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Enhancing Acetophenone Tolerance of Anti-Prelog Short-Chain Dehydrogenase/Reductase EbSDR8 Using a Whole-Cell Catalyst by Directed Evolution

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Abstract: The short-chain dehydrogenase/reductase (SDR) from *Empedobacter brevis* ZJUY-1401 (EbSDR8, GenBank: ALZ42979.1) is a promising biocatalyst for the reduction of acetophenone to (*R*)-1-phenylethanol, but its industrial application is restricted by its insufficient tolerance to acetophenone. In this paper, we developed a chromogenic reaction-based high-throughput screening method and employed directed evolution to enhance the acetophenone tolerance of EbSDR8. The resulting variant, M190V, showed 74.8% improvement over the wild-type in specific activity when catalyzing the reduction of 200 mM acetophenone. Kinetic analysis revealed a 70% enhancement in its catalytic efficiency (k_{cat}/K_m). Molecular docking was conducted to reveal the possible mechanism behind the improved acetophenone tolerance, and the result implied that the M190V mutation is conducive to the binding and release of coenzyme. