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Bioreduction of 3,5-bis(trifluoromethyl)acetophenone using ionic liquid as a co-solvent catalyzed by recombinant *Escherichia coli* cells

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Highlights

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- A novel quaternary ammonium-based ionic liquid was firstly evaluated in bioreduction.
- Reduction of BTAP to (*R*)-BTPE was conducted in a [N1,1,1,1][Cys]-containing system.
- Using [N1,1,1,1][Cys] as co-solvent can improve the whole cell-mediated reduction.
- The product titer, yield and productivity are 252.7 g/L, 98.7 % and 21.058 g/(L · h).
- The bioreduction process is feasible on a 5-litre fermentor preparative scale.

Abstract

We investigated the asymmetric bioreduction of 3,5-bis(trifluoromethyl)acetophenone (BTAP) to (*R*)-[3,5-bis(trifluoromethyl)phenyl] ethanol ((*R*)-BTPE) in a hydrophilic quaternary ammonium-based ionic liquid (IL)-containing system to improve the efficiency of bioreduction catalyzed by recombinant *Escherichia coli* cells overexpressing carbonyl reductase. Based on the low toxicity to microbial cells and moderately increased cell membrane permeability, tetramethylammonium cysteine ([N1,1,1,1][Cys]) was selected and employed as co-solvent. Some key reaction parameters involved in the bioreduction were also investigated in the [N1,1,1,1][Cys]-containing system. The optimum conditions for the process were found to be: 3.5 % (w/v) [N1,1,1,1][Cys], 20 % (v/v) isopropanol, 1 M BTAP, 12.7 g/L of recombinant *E. coli* cells, pH 6.8, reaction for 12 h at 30 °C. A 98.7 % yield (with > 99 % of enantiomeric excess (ee)) was obtained under the optimum conditions. The biocatalytic process was scaled up to a 5-litre fermentor afforded high reaction yield in IL-containing system. The results demonstrated that the IL [N1,1,1,1][Cys] is a useful co-solvent to improve bioreduction process and may has potential applications in

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various biocatalytic reactions.

Keywords

Biocatalysis; Asymmetric bioreduction; Recombinant DNA; Bioprocess design; Ionic liquid-containing system; Fed-batch culture

1. Introduction

Chiral alcohols are among the most valuable building blocks for the manufacture of pharmaceuticals. (*R*)-[3,5-bis(trifluoromethyl)phenyl] ethanol ((*R*)-BTPE) is a key chiral intermediate for the synthesis of Aprepitant (Emend®) with an annual sale of approximately 387 million dollars in 2010 and Fosaprepitant (Ivemend®), a tachykinin NK1 receptor antagonist widely administered to cancer patients for the treatment of chemotherapy-induced nausea and vomiting [1-3]. Currently, (*R*)-BTPE is obtained mainly through the transitional metal-catalyzed asymmetric reduction of the 3,5-bis(trifluoromethyl)acetophenone (BTAP), while the drawbacks of this method are the utilization of air sensitive and expensive catalysts, and/or hazardous reagents. In light of the above description, the search for novel approaches for highly efficient synthesis of enantiometrically pure BTPE is becoming increasingly important. Recently, the asymmetric reduction of BTAP using microbial resting cells and various enzymes have attracted more attention due to their environmental friendliness, mild reaction conditions, and excellent regio-, stereo-, and chemoselectivity. Several studies on microbial reduction of BTAP to (*R*)-BTPE have been reported so far by us and other groups [4-9]. However, the product yield is unsatisfactory at high BTAP concentration, possibly owing to the problems arisen from the relatively poor solubility of the substrate

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in aqueous media, and/or its toxicity to the biocatalyst, which cumbers the practical application of this bioprocess [5, 6, 10].

Over the past decades, it was found that biocatalysts were also able to work in non-aqueous media such as organic solvents and ionic liquids (ILs) [11-15]. In particular, as clean alternative to conventional organic solvents, ILs can be used as reaction media for performing biocatalytic reactions [16, 17]. ILs are a promising new class of functional solvents with unique properties (nonvolatile, non-flammable and highly stable), and able to dissolve a variety of polar and nonpolar compounds, thus exhibiting great potential for environment-friendly green solvents. Moreover, the properties of ILs can be easily tuned to match the specific requirements of particular process by altering the cations and/or anions. As a result, the catalytic performance of enzyme or microbial cells may be improved in IL-containing systems [18-19]. Recently, the biocatalytic asymmetric reduction with ILs in reaction systems has been investigated, and some valuable results have been obtained in terms of the activity [15, 20], enantioselectivity [21] and stability [22] of biocatalysts. However, the reported types of ILs used in reaction systems for whole cell-mediated biocatalysis remain limited, and mainly focus on conventional imidazolium and pyridinium-based ILs. Moreover, most of the ILs used, with recombinant *E. coli* cells as biocatalyst, were hydrophobic ILs and mainly selected for the IL/water two-phase system [23, 24]. Hydrophilic ILs, especially used as co-solvent in aqueous system for recombinant *E. coli*-mediated biocatalytic reaction, has largely been unexplored.

Hydrophilic surfactant ILs (e.g. alkylimidazolium-based ILs), as a new type of functional ILs and co-solvent, have been investigated in the bioreduction sometimes with remarkable results. The surfactant ILs employed in reaction system can make cell membrane more permeable, not only change the intracellular concentration of substrate

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and product, but also reduce their toxicity to the biocatalysts, thus enhancing the biocatalytic efficiency. He et al. [25] described the use of hydrophilic IL 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) as co-solvent for asymmetric bioreduction of ethyl acetoacetate to ethyl (*R*)-3-hydroxybutyrate catalyzed by *Pichia membranaefaciens* Hansen ZJPH07 cells, and it was found that the addition of [BMIM][BF_4] in aqueous system can markedly reduce the substrate inhibition and moderately improve the enantioselectivity. Similarly, the presence of 1-(2'-hydroxyl)ethyl-3-methylimidazolium nitrate (C_2OHMIM·NO_3) as co-solvent in reaction system can facilitate the biocatalytic reduction of 4'-methoxyacetophenone to (*S*)-1-(4-methoxyphenyl)ethanol with immobilized *Rhodotorula* sp. AS2.2241, and provide significant increase in product yield [26]. In some cases, hydrophilic ILs can not only improve the enzyme stability but also act as enzyme activators, and lead to the improvement of productivity [13, 16]. Therefore, it seems that hydrophilic IL is a promising and attractive co-solvent for whole cell-mediated biocatalytic processes.

