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# Enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate to ethyl (R)-2-hydroxy-4-phenylbutyrate using stereospecific carbonyl reductase KmCR: optimization of cultivation and reaction conditions

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**Abstract**
Ethyl (R)-2-hydroxy-4-phenylbutyrate ((R)-HPBE) is used as an intermediate in the production of angiotensin converting enzyme (ACE) inhibitors for treating various medical conditions. The study used Stereospecific carbonyl reductase (KmCR) enzyme for the asymmetric synthesis of (R)-HPBE. The optimization process involved varying IPTG concentration, induction temperatures, and induction times to determine the optimal culture conditions for producing (R)-HPBE with increased yields. The most efficient culture conditions were determined as 0.2 mM IPTG concentration, 17 °C temperature, and 10 hours of induction. Additionally, the study explored various reaction parameters, including temperature, substrate concentration, enzyme concentration, co-solvents, and their ratios, to optimize the reaction conditions for synthesizing (R)-HPBE with higher productivity. The optimal reaction conditions were found at 25 °C, substrate concentration of 10.3 $g \cdot L^{-1}$, enzyme concentration of 50 $g \cdot L^{-1}$, and isopropanol co-solvent with a ratio of 10%. The optimized conditions produced 62% yield of (R)-HPBE with an optical purity of ≥99.9% and a reaction time of 20 minutes. These findings provide valuable insights for potential large-scale production using the identified optimal parameter concentrations.

**Keywords** *Kluyveromyces marxianus* carbonyl reductase (KmCR), (R)-2-hydroxy-4-phenylbutyrate, ethyl-2-oxo-4-phenylbutyrate, asymmetric transformation, stereospecificity

## 1. Introduction

Ethyl (*R*)-2-hydroxy-4-phenylbutanoate ((*R*)-HPBE) is one of the important intermediate of angiotensin converting enzyme inhibitors [1]. ACE inhibitors is the key and prominent group of drugs that are used for controlling different diseases such as treating heart failure, blood pressure, preventing kidney damage, and preventing strokes, in people with diabetes or hypertension [2]. (*R*)-HPBE can be synthesized by resolution of racemic ethyl 2-hydroxy-4-phenylbutyrate (HPBE) with a chemical catalyst or lipase [3, 4]. The resolution conversion of the racemate can be as high as 50%. (*R*)-HPBE can be obtained by asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate using a microorganism as a catalyst, while the conversion of the substrate can theoretically reach 100%. The microorganism can convert a small amount of substrate because the reducing ability of cells can be suppressed by a large amount of substrate.

In the current year different approaches have been reported for the preparation of (*R*)-HPBE, mostly in two ways: synthesis and kinetic resolution. However the resolution methods are very low because the maximum yield can be reached to 50% theoretically [4], and the chemical synthesis method normally involve in many stages and the reaction condition is stringent [5]. Ethyl 2-oxo-4-phenyl-butyrate (OPBE) is a prominent way to produce optically active (*R*)-HPBE, by microbial and enzymatic reduction process. meanwhile ethyl 2-oxo-4-phenyl-butyrate can be synthesized easily and quietly cheap. Numerous biocatalysts has been used for the synthesis of (*R*)-HPBE, such as hydrolysis and transesterification by lipase [6] and the OPBE reduction catalyzed by whole cells [5, 7] and dehydrogenase [8]. Stoichiometric volumes of NADH cofactors is require for the reduction reaction, isolated enzyme rather than whole cell were used specially to avoid cofactor addition and purification of enzyme [9].

However, during the last decade, numerous microorganisms have been recognized as an effective biocatalyst in OPBE reduction to (*R*)-HPBE. Chadha et al reported that, the reduction of enantioselective of OPBE to (*R*)-HPBE can be achieved using callus cell-free aqueous extracts of *Daucus carota* (wild carrot) in high yield (90%) and enantiomeric excess (ee) (99%) [10]. However, their process required a high cell/substrate ratio of 100:1, high cell numbers, and a long reaction time of 10 days. Dao et al. and Lacerda et al. reported and achieved an effective reduction of OPBE with *Saccharomyces cerevisiae* and *Pichia angusta*, respectively, producing (*R*)-HPBE with a reasonable enantioselectivity (81% ee) [7, 11]. Recently, Chen et al. using *Candida boidinii* CIOC21 [12] and described the preparation of successful (*R*)-HPBE by a yield (92%) and favorable ee (99%). However, comparatively low substarte concentrations is about 4.1 $g \cdot L^{-1}$ and product 3.8 g/L in their process, which limits their use in large-scale production. Furthermore, Zhang et al. obtained (*R*)-HPBE from 20 g/L OPBE with moderate yield (82%) and excellent ee (97.4%) [5].

