *e.e.* (%)
C_4mim·PF_6/buffer	2.7	87.5	>99.0
C_5mim·PF_6/buffer	2.5	75.4	>99.0
C_6mim·PF_6/buffer	2.1	60.0	>99.0
C_7mim·PF_6/buffer	1.7	42.8	>99.0
iC_4mim·PF_6/buffer	2.2	61.6	>99.0
C_4mim·Tf_2N/buffer	1.8	59.2	>99.0
C_6mim·Tf_2N/buffer	1.5	52.3	>99.0
PP_{14}·Tf_2N/buffer	1.5	50.9	>99.0
Py_{14}·Tf_2N/buffer	1.6	56.7	>99.0
ethyl acetate/buffer	0.6	48.6	>99.0
methylbenzene/buffer	0.8	33.1	>99.0
n-octanol/buffer	1.1	31.4	>99.0
cyclohexane/buffer	1.3	45.6	>99.0
n-hexane/buffer	1.5	50.1	>99.0
isooctane/buffer	1.4	38.9	>99.0
n-octane/buffer	1.1	34.7	>99.0

[a]Reaction conditions: TEA-HCl buffer (100 mmol/L, pH 5.5)/ IL or organic solvent volume ratio of 1/1, 35.0 mmol/L EAA, 0.45 g/mL immobilized cells, 80.0 mmol/L glucose, 35 °C, 200 rpm.

of frequently used anionic and cationic moieties. In order to allow efficient use in the biotransformation reaction, the ILs that were chosen were liquid at ~30 °C,[24] had densities of >1.2 g/cm^3 (to allow effective separation of the phases after the reaction)[24] and when saturated water had a viscosity of less than ~400 mm^2/s (in order to minimize mass-transfer limitations). Also, the ILs were chosen that could well dissolve the substrate EAA. Furthermore, the nine chosen ILs have been used for other biocatalytic transformations in our previous reports.[26,38]

The immobilized *Acetobacter sp.* CCTCC M209061 cells were capable of catalyzing the asymmetric reduction of EAA in the various IL-based biphasic systems with a high product *e.e.* of above 99%. For biphasic systems involving $C_n$mim·PF_6 (n = 4−7), both the initial reaction rate and the maximum yield clearly decreased as the alkyl chain of the IL chain elongated (i.e.,

increasing n value), possibly partly because of the viscosity increase of the IL with increasing n value,[26,39,40] which may have led to a decrease of the substrate and product mass-transfer rate between the two phases. When the n-butyl group attached to the imidazolium cation and $C_4mim{\cdot}PF_6$ was replaced by *iso*-butyl ($iC_4mim{\cdot}PF_6$), the initial reaction rate and the maximum yield declined greatly, indicating that a minor change of the IL structure exerted a substantial influence on catalyst performance. For the $C_nmim{\cdot}Tf_2N$/buffer (n = 4, 6) biphasic systems, the changes of the initial reaction rate and maximum yield with the elongation of the alkyl chain agreed with those observed in the $C_nmim{\cdot}PF_6$/buffer (n = 4−7) media (see Table 1). In the last two Tf_2N-based ($PP_{14}{\cdot}Tf_2N$, $Py_{14}{\cdot}Tf_2N$) biphasic systems, the bioreduction efficiency changed as the IL cation changed. The reaction efficiency was accelerated when the piperidine group attached to the cation of $PP_{14}{\cdot}Tf_2N$ was replaced by the pyridine ($Py_{14}{\cdot}Tf_2N$). In addition, the product *e.e.* was slightly affected.

Traditionally, biphasic systems consisting of an aqueous phase and a water-immiscible organic phase are often used to avoid inhibiting reactions between the reactants and biocatalysts that occur in aqueous systems. In this case, the microbial cells remain in the aqueous phase, while the substrate and product mainly stay in the organic phase, allowing for easy isolation of the product and reuse of the catalyst.[13−16] We initially performed asymmetric reduction of EAA to (*R*)-EHB, catalyzed by immobilized *Acetobacter sp.* CCTCC M209061 cells in various organic-based biphasic systems (see Table 1). In general, the initial reaction rate (0.15−0.27 mmol/(L min)) and the maximum yield (50.9−87.5%) in the IL-based biphasic systems were much higher than in the organic-based biphasic system (0.06−0.14 mmol/(L min) and 31.4%−50.1%, respectively) under the same reaction conditions. These results were possibly caused by the toxicity of the organic solvents used to the cells and the low partition coefficients of EAA and EHB between the two phases (see Table 2). The volatile nature of such solvents is also a serious threat to the operator, particularly when the solvent is used on a large scale. Hence, there is an increasing need for ionic liquids as alternatives to traditional organic solvents.