Similar to alkylimidazolium-based ILs, quaternary ammonium-based ILs also exhibited surface-active properties, and were regarded as surfactant ILs [27, 28]. To date, only a few studies have been reported about the performances of quaternary ammonium-based surfactant ILs in biocatalytic reactions [29].

In the present study, we focus on the performance evaluation of eight quaternary ammonium-based hydrophilic ILs for efficient synthesis of (*R*)-BTPE by recombinant *E. coli* cells overexpressing an engineered carbonyl reductase (LXCAR-S154Y) from *Leifsonia xyli* HS0904 for the first time. It was found that the bioreduction of BTAP to (*R*)-BTPE could be markedly improved by adding tetramethylammonium cysteine ([N1,1,1,1][Cys]) as co-solvent in aqueous system. To optimize the bioreduction in this developed [N1,1,1,1][Cys]-containing buffer system, the effects of some crucial

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reaction parameters for the synthesis of (*R*)-BTPE were subsequently investigated, such as [N1,1,1,1][Cys] content, buffer pH, reaction temperature, substrate concentration and cell concentration. Moreover, a comparative study was performed either in the presence or in the absence of [N1,1,1,1][Cys], higher reaction yield was achieved in [N1,1,1,1][Cys]-containing buffer system.

2. Material and methods

2.1. Chemicals

Substrate BTAP was supplied by Beijing Golden Olive Company, China. Product (*R*)-BTPE and (*S*)-BTPE were purchased from Capot Chemical Co., Ltd., China. The ILs synthesized in this research were entrusted to Shanghai Chengjie Chemical Co., Ltd., China. All other chemicals were from commercial sources and were of analytical grade.

2.2. Strain and fed-batch cultivation

Recombinant *E. coli* BL21 (DE3), overexpressing an engineered carbonyl reductase (LXCAR-S154Y) from *L. xyli* HS0904, was obtained by directed evolution in our previous study [30].

For the cultivation of recombinant *E. coli* as whole-cell biocatalyst, a fed-batch process was developed. Single colony of recombinant *E. coli* was selected from agar plate, and inoculated into a 500 mL flask containing 100 mL Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) supplemented with 50 μg/mL kanamycin, and incubated for 12 h on a rotary shaker at 37 °C and 200 rpm as the seed culture. The obtained seed culture (4 %, v/v) was then transferred into a 5-litre Biostat® B bioreactor (Braun Biotech Int., Germany) containing 2.5 L fermentation medium, and incubated at 37 °C. The fermentation medium contained: citric acid 3 g/L, glucose 20 g/L, tryptone 30 g/L, yeast extract 20 g/L, NH_4Cl 0.1 g/L, $Na_2HPO_4 \cdot 12H_2O$ 3 g/L,

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KH_2PO_4 6.75 g/L, $(NH_4)_2SO_4$ 5 g/L, $MgSO_4 \cdot 7H_2O$ 1.5 g/L and 6 mL/L of trace metal solution ($FeSO_4 \cdot 7H_2O$ 10 g/L, $ZnSO_4 \cdot 7H_2O$ 2.25 g/L, $MnSO_4 \cdot 5H_2O$ 0.5 g/L, $CaCl_2 \cdot 2H_2O$ 1.35 g/L, $CuCl_2 \cdot 2H_2O$ 1 g/L, $NaMoO_4 \cdot 2H_2O$ 0.1 g/L and H_3BO_3 0.2 g/L). The agitation speed and aeration was set at 800 rpm and 5 L/min. The expression of carbonyl reductase was induced at 33 °C by the addition of lactose to a final concentration of 20 g/L when the OD_{600} reached around 25 (about at 5 h). The pH value of the culture was kept constant at 7.1 throughout the whole process by adding ammonia water or nutrient feeding solution. When an increase in pH signalled the complete consumption of glucose, the nutrient feeding solution was fed automatically by peristaltic pump. The nutrient feeding solution contained 700 g/L of glucose. After induction for 28 h, the incubated recombinant *E. coli* cells were harvested by centrifugation at 4 °C, 9000 rpm for 10 min, and washed twice with saline, and then subjected to biocatalytic reduction.

2.3. Selection of ILs

Eight quaternary ammonium-based hydrophilic ILs (shown in Table 1) were evaluated for their performances in the asymmetric reduction of BTAP to (*R*)-BTPE catalyzed by recombinant *E. coli* cells. The bioreduction was performed in 50-mL Erlenmeyer flasks. The reaction mixtures (total volume of 5 mL) contained 3.82 mL phosphate buffer (200 mM, pH 7.2), 5 g/L recombinant *E. coli* cells (DCW), 1.0 mL isopropanol (20 %, v/v), 200 mM BTAP, and 1.5 % (w/v) ILs. The reaction mixtures were incubated at 30 °C and 200 rpm. After reaction for 10 min and 2 h, samples were taken to determine the initial reaction rate and yield. The yield and ee value of the product were assayed by chiral-GC analysis described in our previous report [9].

2.4. Cell viability assay

The viability of recombinant *E. coli* cells was defined as the ratio of the consumed

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glucose amount by cells pretreated in various IL-containing buffer systems [16, 25]. The recombinant *E. coli* cells were pre-incubated at 30 °C, 200 rpm for 6 h in various 1.5 % (w/v) IL-phosphate buffer (200 mM, pH 7.2) systems in the presence of 200 mM BTAP or not, then adding 10 g/L glucose and cultured for an additional 4 h. The glucose concentrations in the medium were then assayed using biological sensing analyzer.

2.5. Cell membrane permeability assay

The recombinant *E. coli* cells were incubated at 30 °C, 200 rpm in various IL-phosphate buffer (200 mM, pH 7.2) systems, or phosphate buffer (200 mM, pH 7.2) solution only. The cell-free supernatants containing ILs and released intracellular components (primarily nucleic acids and proteins) were withdrawn from the systems at 0 h (as controls) and 6 h respectively, for the measurement of medium OD value. The OD_{260nm} and OD_{280nm} values of samples were determined using ultraviolet spectrophotometer, and the OD value was taken as a direct measure of the ILs' effect on the permeability of cell membrane [20, 26].

2.6. Viscosity of IL and substrate solubility assay

Viscosity of IL and reaction system were measured at the temperature $T = 298.15 \pm 0.02$ K by a rotational automated viscometer Anton Paar Stabinger AMA 5000 M and viscometer ND58S, Shanghai Inspection & Measurement Co. Ltd., respectively. Each data point of the viscosity is the average value of three measurements.

For the determination of substrate solubility, calibration curves were firstly established using the corresponding standard dissolved in n-hexane as the reference. The solubility of the substrate BTAP in the reaction system was determined with a similar method described in our previous report [10].