In past [13] *Rhodotorula mucilaginosa* CCZU-G5 yeast strain was isolated from vineyard soil samples and used it for the preparation of (*R*)-HPBE with high enantiomeric excess and high yields. However, strong substrate inhibition was detected when the concentration of test OPBE in the monophasic aqueous system was high due to the strong hydrophobicity of the substrate and its toxicity to cells, and the concentration of substrate was high and that the bacteria transformed was only 50 mM. A two-phase water/organic solvent system is a good alternative to solve the above problems in an aqueous system. In a biphasic system, the organic solvent phase serves as a substrate reservoir and protects the cells in the aqueous phase from being harmed by a high substrate concentration.This two-stage system has attracted a lot of attention in the last decades and several successful examples have been reported [14, 15].

In a preliminary study, Gene mining was used to identify the carbonyl-reducing enzymes from *Candida albicans* (CaCR), *Saccharomyces cerevisiae* (ScCR), *Kluyveromyces marxianus* (KmCR), and *Candida parapsilosis* (CPR-C1, CPRC2). These enzymes are anticipated to solve the issue of enzymatic reactions involving large substrates, such as ketoesters and heterocyclic ketones, which serve as precursors for the synthesis of crucial pharmacological intermediates. The newly identified enzymes' biocatalytic abilities towards the tested carbonyl substrates were also assessed [16]. In this study, the biocatalytic efficiency was improved by optimize the conditions for culturing the recombinant *E. coli* that which temperature, time, and inducer were optimized for high production of enzyme (KmCR), to ensure a balance between expression and growth. Furthermore, the enzymatic reaction conditions were optimized for catalyzing the substrate under increased concentration and producing high yield of corresponding

chiral product. Substrate feeding and optimum condition for culturing of *E. coli* were successfully adopted, achieving an unprecedented catalyst yield up to 80 % in 20 min reaction time.

## 2. Materials and methods

### 2.1 Chemicals

(*R*)-HPBE was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Wujin Fine Chemical Factory Co., Ltd. (Changzhou, Jiangsu, China) provided the OPBE. All remaining reagents and solvents were commercially accessible and had analytical grade purity.

### 2.2 Media preparation

The preparation of the LB liquid media needs tryptone 10 $g \cdot L^{-1}$, yeast extract 5 $g \cdot L^{-1}$ and NaCl 10 $g \cdot L^{-1}$, and for solid media 2% agar (20 $g \cdot L^{-1}$) was added. The media should be sterilized at 120 °C for 20 minutes before use.

### 2.3 Strain and cultivation condition

The recombinant strain *E. coli* BL21 (DE3)/PET28a+KmCR was constructed and preserved by our laboratory. It was cultured on LB plate containing 50 $\mu g \cdot mL^{-1}$ kanamycin and incubated upside down at 37 °C for 12 hours. A single colony was picked from the plate and inoculated on 5 ml of LB liquid medium containing (kanamycin 50 $\mu g \cdot mL^{-1}$) at 37 °C for 8–10 h, and then transferred in the liquid medium of 50 mL LB containing 50 $\mu g \cdot mL^{-1}$ kanamycin in a 100 mL shaker flask, shaking at 37°C for 2-3 h, and then IPTG 0.5 $mmol \cdot L^{-1}$ added to a different final concentration when the $OD_{600}$ of the culture solution was 0.6–0.8 and the expression was induced at 17 °C. After that, the cells were collected by centrifugation at 9000 rpm for 10 min, washed twice with 0.9% NaCl in water, and stored at -20 °C before using.

### 2.4 SDS-PAGE analysis

To evaluate the molecular weight of the KmCR enzyme produced. The supernatant sample was separated by SDS-PAGE. The sample where mixed with 4 × SDS loading buffer at the ratio of 3:1 and incubated for 10 min at 80 °C, then centrifuged at 12000 rpm for 10 min, and 10 µL supernatant of the processed protein sample was taken for the electrophoresis with the voltage 80 V. After the sample entered the lower separating gel, the voltage was adjusted to 120 V. When the indicator reached the bottom of the gel, turned off the electrophoresis apparatus, took out the gel, shaked and dyed it for 15 minutes, then discarded the dye solution, shaked and decolorized it for 30 minutes, then shaked and decolorized it overnight. The relative expression level of the interested enzyme was analyzed by image lab software (Bio-Rad, USA).

### 2.5 Analytical method

Using a Chiralcel OD-H column (4.6 mm × 250 mm × 5 m, Diacel, Hyogo, Japan) and an Agilent 1260 HPLC system (Sta. Clara, CA, USA) utilizing n-hexane:isopropanol (95:5, v/v) with an eluent flow rate of 1.0 mL/min, the (R)-HPBE and (S)-HPBE were both examined. At 225 nm, the detection was carried out [13].