Table 2. Partition Coefficients of EAA and EHB between the Two Phases

	Partition Coefficients	
biphasic system	EAA	EHB
$C_4mim{\cdot}PF_6$/buffer	13.2	1.6
$C_5mim{\cdot}PF_6$/buffer	9.7	1.4
$C_6mim{\cdot}PF_6$/buffer	8.2	1.3
$C_7mim{\cdot}PF_6$/buffer	6.2	1.2
$iC_4mim{\cdot}PF_6$/buffer	4.7	1.3
$C_4mim{\cdot}Tf_2N$/buffer	11.0	1.8
$C_6mim{\cdot}Tf_2N$/buffer	10.5	1.5
$PP_{14}{\cdot}Tf_2N$/buffer	9.0	1.4
$Py_{14}{\cdot}Tf_2N$/buffer	11.8	1.4
ethyl acetate/buffer	12.5	4.6
methylbenzene/buffer	6.5	1.2
n-octanol/buffer	2.5	1.1
cyclohexane/buffer	1.6	1.0
n-hexane/buffer	1.6	1.0
isooctane/buffer	1.8	0.6
n-octane/buffer	1.0	0.1

Many studies have shown that the second phase has been found to be toxic toward biocatalysts, regardless of whether the second phase consists of water-immiscible ILs or organic solvents.[27,32] Therefore, it is necessary to evaluate the biocompatibility of the used ILs or organic solvents with *Acetobacter sp.* CCTCCM209061 by directly measuring the sugar metabolic activity retention (MAR) of the microbial cell, which is dependent on its tolerance to ILs or organic solvents and is taken as an easy indicator of cell viability.[32,33] As shown in Figure 2A and 2B, the MAR value of the cells clearly

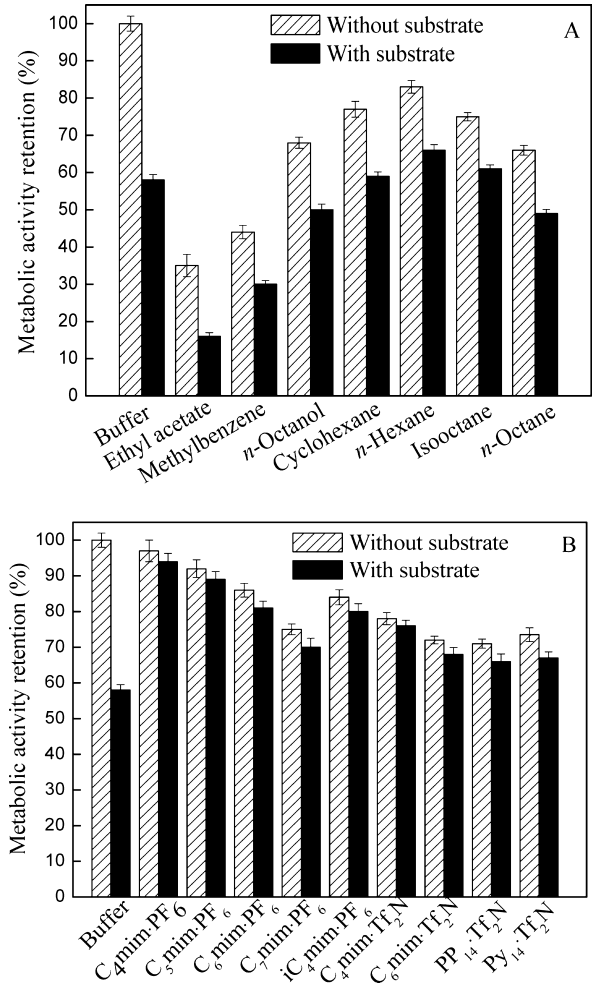

Figure 2. Metabolic activity retention of immobilized *Acetobacter sp.* CCTCC M209061 cells after 12 h of exposure to biphasic systems containing various hydrophobic (A) organic solvents and (B) ILs, with and without substrate (35 mmol/L EAA).

decreased in the presence of substrate, compared to the value obtained in the absence of substrate in both organic-based and ILs-based biphasic systems, suggesting that EAA is substantially toxic to immobilized *Acetobacter sp.* CCTCC M209061 cells. Thus, a high EAA partition coefficient between two phases could effectively eliminate the substrate toxicity to the cells. In the presence of substrate, the MAR value of the immobilized cells was significantly higher in most of the IL-based biphasic systems than in the organic-based biphasic systems. In the absence of substrate, the MAR value was lower in all tested IL-based biphasic systems than in the aqueous monophasic system but higher than most of the organic-based biphasic systems. This result indicates that the tested ILs were more

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biocompatible with the biocatalysts than the organic solvents, although they were both toxic to the cells. To better understand the toxic or inhibitory effects of the product in the various organic-based and ILs-based biphasic systems, the deactivation profiles of the immobilized cells in different media exposed to 35 mmol/L EHB product were investigated (see Figures 3A and 3B). After incubation in the aqueous system with the product for 12 h, the immobilized cells retained only 65% of their original activity, clearly showing the severe toxic or inhibitory effect of the product. However, the immobilized cells in IL-based biphasic systems retained higher relative activities (compared to the immobilized cells in the aqueous monophasic system and all the organic solvent-based biphasic systems) after incubation for the same period. Based on these results, water-immiscible ILs can be used as an excellent second liquid phase in the bioreduction process, acting as a substrate reservoir and in situ extractant for the product.

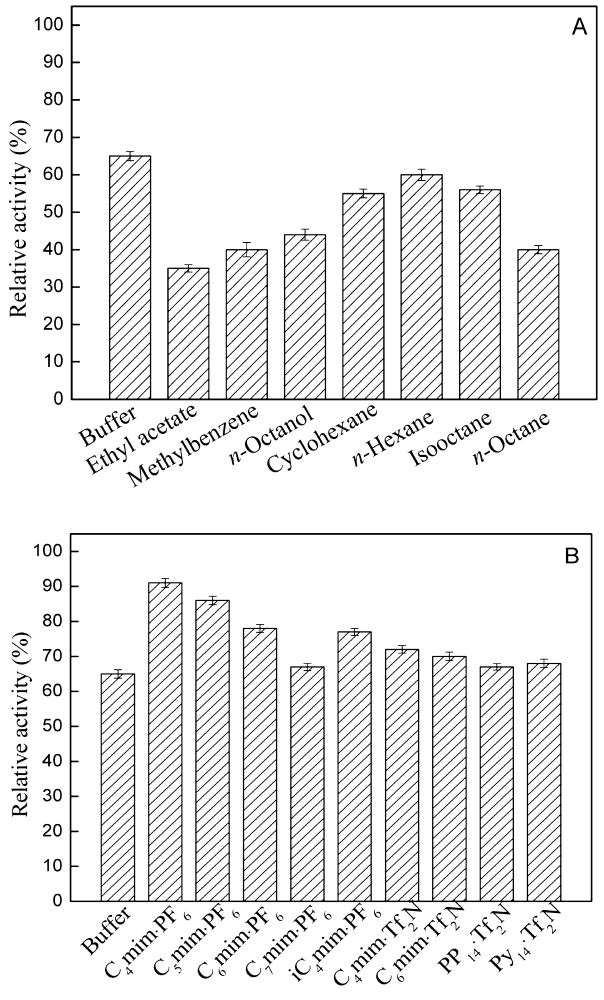

Figure 3. Deactivation of immobilized *Acetobacter sp.* CCTCC M209061 cells in the presence of EHB product (35 mmol/L) in various biphasic systems containing (A) organic solvents and (B) ILs. In each case, the 100% relative activity corresponded to the initial activity of the cells.