2.7. Effects of key variables on the asymmetric reduction of BTAP to (R)-BTPE

To determine appropriate conditions for the bioreduction of BTAP to (*R*)-BTPE in the

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[N1,1,1,1][Cys]-containing system, some crucial parameters, including [N1,1,1,1][Cys] content, buffer pH, reaction temperature, substrate concentration and cell concentration, were investigated individually. The reaction mixtures contained phosphate buffer (200 mM), isopropanol (20 %, v/v), certain amount of recombinant *E. coli* cells, [N1,1,1,1][Cys] and substrate BTAP, and were incubated at 30 °C, 200 rpm. Samples were taken to determine the initial reaction rate (reaction for 10 min) and product yield (reaction for 2 h). The yield and product ee were assayed by GC analysis.

2.8. Bioreduction of BTAP to (R)-BTPE in the developed reaction system

The asymmetric reduction of BTAP to (*R*)-BTPE catalyzed by recombinant *E. coli* cells using the developed reaction system either in the presence or in the absence of [N1,1,1,1][Cys] were performed in 50 mL Erlenmeyer flasks (working volume of 5 mL) and a 5-litre bioreactor (working volume of 2 L), respectively. The reaction mixture contained 200 mM phosphate buffer (initial buffer pH 6.8 with 35 g/L [N1,1,1,1][Cys] or initial buffer pH 7.2 without 35 g/L [N1,1,1,1][Cys]), isopropanol (20 %, v/v), 12.7 g/L recombinant *E. coli* cells (DCW) and 800 mM or 1 M BTAP, with or without the addition of 35 g/L [N1,1,1,1][Cys]. The asymmetric reduction was conducted at 30 °C and 200 rpm (Erlenmeyer flask) or 500 rpm (5 L fermentor). The samples were taken from the reaction system at certain time intervals, and assayed by GC analysis for the yield and product ee.

3. Results and discussion

3.1. Effects of various ILs on the asymmetric reduction of BTAP to (R)-BTPE

Eight hydrophilic ILs were evaluated for their performances in the asymmetric reduction of BTAP catalyzed by recombinant *E. coli* cells. As shown in Table 2, the recombinant *E. coli* cells were capable of catalyzing the bioreduction of BTAP in all the

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tested IL-containing buffer systems with above 99 % product ee, but the initial reaction rates and yields displayed great difference for various IL-containing systems. The addition of amino acid-based ILs in reaction system accelerated the bioreduction and resulted in the increased yields (Table 2, entries 2-5), especially [N1,1,1,1][Cys] afforded the highest initial reaction rate and the best yield. However, the initial reaction rate and achieved yield substantially decreased in the presence of $[BF_4]^-$-based ILs (Table 2, entries 6-9). Moreover, it was found that the initial reaction rate and yield decreased with the elongation of the alkyl chain attached to the cation. Our experimental results were similar with the previous reports, in which the relationships between the increasing length of alkyl chain of imidazolium cation and the increasing toxicity of IL to microorganisms had been investigated [15, 20].

Based on the results described above, [N1,1,1,1][Cys] turned out to be the most powerful for the bioreduction. Additionally, various ILs exerted different influences on the recombinant *E. coli* cell-mediated bioreduction, possibly owing to the fact that various ILs display different effects on cell viability and/or cell membrane permeability of recombinant *E. coli* cells, and/or ILs might enter the cells and then affect the enzymes responsible for the bioreduction, which activate or inactivate enzyme activity.

3.2. Cell viability of recombinant E. coli cells in various IL-containing buffer systems

It was reported that ILs had great impact on cell viability and most of ketone substrates and/or their corresponding reduction products exhibit pronounced toxicity to microbial cells [15, 17, 20, 26]. Therefore, the cell viabilities of recombinant *E. coli* cells were examined in various IL-containing buffer systems, with or without the addition of substrate BTAP. As shown in Fig. 1, in the absence of BTAP, the cell viabilities in all the tested IL-containing buffer systems are lower than that in buffer system, suggested that the examined ILs exhibit more or less toxicity to the recombinant *E. coli* cells.

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Moreover, cell viability decreased slightly in all the systems in the presence of BTAP compared with that in the absence of BTAP, possibly due to the substrate toxicity to the cells. However, it is worth noting that the cell viability was markedly improved in the presence of BTAP in [N1,1,1,1][Cys]-containing buffer system compared with that in phosphate buffer only. The results indicated that the addition of [N1,1,1,1][Cys] in reaction system seemed to be able to reduce the substrate toxicity to microbial cells, thus affording the highest initial reaction rate and the best yield in the [N1,1,1,1][Cys]-containing system (Table 2). Similar results were also obtained by Xiao et al. [20] that the toxic effect of 4-(trimethylsilyl)-3-butyn-2-one on *Acetobacter* sp. CCTCC M209061 cells could be reduced in hydrophilic IL C_2OHMIM·NO_3-containing system. Additionally, it was found that the cell viabilities clearly decreased with the alkyl chain of the cation elongated for tested eight hydrophilic ILs, and the results are consistent with the data of initial reaction rates and yields in Table 2.

3.3. Effect of various ILs on cell membrane permeability of recombinant E. coli cells

ILs might increase the cell membrane permeability and thus allow the substrate and product to pass more quickly in and out of cells, leading to the acceleration of the bioreduction. On the other hand, the increase in cell membrane permeability caused by the addition of ILs might give rise to a detrimental effect on the biocatalysts because the damage to cell membrane may result in cell death and lower the availability of reducing equivalents for the reaction [20, 26, 31]. Thus, it is of great importance to investigate the effect of various ILs on cell membrane permeability of recombinant *E. coli* cells. The results showed that the OD_{260nm} and OD_{280nm} values increased clearly in IL-buffer systems (Table 3), indicating that the cell membrane becomes permeabilized in eight examined IL-containing systems. Of all the tested ILs, $[BF_4]^-$-based quaternary ammonium ILs display greatly increase in cell membrane permeability (Table 3, entries

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6-9), but afford lower reaction efficiency (Table 2, entries 6-9), probably due to the fact that the significant toxicity of $[BF_4]^-$-based ILs to cells resulted in the termination of the reactions (Fig. 1). However, amino acid-based quaternary ammonium ILs can moderately improve the cell membrane permeability (Table 3, entries 2-5) and enhance the reduction efficiency (Table 2, entries 2-5). As shown in Table 3, among eight hydrophilic ILs tested, the lowest OD_{260nm} and OD_{280nm} values were observed with the addition of [N1,1,1,1][Cys], suggesting that [N1,1,1,1][Cys] could modestly increase the cell membrane permeability with negligible toxicity to *E. coli* cells (Fig. 1). These results could well explain the superior performance of cells in the [N1,1,1,1][Cys]-containing buffer system for the bioreduction (Table 2).