As seen below, the ee value was calculated.

$$ee = \frac{[R]-[S]}{[R]+[S]} \times 100\%$$

where [R] is the peak area of (*R*)-HPBE and [S] is the peak area of (*S*)-HPBE, respectively. The final molar concentration of the product, Cp, and the starting molar concentration of the substrate, Cs, were used to compute the yield of (R)-HPBE as follows:

$$\text{yield} = \frac{[\text{Cp}]}{[\text{Cs}]} \times 100\%$$

## 3. Results and Discussion

### 3.1 Optimization of culture conditions

#### 3.1.1 Effects of IPTG concentration, temperatures, and cultivation time

The investigation aimed to determine the optimal concentration of IPTG for inducing protein expression and subsequent substrate conversion. Various IPTG concentrations (0.1, 0.2, 0.5, and 1.0 mM) were tested in LB medium. Figure 1 (A) shows the comparison of high KmCR production at different IPTG concentrations. Based on the results, 0.2 mM IPTG resulted in the highest production (62.30%) as confirmed by SDS-PAGE analysis Figure 1

(B). Increasing IPTG concentrations did not enhance the production, suggesting potential metabolic burden effects that can reduce productivity [17, 18]. It is crucial to optimize the IPTG concentration due to the unpredictable response of protein expression[17, 19].

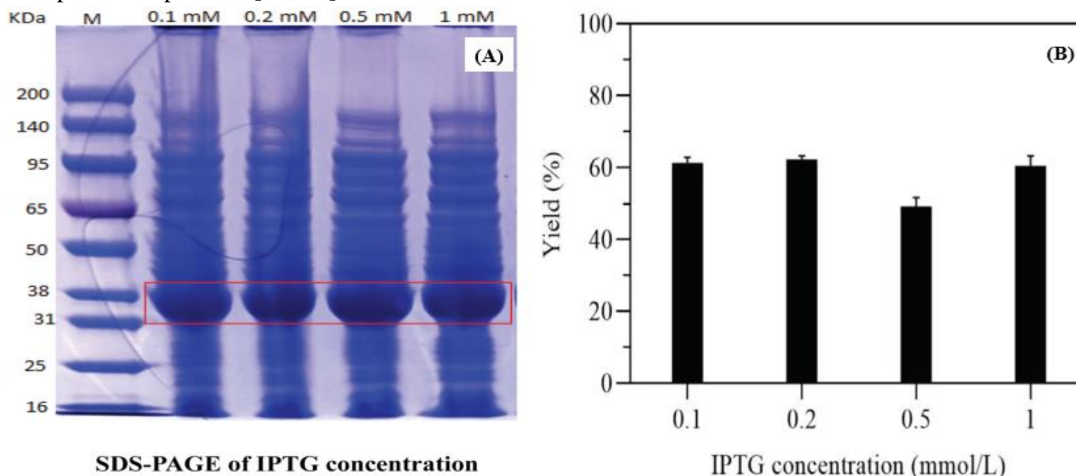


*Figure 1: (A) SDS-PAGE analysis of expressing KmCR at different IPTG concentration (B) effects of IPTG concentrations on catalytic efficiency of KmCR*

The recombinant E. coli strain BL21(DE3)/pET28a (+)-KmCR was found to produce soluble proteins with high activity. The effect of induction temperature on soluble protein expression was investigated by incubating cultures at different temperatures (17, 26, 30, and 37 °C) for 16 hours after induction with 0.5 mM IPTG. The results showed that the highest expression of soluble protein was achieved at 17 °C, while the level of soluble protein decreased with increasing temperature. Simultaneously, cell growth decreased with higher temperatures. SDS-PAGE analysis confirmed these findings in Figure 2 (A). When crude enzyme bioconversion was determined by HPLC, the highest productivity of (*R*)-HPBE was observed at 17 °C in Figure 2 (B). Interestingly, even when using the supernatant of cell lysate as a catalyst, the highest productivity was still achieved at 17 °C. This suggests that higher temperatures may negatively affect productivity and E. coli growth. Choosing the right induction temperature is critical for optimizing recombinant protein production [20].