Partition coefficients have been identified as important criteria for the preliminary screening of the second phase. Higher partition coefficients between the second phase and aqueous phase could reduce the effect of the toxic substrate and product on the cells. They could also reduce the pronounced inhibition of the reaction by the substrate and the product in the aqueous monophasic system.[41,42] From the data summarized in Table 2, most of the tested ILs possessed considerably higher EAA and EHB partition coefficients than the tested organic solvents, except for trichloromethane and ethyl acetate. In the tested biphasic systems, all the partition coefficients of EAA were significantly higher than those of EHB, because of the stronger lipophilic property of the former. Moreover, combined with the results in Table 1, a higher partition coefficient of the substrate and product between two phases, which less toxic reactants stayed with biocatalysts in the aqueous phase and this brought less damage to cells, was correlated with higher biocompatibility of ILs with the biocatalysts, which has an outstanding effect on the overall process efficiency.

Overall, the diverse ILs showed significant but varying effects on the catalytic performance of immobilized *Acetobacter sp.* CCTCC M209061 cells and the bioreduction reaction. Interestingly, the initial reaction rate, yield, and metabolic activity retention are tightly associated with the partition coefficients of the substrate EAA (Ps) in the IL/aqueous system. Indeed, an approximate linear relationship can be obtained by drawing the initial reaction rate, yield, and cell viability against the partition coefficients (Figure 4). As illustrated in Figure 4, the initial reaction rate, yield, and metabolic activity retention all increase as Ps rises, which indicates that the ILs affect the activity of the biocatalyst by greatly influencing the substrate concentration in the aqueous layer around the biocatalyst. Therefore, the superior performance of the biocatalyst found in the IL-containing system could be attributed to the markedly reduced effect of the toxic substrate on the immobilized cells and substrate inhibition, since the IL could effectively extract the substrate from the aqueous phase. This finding was consistent with that of Zhang, Yang, and Robb, in which Ps had a systematic relationship with the activity of biocatalysts in organic solvent/aqueous systems and water-immiscible IL/aqueous systems.[43,44]

In this study, the best bioreduction results and excellent cell biocompatibility were observed in the C_4mim·PF_6/buffer biphasic system, which was consistent with many other published papers.[10,26,42] Hence, C_4mim·PF_6 was chosen as the best second phase in the IL/buffer biphasic system for subsequent experiments.

3.2. Effects of Key Variables on the Biocatalytic Reduction of EAA. For a better understanding of the bioreduction performed in the biphasic system containing C_4mim·PF_6, the effects of several influential variables on the reaction were studied.

As shown in Figure 5A, the volume ratio of the aqueous phase to the IL phase (V_{aq}/V_{IL} (mL/mL)) substantially affected the initial reaction rate and the yield, but only slightly affected the product *e.e.* The obvious enhancement in the initial reaction rate and the maximum yield with the increase of V_{aq}/V_{IL} to 1/2 may be due to the lesser chance for the cells to come into contact with the IL. Further increasing V_{aq}/V_{IL} led to a decline in the initial reaction rate. Therefore, the optimum V_{aq}/V_{IL} value for the reaction is 1/2.

The initial reaction rate in the C_4mim·PF_6-based biphasic system markedly increased as the substrate concentration increases from 5 mmol/L to 55 mmol/L (based on the volume of the entire system), while the maximum yield did not vary significantly (Figure 5B). However, further increasing substrate concentration above 55 mmol/L led to a clear drop in the

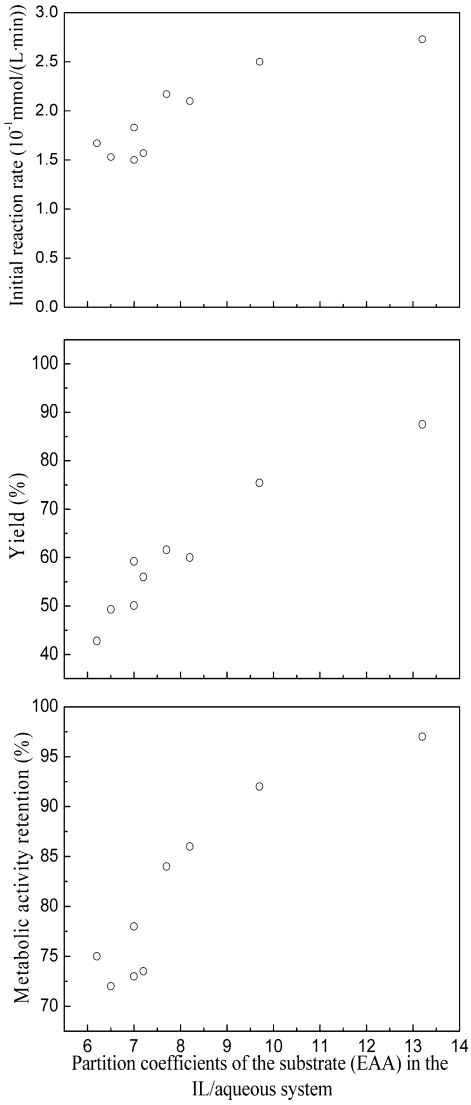

Figure 4. Effect of partition coefficients of the substrate (EAA) on the initial reaction rate, yield, and cell viability.