3.4. Effects of key variables on asymmetric reduction of BTAP to (R)-BTPE in the [N1,1,1,1][Cys]-containing system

Bioreduction of BTAP to (*R*)-BTPE catalyzed by recombinant *E. coli* cells was optimized in terms of various reaction parameters, such as [N1,1,1,1][Cys] content, reaction temperature, buffer pH, substrate concentration, cell concentration and reaction time.

It was reported that the ionic liquid content in IL-containing buffer system has a great impact on biocatalytic process. The presence of the IL in proper concentration can boost the activity, enantioselectivity and stability of enzymes and microbial cells [18-22]. Thus, the effect of [N1,1,1,1][Cys] content on asymmetric reduction of BTAP with recombinant *E. coli* cells was investigated, and the results are illustrated in Fig. 2. The initial reaction rate and product yield (reaction for 2 h) increased obviously with the increasing content of [N1,1,1,1][Cys] up to 3.5 % (w/v). Further increase in [N1,1,1,1][Cys] content resulted in sharp decrease in both the initial reaction rate and product yield. The results suggested that excessive amount of [N1,1,1,1][Cys] possibly

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caused a high ionic strength of the reaction medium which might inactivate the cells or the enzymes. Throughout the range of [N1,1,1,1][Cys] content (0-5 %, w/v) examined, the product ee remains almost constant (> 99 %). Thus, the optimal [N1,1,1,1][Cys] content was 3.5 % (w/v), with an initial reaction rate of 436.4 μmol/min/g_{cell} and 77.0 % yield. Additionally, the viscosities of [N1,1,1,1][Cys], buffer system and [N1,1,1,1][Cys]-containing system were respectively determined. The results showed that [N1,1,1,1][Cys] exhibits relatively high viscosity (3.24×10^4 mPa·s) compared with that of the buffer system (1.12 mPa·s, 298.15 K). However, the viscosity of the developed [N1,1,1,1][Cys]-containing reaction system in the present study is 1.94 mPa·s. It is demonstrated that the reaction system contained only a minor fraction (3.5 %, w/v) of [N1,1,1,1][Cys] exert negligible effect on mass-transfer of substrate and product in the reduction process. The solubility of substrate BTAP in the developed [N1,1,1,1][Cys]-containing system was also determined. It was found that the solubility of BTAP (51 mg/L) in [N1,1,1,1][Cys] (3.5 %, w/v) with isopropanol (20 %, v/v) was slightly higher than that in isopropanol (20 %, v/v) only (48 mg/L). Therefore, the IL [N1,1,1,1][Cys] is indispensable for improving the bioreduction efficiency catalyzed by recombinant *E. coli* cells. Additionally, the effect of cysteine concentration (0-5 %, w/v) on asymmetric reduction of BTAP was also investigated (data no shown). The results indicated that, compared with the buffer solution, adding 3.5% cysteine in reaction system exhibit no appreciable impact on both the initial reaction rate (314.3 μmol/min/g_{cell} versus 318.7 μmol/min/g_{cell}) and the yield (51.1% versus 52.2%).

The effects of reaction temperature and buffer pH on asymmetric reduction were also investigated in buffer systems with or without the addition of [N1,1,1,1][Cys]. As illustrated in Fig. 3a, the initial reaction rate and the yield were both boosted with increasing reaction temperature from 20 °C to 30 °C. However, the initial reaction rate

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and yield decreased when the temperature was above 30 °C, possibly resulting from the partial inactivation of enzyme within the cells at high temperature. Thus, the optimal reaction temperature was 30 °C. Fig. 3b depicts the significant effect of buffer pH on the bioreduction. It was found that the bioreduction was markedly accelerated with increasing buffer pH from 6.0 to 6.8 in the presence of [N1,1,1,1][Cys], and the initial reaction rate and yield increased significantly. Further increase in buffer pH resulted in decline in both the initial reaction rate and the yield. Similar results were also observed in buffer system without the addition of [N1,1,1,1][Cys]. The optimal buffer pH was 6.8 for the system in the presence of [N1,1,1,1][Cys], which is lower than that in the absence of [N1,1,1,1][Cys] (the optimal buffer pH was 7.2). It is likely that [N1,1,1,1][Cys] as an alkaline compound led to the pH value of reaction system increased to the optimum for the bioreduction. Within the assayed reaction temperature and buffer pH range, the product ee remains intact (> 99 %).

The effects of substrate concentration and cell concentration on the asymmetric reduction of BTAP were also investigated. The results indicated that the initial reaction rate enhanced obviously with the increasing substrate concentration up to 1 M (256 g/L), beyond which further increase in substrate concentration decrease the initial reaction rate due to substrate inhibition (Fig. 3c). Fig. 3d shows the effect of cell concentration on the asymmetric reduction, and the maximal initial reaction rate was obtained at 12.7 g/L (DCW) of cell concentration. Both substrate concentration and cell concentration showed no influences on the enantioselectivity (remained > 99 %).

3.5. Bioreduction of BTAP to (R)-BTPE in the developed reaction system

The time courses of BTAP reduction catalyzed by recombinant *E. coli* cells using the developed reaction system in Erlenmeyer flasks were illustrated in Fig. 4. The results indicated that a best yield of 99.1 % for (*R*)-BTPE was obtained at 800 mM of BTAP in

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12 h in the [N1,1,1,1][Cys]-buffer system. Further increase of BTAP concentration up to 1 M, the maximal concentration of 987 mM (252.7 g/L) for (*R*)-BTPE was achieved in 12 h in the [N1,1,1,1][Cys]-buffer system, an increase of 173 mM compared with that in buffer system in the absence of [N1,1,1,1][Cys]. To our knowledge, this is the highest product titer (252.7 g/L) and productivity (21.058 g/(L·h)) reported so far for the biocatalytic asymmetric reduction of BTAP to (*R*)-BTPE (Table 4). Additionally, the product ee remains almost constant (> 99 %) throughout the whole process. The results suggested that the recombinant *E. coli* whole cell-mediated biocatalytic process with [N1,1,1,1][Cys] is promising for efficient synthesis of (*R*)-BTPE.

A comparative study on preparative scale bioreduction of BTAP to (*R*)-BTPE using the developed reaction system with or without the addition of [N1,1,1,1][Cys] was carried out in a 5-litre fermentor (working volume of 2 L). Higher yield (93.9 % versus 80.9 %) could be achieved at 1 M BTAP concentration after reaction for 12 h in the IL-containing system. In spite of being slightly lower than that obtained in shaking culture with total volume of 5.0 mL, the achieved yield of (*R*)-BTPE is much higher than the previously reported [30]. The product ee remained above 99 % in reaction systems both with or without the addition of [N1,1,1,1][Cys]. Based on above results, the bioredcution process in the developed reaction system was shown to be feasible on a 5-litre fermentor preparative scale.

