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Efficient Biocatalytic Preparation of Optically Pure (*R*)-1-[4-(Trifluoromethyl)phenyl]ethanol by Recombinant Whole-Cell-Mediated Reduction

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Abstract: (*R*)-1-[4-(Trifluoromethyl)phenyl]ethanol is an important pharmaceutical intermediate of a chemokine CCR5 antagonist. In the present study, a bioprocess for the asymmetric reduction of 4-(trifluoromethyl)acetophenone to (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol was developed by recombinant *Escherichia coli* cells with excellent enantioselectivity. In order to overcome the conversion limitation performed in the conventional buffer medium resulting from poor solubility of non-natural substrate, we subsequently established a polar organic solvent-aqueous medium to improve the efficacy. Isopropanol was selected as the most suitable cosolvent candidate, based on the investigation on a substrate solubility test and cell membrane permeability assay in different organic solvent-buffer media. Under the optimum conditions, the preparative-scale asymmetric reduction generated a 99.1% yield with >99.9% product enantiomeric excess (ee) in a 15% (*v*/*v*) isopropanol proportion, at 100 mM of 4-(trifluoromethyl)acetophenone within 3 h. Compared to bioconversion in the buffer medium, the developed isopropanol-aqueous system enhanced the substrate concentration by 2-fold with a remarkably improved yield (from 62.5% to 99.1%), and shortened the reaction time by 21 h. Our study gave the first example for a highly enantioselective production of (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol by a biological method, and the bioreduction of 4-(trifluoromethyl)acetophenone in a polar organic solvent-aqueous system was more efficient than that in the buffer solution only. This process is also scalable and has potential in application.

Keywords: (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol; asymmetric bioreduction; recombinant whole-cell catalysis; polar organic solvent-aqueous system

1. Introduction

Enantiopure alcohols are valuable and versatile building blocks for the manufacturing of chiral pharmaceuticals [1]. (*R*)-1-[4-(Trifluoromethyl)phenyl]ethanol is a crucial enantiomerically enriched intermediate for the production of AD101 (SCH-350581), a chemokine CCR5 antagonist widely used in AIDS patients to inhibit the replication of HIV-1 *via* blockade of its entry into cells [2]. A chemical approach for the (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol synthesis has been reported through the asymmetric reduction with BH_3Me_2S in the presence of a chiral catalyst oxazaborolidine, while the drawbacks of this method are the expensive chiral reagent and environmental pollution [3]. Therefore, the search for alternative biocatalytic processes for efficient preparation of enantiometrically pure (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol attracts more attention. An alternative way is the enantioselective reduction of prochiral compounds using enzymes or enzyme-containing cells [4]. Furthermore, the use of microbial whole-cells with metabolic activity as catalysts provides several merits over isolated enzymes, including the cofactor recycling *in situ* and better protecting target enzymes against inactivation, thereby significantly cutting down the process cost [5,6].

In our previous study, we found a carbonyl reductase derived from the *Leifsonia xyli* HS0904 isolate exhibiting catalytic activity toward 4-(trifluoromethyl)acetophenone by investigating the substrate specificity of this purified enzyme for several aromatic ketones, alkyl ketones and keto esters [7]. Subsequently, a robust recombinant *Escherichia coli* LXCAR-S154Y mutant, an engineered reductase from *L. xyli* HS0904, was obtained through a directed evolution strategy, and used for the efficient asymmetric reduction of 3,5-bis(trifluoromethyl) acetophenone [8].

In the present study, we first reported the preparation of the (*R*)-1-[4-(trifluoromethyl) phenyl]ethanol through the 4-(trifluoromethyl)acetophenone reduction using a biocatalytic method. The reaction scheme is shown in Figure 1. By screening the strains isolated from soil samples or preserved in our laboratory, the recombinant *E. coli* LXCAR-S154Y was selected and used as a suitable biocatalyst for the (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol production, with excellent enantioselectivity (over 99.9% ee).

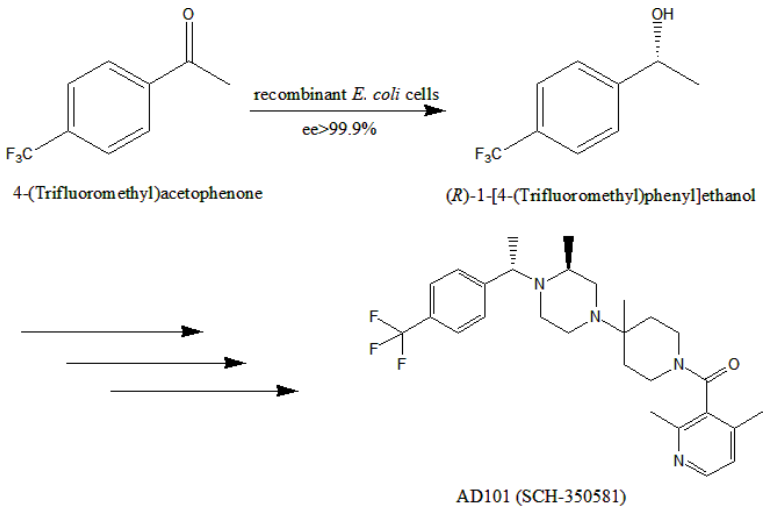

Figure 1. The biological asymmetric reduction route.