initial rate and maximum yield, possibly due to the relatively pronounced inhibition of the substrate when the substrate concentration exceeded 55 mmol/L. Among the tested range, the product *e.e.* remained above 99%.

It is well-known that buffer pH plays an important role in bioreduction.[45] Figure 5C illustrates the significant effect of buffer pH on the reaction in the C_4mim·PF_6/buffer biphasic system. When buffer pH increased from 4.0 to 5.5, the reaction accelerated and the maximum yield increased. Further increasing buffer pH led to a poor reaction rate and maximum production yield. Clearly, the optimal buffer pH for the reaction is 5.5.

As depicted in Figure 5D, increasing the reaction temperature from 25 °C to 35 °C caused the initial reaction rate to increase from 0.28 mmol/(L min) to 0.35 mmol/(L min), while the maximum yield markedly increased from 68.5% to 90.8%. However, further increasing reaction temperature led to a clear drop in the initial reaction rate and maximum yield, probably because of the inactivation of the cells at a higher temperature for a prolonged period. The product *e.e.* kept above 99% within the tested range. Thus, 35 °C was selected as the most suitable reaction temperature.

The initial reaction rate increased rapidly as the shaking rate was increased to 220 rpm, suggesting that mass transfer was the rate-limiting step (data not shown). However, further increasing the shaking rate had little effect on the initial reaction rate, maximum yield, and product *e.e.*, indicating that 220 rpm was the optimal shaking rate.

Figures 6A−C depicts time-course profiles of the biocatalytic reduction of EAA by immobilized *Acetobacter sp.* CCTCC M209061 cells in the aqueous monophasic system, the *n*-hexane/buffer biphasic system, and the C_4mim·PF_6-containing biphasic system under the optimal conditions for each medium. The initial reaction rate was clearly highest in the C_4mim·PF_6/buffer biphasic system {0.39 mmol/(L·min) vs 0.25 or 0.29 mmol/(L·min)}. This difference might be attributable to the most appropriate substrate concentration in the aqueous phase of the IL-containing biphasic system and the severe inhibiting reactions between the substrate and biocatalysts that occurred in another two systems. The reaction rate decreased sharply as the reacting time increased in the aqueous monophasic system (Figure 6A), possibly because of the pronounced inhibition of the reaction by the reactants. However, the reaction rate decreased relatively slowly over time in the two biphasic systems (Figures 6B and 6C), probably because of in situ extraction of the substrate and the formed product into the second phase. As a result of the higher partition coefficients of EAA and EHB between C_4mim·PF_6 and buffer than that between *n*-hexane and buffer, the reaction time required to reach chemical equilibrium in the C_4mim·PF_6-containing biphasic system was longer than that in the *n*-hexane/buffer biphasic system. However, the highest product yield (90.8%) was obtained in the C_4mim·PF_6/buffer biphasic system within 4 h. The product *e.e.* remained near 99% constantly. Therefore, using the C_4mim·PF_6/buffer biphasic system instead of other two reaction systems can markedly improve the biocatalytic reduction of EAA.

