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Using Choline Chloride-Based DESs as Co-Solvent for 3,5-Bis(trifluoromethyl) Acetophenone Bioreduction with *Rhodococcus erythropolis* XS1012

Hejie Chen [1], Feng Qian [1], Hongxin Lin [1], Weiqing Chen [2] and Pu Wang [1,*]

[1] College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, China; chenhejiezjut@163.com (H.C.); qianfengzjut@163.com (F.Q.); linhongxinzjut@163.com (H.L.)

[2] College of Biological and Environmental Engineering, Zhejiang Shuren University, Hangzhou 310015, China; cwq135790@sina.com

* Correspondence: wangpu@zjut.edu.cn; Tel.: +86-571-8832-0389

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Abstract: (*S*)-3,5-Bistrifluoromethylphenyl ethanol((*S*)-BTPE) is a key pharmaceutical intermediate of the NK-1 receptor antagonist. The asymmetric bioreduction of 3,5-bis(trifluoromethyl) acetophenone (BTAP) to (*S*)-BTPE using *Rhodococcus erythropolis* XS1012 has been established in a phosphate buffer system. To overcome the problem of unsatisfactory yields at high substrate concentration, deep eutectic solvents (DESs) have been introduced to the buffer system. After screening 13 kinds of choline chloride-based DESs, [choline chloride][urea] ([ChCl][U]) showed great influence on the cell activity and significantly increased the cell membrane permeability. Subsequently, some major parameters for this reaction were determined. A remarkable (*S*)-BTPE yield of 91.9% was gained at 150 mM substrate concentration under optimized reaction conditions with >99.9% product enantioselectivity. Compared to reduction in a buffer system, the developed [ChCl][U]-containing system increased the yield from 82.6% to 91.9%. It maintains a yield of 80.7% with the substrate concentration up to 300 mM, compared to only 63.0% in buffer system. This study demonstrated that [ChCl][U] is a feasible co-solvent to improve the bioreduction process.

Keywords: (*S*)-3,5-bistrifluoromethylphenyl ethanol; asymmetric reduction; deep eutectic solvent; biocatalysis

1. Introduction

Chirality is a significant characteristic of drugs and drug candidates that mainly makes a difference in pharmacokinetics, pharmacodynamics, and toxicity [1,2]. Both optical pure 3,5-bistrifluoromethylphenyl ethanol ((*R*)- and (S)-BTPE) is an intermediate for the synthesis of NK-1 receptor antagonist, while (*S*)-BTPE is included in the antagonists currently under clinical evaluation [3]. The chemical method for BTPE isomer preparation always employs transition metals as catalysts, which are expensive and highly toxic. Furthermore, they also have strict requirements for reaction conditions [4]. The biocatalytic process for chiral alcohol synthesis uses whole cells or isolated enzymes from the cells for implementation [5]. Compared to traditional chemical routes, the preparation of chemicals by biocatalysis has many merits, such as high enantioselectivity, energy effectiveness, environment protection, mild reaction conditions, and so on [6,7]. Immobilized *Saccharomyces rhodotorula* cells were used for asymmetric reduction of BTAP to (*S*)-BTPE in the aqueous-organic system with 1.42 g/L substrate, but only achieved the conversion rate at 93.3% with e.e. value of 95.9% [8]. In our previous report, the asymmetric production of (*S*)-BTPE by *Candida tropicalis* 104 cells gained a high yield (70.3%) and excellent enantioselectivity at 50 mM substrate concentration [9]. The above literature indicates that the biocatalytic reduction of (*S*)-BTPE in the

aqueous phase is generally unsatisfied when at high substrate concentration. In a previous study, Li et al. introduced the ionic liquid [N1,1,1,1][Cys] to the bioreduction of (*S*)-BTPE by *Candida tropicalis* 104, which increased BTAP concentration from 50 mM to 90 mM, and the yield from 70.3% to 80.4% [10].

Ionic liquids (ILs) display many unique characteristics, such as almost negligible vapor pressure, excellent chemical and thermal stability, low melting points, low toxicity, and good biocompatibility. Because of their superior performance, they have drawn extensive interest and are considered to be replacements for organic solvents [11,12]. Recently, another kind of interesting solvent for biotransformation and organic synthesis has drawn attention, namely deep eutectic solvents (DESs) [13–16]. Due to their similar properties to ionic liquids, they are also regarded as analogs of ILs, but with the advantages of lower cost of synthesis [17] and easier preparation [18]. DESs generally consist of two or three components, such as choline chloride with a hydrogen donor (such as urea and amino acids). For example, a study using [ChCl][U] as co-solvent has improved the reaction efficiency of steroid 1-en-dehydrogenation biotransformation by *Arthrobacter simplex* [19].

However, little attention has been paid to asymmetric reduction using DESs as the reaction medium by whole-cell biocatalysis. In our previous study, *Rhodococcus erythropolis* XS1012 was found to have the ability for the asymmetric reduction of BTAP to (*S*)-BTPE with high selectivity (>99.9%) but provided poor catalytic efficiency [20]. Choosing a co-solvent appropriately can provide a higher reactant concentration [21], so it is of great interest to carry out the investigation of the influences of choline chloride-based DESs on biocatalysis reduction.