We found however, similar to most biocatalytic processes, that the yield for the (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol synthesis by this recombinant whole-cell-mediated reduction to be unsatisfactory at a high substrate concentration performed in conventional buffer medium, possibly due to the poor solubility of 4-(trifluoromethyl)acetophenone in aqueous medium, preventing the interaction between the intracellular enzyme and substrate, then limiting its productivity to some extent [9].

The study on medium engineering for biocatalysis is a prominent topic for improving the bioprocess efficacy [10]. The use of organic cosolvents or organic biphasic systems to enhance activity, stability, and productivity of biocatalysts has been examined, sometimes with positive results [11,12]. It is known that the addition of a water-miscible organic solvent into an aqueous medium could improve the substrate solubility and decrease the mass-transfer limitations in biocatalysis [13]. Yasohara et al. reported the stereoselective synthesis of optically pure alkyl 4-substituted-3-hydroxybutanoates in a water/organic solvent two-phase system by the *E. coli* HB101 transformant co-expressing the carbonyl reductase (S1) from *Candida magnoliae* and glucose dehydrogenase from *Bacillus megaterium* [14]. Liu et al. also examined the effect of solvent properties on the activity of enzymes. They found that the solvent effect might be related to the distribution and diffusion of substrates and products on the surface of the enzyme in the reaction medium [15]. Lanne et al. indicated that the activity greatly depends on solvent properties, especially the octanol-water partition coefficient, log P_{ow}, that provides a reference for optimizing biocatalytic reactions [16].

In this study, we describe an efficient strategy to improve the asymmetric reduction of 4-(trifluoromethyl)acetophenone to (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol by the recombinant *E. coli*

LXCAR-S154Y as a biocatalyst, based on the use of hydrophilic organic solvent as a cosolvent. The substrate loading and bioreductive yield could be markedly enhanced with the isopropanol addition as a cosolvent in an aqueous system. Subsequently, a comparative study was performed with or without isopropanol as a cosolvent, and a greatly improvement in reaction yield was observed in an isopropanol-aqueous medium. Moreover, this developed biocatalytic process is also scalable in the preparative scale, and has potential for practical application.

2. Results

2.1. Screening of Biocatalyst

Microorganisms isolated from soil samples or preserved in our laboratory, including bacterial, yeast and filamentous fungus, were tested for their abilities to produce (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol. The recombinant *E. coli* BL21(DE3)/pET-28a(+)-LXCAR- S154Y, showing an asymmetric reductive activity from 4-(trifluoromethyl)acetophenone to (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol with excellent enantioselectivity (>99.9% ee), was determined as the most powerful strain for further investigation due to its good conversion (65.4% yield at 16 mM 4-(trifluoromethyl)acetophenone). The screening results of some strains are shown in Table 1. We found several strains isolated from soil samples affording reductive activities to 4-(trifluoromethyl)acetophenone, most of which gave the (*S*)-form product. However, some strains preserved in our laboratory produced the (*R*)-form alcohol. The recombinant *E. coli* BL21(DE3)/pET-28a(+)-LXCAR-S154Y gave both good yield and stereoselectivity comparatively. The standards of 4-(trifluoromethyl)acetophenone, its corresponding alcohol products, as well as the bioconverted sample were detected by the gas chromatography (GC) analysis with a chiral stationary phase (Figure S1).

Table 1. The results of some screened strains.

Strain	Yield (%)	ee (%)
CY 1-1	14.9	99.9 (*S*)
HZ 1-3	21.8	99.9 (*S*)
XA 1-4	30.1	99.9 (*S*)
AF 3-4	23.9	99.9 (*S*)
XM 7-1	45.9	60.2 (*S*)
CQ 8-1	34.7	55.7 (*S*)
Escherichia coli BL21(DE3)/pET-28a(+)-LXCAR-S154Y	65.4	99.9 (*R*)
Geotrichum candidum ZJPH1704	47.3	60.9 (*R*)
Candida tropicalis 104	25.0	38.9 (*R*)
Rhodococcus erythropolis XS1012	9.5	35.6 (*R*)

2.2. Effects of Cell Concentration and Substrate Concentration on the Asymmetric Reduction of 4-(trifluoromethyl)acetophenone in Phosphate Buffer

Biomass concentration plays an important role in the asymmetric reduction. As illustrated in Figure 2a, with the increase of cell concentration, the yield enhanced rapidly and then decreased to a certain extent. It may be due to the limited mass transfer resulted from the high concentration of the cell, and thus leading to the decline of the yield. The maximum yield for the (*R*)-form alcohol reached 86.7% at 60 g/L of the wet cell mass (approximately 17 g/L DCW).

Figure 2b shows the impact of the substrate concentration on the enantioselective reduction. The results indicated that the yield decreased obviously with the increase in initial substrate concentration while the yield was dropped to only 62.5% at 100 mM 4-(trifluoromethyl)acetophenone. The yield of the product increased with the reaction time both at 50 mM or 100 mM of the substrate concentration, but this increase slowed down after 24 h (Figure 2c). So we chose 24 h as the optimum

reaction time in terms of efficiency in time and yield. Additionally, the effects of the buffer pH and reaction temperature on the reduction were also examined, pH 7.5 and 30 °C were determined to be the optimal (data no shown). In order to further promote the reaction at a high substrate loading, employing a novel reaction medium was envisaged as an efficient strategy to overcome poor solubility of substrate in phosphate buffer medium and improve the biocatalytic efficacy.