4. Conclusions

The present study demonstrated that using IL [N1,1,1,1][Cys] as co-solvent could substantially improve the biocatalytic asymmetric reduction of BTAP to (*R*)-BTPE with recombinant *E. coli* cells. The addition of [N1,1,1,1][Cys] in whole-cell-catalyzed reaction system can moderately increase the cell membrane permeability and improve

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substrate accessibility to the intracellular carbonyl reductase, reduce the toxicity of substrate to the biocatalyst. The developed biocatalytic process is scalable and has potential applications in other biocatalytic reactions. The related further investigations are now underway in our laboratory, and some valuable results have been obtained. The IL [N1,1,1,1][Cys], as an environment-friendly green solvents which the anion involved is directly derived from non-toxic renewable materials (natural α-amino acid), is a promising and attractive co-solvent for whole cell-mediated biocatalytic processes.

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References

[1] Y. Jin, X. Wu, Y. Guan, D. Gu, Y. Shen, Z. Xu, Efficacy and safety of aprepitant in the prevention of chemotherapy-induced nausea and vomiting: a pooled analysis, J. Chen, Support. Care Cancer 20 (2012) 1815-1822.

[2] S. Nakade, T. Ohno, J. Kitagawa, Y. Hashimoto, M. Katayama, H. Awata, Y. Miyata, Population pharmacokinetics of aprepitant and dexamethasone in the prevention of chemotherapy-induced nausea and vomiting, Cancer Chemoth. Pharm. 63 (2008) 75-83.

[3] C.E. Wu, C.C. Liaw, Using aprepitant as secondary antiemetic prophylaxis for cancer patients with cisplatin-induced emesis, Support. Care Cancer 20 (2012) 2357-2361.

[4] P. Gai, C.G. Tang, J.Y. Liu, Y. Liu, C. Zhang, Z.L. Wu, Asymmetric *anti*-Prelog

ACCEPTED MANUSCRIPT

reduction of 3,5-bis(trifluoromethyl)-acetophenone by *Microbacterium oxydans* C3, Chin. J. Appl. Environ. Biol. 19 (2013) 37-42.

[5] M. Gelo-Pujic, F. Le Guyader, T. Schlama, Microbial and homogenous asymmetric catalysis in the reduction of 1-[3,5-bis(trifluoromethyl)phenyl] ethanone, Tetrahedron: Asymmetry 17 (2006) 2000-2005.

[6] E.B. Kurbanoglu, K. Zilbeyaz, M. Taskin, N.I. Kurbanoglu, Total production of (*R*)-3,5-bistrifluoromethylphenyl ethanol by asymmetric reduction of 3,5-bis (trifluoromethyl)acetophenone in the submerged culture of *Penicillium expansum* isolate, Tetrahedron: Asymmetry 20 (2009) 2759-2763.

[7] J. Li, P. Wang, J.Y. He, J. Huang, J. Tang, Efficient biocatalytic synthesis of (*R*)-[3,5-bis (trifluoromethyl) phenyl] ethanol by a newly isolated *Trichoderma asperellum* ZJPH0810 using dual cosubstrate: ethanol and glycerol, Appl. Microbiol. Biotechnol. 97 (2013) 6685-6692.

[8] Y. Liu, T.X. Tang, X.Q. Pei, C. Zhang, Z.L. Wu, Identification of ketone reductase ChKRED20 from the genome of *Chryseobacterium* sp. CA49 for highly efficient anti-Prelog reduction of 3,5-bis (trifluoromethyl) acetophenone, J. Mol. Catal. B: Enzym. 102 (2014) 1-8.

[9] P. Wang, J.B. Cai, Q. Ouyang, J.Y. He, H.Z. Su, Asymmetric biocatalytic reduction of 3,5-bis (trifluoromethyl) acetophenone to (1*R*)-[3,5-bis (trifluoromethyl) phenyl] ethanol using whole cells of newly isolated *Leifsonia xyli* HS0904, Appl. Microbiol. Biotechnol. 90 (2011) 1897-1904.

[10] Q. Ouyang, P. Wang, J. Huang, J.B. Cai, J.Y. He, Efficient enantioselective synthesis of (*R*)-[3,5-bis (trifluoromethyl) phenyl] ethanol by *Leifsonia xyli* CCTCC M 2010241 using isopropanol as co-substrate, J. Microbiol. Biotechnol. 23 (2013) 343-350.

ACCEPTED MANUSCRIPT

[11] A.M. Klibanov, Improving enzymes by using them in organic solvents, Nature 409 (2001) 241-246.

[12] H. Noritomi, K. Suzuki, M. Kikuta, S. Kato, Catalytic activity of α-chymotrypsin in enzymatic peptide synthesis in ionic liquids. Biochem. Eng. J. 47 (2009) 27-30.

[13] O. Dipeolu, E. Green, G. Stephens, Effects of water-miscible ionic liquids on cell growth and nitro reduction using *Clostridium sporogenes*, Green Chem. 11 (2009) 397-401.

[14] M. Singh, R.S. Singh, U.C. Banerjee, Stereoselective synthesis of (*R*)-1-chloro-3(3,4-difluorophenoxy)-2-propanol using lipases from *Pseudomonas aeruginosa* in ionic liquid-containing system, J. Mol. Catal. B: Enzym. 56 (2009) 294-299.

[15] X.T. Wang, D.M. Yue, M.H. Zong, W.Y. Lou, Use of ionic liquid to significantly improve asymmetric reduction of ethyl acetoacetate catalyzed by *Acetobacter* sp. CCTCC M209061 cells, Ind. Eng. Chem. Res. 52 (2013) 12550-12558.

[16] C. Kohlmann, N. Robertz, S. Leuchs, Z. Dogan, S. Lütz, K. Bitzer, S. Na'amnieh, L. Greiner, Enhanced stability and activity of cellulase in an ionic liquid and the effect of pretreatment on cellulose hydrolysis, J. Mol. Catal. B: Enzym. 68 (2011) 147-153.

[17] Y.N. Li, X.A. Shi, M.H. Zong, C. Meng, Y.Q. Dong, Y.H. Guo, Asymmetric reduction of 2-octanone in water/organic solvent biphasic system with Baker's yeast FD-12, Enzyme Microb. Technol. 40 (2007) 1305-1311.