3.3. Preparative Scale Biotransformation in the C_4mim·PF_6-Based Biphasic System. To test the applicability of the biocatalytic reduction of EAA to (*R*)-EHB using immobilized *Acetobacter sp.* CCTCC M209061 cells in the C_4mim·PF_6-based biphasic system, we also carried out the bioreduction on a 450 mL preparative scale using the optimal reaction conditions {55 mmol/L EAA, C_4mim·PF_6/TEA-HCl buffer (100 mmol/L, pH 5.5), V_{aq}/V_{IL} = 1/2, 80 mmol/L glucose, 0.45 g/mL cell-loaded alginate beads, 35 °C, and 220 rpm}. The reaction process was monitored by GC analysis, and the product was extracted from the reaction mixture with acetic ether upon the exhaustion of the substrate. The bioreduction behavior was similar to that shown in Figure 6C. The isolated yield (89.5%) after reacting for 4 h on the 450 mL scale was much higher than that achieved in the aqueous system (75.7%)[9] and the product *e.e.* was excellent (>99%). Furthermore, no emulsification of the IL-based biphasic system was observed, so the phases could be separated readily by centrifugation. No byproducts accumulated in the IL phase, and the IL could be easily recycled, thus reducing the overall cost of the biocatalytic process. Hence, the whole-cell biocatalytic reduction of EAA to (*R*)-EHB on a preparative scale in the C_4mim·PF_6-based biphasic system is a promising and competitive reaction.

3.4. Operational Stability of Immobilized *Acetobacter sp.* CCTCC M209061 Cells. The operational stability of the biocatalyst was investigated in the C_4mim·PF_6-based biphasic system, *n*-hexane-based biphasic system, and the aqueous