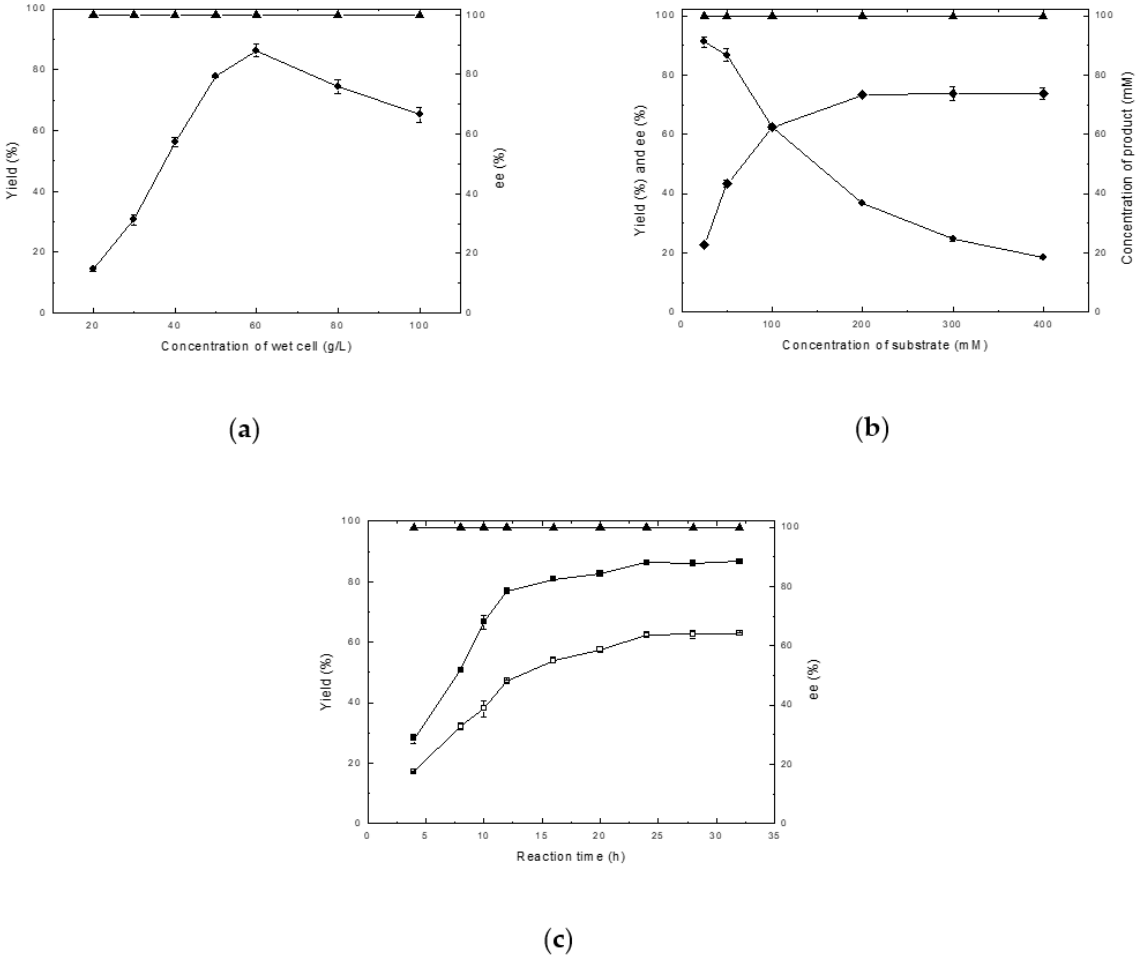

Figure 2. Effects of cell concentration. (**a**) Substrate concentration; (**b**) reaction time (**c**) on the asymmetric reduction of 4-(trifluoromethyl)acetophenone in phosphate buffer. Symbols: (●) Yield and (▲) ee; (◆) product concentration; (■) yield at 50 mM 4-(trifluoromethyl)acetophenone and (□) yield at 100 mM 4-(trifluoromethyl)acetophenone.

2.3. Effect of Organic Cosolvents on the Asymmetric Reduction of 4-(trifluoromethyl)acetophenone

To establish an effective polar organic solvent-aqueous medium for improving the catalytic efficiency of the (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol production by recombinant *E. coli* cells, several polar organic solvents were tried and added into the reaction buffer at a volume fraction of 5%. As shown in Figure 3, only the isopropanol accelerated the bioreduction and resulted in the increased yield with above 99.9% ee. The other five all decreased the yields and acetone was the most reduced. It is likely attributed to the fact that the alterations of enzyme conformation in the presence of organic solvents resulted in the decline in yield [17,18]. Particularly, hydrophilic solvents, penetrating into active sites of target intracellular enzyme, are capable of inducing the changes in secondary and tertiary structure of enzyme [19,20].