2. Results and Discussion

2.1. The Bioreduction Carried out in Phosphate Buffer System

Rhodococcus erythropolis XS1012 was selected as a biocatalyst for the asymmetric reduction of BTAP to (*S*)-BTPE with superior enantioselectivity (>99.9% e.e.). Figure 1 shows the effect of substrate concentration on the bioreduction in the phosphate buffer system after the optimization of various parameters of the reaction (data not shown); the yield was 82.6% at 150 mM BTAP, and only 63.0% under 300 mM BTAP. To further enhance the product yield under high substrate concentration, DESs were introduced to the reaction system.

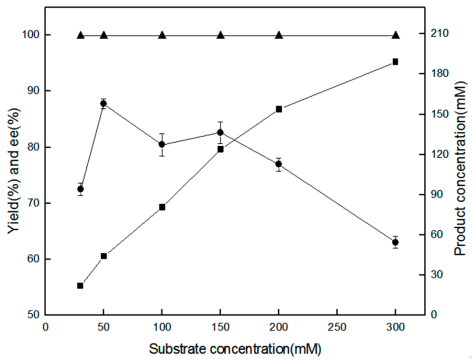


Figure 1. Effect of substrate concentration on the asymmetric reduction of BTAP in the phosphate buffer system. Symbols: (●) yield and (▲) e.e.; (■) product concentration. Reaction conditions: phosphate buffer (Na_2HPO_4-NaH_2PO_4, 100 mM, pH 7.0), 45 g (DCW)/L *R. erythropolis* XS1012, 50 g/L glucose, 15% (*v*/*v*) isopropanol content, various BTAP concentration, and 30 °C, 200 rpm for 24 h.

2.2. Screening of Choline Chloride-Based Deep Eutectic Solvents (DES)

To improve the reduction efficiency of BTAP to (*S*)-BTPE under high substrate concentration, 13 varieties of DESs (in Table S1) were assessed for their behaviors in bioreduction at a weight-to-volume ratio of 1%. The reaction mixture without DES addition was set as the control group. As illustrated in Table 1, [ChCl][U](1:1) and [ChCl][U](1:2) displayed significant promotion of the asymmetric reduction with a yield of 64.9% and 64.3%, respectively, much higher than the control (53.2%). In addition, [ChCl][Cys] and

[ChCl][Tyr] also brought increasing effects, with a yield of 57.7% and 57.6%, respectively. However, the other 9 types of DESs affected the reduction only slightly, and some DES addition in the reaction system even reduced the yield.

Table 1. Effect of DESs on the asymmetric reduction of BTAP. Reaction conditions: 9 mL phosphate buffer (100 mM, pH 7.0), 1% (*w/v*) various DESs, 45 g (DCW)/L *R. erythropolis* XS1012, 1 mL isopropanol (10%, *v/v*), 50 g/L glucose, 150 mM BTAP, 30 °C and 200 rpm for 24 h.

DESs	Yield (%)	e.e. (%)
Control	53.2	>99.9
[ChCl][Ala](1:1)	54.9	>99.9
[ChCl][Cys](1:1)	57.7	>99.9
[ChCl][EG](1:1)	50.0	>99.9
[ChCl][Glu](1:1)	52.7	>99.9
[ChCl][Gly](1:1)	50.5	>99.9
[ChCl][GSH](1:1)	54.9	>99.9
[ChCl][IPA](1:1)	50.1	>99.9
[ChCl][Lys](1:1)	52.1	>99.9
[ChCl][Trp](1:1)	49.4	>99.9
[ChCl][Tyr](1:1)	57.6	>99.9
[ChCl][U](1:1)	64.9	>99.9
[ChCl][U](1:2)	64.3	>99.9
[ChCl][U](2:1)	55.3	>99.9

2.3. Influence of Various DESs on Cell Membrane Permeability

Although the product yield rose after introducing DESs, the drawbacks of DESs such as high viscosity, unknown corrosivity, and instability of mixture [22] may influence the mass transfer of the substrate and product in the DES-containing buffer systems, therefore influencing the efficiency of the reduction. Cell membrane permeability can be represented by leakage of nucleic acids and proteins, and they exhibited maximum ultraviolet absorption peak at 260 and 280 nm, respectively. The cells suspended in phosphate buffer without DES were set as control. The results in Table 2 demonstrate that the OD_{260nm} values all increased in the DES-containing buffer systems. [ChCl][GSH] and [ChCl][U] gave the lowest and highest values, respectively, of the OD_{280nm} compared to the control. We speculate that DESs may cause cell permeability alteration or membrane expansion [23], which increases the frequency of the substrate transformation with the enzyme in cells, and promotes the substrate and product that comes in and out of the cells [24].

Table 2. Effect of various DESs on cell membrane permeability. Reaction conditions:10 mL phosphate buffer (100 mM, pH 7.0), 1% (*w/v*) various DESs, 45 g (DCW)/L *R. erythropolis* XS1012, 30 °C and 200 rpm for 24 h.