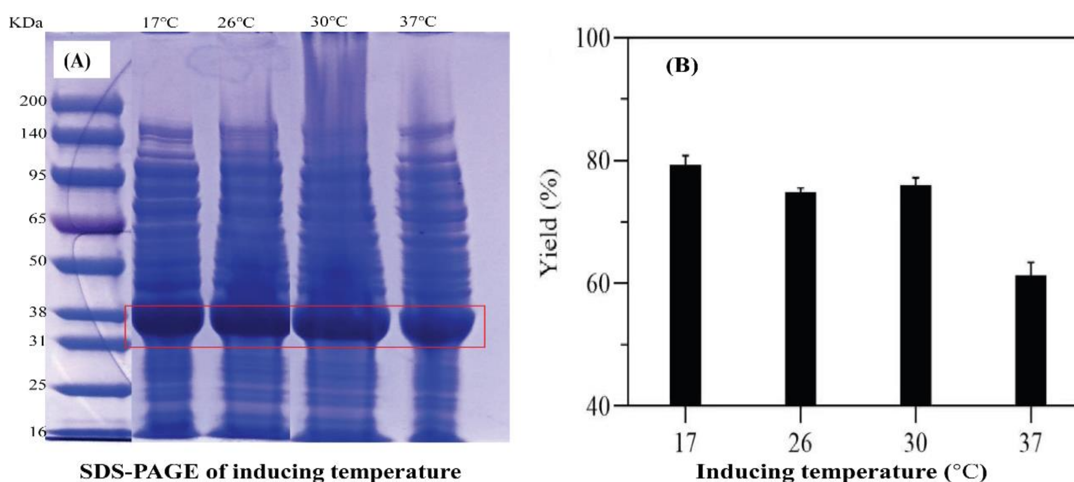


*Figure 1: (A) SDS-PAGE analysis of expressing KmCR at different induction temperature (B) effects of induction temperature on catalytic efficiency of KmCR*

The optimal induction time for the growth of recombinant E. coli BL21(DE3)/pET28a (+)-KmCR was investigated. Four induction time gradients (8, 10, 12, and 16 hours) were selected for analysis. The growth curve analysis showed that the bacterial mass increased with increasing induction temperature, and the optimal culture time was determined to be 10 hours. The cell concentration reached 0.7 at 10 hours of induction, while it reached 0.6 at 8 hours, 0.8 at 12 hours, and 0.9 at 12 hours as shown in Figure 3 (A). In contrast to E. coli BL21, which is typically induced at an OD600 of 0.6, it was possible to induce protein expression at a later stage using *E. coli* BL21(DE3)/pET28a(+)-KmCR due to its higher optical density before induction. However, for the enzyme synthesis reaction, an optimum induction time of 10 hours was determined based on the analysis shown in Figure 3 (B) and (C). The levels of soluble protein expression remained constant at different induction times, while slight decreases in yield production were observed with increasing induction times [21].This indicates that induction time is a critical factor affecting recombinant E. coli growth and protein yield production [22].