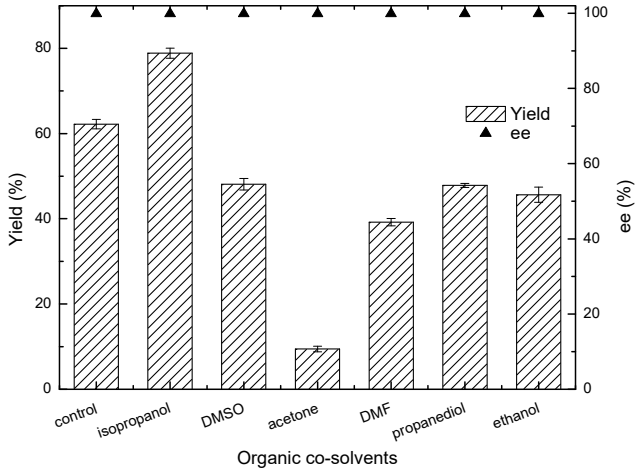

Figure 3. Effect of various organic cosolvents on the asymmetric reduction of 4-(trifluoromethyl)acetophenone.

2.4. Effects of Key Variables on Asymmetric Reduction of 4-(trifluoromethyl)acetophenone in Polar Organic Solvent–Aqueous System

The biocatalytic preparation of (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol in a polar organic solvent–aqueous system catalyzed by recombinant *E. coli* cells was optimized in terms of several key reaction parameters, such as isopropanol proportion, buffer pH and ionic strength, reaction temperature, substrate concentration and reaction time.

Figure 4 depicts the influence of the isopropanol proportion in buffer solution on the yield and ee of the product. In the range of the isopropanol concentration from 1% to 25% (*v*/*v*) in the reaction buffer, the highest yield of 93.1% was achieved at a volume fraction of 15% isopropanol. A further increase in the isopropanol content resulted in the slight decline of product yield. It is likely that the excessive amount of isopropanol possibly causes higher conformational mobility, thus increasing the tendency of enzyme denaturation [21]. The ee value of the product remained above 99.9% with the addition of isopropanol. Thus, isopropanol at a volume fraction of 15% showed the best effect on the yield.

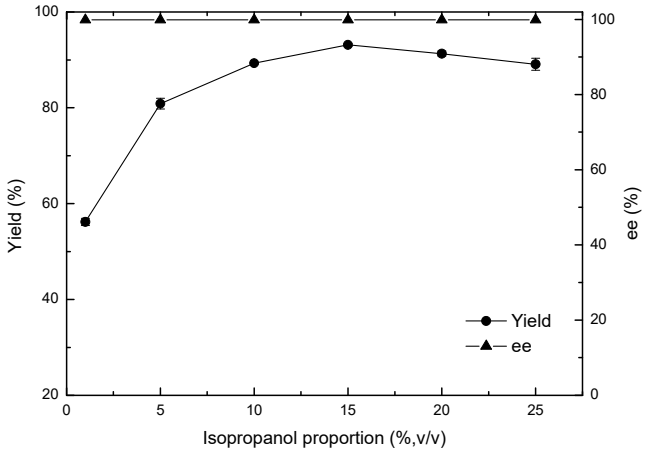

Figure 4. Effect of isopropanol proportion in reaction system on the asymmetric reduction of 4-(trifluoromethyl)acetophenone.

The effects of the buffer pH and ionic strength on the asymmetric reduction were also investigated. The yield was relatively stable with pH within the scope of our study (Figure 5a). The optimal buffer pH for this reduction is 7.5. As seen in Figure 5b, the optimal ionic strength of the buffer solution is 100 mM. The yield of the (*R*)-form alcohol was reduced as the buffer ionic strength increased. Within the assayed buffer pH and ionic strength range, the product ee remained intact (all above 99.9%).

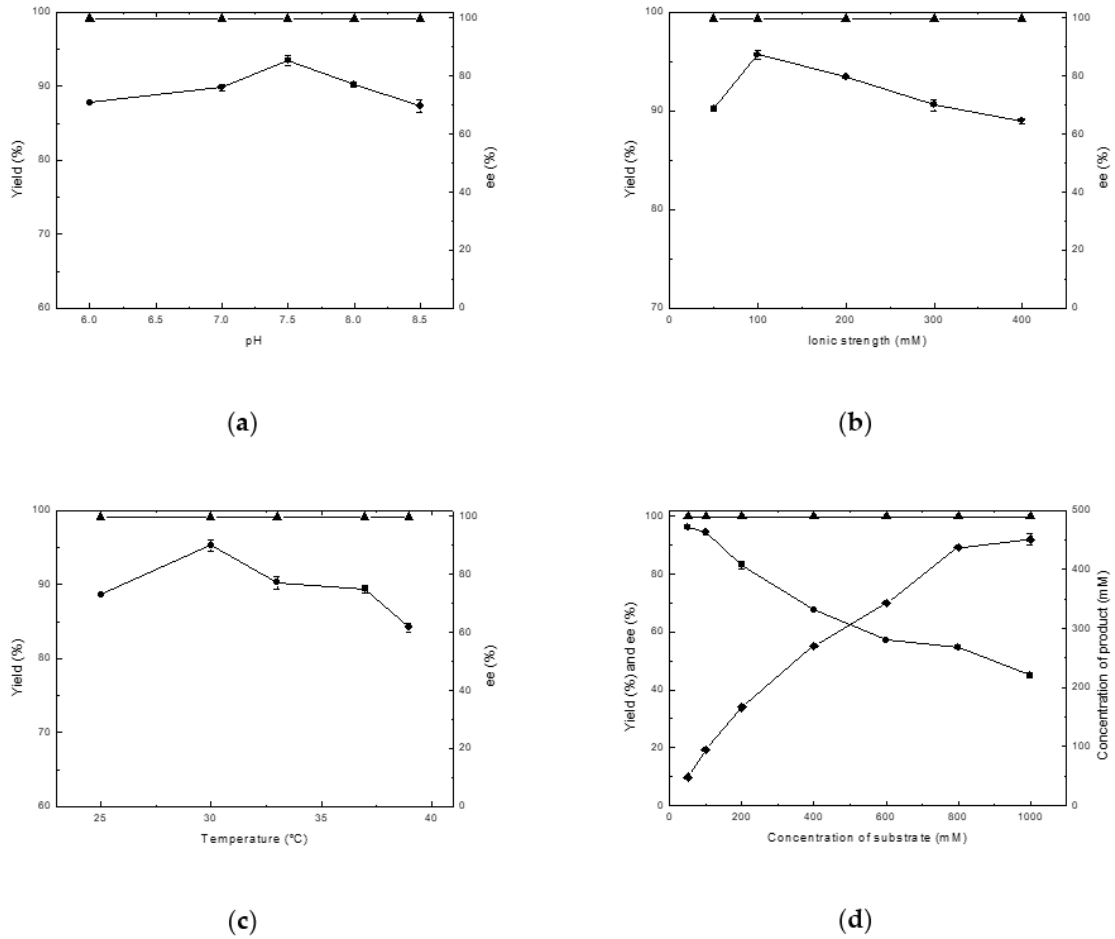

Figure 5. Effects of pH. (**a**) Ionic strength; (**b**) temperature (**c**) and substrate concentration (**d**) on the asymmetric reduction of 4-(trifluoromethyl)acetophenone. Symbols: (●) Yield, (▲) ee and (◆) product concentration.

The effects of the reaction temperature and substrate concentration on the bioreduction were also examined. As illustrated in Figure 5c, the yield was boosted with the increase in the reaction temperature from 25 °C to 30 °C. However, the yield decreased slightly when the temperature was above 30 °C, possibly resulting from the partial inactivation of enzyme within the cells at high temperature. The optimal reaction temperature is 30 °C. The effect of the substrate concentration on the product yield was tested from 50 mM to 1000 mM. As shown in Figure 5d, the concentration of the product markedly increased with the increase in substrate loading, while the yield of product accordingly decreased. The yield and product concentration varied significantly at a low substrate concentration and became stable at a high substrate loading. This implies that the reaction might be inhibited at higher substrate concentration. When the substrate concentration reached 100 mM, a yield of 95.2% was obtained for the (*R*)-form alcohol at 24 h, with above 99.9% ee. The substrate concentration had no influence on the product ee value.

2.5. Relationship between Bioreduction and Cosolvent Characteristics

With the addition of organic cosolvents in the reaction medium, the cell membrane permeability might be modulated, thereby promoting across-membrane transport of the substrate and product, facilitating the biocatalytic process and accelerating the reaction [22]. On the other hand, excessive organic cosolvents exert more toxicity to biocatalysts and make the reaction terminated [23]. The results in Table 2 demonstrated that the OD_{260} nm and OD_{280} nm values all increased in an organic solvent-aqueous medium, implying that the cell membrane became more permeabilized compared

with the buffer solution only. Of all the tested organic cosolvents, acetone displayed the greatest increase in cell membrane permeability, but afforded the lowest reaction efficiency, probably owing its significant toxicity to cells resulting in the termination of the reaction. It was found however, that isopropanol can moderately improve the permeability of the cell membrane and promote the reaction. Meanwhile, the solubility of 4-(trifluoromethyl)acetophenone in an isopropanol-aqueous medium (663.6 mg/L) was higher than that in the buffer solution only (410.9 mg/L). Our results gave a good explanation for the superior performance of recombinant microbial whole cells in the isopropanol-aqueous medium for 4-(trifluoromethyl)acetophenone reduction.

Table 2. Solubility of 4-(trifluoromethyl)acetophenone in different organic solvent-aqueous media and effect of various cosolvents on cell membrane permeability.

Medium	Net OD_{260nm}	Net OD_{280nm}	Solubility (mg/L)
15% (*v*/*v*) Isopropanol-buffer	2.560	1.225	663.6
15% (*v*/*v*) DMF-buffer	1.394	0.651	1416.4
15% (*v*/*v*) DMSO-buffer	1.614	0.597	709.3
15% (*v*/*v*) Ethanol-buffer	1.648	0.837	641.0
15% (*v*/*v*) Propylene glycol-buffer	0.954	0.520	535.5
15% (*v*/*v*) Acetone-buffer	2.969	2.339	1067.7
Phosphate buffer	0.914	0.485	410.9

2.6. Comparison of Asymmetric Reduction in an Isopropanol-Aqueous System and Buffer Solution

The time course of the enantioselective reduction of 4-(trifluoromethyl)acetophenone in the buffer solution (pH 7.5), containing isopropanol at a volume fraction of 15%, is shown in Figure 6.