DESs	Net OD_{260nm}	Net OD_{280nm}
Control	0.232	0.873
[ChCl][Ala](1:1)	0.274	0.866
[ChCl][Cys](1:1)	0.279	0.932
[ChCl][EG](1:1)	0.242	0.846
[ChCl][Glu](1:1)	0.283	0.860
[ChCl][Gly](1:1)	0.257	0.844
[ChCl][GSH](1:1)	0.274	0.834
[ChCl][IPA](1:1)	0.287	0.844
[ChCl][Lys](1:1)	0.347	0.953
[ChCl][Trp](1:1)	0.275	0.871
[ChCl][Tyr](1:1)	0.241	0.897
[ChCl][U](1:1)	0.292	0.956
[ChCl][U](1:2)	0.296	0.934
[ChCl][U](2:1)	0.281	0.935

2.4. Effect of Various DESs on Cell Activity

To further understand the effect of DESs on *R. erythropolis* XS1012 cells, cell activity was estimated by the value of sugar metabolic retention (MAR), which can be altered with cell tolerance to the DESs and substrate. The cells suspended in phosphate buffer without DESs were set as the control group.

As shown in Figure 2, not all the DESs in the buffer system (with substrate) had a negative influence on cell activity, but the MAR values in all tested DES-containing systems (without substrate) were lower than the control, suggesting that DESs exhibited their toxicity in cells to varying degrees. [ChCl][GSH](1:1) and [ChCl][Glu](1:1) showed serious damage to cell activity, giving lower MAR values of 27.2% and 31.3%, respectively. However, the addition of [ChCl][U](1:1) and [ChCl][U](1:2) exhibited pretty good biocompatibility with the cells at MAR values of more than 76.2% in the absence of substrate, and the MAR value of the [ChCl][Lys](1:1)-containing system showed a maximum value of 98.0%. Hayyan et al. found that choline chloride-based DESs (choline chloride-based with glycerine (GI), ethylene glycol (EG), triethylene glycol (TEG), and urea (U)) had a benign effect on the tested bacteria (including two Gram-positive bacteria, i.e., *Bacillus subtilis* and *Staphylococus aureus*, and two Gram-negative bacteria, i.e., *E. coli* and *Pseudomonas aeruginosa*) and no inhibition from them [25]. That had similar results to our study to some extent.

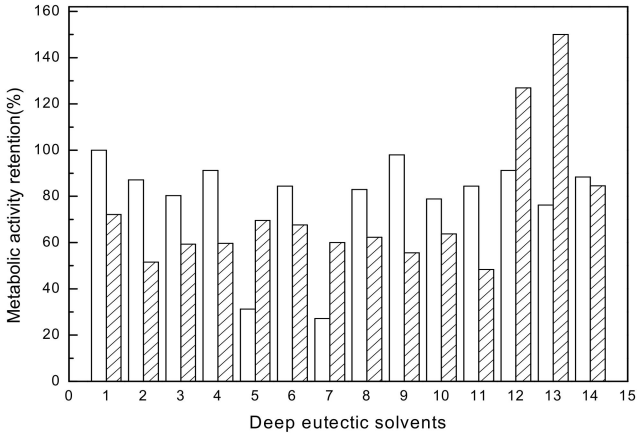


Figure 2. The sugar metabolism activity retention (MAR) of *R. erythropolis* XS1012 cells in various DESs-containing buffer systems without (white) and with (pattern) substrate. (1) Control, (2)[ChCl][Ala](1:1), (3) [ChCl][Cys](1:1), (4) [ChCl][EG](1:1), (5) [ChCl][Glu](1:1), (6) [ChCl][Gly](1:1), (7) [ChCl][GSH](1:1), (8) [ChCl][IPA](1:1), (9) [ChCl][Lys](1:1), (10) [ChCl][Trp](1:1), (11) [ChCl][Tyr](1:1), (12) [ChCl][U](1:1), (13) [ChCl][U](1:2), (14) [ChCl][U](2:1). Conditions: 10 mL phosphate buffer (100 mM, pH 7.0), 1% (*w/v*) various DESs, 45 g (DCW)/L *R. erythropolis* XS1012, with or without 150 mM BTAP, 30 °C and 200 rpm for 3 h, 10 g/L glucose solution for another 3 h.

Obviously, the substrate and DESs both caused cell damage, and all the MAR values in the presence of DESs and substrate individually declined to varying degrees; however, the MAR value increased greatly from 76.2% up to 150% with substrate addition in the [ChCl][U](1:2)-containing system. Also, [ChCl][U](1:1) displayed excellent cell activity compared to those in the absence of substrate, and the MAR value increased from 91.2% to 126.9%. It is possible that the toxicity to cells was weakened under the joint action by [ChCl][U](1:1)/[ChCl][U](1:2) and substrate, or maybe the substrate toxicity to cells was alleviated by DES.

2.5. Effects of [ChCl][U] and Its Components on the Asymmetric Reduction

To examine the effect of [ChCl][U] and its components on the reduction, bioreductions were compared in the presence of ChCl, urea, ChCl and urea, or in a [ChCl][U]-containing system. As shown in Figure 3, all the evaluated systems exhibited improved product yields compared to the control in the phosphate buffer system except for the ChCl-containing system. The [ChCl][U]-containing

system gave the highest yield in *R. erythropolis* XS1012 catalyzed bioreduction. However, ChCl showed a negative influence on bioreduction compared to the phosphate buffer system. The addition of ChCl, urea, ChCl and urea, or [ChCl][U] did not affect the product e.e. values, which were all above 99.9%.