[18] M. Naushad, Z.A. ALOthman, A.B. Khan, M. Ali, Effect of ionic liquid on activity, stability, and structure of enzymes: a review, Int. J. Biol. Macromol. 51 (2012) 555-560.

[19] M. Moniruzzaman, K. Nakashima, N. Kamiya, M. Goto, Recent advances of

ACCEPTED MANUSCRIPT

enzymatic reactions in ionic liquids, Biochem. Eng. J. 48 (2010) 295-314.

[20] Z.J. Xiao, P.X. Du, W.Y. Lou, H. Wu, M.H. Zong, Using water-miscible ionic liquids to improve the biocatalytic anti-Prelog asymmetric reduction of prochiral ketones with whole cells of *Acetobacter* sp. CCTCC M209061, Chem. Eng. Sci. 84 (2012) 695-705.

[21] T. Itoh, Y. Matsushita, Y. Abe, S.H. Han, S. Wada, S. Hayase, Y. Hirose, Increased enantioselectivity and remarkable acceleration of lipase-catalyzed transesterification by using an imidazolium PEG–alkyl sulfate ionic liquid, Chem. Eur. J. 12 (2006) 9228-9237.

[22] S. Bose, C. A. Barnes, J. W. Petrich, Enhanced stability and activity of cellulase in an ionic liquid and the effect of pretreatment on cellulose hydrolysis, Biotechnol. Bioeng. 109 (2012) 434-443.

[23] H.J. Choi, K.N. Uhm, H.K. Kim, Production of chiral compound using recombinant *Escherichia coli* cells co-expressing reductase and glucose dehydrogenase in an ionic liquid/water two phase system, J. Mol. Catal. B: Enzym. 70 (2011) 114-118.

[24] S. Bräutigam, D. Dennewald, M. Schürmann, J. Lutje-Spelberg, W.R. Pitner, D. Weuster-Botz, Whole-cell biocatalysis: Evaluation of new hydrophobic ionic liquids for efficient asymmetric reduction of prochiral ketones, Enzyme Microbl. Technol. 45 (2009) 310-316.

[25] J.Y. He, L.M. Zhou, P. Wang, L. Zu, Microbial reduction of ethyl acetoacetate to ethyl (*R*)-3-hydroxybutyrate in an ionic liquid containing system, Process Biochem. 44 (2009) 316-321.

[26] W.Y. Lou, W. Wang, R.F. Li, M.H. Zong, Efficient enantioselective reduction of 4′-methoxyacetophenone with immobilized *Rhodotorula* sp. AS2.2241 cells in a

ACCEPTED MANUSCRIPT

hydrophilic ionic liquid-containing co-solvent system, J. Biotechnol. 143 (2009) 190-197.

[27] P. Brown, C. Butts, R. Dyer, J. Eastoe, I. Grillo, F. Guittard, R. Heenan, Anionic surfactants and surfactant ionic liquids with quaternary ammonium counterions, Langmuir 27 (2011) 4563-4571.

[28] J. Pernak, M. Smiglak, S.T. Griffin, W.L. Hough, T.B. Wilson, A. Pernak, R.D. Rogers, Long alkyl chain quaternary ammonium-based ionic liquids and potential applications, Green Chem. 8 (2006) 798-806.

[29] J. Li , P. Wang , J. Huang , J. Sun, Design and application of a novel ionic liquid with the property of strengthening coenzyme regeneration for whole-well bioreduction in an ionic liquid-distilled water medium, Bioresour. Technol. 175 (2015) 42-50.

[30] N.Q. Wang, J. Sun, J. Huang, P. Wang, Cloning, expression, and directed evolution of carbonyl reductase from *Leifsonia xyli* HS0904 with enhanced catalytic efficiency, Appl. Microbiol. Biotechnol. 98 (2014) 8591-8601.

[31] S.A. Gangu, L.R. Weatherley, A.M. Scurto, Whole-cell biocatalysis with ionic liquids, Curr. Org. Chem. 13 (2009) 1242-1258.

Figure Captions

Fig. 1. The cell viability of recombinant *E. coli* cells in buffers containing various ILs.

Fig. **2.** Effect of [N1,1,1,1][Cys] content on asymmetric reduction of BTAP with recombinant *E. coli* cells. Symbols: (○) yield; (●) initial reaction rate. Reaction conditions: 200 mM of BTAP, 5 g/L recombinant *E. coli* cells (DCW), 20 % (v/v)

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isopropanol.

Fig. **3.** Effects of reaction temperature (a), buffer pH (b), substrate concentration (c) and cell concentration (d) on asymmetric reduction of BTAP with recombinant *E. coli* cells. Symbols: (○) yield with [N1,1,1,1][Cys] and (□) yield without [N1,1,1,1][Cys]; (●) initial reaction rate with [N1,1,1,1][Cys] and (■) initial reaction rate without [N1,1,1,1][Cys]. Reaction conditions: 3.5 % (w/v) [N1,1,1,1][Cys], 20 % (v/v) isopropanol, 200 mM of BTAP (a and b) or 1 M of BTAP (d), 5 g/L recombinant *E. coli* cells (DCW) (a, b and c).

Fig. **4.** Time courses of asymmetric reduction of BTAP with recombinant *E. coli* cells in phosphate buffer systems with or without the addition of [N1,1,1,1][Cys]. Symbols: (●) 1 M BTAP with [N1,1,1,1][Cys]; (○) 1 M BTAP without [N1,1,1,1][Cys]; (■) 800 mM BTAP with [N1,1,1,1][Cys]; (□) 800 mM BTAP without [N1,1,1,1][Cys].

Table 1

ILs used in this work and their nuclear magnetic resonance (HMR)

Ionic liquid	Abbreviation	^{1}H NMR (400 MHz, D_2O) and ^{13}C NMR (100 MHz, D_2O)
Tetramethylammonium cysteine	[N1,1,1,1][Cys]	δ/ppm (^{1}H): 3.64–3.61 (q, *J* = 3.6 Hz, 1H, CH), 3.09 (s, 12H, CH_3 × 4), 2.8 (^{13}C): 179.8 (COOH), 57.9 (CH), 55.7 (CH_3 × 4), 27.2 (CH_2).
Tetraethylammonium cysteine	[N2,2,2,2][Cys]	δ/ppm (^{1}H): 3.47–3.44 (q, *J* = 3.2 Hz, 1H, CH), 3.18–3.13 (q, *J* = 6.0 Hz, 8H (t, *J* = 5.2 Hz, 12H, CH_3 × 4); δ/ppm (^{13}C): 176.8 (COOH), 64.6 (CH), 52.5 (
Tetramethylammonium glutamate	[N1,1,1,1][Glu]	δ/ppm (^{1}H): 3.05 (s, 12H, CH_3 × 4), 2.10–2.06 (t, *J* = 8.0 Hz, 1H, CH), 1.7 CH_2); δ/ppm (^{13}C): 182.5 (COOH), 182.2 (COOH), 57.8 (CH), 56.0 (CH_3 × 4
Tetraethylammonium glutamate	[N2,2,2,2][Glu]	δ/ppm (^{1}H): 3.18–3.14 (q, *J* = 5.6 Hz, 8H, CH_2 × 4), 2.15–2.07 (m, 1H, CH), 2H, CH_2), 1.16 (t, *J* = 6.0 Hz, 12H, CH_3 × 4); δ/ppm (^{13}C): 182.7 (COOH), 1 35.0 (CH_2), 32.6 (CH_2), 7.4 (CH_2 × 4).