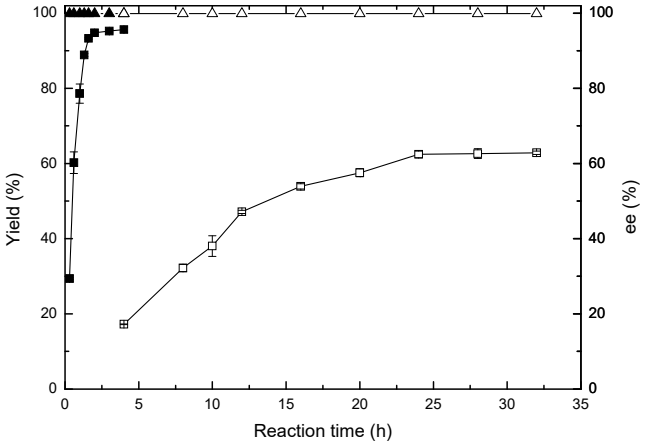

Figure 6. Comparison of asymmetric reduction of 4-(trifluoromethyl)acetophenone in isopropanol-buffer medium and aqueous system. Symbols: (■) Yield in 15% (*v*/*v*) isopropanol-buffer solution and (□) yield in buffer solution, (▲) ee in 15% (*v*/*v*) isopropanol-buffer solution and (△) ee in buffer solution.

The yield of the product increased with the reaction time but this increase performed completely different in the 15% (*v*/*v*) isopropanol-aqueous system and buffer solution. Under the optimal conditions (15% (*v*/*v*) isopropanol, 50 g/L maltose as co-substrate, 17 g/L recombinant *E. coli* dry cell mass, 100 mM phosphate buffer, pH 7.5, 100 mM 4-(trifluoromethyl)acetophenone, 30 °C), (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol was generated with a 95.2% yield and above 99.9% ee value within 3 h. Comparatively, only a 62.5% yield was observed after the reaction for 24 h in an aqueous system under the same conditions. With the asymmetric reduction in an isopropanol-aqueous medium, the optimal reaction time was shortened by 21 h and the yield of product sharply increased (from 62.5% to 95.2%). These positive results may be explained by an increase of membrane permeability

with the help of isopropanol as a cosolvent. Enhancement of the reaction efficiency by the use of an hydrophilic organic-aqueous system may be of practical importance for highly efficient synthesis of the (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol by the biocatalytic asymmetric reduction.

2.7. Preparative Scale Bioreduction of 4-(trifluoromethyl)acetophenone to (R)-1-[4-(trifluoromethyl) phenyl]ethanol in the Developed Isopropanol-Aqueous System

A preparative-scale bioreduction of 4-(trifluoromethyl)acetophenone to (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol with the addition of isopropanol as a cosolvent was carried out in a 500 mL Erlenmeyer flask (working volume of 100 mL). A yield of 99.1% was achieved at 100 mM substrate concentration after reaction within 3 h in the developed reaction system (Figure S2). The product ee remained above 99.9%. Our results indicated that the bioreductive process in the developed reaction system was scalable at a preparative scale of a 500 mL Erlenmeyer flask.

3. Discussion

Optically active aromatic alcohols are the kinds of valuable chiral building blocks of many natural products and chiral drugs [24]. In comparison with the general chemical approach for chiral aryl alcohols synthesis, the biocatalysis method is more preferable because of its high substrate specificity, mild reaction conditions, and environmental sustainability [25,26]. In our study, a recombinant *E. coli* LXCAR-S154Y was adopted for the asymmetric production of (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol because of its high activity toward 4-(trifluoromethyl)acetophenone. Based on results from key parameters optimized in a phosphate buffer medium, we got a 86.7% yield of product at 50 mM substrate concentration with perfect enantioselectivity of (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol. After that, an isopropanol-containing aqueous system was established overcoming poor solubility of 4-(trifluoromethyl) acetophenone to improve the conversion at a high substrate concentration, a yield of 95.2% was achieved at 100 mM substrate. Furthermore, the enantiopure (*R*)-form alcohol was produced in a preparative-scale synthesis with higher yield of 99.1%. Meanwhile, the optimal reaction time for this bioprocess was cut down by 21 h compared with that in the aqueous buffer solution.

Aqueous solutions are considered as a conventional reaction media for biocatalytic reaction [27]. Unfortunately, industrial attractive substrate for biocatalysis usually are non-natural compounds [28]. Water sometimes is a poor solvent for most synthetic reactions even though it is considered as a solvent for life. Organic solvents are often required to increase the solubility of the hydrophobic substrate, alter the thermodynamic equilibrium to promote synthesis, and inhibit water-dependent side reactions. But rapid denaturation of enzymes occurs when the volume fraction of most water-miscible solvents exceeds 20–50% (*v*/*v*) [29]. Therefore, the isopropanol concentration in the reaction medium is the certain issue of the asymmetric reduction. In our study, we found that the recombinant *E. coli* cells afforded the highest yield at a 15% (*v*/*v*) isopropanol proportion. Higher concentration of isopropanol probably causes the loss of enzyme activity resulted from the loss of crucial water molecules. Our results indicated that the adopted recombinant *E. coli* cells had much tolerance of isopropanol to overcome the toxicity and destructive effect of organic solvent. In regard to optimized results of temperature and pH, we observed that the recombinant *E. coli* cells had a wide catalytic range for asymmetric reduction because yields were not significantly changed with the temperature and pH within the tested range. This recombinant *E. coli* mutant was a powerful strain that may be suitable for a variety of asymmetric reduction reactions.