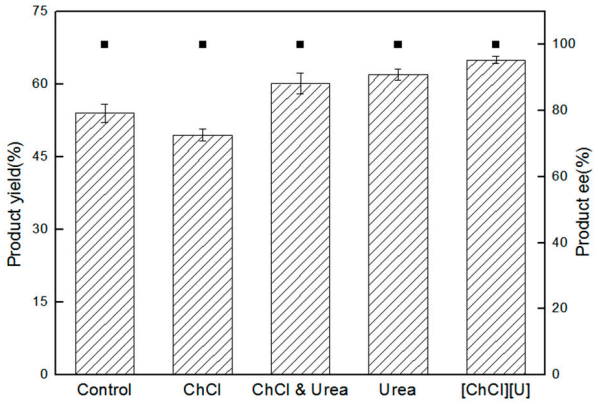


Figure 3. Effect of [ChCl][U] components on the bioreduction of BTAP to BTPE catalyzed by *R. erythropolis* XS1012. Reaction conditions: 9 mL buffer (Na_2HPO_4-NaH_2PO_4, 100 mM, pH 7.0), 45 g (DCW)/L *R. erythropolis* XS1012, 150 mM BTAP, 50 g/L glucose, 10% (*v/v*) isopropanol content, 30 °C and 200 rpm for 24 h. 1% *w/v* ChCl&U, ChCl and Urea were added according to the content of 1% *w/v* [ChCl][U](1:1).

2.6. Effects of [ChCl][U](1:1) Content, Diverse Buffer Systems, Buffer pH on Asymmetric Reduction

As shown in Figure 4a, there was an increase in [ChCl][U](1:1) content up to 1% (*w/v*) with a clear progress in yield, while a further rise [ChCl][U](1:1) content led to a decline in yield. This may have been caused by the high viscosity of the DESs, which led to some problems in transporting; moreover, this could have reduced the mass transfer efficiency [26,27].

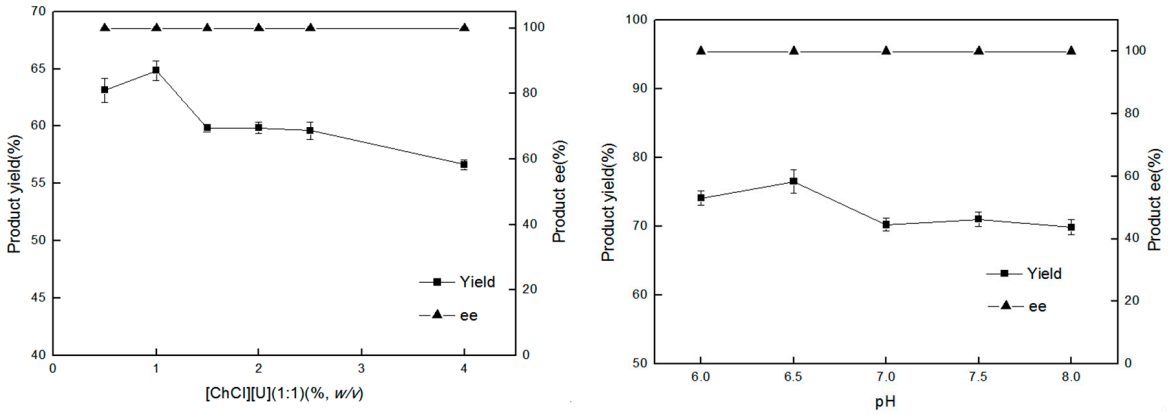


Figure 4. Effect of [ChCl][U](1:1) content (**a**), pH (**b**) on the asymmetric reduction of BTAP. Reaction conditions: 9 mL phosphate buffer (100 mM, pH 7.0) (**a**), various (*w/v*) [ChCl][U](1:1) (**a**), various pH of K_2HPO_4-KH_2PO_4 buffer (**b**), 1% (*w/v*) [ChCl][U](1:1) (**b**), 45 g (DCW)/L *R. erythropolis* XS1012, 1 mL isopropanol (10%, *v/v*), 50 g/L glucose, 150 mM BTAP, 30 °C and 200 rpm for 24 h.

The results in Table 3 imply the influence of diverse buffer systems on bioreduction. After screening five types of buffer systems, the reaction in K_2HPO_4-KH_2PO_4 buffer gave the highest yield of 70.2%. The reaction also gained a yield of 61.3% in the distilled water system. The optimal pH of K_2HPO_4-KH_2PO_4 buffer was also investigated; it revealed that the maximum yield appeared at pH 6.5 (Figure 4b). When there was a continued increase in pH, there was an obvious decrease in the product yield; the product e.e. values were above 99.9% across the range.

Table 3. Effect of different buffer systems on the asymmetric reduction of BTAP. Reaction conditions: 9 mL different buffer (100 mM, pH 7.0), 1% (*w/v*) [ChCl][U](1:1), 45 g (DCW)/L *R. erythropolis* XS1012, 1 mL isopropanol (10%, *v/v*), 50 g/L glucose, 150 mM BTAP, 30 °C and 200 rpm for 24 h.