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Tetramethylammonium tetrafluoroborate	[N1,1,1,1][BF_4]	δ/ppm (^{1}H): 3.05 (s,CH_3× 4); δ/ppm (^{13}C): 55.6 (CH_3 × 4).
Tetraethylammonium tetrafluoroborate	[N2,2,2,2][BF_4]	δ/ppm (^{1}H): 3.23-3.19 (q, *J* = 5.2 Hz, 8H, CH_2 × 4), 1.24-1.21 (m, 12H, CH (CH_2 × 4).
Tetrapropylammonium tetrafluoroborate	[N3,3,3,3][BF_4]	δ/ppm (^{1}H): 3.06 (s, 8H, CH_2 × 4), 1.61 (s, 8H, CH_2 × 4), 0.86 (s, 12H, CH (CH_2 × 4),10.0 (CH_2 × 4).
Tetrabutylammonium tetrafluoroborate	[N4,4,4,4][BF_4]	δ/ppm (^{1}H): 3.12-3.09 (t, *J* = 6.0 Hz, 8H, CH_2 × 4), 1.58-1.54 (t, *J* = 8.0 Hz 8H, CH_2 × 4), 0.87-0.84 (t, *J* = 6.0 Hz, 12H, CH_3 × 4); δ/ppm (^{13}C): 58.3 (12.9 (CH_2 × 4).

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Table 2

Effect of various ILs on asymmetric reduction of BTAP with recombinant *E. coli* cells

Entries	Medium	V_0/μmol/min/g	Yield/%	Product ee/%
1	Phosphate buffer	318.7±3.98	52.2±0.78	>99
2	[N1,1,1,1][Cys]-buffer	373.6±4.23	61.4±0.92	>99
3	[N2,2,2,2][Cys]-buffer	338.5±4.42	54.9±1.07	>99
4	[N1,1,1,1][Glu]-buffer	343.9±4.16	56.2±0.82	>99
5	[N2,2,2,2][Glu]-buffer	328.9±3.92	52.4±0.56	>99
6	[N1,1,1,1][BF_4]-buffer	291.1±4.89	47.5±1.08	>99
7	[N2,2,2,2][BF_4]-buffer	277.6±4.04	46.7±0.72	>99
8	[N3,3,3,3][BF_4]-buffer	272.1±4.31	45.5±0.88	>99
9	[N4,4,4,4][BF_4]-buffer	250.0±3.94	43.6±0.82	>99

Reaction conditions: 1.5 % (w/v) various ILs, 5 g/L recombinant *E. coli* cells (DCW), 20 % (v/v) isopropanol, and 200 mM BTAP, buffer pH 7.2, 30 °C and 200 rpm, reaction for 10 min (V_0) and 2 h (yield).

Table 3

Effect of various ILs on cell membrane permeability of recombinant *E. coli* cells

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Entries	Medium	Net OD_{260nm}	Net OD_{280nm}
1	Phosphate buffer	0.257±0.008	0.235±0.005
2	[N1,1,1,1][Cys]-buffer	0.289±0.006	0.295±0.004
3	[N2,2,2,2][Cys]-buffer	0.304±0.007	0.389±0.009
4	[N1,1,1,1][Glu]-buffer	0.384±0.006	0.510±0.007
5	[N2,2,2,2][Glu]-buffer	0.418±0.011	0.566±0.012
6	[N1,1,1,1][BF_4]-buffer	0.738±0.012	0.842±0.016
7	[N2,2,2,2][BF_4]-buffer	0.718±0.014	0.849±0.012
8	[N3,3,3,3][BF_4]-buffer	0.795±0.011	1.011±0.018
9	[N4,4,4,4][BF_4]-buffer	0.508±0.016	0.864±0.021

Reaction conditions: 200 mM phosphate buffer (pH 7.2), 1.5 % (w/v) various ILs, and 5 g/L recombinant *E. coli* cells (DCW), 30 °C and 200 rpm for 6 h.

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Table 4

Comparison of biosynthesis of (*R*)-BTPE by asymmetric reduction using BTAP as substrate

yst	Product titer/g/L	Yield/ %	ee /%	Productivity /g/(L·h)	Referenc
cillus kefir (20-24 g/L, DCW)	1.20	100.0	> 99.0	0.075	[5]
um expansum EBK-9	2.6	76.0	> 99.0	0.046	[6]
a xyli HS0904 (300 g/L, wet cell mass)	47.0	91.8	> 99.0	1.567	[10]
cterium oxydans C3 (33 g/L, DCW)	28.0	56.0	>99.0	0.700	[4]
rma asperellum ZJPH0810 (60 g/L, DCW)	11.9	93.4	> 98.0	0.567	[7]
bacterium sp. CA49 derived recombinant *E.* e enzyme lyophilized powder (2 g/L)	149.9	>99.9	>99.9	6.246	[8]
nant *E. coli* cells expressing LXCAR-S154Y , DCW)	252.7	98.7	>99.0	21.058	Our pres