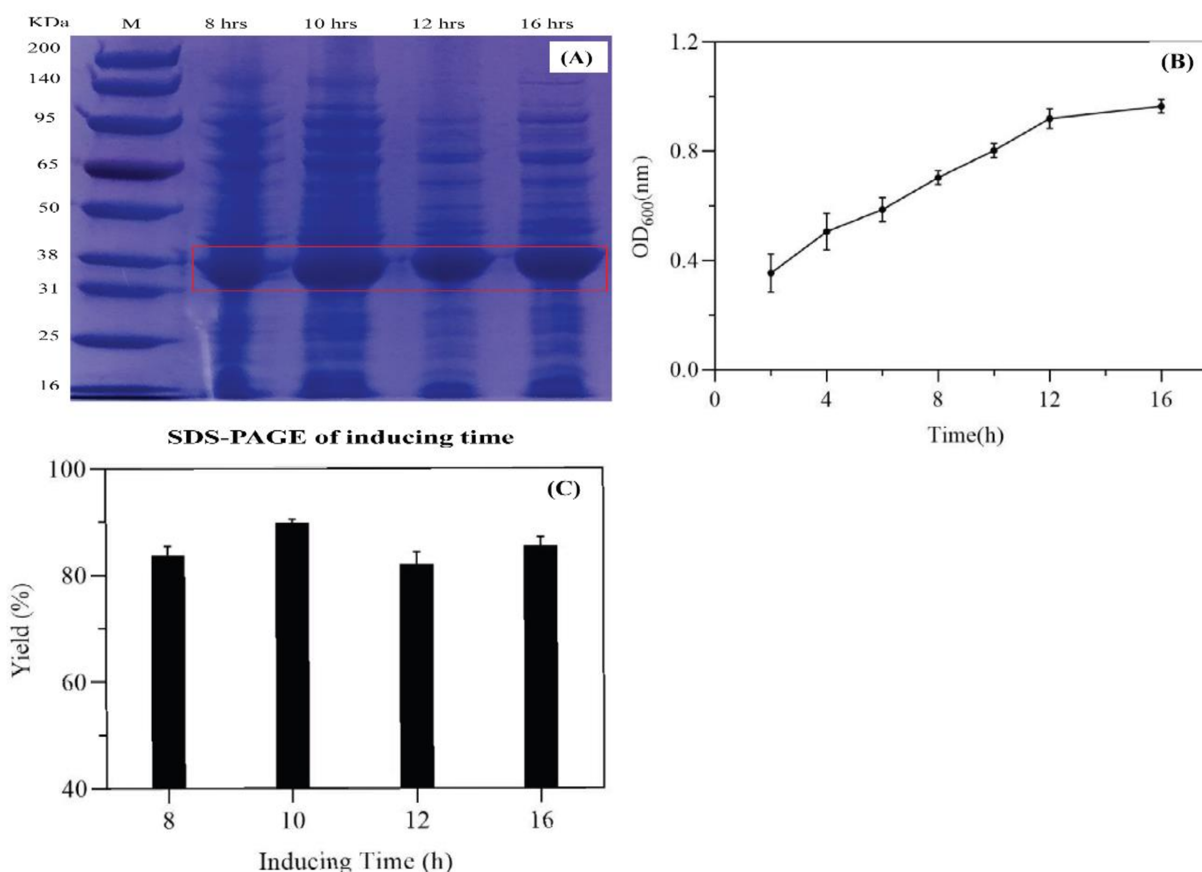


*Figure 2: (A) SDS-PAGE analysis of expressing KmCR at different induction time (B) Growth curve of E. coli BL21(DE3)/pET28a (+)-KmCR at different induction time (C) effects of induction time on catalytic efficiency of KmCR*

In summary, the study concluded that the optimal conditions for inducing protein expression and substrate conversion in recombinant *E. coli* BL21(DE3)/pET28a(+)-KmCR were 0.2 mM IPTG concentration, an induction temperature of 17 °C, and an induction time of 10 hours. These findings highlight the importance of carefully optimizing these factors to maximize protein yield production and ensure proper growth of the recombinant bacteria.

### 3.2 Effect of catalyst form on catalytic efficiency

The passage describes the use of a biocatalyst, specifically an enzyme with exceptional stability, to catalyze enzymatic reactions using whole cells. The approach involves intracellular coenzyme regeneration, which allows for the renewal of the coenzyme cycle by providing a low-cost auxiliary substrate instead of expensive coenzymes. However, this method presents challenges such as complex reaction mechanisms, the presence of various byproducts, and limitations imposed by the cell wall membrane, which affects substrate and catalyst mass transfer and leads to longer reaction times, lower catalytic efficiency, and lower space-time yield [23].

Alternatively, using free enzymes as biocatalysts simplifies downstream product separation and purification due to fewer side reactions and higher product purity [24] [25]. However, free enzymes are not resistant to high substrate/product concentrations and organic solvents, requiring the addition of expensive coenzymes. The process of producing biocatalysts using free enzymes is labor-intensive, complex, and expensive [24]. In the context of the study, a fusion enzyme called KmCR was used as the biocatalyst. The reaction conditions involved the use of a whole cell catalyst with a bacterial suspension of 100 g·L-1, and a crude enzyme catalyst obtained by crushing the bacterial suspension at the same concentration. The aim was to investigate the best form of the biocatalyst for the asymmetric synthesis of (*R*)-HPBE. The results showed that the whole cell catalytic form had a low yield of the desired product, (*R*)-HPBE, at 10.0% or lower after a 10-minute reaction at 30°C. In contrast, the crude enzyme form relieved the barrier effect of the cell wall membrane and exhibited higher catalytic activity [26]. The yield of (*R*)-HPBE in the crude enzyme form reached approximately 62-68%, which was five times higher than that of the whole cell form as shown in Figure 4. This led to the determination that the asymmetric conversion reaction should be catalyzed using the crude enzyme form. Overall, the study highlights the advantages and limitations of using whole cells versus free enzymes as biocatalysts, with the crude enzyme form demonstrating higher catalytic efficiency and product yield in the asymmetric reduction reaction of OPBE to (*R*)-HPBE.

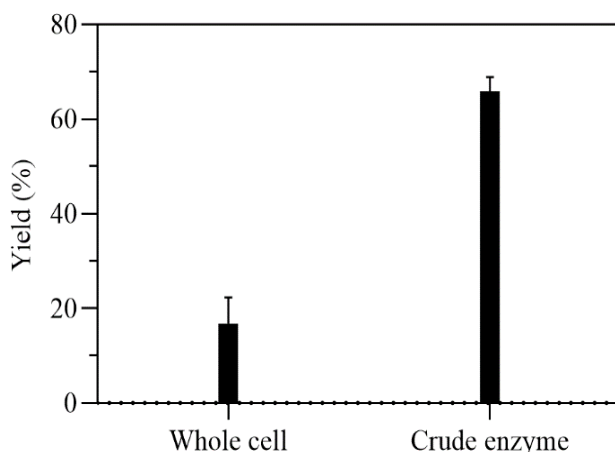


*Figure 3: Time course of KmCR asymmetric synthesis of (R)-HPBE*

### 3.3 Optimization of reaction conditions

#### 3.3.1 Effect of pH on asymmetric synthesis of (R)-HPBE by KmCR during reaction

The pH of a buffer solution within a reaction system exerts a significant influence on the conformation of enzyme molecules, the dissociation state of specific amino acid residues in the enzyme's active center, and the dissociation state of the enzyme-substrate complex. These factors subsequently impact the enantioselectivity, activity, coenzyme regeneration, and hydrogen proton transfer within the reaction [27, 28]. To explore the impact of pH on the conversion reaction, various pH conditions were tested using Tris-HCl buffer (0.1 M, pH 8.0 and 9.0), sodium acetate buffer (0.1 M, pH 4.0), and PBS buffer (0.1 M, pH 5.5, 6.0, 6.5, and 7.0). Experimental results indicated that the reaction yield exhibited an increasing trend with rising pH when pH was below 7.0. However, at pH 7.0, the yield reached its maximum level. Beyond pH 7.0, the yield decreased as pH increased. Consequently, the optimal pH for the reaction was determined to be 7.0, resulting in an approximate productivity of 89% as shown in Figure 5.