Buffer System	Yield (%)	e.e. (%)
Na_2HPO_4-NaH_2PO_4	64.9	>99.9
K_2HPO_4-KH_2PO_4	70.2	>99.9
Tris-HCl	66.9	>99.9
Na_2HPO_4-KH_2PO_4	59.4	>99.9
Distilled water	61.3	>99.9

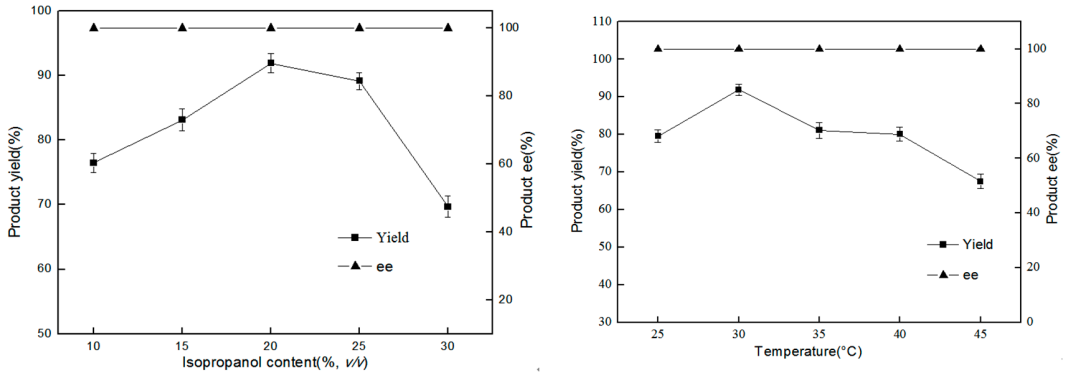
2.7. Effects of the Type and Content of Co-Substrates, Reaction Temperature on the Asymmetric Reduction of BTAP in [ChCl][U]-Containing Phosphate Buffer System

Although bioreduction has many advantages for chiral asymmetric reduction, there still exists a problem regarding the reduction of equivalents in the form of NAD(P)H. The addition of NAD(P)H is neither economically nor technically feasible. Therefore, it is necessary to seek cheap co-substrates to replace the NAD(P)H [28]. Several sugars and alcohols were tested in the reaction as co-substrates. As illustrated in Table 4, after the co-substrate was added, all employed varieties of co-substrates except for methanol and ethanol could promote the reaction to varying degrees. In particular, isopropanol brought the yield markedly up from 0.2% to 76.1%. It is possible that isopropanol acted as the co-substrate, or that isopropanol can enhance the cell membrane permeability and increase the solubility of the substrate that promotes the reaction [29]. However, the joint addition of glucose and isopropanol did not make an obvious difference when compared with the addition of isopropanol individually.

Table 4. Effect of different co-substrates on the asymmetric reduction of BTAP. Reaction conditions: 9 mL phosphate buffer (K_2HPO_4-KH_2PO_4, 100 mM, pH 6.5), [ChCl][U](1:1) 1% (*w/v*), 45 g (DCW)/L *R. erythropolis* XS1012, various co-substrate content, 150 mM BTAP, 30 °C and 200 rpm for 24 h.

Co-Substrates	Content	Yield (%)	e.e. (%)
Control	—	0.2	>99.9
Fructose	50 g/L	12.0	>99.9
Maltose	50 g/L	15.8	>99.9
Glucose	50 g/L	15.8	>99.9
Sucrose	50 g/L	13.5	>99.9
Methanol	10% (*v/v*)	—	>99.9
Ethanol	10% (*v/v*)	2.8	>99.9
Glycerin	10% (*v/v*)	—	>99.9
Isopropanol	10% (*v/v*)	76.1	>99.9
Isopropanol + glucose	10% (*v/v*) + 50 g/L	76.5	>99.9

It is known that organic solvents have toxicity. Isopropanol is less toxic than methanol and ethanol, but can cause impaired gluconeogenesis [30]. In our investigations, the appropriate content of isopropanol (10-30%, *v/v*) (Figure 5a) was measured; the highest yield of 91.9% was obtained with the addition of 20% (*v/v*) isopropanol. Additionally, the impact of the reaction temperature (Figure 5b) on the reaction was also investigated, and 30 °C was determined to be optimal, beyond which there will be a reduction in the product yield. It seems that high reaction temperature is beneficial to the formation of enzyme-substrate complexes and improves the colliding probability between the substrate and enzyme [31]. However, too high a temperature may damage the enzyme structure and lower the enzyme catalytic efficiency.



(**a**) (**b**)

Figure 5. Effects of isopropanol content (**a**), reaction temperature (**b**) on the asymmetric reduction of BTAP. Reaction conditions: phosphate buffer (K_2HPO_4-KH_2PO_4, 100 mM, pH 6.5), [ChCl][U](1:1) 1% (*w/v*), 45 g (DCW)/L *R. erythropolis* XS1012, isopropanol content (20%, *v/v*) (**b**), 150 mM BTAP, 30°C (**a**) and 200 rpm for 24 h.