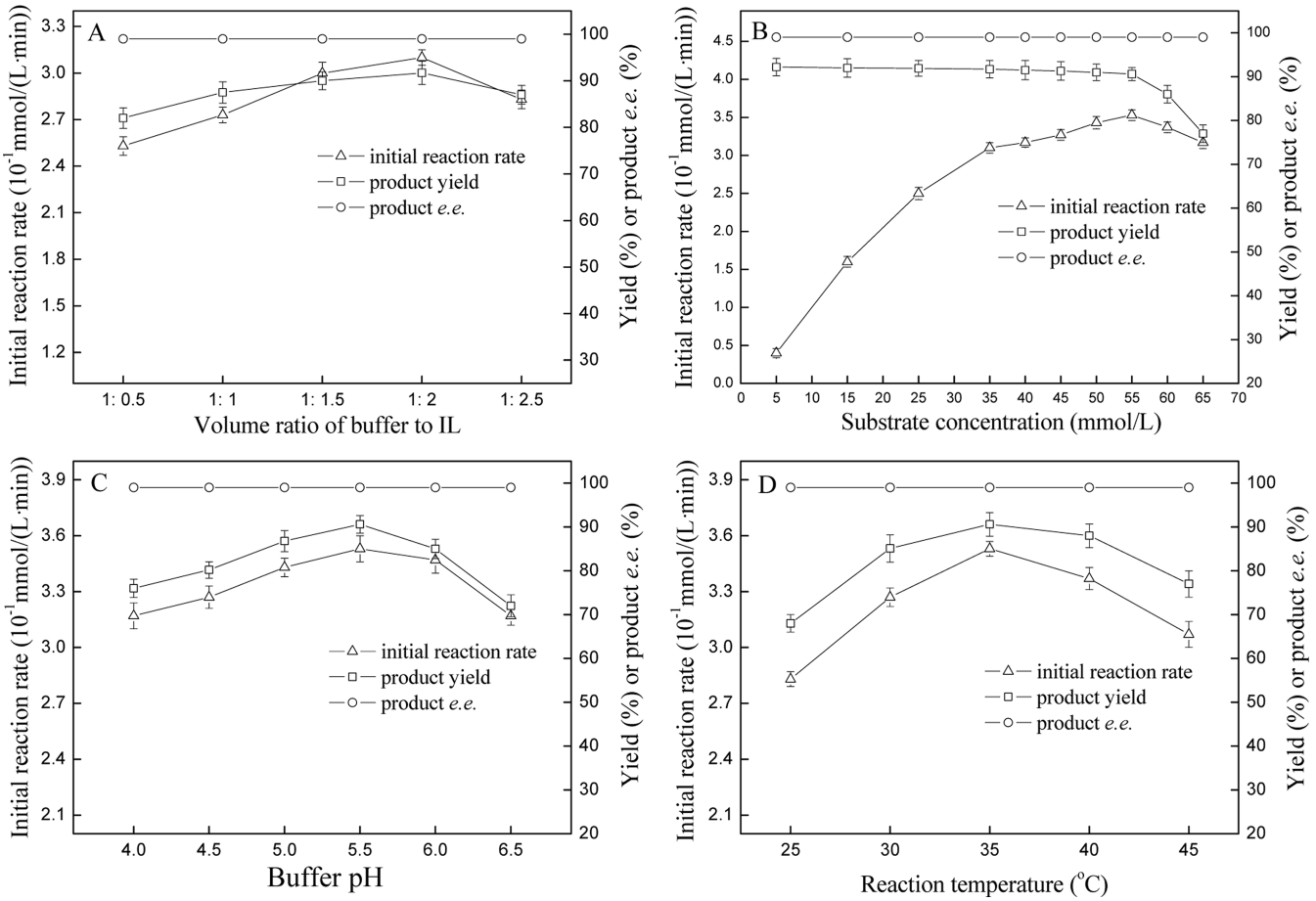

Figure 5. Effects of key variables on the biocatalytic asymmetric reduction of EAA catalyzed by immobilized *Acetobacter sp.* CCTCC M209061 cells in IL-containing biphasic systems: (A) effect of volume ratio of buffer to IL {35 mmol/L EAA; pH 5.5; 80 mmol/L glucose; 0.45 g/mL cell-loaded alginate beads; 35 °C; 200 rpm}; (B) effect of substrate concentration {TEA-HCl buffer (100 mmol/L, pH 5.5)/IL volume ratio of 1/2; 80 mmol/L glucose; 0.45 g/mL cell-loaded alginate beads; 35 °C; 200 rpm}; (C) effect of buffer pH {55 mmol/L EAA; TEA-HCl buffer (100 mmol/L)/IL volume ratio of 1/2; 80 mmol/L glucose; 0.45 g/mL cell-loaded alginate beads; 35 °C; 200 rpm}. (**D**) Effect of reaction temperature {55 mmol/L EAA; TEA-HCl buffer (100 mmol/L, pH 5.5)/IL volume ratio of 1/2; 80 mmol/L glucose; 0.45 g/mL cell-loaded alginate beads; 200 rpm}.

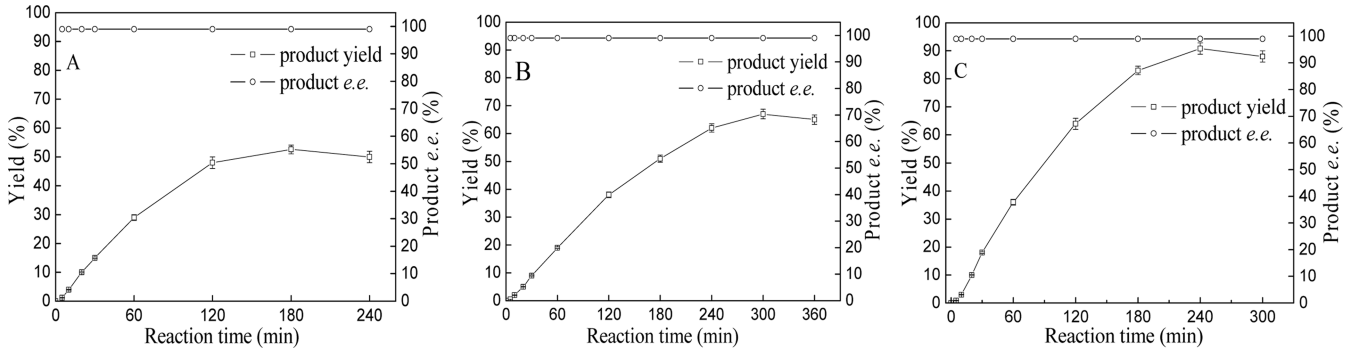

Figure 6. Time-course profiles of the biocatalytic asymmetric reduction of EAA with immobilized *Acetobacter sp.* CCTCC M209061 cells (A) in the aqueous monophasic system {55 mmol/L EAA; 80 mmol/L glucose; 0.45 g/mL immobilized cells; 35 °C, 220 rpm}, (B) in the *n*-hexane/buffer biphasic system {55 mmol/L EAA; TEA-HCl buffer (100 mmol/L, pH 5.5)/*n*-hexane volume ratio of 1/2; 80 mmol/L glucose; 0.45 g/mL immobilized cells; 35 °C; 220 rpm}, and (C) in the C_4mim·PF_6/buffer biphasic system {55 mmol/L EAA; TEA-HCl buffer (100 mmol/L, pH 5.5)/C_4mim·PF_6 volume ratio of 1/2; 80 mmol/L glucose; 0.45 g/mL immobilized cells; 35 °C; 220 rpm}, respectively.