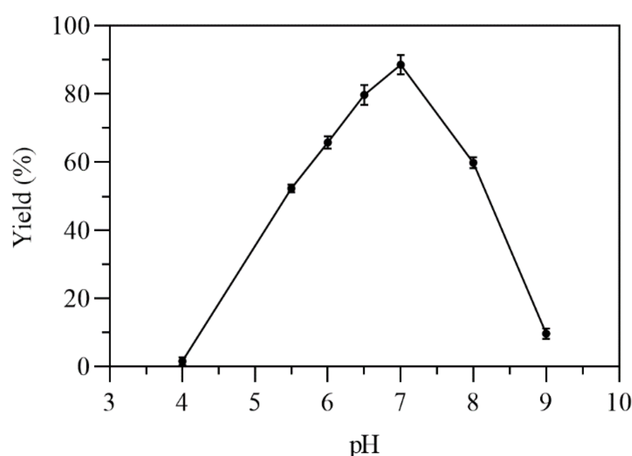


*Figure 4: Effect of pH on KmCR asymmetric synthesis of (R)-HPBE*

#### 3.3.2 Effect of reaction temperature on asymmetric synthesis of (R)-HPBE

The effect of reaction temperature on the conversion reaction was investigated over a range of temperatures from 20 °C to 45 °C. Temperature plays a crucial role in influencing both the activity and stability of the enzyme, as well as the overall reaction balance. Higher temperatures were found to inhibit enzyme activity but also facilitate the collision between enzymes and substrate molecules, thereby enhancing reaction efficiency [29, 30]. The temperature increase was observed to result in a substantial improvement in the reaction yield, with the highest yield achieved at 25 °C as depicted in Figure 6. However, beyond 25 °C, the yield exhibited a sharp decline, indicating a possible decrease in enzyme activity or even enzyme inactivation due to the adverse effects of high temperatures. Notably, the temperature did not impact the optical purity of the product, which remained consistently above 99%. Thus, the optimum reaction temperature was determined to be 25 °C, and the productivity determined by HPLC analysis was approximately 63%.

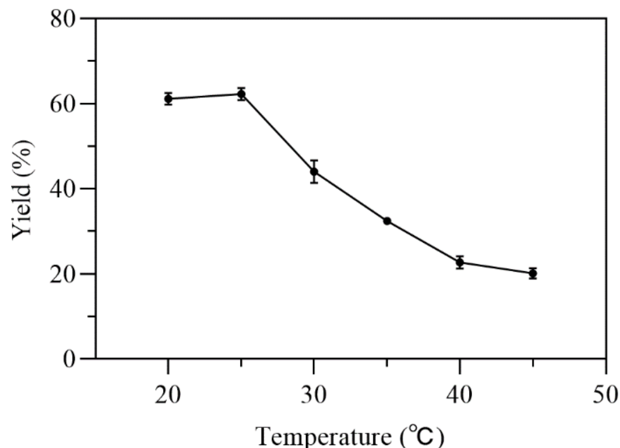


*Figure 5: Effect of temperature on KmCR asymmetric synthesis of (R)-HPBE*

### 3.3.3 Effect of enzyme concentration on asymmetric synthesis of (R)-HPBE

The impact of varying enzyme concentrations on the reduction of OPBE in a reaction system was investigated. Enzyme concentrations ranging from 10 g·L-1 to 50 g·L-1 were utilized. The productivity of the reaction showed an improvement from approximately 45% at 10 g·L-1 to 61% at 50 g·L-1 enzyme concentration. Although the enantiomeric excess (ee) value remained unaffected by changes in enzyme concentration, both the reaction rate and yield were significantly influenced [31]. The yield exhibited an increasing trend with increasing enzyme concentration, reaching a maximum of approximately 61.54% at 50 g·L-1. However, further increases in enzyme concentration beyond 50 g·L-1 did not lead to any additional improvement in the yield. These findings underscore the critical role of enzyme concentration as a key parameter in enzymatic reactions, impacting both the reaction rate and efficiency [13]. Therefore, the optimal enzyme concentration determined in this study was found to be 50 g·L-1, resulting in an impressive productivity of approximately 62% for (*R*)-HPBE as shown in Figure 7.