2.8. Effects of Cell Concentration, Substrate Concentration and Reaction Time on the Asymmetric Reduction of BTAP in [ChCl][U](1:1)-Containing Phosphate Buffer System

Cell concentration is significant for biocatalysis because excessive biomass may make the reaction transformation difficult, while a lack of cells led to a low amount of enzyme. Based on the cell activity experiment, the substrate showed toxicity to the cells, and it is necessary to determine the proper concentration of the substrate. Figure 6a shows the effect of cell concentration on the asymmetric reduction. The yield rose with increasing cell concentration up to 45 g (DCW)/L, further increasing the cell concentration, which accounts for an appreciable loss of product yield. The results in Figure 6b show the influence of substrate concentration on reduction, and the maximum yield of 91.9% was obtained at 150 mM substrate concentration. Additionally, the results in Figure 6c show the impact of the reaction time on the reaction; the product yield rose rapidly from 0 to 24 h, and the best yield of 80.7% for (*S*)-BTPE was achieved at 300 mM of BTAP after a 24 h reaction in the [ChCl][U](1:1)-containing phosphate buffer system, further increasing the reaction time, at which point the product yield started to decrease. During the examinations above, there was no impact on the product e.e. value (>99.9%).

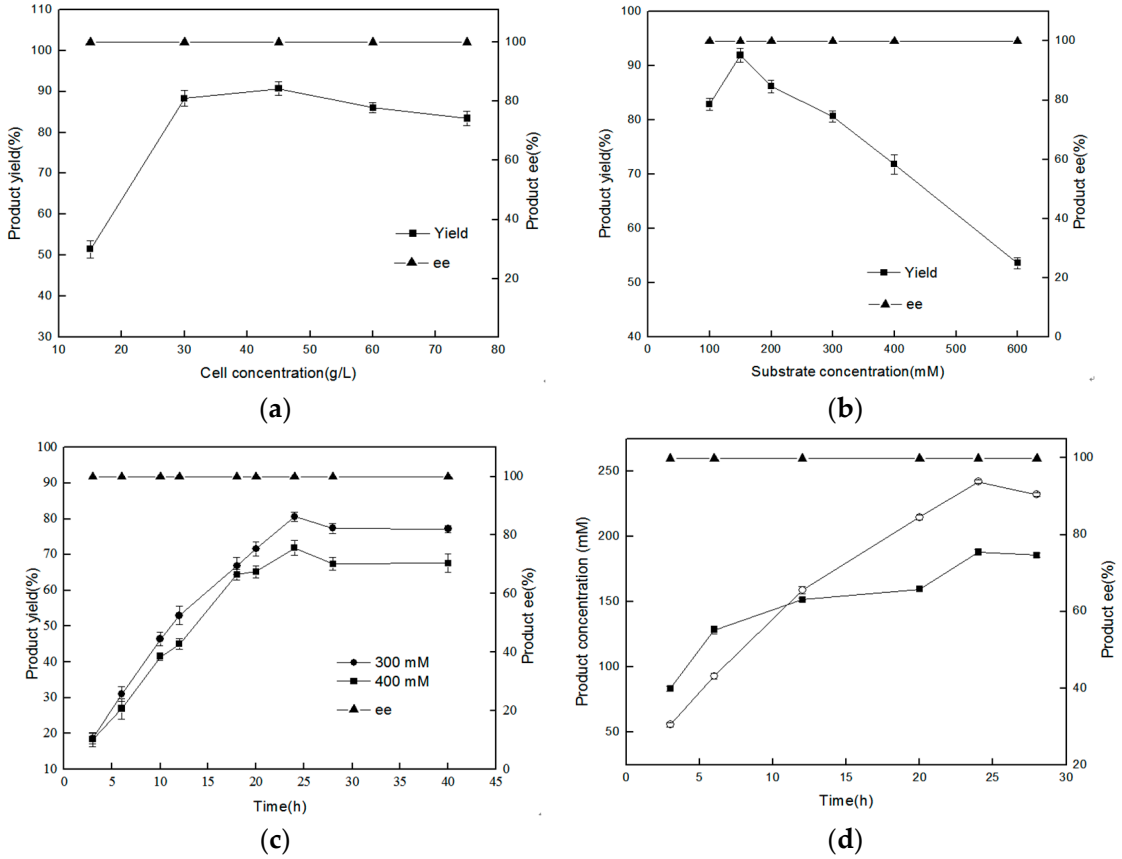


Figure 6. Effects of cell concentration (**a**), substrate concentration (**b**) and reaction time (**c**) on the asymmetric reduction of BTAP. Symbol: (**c**) (●) yield in 300 mM BTAP; (■) yield in 400 mM BTAP; (▲) e.e. in both 300 mM and 400 mM BTAP. (**d**) The effect of reaction time on the asymmetric reduction of BTAP at 300 mM (■) product concentration in phosphate buffer system; (□) product concentration in [ChCl][U](1:1)-containing phosphate buffer system; (▲) e.e. Reaction conditions: 8 mL phosphate buffer (K_2HPO_4-KH_2PO_4, 100 mM, pH 6.5), 45 g (DCW)/L *R. erythropolis* XS1012 (**b**–**d**), [ChCl][U] 1% (*w*/*v*), isopropanol content (20%, *v*/*v*), 150 mM BTAP **(a)**, 30 °C, 200 rpm for 24 h.