Fig. 1

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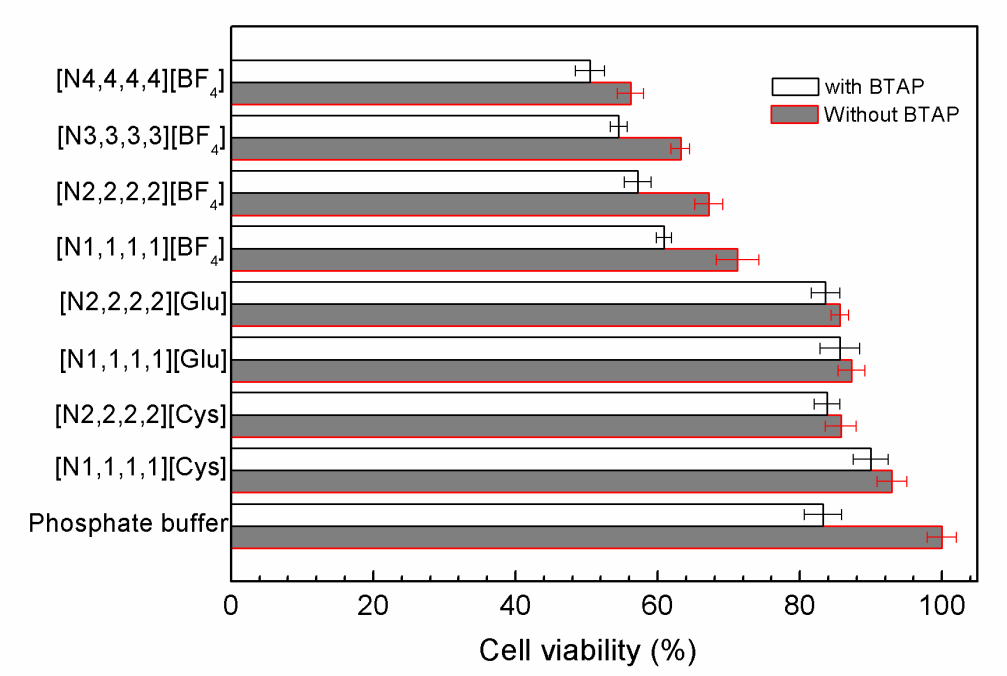

Fig. 2

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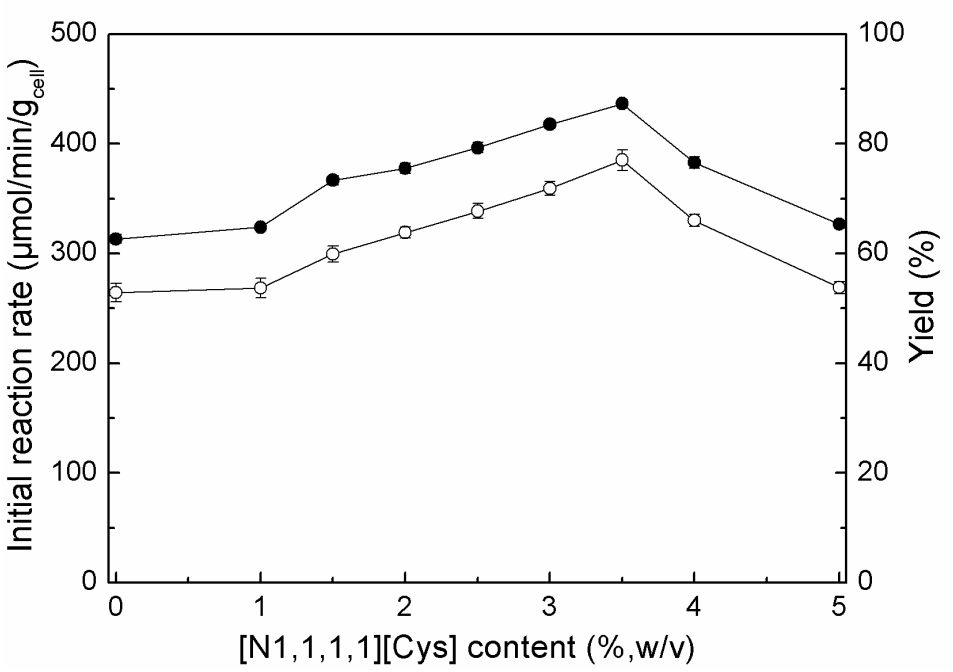

Fig. 3

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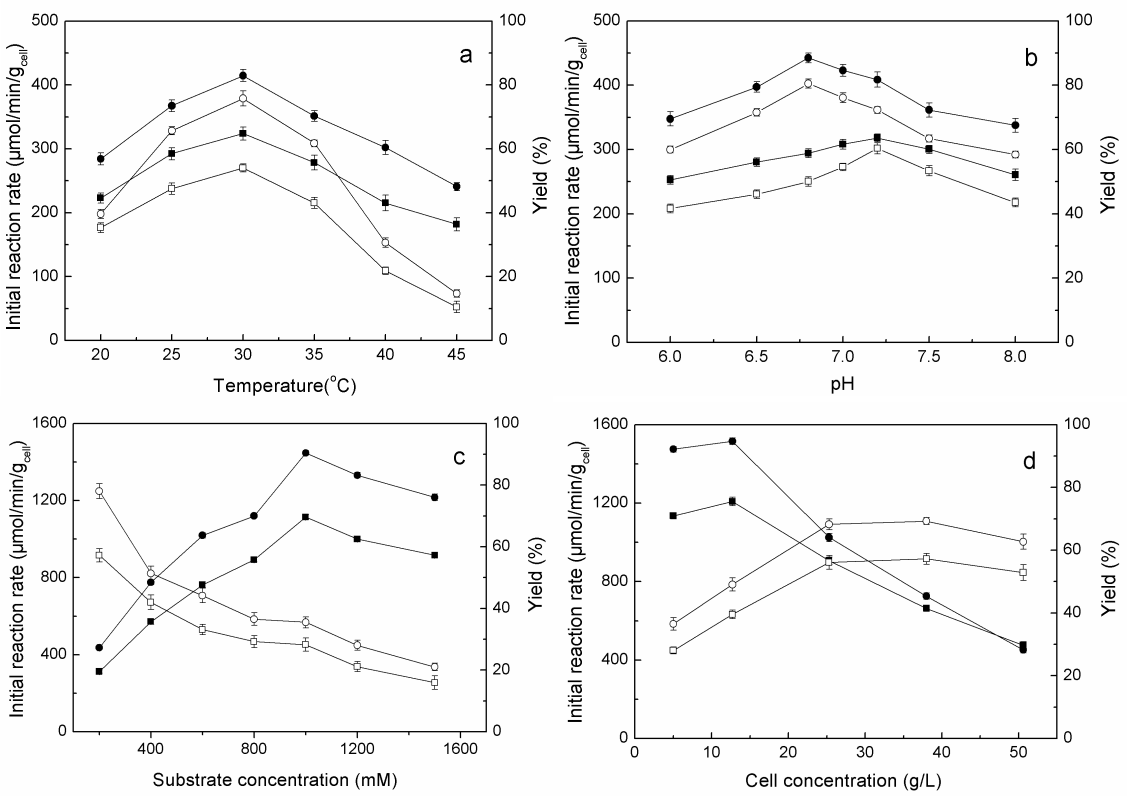

Fig. 4

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