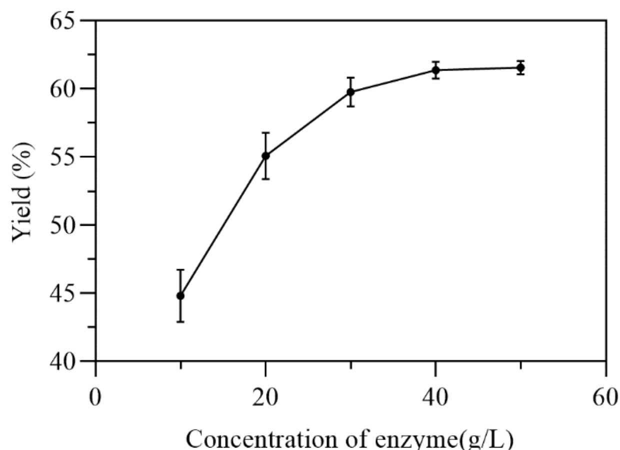


*Figure 6: Effect of enzyme concentrations on KmCR asymmetric synthesis of (R)-HPBE*

### 3.3.4 Effect of substrate concentration on asymmetric synthesis of (R)-HPBE

The substrate concentration in a reaction system plays a crucial role in determining both the reaction rate and enantioselectivity [32], as observed in the case of OPBE asymmetric reduction. A study investigated the effect of varying substrate concentrations ranging from 2.06 g·L-1 to 20.6 g·L-1. The highest productivity, around 67.64%, was achieved at a substrate concentration of 10.3 g·L-1, while lower concentrations resulted in lower yields (51.63% at 2.06 g·L-1, 47% at 4.12 g·L-1, etc.) as shown in Figure 8. However, it was observed that the optical purity of the product was not significantly affected by changes in substrate concentration. This indicates that although OPBE, being an unnatural substrate for enzyme asymmetric reduction, can inhibit the catalytic activity of the mutant enzyme, it does not compromise the enantioselectivity of the reaction [33]. These findings highlight the importance of optimizing substrate concentration in biocatalytic organic synthesis to overcome substrate inhibition and enhance overall reaction efficiency.

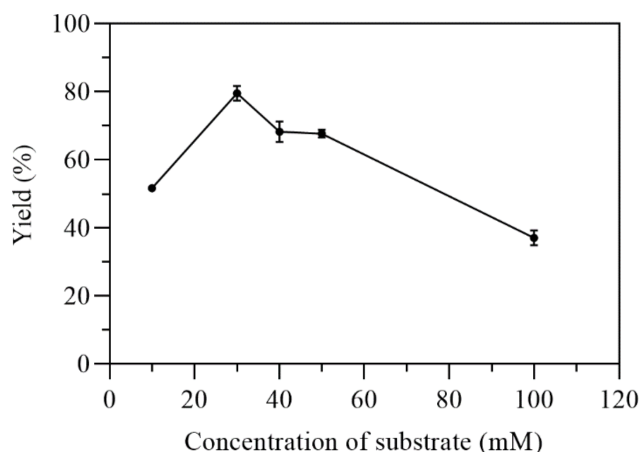


*Figure 7: Effect of OPBE concentration on KmCR asymmetric synthesis of (R)-HPBE*

### 3.3.5 Effect of different cosolvent on asymmetric synthesis of (R)-HPBE

The use of organic molecules as cosolvents in biocatalytic processes can enhance the applicability of hydrophobic substrates. By incorporating hydrophilic solvents, the solubility of the substrates can be increased, facilitating the interaction between enzymes and substrates [34]. Additionally, cosolvents have been shown to improve the yield and enantioselectivity of enzyme conversions [35]. In a study investigating the effect of various solvents on substrate dissolution, including isopropanol, ethanol, acetonitrile, dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF), isopropanol exhibited the highest reaction yield at 62%, followed by ethanol and DMSO at 50% as shown under in Figure 9. This observation can be attributed to several factors. Firstly, polar solvents like isopropanol exhibit superior solubility in the hydrophobic substrate compared to nonpolar solvents. Secondly, isopropanol and ethanol demonstrate better biocompatibility and exert a lesser impact on enzyme catalytic activity. Lastly, the enzyme can oxidize isopropanol, thereby utilizing it as a hydrogen donor to facilitate the regeneration cycle of the coenzyme. Previous reports have documented the inclusion of isopropanol in reaction systems, where the enzyme regenerates the coupling cofactor of the substrate through isopropanol oxidation [15, 26, 36]. However, when the solvent possesses a low log P value, indicating high polarity, cells exhibit reduced metabolic activity. The damaging

effect of polar organic solvents with low log P values on the cell membrane resulted in metabolic activity retention of 14%, 44%, and 19% in a biphasic system consisting of no cosolvent, acetonitrile, and THF, respectively [37].