2.9. Comparison of the Bioreduction in [ChCl][U](1:1)-Containing Phosphate Buffer System and Phosphate Buffer System

The yield of (*S*)-BTPE at 300 mM BTAP in the optimal [ChCl][U](1:1)-containing phosphate buffer system is completely different than in phosphate buffer system. Under optimal conditions (50 g/L glucose and isopropanol 15% (*v*/*v*) as dual co-substrates, 45 g (DCW)/L *R. erythropolis* XS1012 cells, 300 mM BTAP, 100 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 30 °C and 200 rpm for 24 h), the maximum yield and product e.e. values were 63.0% and over 99.9%, respectively. Comparatively, an increase of 17.7% in yield was achieved with the presence of [ChCl][U](1:1) under the conditions (isopropanol 20% (*v*/*v*), 45 g (DCW)/L *R. erythropolis* XS1012 cells, 300 mM BTAP, 100 mM K_2HPO_4-KH_2PO_4, pH 6.5, 30 °C and 200 rpm for 24 h). These positive results can be explained by the improvement of membrane permeability with the addition of [ChCl][U](1:1). In addition, the addition of [ChCl][U](1:1) also enhanced the cell activity proved by the MAR values. Otherwise, it could be seen that cell tolerance for isopropanol was strengthened in the [ChCl][U](1:1)-containing phosphate buffer system.

3. Materials and Methods

3.1. Chemicals

The substrate BTAP (purity >99%) was purchased from Beijing Golden Olive Company, China. Product (*S*)-BTPE and (*R*)-BTPE (purity >99%) were provided by Hangzhou Xinhai Biotechnology

Co., Ltd., Hangzhou 310052 China. DESs (purity >98%) used in this research were synthesized by Shanghai Fujie Chemical Co., Ltd., Shanghai 201203 China. All other chemicals were from commercial sources and were of analytical grade.

3.2. Culture of the Strain

The strain *R. erythropolis* XS1012 (CCTCC M 2013650) stored in our laboratory can catalyze the reduction of substrate BTAP to (*S*)-BTPE with high enantioselectivity. The single colony of *R. erythropolis* XS1012 was selected from the agar plate after being cultivated for 2–3 days at 30 °C, then inoculated into a 250 mL flask containing 100 mL medium (glucose 15 g/L, yeast extract 6 g/L, peptone 7.5 g/L, $(NH_4)_2SO_4$ 3 g/L, KH_2PO_4 1.5 g/L, NaCl 0.75 g/L, $MgSO_4{\cdot}7H_2O$ 0.75 g/L, pH 6.5). After that, it was incubated at 30 °C, 200 rpm for 24 h as the seed culture. Subsequently, seed culture was transferred into another 100 mL of fermentation medium (glucose 15 g/L, yeast extract 6 g/L, peptone 7.5 g/L, KH_2PO_4 1.5 g/L, $(NH_4)_2SO_4$ 3 g/L, NaCl 0.75 g/L, $MgSO_4{\cdot}7H_2O$ 0.75 g/L, pH 6.5) at a volume ratio of 6% and cultured at 30 °C and 200 rpm for another 48 h. The incubated cells were harvested after centrifugation at 9000 rpm and 4 °C for 10 min, and subjected to biocatalytic reduction.

3.3. Bioreduction Process and Screening of DESs

13 kinds of choline chloride-based DESs (listed in Table 1) were assessed for their behaviors in the reaction. The bioreduction was carried out in 50 mL flasks containing the reaction mixture (total volume of 10 mL) including Na_2HPO_4-NaH_2PO_4 (100 mM, pH 7.0), 45 g (DCW)/L *R. erythropolis* XS1012, 1% (*w/v*) DESs, 150 mM BTAP and used 50 g/L glucose and isopropanol (10%, *v/v*) as co-substrates. The reaction mixture was incubated at 30 °C and 200 rpm for 24 h, and the product yield and e.e. values were determined by chiral gas chromatography analysis. Triplicate runs were performed for each trial.

3.4. GC Analysis Methods

Agilent Technologies 7820A GC system was used to determine the product yield and e.e. values, using a flame ionization detector and Varian CP-Chirasil-Dex CB column (25 m × 0.25 mm × 0.25 μm) with the split ratio at 15:1. The injector and detector temperature were both kept at 250 °C. The column temperature was maintained at 80 °C and kept constant for 2 min, then raised up to 180 °C with a heating rate of 5 °C/min. Nitrogen was used as a carrier gas at a flow rate of 2.0 mL/min. An internal standard method was adopted for the calculations. The results of chiral analysis of the bioconverted sample and standard reference were shown in Figure S1. The retention times for BTAP, dodecane, (*S*)-BTPE, and (*R*)-BTPE were 4.1 min, 8.8 min, 10.1 min, and 10.6 min, respectively.

3.5. Cell Membrane Permeability Assay

The cell membrane permeability of *R. erythropolis* XS1012 cells was assessed in various DESs-containing buffer systems. Conditions: 45 g (DCW)/L *R. erythropolis* XS1012 cells were suspended in 10 mL of Na_2HPO_4-NaH_2PO_4 (100 mM, pH 7.0) in the presence of various choline chloride-based DESs at a content of 1% (*w/v*) or Na_2HPO_4-NaH_2PO_4 buffer (100 mM, pH 7.0), 30 °C and 200 rpm for 24 h.

3.6. Metabolic Activity Retention (MAR) Assay

R. erythropolis XS1012 cells were pre-treated by 13 DESs (1%, *w/v*) respectively, with the addition of 150 mM BTAP or not, substrate or without for 3 h in 10 mL of Na_2HPO_4-NaH_2PO_4 (100 mM, pH 7.0), respectively. Cells (DESs-treatment with the substrate or without) were resuspended in 10 g/L glucose solution to start the sugar metabolism at 30 °C and 200 rpm for another 3 h. Then centrifugation at 9000 rpm and 4 °C for 10 min, the rest of the glucose content was analyzed by the biological sensing analyzer.