4. Materials and Methods

4.1. Chemicals

The substrate 4-(trifluoromethyl)acetophenone (purity > 98%) was provided by the Zhengzhou Alfa Company, Zhengzhou, China. The product (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol (purity > 98%) was purchased from Aikonchem, Nanjing, China. The (*R*, *S*) 4-(trifluoromethyl)

phenylethanol was synthesized from 4-(trifluoromethyl)acetophenone by sodium borohydride reduction. All other chemicals were obtained from local suppliers and were of analytical grade.

4.2. Strain and Cultivation

One gram of each soil sample was suspended in 100 mL of saline (0.85%, *w*/*v*) and vibrated for 30 min. One milliliter of soil mixture was taken into the screening medium ($(NH_4)_2SO_4$ 2 g/L, $KH_2PO_4$1g/L, NaCl 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, 16 mM 4-(trifluoromethyl)acetophenone with initial pH of 6.5) for a substrate-directed screening at 30 °C for 3–5 days. After several rounds of enriched culture and being diluted appropriately, the diluents (200 µL) were respectively plated over the screening medium agar, and then incubated at 30 °C for 3–5 days. The pure colonies were resulted by continuous streaking of obtained single colony on an agar plate of the all nutrition culture medium (glucose 15 g/L, peptone 5 g/L, yeast extract 5 g/L, K_2HPO_4 0.5 g/L, KH_2PO_4 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, NaCl 1 g/L, $(NH_4)_2SO_4$ 1 g/L) at 30 °C for 2–3 days. The isolated pure colonies were then cultured on the all nutrition culture medium and used for further evaluation.

Strain *Rhodococcus erythropolis* XS1012 (CCTCC M 2013650), *Candida tropicalis* 104 (CCTCC M 209034), and *Geotrichum candidum* ZJPH1704 (CCTCC M 2017380) were isolated from the soil by our research group. The recombinant *E. coli* LXCAR-S154Y, an engineered reductase from *L. xyli* HS0904, was obtained by a directed evolution strategy in our previous study [8]. *Rhodococcus erythropolis* XS1012 (bacterial) and *Candida tropicalis* 104 (yeast) were cultivated according to our previous reports [30,31]. The fermentation medium for the *Geotrichum candidum* ZJPH1704 cultivation contained the following components: Glucose 24.45 g/L, peptone 15.75 g/L, $(NH_4)_2SO_4$ 21.39 g/L, $CaCl_2$ 0.111 g/L. The initial pH of medium was 6.5. After cultivation for 24 h at 30 °C with shaking at 200 rpm, the cells were harvested by centrifugation at 9000 rpm and 4 °C for 20 min. Cultivation of recombinant *E. coli* LXCAR-S154Y: First, it was inoculated into a 250 mL flask containing 50 mL Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 9 g/L) supplemented with 50 µg/mL of kanamycin, and cultured at 37 °C, 200 rpm for 10 h on a rotary shaker. The obtained seed culture was transferred into a 500 mL flask containing 100 mL LB medium with 2% (*v*/*v*) inoculation ratio, and cultivated with shaking for 2 h under the same conditions. Then, 0.5 mM IPTG was added for each flask and further incubated at 33 °C for 10 h. The cultivated cells were collected by the centrifugation at 8000 rpm, 4 °C for 10 min, followed by the biocatalytic reaction.

4.3. General Bioreduction Process and Selection of Organic Cosolvents

The bioreduction was conducted in 50 mL Erlenmeyer flasks containing 10 mL of phosphate buffer, 17 g/L of recombinant *E. coli* cells (DCW), certain amount of 4-(trifluoromethyl)acetophenone, 50 g/L maltose as co-substrate for coenzyme regeneration. The reaction mixtures were shaken for 24 h at 30 °C and 200 rpm, and samples were taken for GC analysis. The performances of six hydrophilic organic cosolvents were assessed respectively by adding them individually into the reaction buffer at a volume fraction of 5%.

4.4. GC Analysis

The yield and ee value of the product were determined using Agilent Technologies 7820A GC system, using a flame ionization detector and Varian CP-Chirasil-Dex CB column (25 m × 0.25 mm × 0.25 µm). The split ratio was 15:1. The temperature of the injector and detector were both of 250 °C. The column temperature was maintained at 100 °C for 3 min, then raised up to 160 °C at a rate of 10 min^{-1} and remained constant for 1 min. Nitrogen was used as a carrier gas at a flow rate of 2.0 mL min^{-1}. An internal standard method was adopted for the calculations. The retention times for 4-(trifluoromethyl)acetophenone and (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol were 5.0 min and 7.7 min, respectively.

The yield was calculated as follows:

$$\text{Yield } (\%) = \frac{Cp}{Co} \times 100\%$$

where *Co* is the initial concentration of 4-(trifluoromethyl)acetophenone, *Cp* is the final concentration of the resulting (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol.

4.5. Cell Membrane Permeability Assay

The cell membrane permeability of recombinant *E. coli* cells was evaluated in different hydrophilic organic cosolvents-phosphate buffer systems. The method for this measurement was described in our previous work [32]. Conditions: 17 g/L recombinant *E. coli* cells (DCW) was added into a 10 mL phosphate buffer (100 mM, pH 7.5) containing different organic cosolvents at a volume fraction of 15%, 30 °C, 200 rpm, incubated for 24 h.