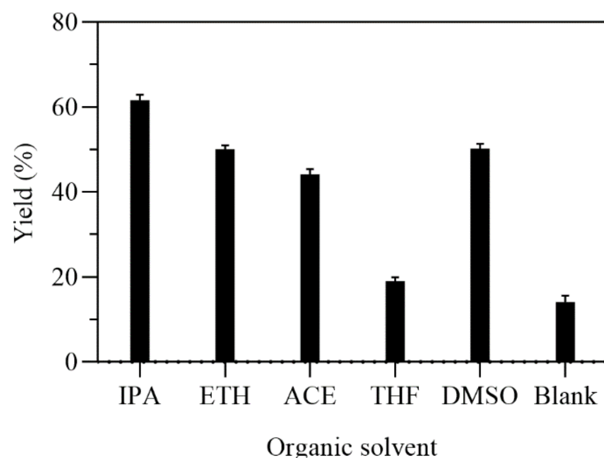


*Figure 8: Effect of cosolvents on KmCR asymmetric synthesis of (R)-HPBE*

### 3.3.6 Effect of different ratio of isopropanol on asymmetric synthesis of (R)-HPBE

The addition of organic solvents was found to enhance the dissolution of the substrate in a reaction. However, it was observed that a solvent with a higher concentration exhibited high toxicity towards organisms. The toxicity of the solvent was not solely dependent on its polarity, but also on the percentage of solvent added to the reaction system [15]. By examining Figure 10, it was evident that increasing the ratio of the cosolvent, isopropanol, had minimal impact on the stereoselectivity of the reaction, but resulted in a significant increase in yield. When the ratio reached 10%, the yield reached an approximate value of 68%. However, further increasing the proportion of isopropanol led to a decrease in yield. This decline was speculated to be a consequence of the solvent concentration surpassing the permissible limit for enzyme activity. Consequently, a ratio of 10% was determined to be the optimal concentration of isopropanol in the reaction system, as depicted in Figure 10.

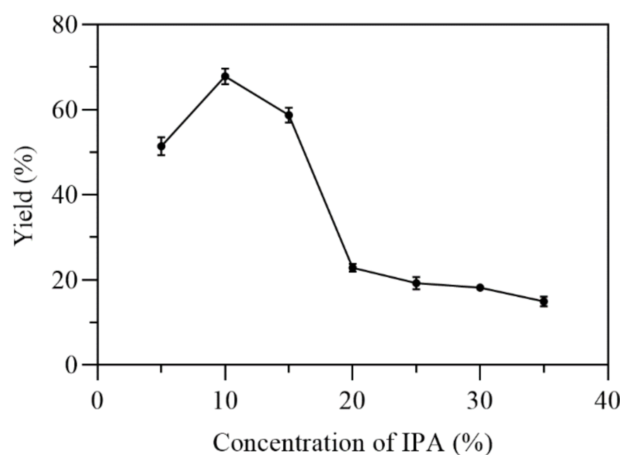


*Figure 9: Effect of ratio of isopropanol (IPA) on KmCR asymmetric synthesis of (R)-HPBE*

## 4. Conclusion

The current study focused on optimizing the culture conditions of recombinant *E. coli* and the reaction conditions to enhance the productivity of (*R*)-ethyl-2-hydroxy-4-phenylbutyrate ((*R*)-HPBE) through asymmetric synthesis. The stereospecific carbonyl reductase (KmCR) was employed to selectively reduce the prochiral ketone group in ethyl 2-oxo-4-phenylbutyrate (OPBE) and produce (*R*)-HPBE with high enantioselectivity. (*R*)-HPBE is a valuable chiral building block in the synthesis of pharmaceuticals and biologically active compounds. To optimize the culture conditions of recombinant E. coli, three parameters were considered: IPTG concentration, induction temperature, and induction time. HPLC analysis revealed that an IPTG concentration of 0.2 mM resulted in a yield of 62.32%, while an induction temperature of 17 °C achieved a yield of 79.32%. Additionally, an induction time of 10 hours led to a yield of approximately 90%. The study compared the reaction efficiency of different catalyst forms, namely crude enzyme, and whole-cell catalytic forms. The crude enzyme catalytic form exhibited a higher product yield of 68.42% compared to the whole-cell catalytic form, thus determining the crude enzyme as the preferred catalyst. The stability of KmCR enzyme and substrate product in the reaction environment was also investigated, showing that the relative enzyme activity remained above 90% after 10 hours, and the substrate product remained stable. Optimizing the reaction conditions involved exploring parameters such as reaction temperature, enzyme amount, substrate amount, co-solvents, and their ratios. The optimum reaction temperature was determined to be 25 °C, resulting in a productivity of 62.26%. An enzyme amount of 50 g·L-1 yielded a productivity of 61.54%, while a substrate amount of 10.3 g·L-1 achieved a productivity of 67.64%. Isopropanol was identified as the optimal co-solvent, with a productivity of 62% at a ratio of 10%. Overall, this study successfully optimized the expression conditions for recombinant E. coli and the reaction conditions to enhance the yield of (*R*)-HPBE, providing valuable insights for the efficient synthesis of this chiral building block.

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