system. As depicted in Figure 7, the immobilized cells retained above 80% of their initial activity after being used repeatedly for 10 batches (4 h per batch) in the C_4mim·PF_6/buffer biphasic system. By contrast, the relative activity of the immobilized cells was only 62.5% after being reused for the same period of time in the aqueous monophasic system and 3.1% in the *n*-hexane/buffer biphasic system after seven batches. The excellent solvent properties of the IL C_4mim·PF_6 for the toxic substrate and product (Table 2) and the good biocompatibility of C_4mim·PF_6 (Figure 2B and Figure 3B) could partly account for these observations. The interactions between the IL and the carrier (calcium alginate)[46] used for the immobilization of

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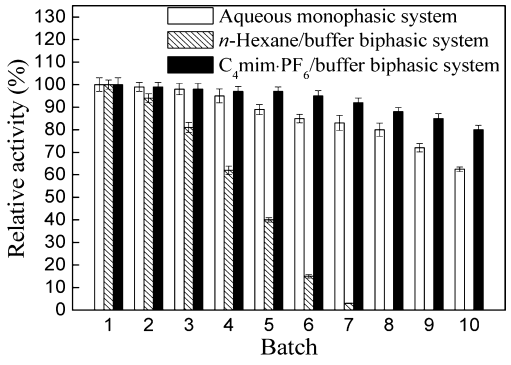

Figure 7. Operational stability of immobilized *Acetobacter sp.* CCTCC M209061 cells in various reaction systems. Reaction conditions with C_4mim·PF_6/buffer biphasic system: 55 mmol/L EAA, C_4mim·PF_6/TEA-HCl buffer (100 mmol/L, pH 5.5) volume ratio = 2/1, 80 mmol/L glucose, 0.45 g/mL cell-loaded alginate beads, 35 °C, 220 rpm, 4 h per batch. Reaction conditions with *n*-hexane/buffer biphasic system: 40 mmol/L EAA, *n*-hexane/TEA-HCl buffer (100 mmol/L, pH 5.5) volume ratio = 2/1, 80 mmol/L glucose, 0.45 g/mL cell-loaded alginate beads, 35 °C, 220 rpm, 4 h per batch. Reaction conditions with aqueous system: 35 mmol/L EAA, 3 mL TEA-HCl buffer (100 mmol/L, pH 5.5), 80 mmol/L glucose, 0.45 g/mL cell loaded alginate beads, 35 °C, 200 rpm, 4 h per batch. The relative activity of the immobilized cells in the first batch was defined as 100%.

Acetobacter sp. CCTCC M209061 cells may also contribute to the good operational stability of the immobilized cells in the C_4mim·PF_6-containing biphasic system.

4. CONCLUSIONS

The synthesis of (*R*)-EHB can be successfully conducted with high yield and excellent product *e.e.* by the biocatalytic asymmetric reduction of EAA using immobilized *Acetobacter sp.* CCTCC M209061 cells in water-immiscible IL-based biphasic systems. Different ILs had significant but varied influence on the bioreduction. Of all the examined ILs, C_4mim·PF_6 was the most effective for the bioreduction. Improved substrate concentration (55 mmol/L vs 35 mmol/L) and yield (90.8% vs 82.6%) were achieved in the C_4mim·PF_6-based system compared to the aqueous system. The cells also retained a much higher relative activity in the C_4mim·PF_6-based system than those in the aqueous system after each set was used repeatedly for 10 batches (80% vs 65%), showing excellent operational stability in the presence of C_4mim·PF_6. The good performance of the biocatalyst in the presence of the IL may be due to the IL's excellent solvent properties for the substrate and product, as well as its good biocompatibility with the cells. Furthermore, the results described here clearly show that the whole-cell biocatalytic process in the presence of C_4mim·PF_6 is feasible up to a scale of 450 mL. If further scaleup is possible, the reaction will be attractive for large-scale industrial applications.

■ ASSOCIATED CONTENT

Supporting Information

Water-immiscible ILs used for the biocatalytic reduction of EAA and their abbreviations, NMR, GC, and HPLC figures of reactants. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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