3.7. Effect of [ChCl][U] and Its Components on the Asymmetric Reduction

The results of [ChCl][U] and its components on the reduction are shown in Figure 3. Reaction conditions: 9 mL buffer (Na_2HPO_4-NaH_2PO_4, 100 mM, pH 7.0), 45 g (DCW)/L *R.* erythropolis XS1012, 150 mM BTAP, 50 g/L glucose, 10% (*v/v*) isopropanol content, 30 °C and 200 rpm for 24 h. 1% *w/v* ChCl&U, ChCl and Uera were added according to the content of 1% *w/v* [ChCl][U](1:1).

3.8. Impact of Vital Parameters on the Bioreduction

To determine the suitable conditions for the bioreduction of BTAP to (*S*)-BTPE in the [ChCl][U]-containing system, some key parameters, such as the content of the [ChCl][U], the suitable sort and addition of co-substrates, the type of buffer system, buffer pH, the concentration of substrate, and cell and reaction temperature were investigated. The reaction mixtures consisted of buffer (100 mM), the appropriate co-substrates, certain amount of *R. erythropolis* XS1012 cells, [ChCl][U](1:1), and substrate BTAP, incubated at 30 °C, 200 rpm for 24 h. The yield and product e.e. values were assayed by GC analysis.

3.8.1. Optimization of [ChCl][U](1:1) Content, Selection of Buffer Systems and Buffer pH

The optimized results of [ChCl][U] content, the type and pH of the buffer are shown in Table 3 and Figure 4. Reaction conditions: buffer (100 mM), 45 g (DCW)/L *R. erythropolis* XS1012 cells, 150 mM BTAP, 50 g/L glucose, 10% (*v/v*) isopropanol, 30 °C, 200 rpm for 24 h.

3.8.2. Screening of Co-Substrate, Optimization of Co-Substrate Content, Reaction Temperature in [ChCl][U](1:1)-Containing Phosphate Buffer System

The optimized results of the kind and content of co-substrate and the reaction temperature are shown in Table 4 and Figure 5. Reaction conditions: K_2HPO_4-KH_2PO_4 buffer (100 mM, pH 6.5), 45 g (DCW)/L *R. erythropolis* XS1012 cells, 150 mM BTAP, 30 °C, 200 rpm for 24 h.

3.8.3. Optimization of Cell Concentration, Substrate Concentration and Reaction Time in [ChCl][U](1:1)-Containing Phosphate Buffer System

The optimized results of the cell concentration, substrate concentration, and the reaction time are shown in Figure 6. Reaction conditions: K_2HPO_4-KH_2PO_4 (100 mM, pH 6.5), 45 g (DCW)/L *R. erythropolis* XS1012 cells, 150 mM BTAP, 20% isopropanol (*v/v*), 30 °C, 200 rpm for 24 h.

3.8.4. The Comparison of Bioreduction in [ChCl][U](1:1)-Containing Phosphate Buffer System and Phosphate Buffer System

Figure 6d shows the results for the effect of reaction time in phosphate buffer system with [ChCl][U] or not. Reaction conditions: 45 g (DCW)/L *R. erythropolis* XS1012 cells, 300 mM BTAP, 30 °C and 200 rpm, K_2HPO_4-KH_2PO_4 buffer (100 mM, pH 6.5), 20% isopropanol (*v/v*), in [ChCl][U](1:1)(1%, *w/v*)-containing system and Na_2HPO_4-NaH_2PO_4 (100 mM, pH 7.0), 50 g/L glucose and 15% isopropanol (*v/v*) as dual co-substrates in phosphate buffer system.

4. Conclusions

In the study, we screened 13 kinds of choline chloride-based DESs to examine whether they can enhance the product yield of the reduction, and investigated the effect of DESs on cell membrane permeability *via* the value of MAR to represent the cell activity. The [ChCl][U](1:1) was regarded as the most suitable DES. Subsequently, we investigated the key variables of the asymmetric reduction to make the reaction system more efficient. Isopropanol was agreed to be the co-substrate with the highest concentration, of 20% (*v/v*), which indicates that *R. erythropolis* XS1012 has benign tolerance to isopropanol. The optimal conditions were determined: 45 g (DCW)/L *R. erythropolis* XS1012, 150 mM BTAP, K_2HPO_4-KH_2PO_4 buffer (100 mM, 6.5), 20% (*v/v*) isopropanol as co-substrate, the addition of [ChCl][U](1:1) was 1% (*w/v*). The reaction was conducted at 30 °C, 200 rpm for 24 h. A yield of 80.7%

was achieved under 300 mM BTAP. Through the research, it was proved that by introducing the DESs to the reaction medium could make a difference to the bioreduction. According to the research into the effect of the DESs on cells, this is probably due to DESs improving cell membranes or weakening the substrates' toxicity to cells. This research may enrich the investigation regarding the toxicity of DESs to cells and provide a valuable reference regarding the performance of DESs in bioreduction.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/1/30/s1, Figure S1: Chiral analysis of the bioconverted sample. Table S1: Abbreviations of DESs used in this study.

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