4.6. Substrate Solubility Assay

In order to determine the solubility of substrate, the corresponding standard was dissolved in ethyl acetate and used as the reference to establish the calibration curve. The solubility of substrate 4-(trifluoromethyl)acetophenone in different reaction systems was examined with a similar method reported by Wang et al. [33]. Conditions: Excessive 4-(trifluoromethyl)acetophenone was added into a 10 mL phosphate buffer (100 mM, pH 7.5) containing different organic cosolvents at a volume fraction of 15%, 30 °C, 200 rpm, incubated for 24 h.

4.7. Effects of Key Variables on the Asymmetric Reduction

In the quest of determining suitable conditions for the bioreduction of 4-(trifluoromethyl) acetophenone to (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol in the phosphate buffer system, some crucial reaction parameters, including the buffer pH, cell concentration, reaction temperature, substrate concentration and reaction time, were optimized individually. In the isopropanol-aqueous system, key variables involved in the asymmetric reduction were also optimized, especially the proportion of isopropanol. The reaction mixtures were composed of the phosphate buffer, maltose as a co-substrate, certain amount of recombinant *E. coli* cells and substrate 4-(trifluoromethyl)acetophenone, appropriate organic cosolvent or not, incubated at 30 °C, 200 rpm for 24 h. The yield and product ee were assayed by the GC analysis.

4.7.1. Optimization of Cell Concentration, Substrate Concentration, and Reaction Time in Phosphate Buffer System

The optimized results of cell concentration, substrate concentration, and reaction time in the phosphate buffer are shown in Figure 2. Reaction conditions: 10 mL of 200 mM phosphate buffer (pH 7.5), 50 g/L maltose as co-substrate, 50 mM 4-(trifluoromethyl)acetophenone (**a**), 30 °C and 200 rpm for 24 h (**a**, **b**).

4.7.2. Screening of Organic Cosolvents

The screening results of the organic cosolvent are shown in Figure 3. Reaction conditions: 10 mL phosphate buffer (200 mM, pH 7.5) containing various organic cosolvents at a volume fraction of 5%, 17 g/L recombinant *E. coli* cells (DCW), 50 g/L maltose as co-substrate, 100 mM 4-(trifluoromethyl)acetophenone, 30 °C, 200 rpm, reaction for 24 h.

4.7.3. Effect of Isopropanol Proportion on the Asymmetric Reduction

The optimized result of isopropanol proportion in the reaction system is shown in Figure 4. Reaction conditions: 10 mL phosphate buffer (200 mM, pH 7.5) using isopropanol as cosolvent with a

volume fraction of 1% to 25%, 17 g/L recombinant *E. coli* cells (DCW), 50 g/L maltose as co-substrate, 100 mM 4-(trifluoromethyl)acetophenone, 30 °C, 200 rpm, reaction for 24 h.

4.7.4. Optimization of Other Key Variables in Polar Organic Solvent–Aqueous System

The optimized results of other key variables are shown in Figure 5. Reaction conditions: 10 mL phosphate buffer containing isopropanol at a volume fraction of 15%, 17 g/L recombinant *E. coli* cells (DCW), 50 g/L maltose, 100 mM 4-(trifluoromethyl)acetophenone (**a**, **b**, **c**), 30 °C (**a**, **b**, **d**) and 200 rpm for 24 h.

4.7.5. Comparison of Asymmetric Reduction in Isopropanol-Buffer Medium and Aqueous System

The optimized results of the reaction time in an isopropanol-buffer medium and aqueous system are shown in Figure 6. Reaction conditions: 10 mL of 100 mM phosphate buffer (pH 7.5) containing 15% (*v*/*v*) isopropanol in organic solvent-buffer medium, or 10 mL of 200 mM phosphate buffer (pH 7.5) in an aqueous system, 17 g/L recombinant *E. coli* cells (DCW), 50 g/L maltose, 100 mM 4-(trifluoromethyl)acetophenone, 30 °C and 200 rpm.

4.8. Preparative Scale Bioreduction in the Developed Isopropanol-Aqueous System

The preparative-scale enantioselective synthesis of (*R*)-1-[4-(trifluoromethyl)phenyl]ethanol by recombinant *E. coli* cells was conducted in 500 mL Erlenmeyer flasks. The reaction mixture (total volume of 100 mL) consisted of 100 mM phosphate buffer with pH 7.5 and 15% (*v*/*v*) isopropanol, 17 g/L recombinant *E. coli* cells, 50 g/L maltose as co-substrste, 100 mM 4-(trifluoromethyl)acetophenone. The reaction mixtures were incubated at 30 °C and 200 rpm for a certain time. Samples were withdrawn periodically to determine the yield and product ee value.

5. Conclusions

In the present study, we successfully developed a biological process for the efficient asymmetric reduction of 4-(trifluoromethyl) acetophenone to its (*R*)-form alcohol by the recombinant *E. coli* whole cells for the first time. Meanwhile, isopropanol is a promising and attractive cosolvent for this recombinant whole-cell-mediated biocatalytic process. We also provide a useful strategy to enhance the substrate loading and improve biocatalytic efficacy for poorly water-soluble non-natural substrates. Although some significant results were obtained in our study, some problems involved in the reaction such as recycling of biocatalysts and developing novel reaction medium of biocatalysis are worth further consideration in future work for larger scale production.

Supplementary Materials: The following are available online at http://www.mdpi.com/xxx/s1, Figure S1: GC chromatogram of the bioconverted sample, Figure S2: Chiral analysis of the bioconverted sample in the preparative-scale bioreduction.

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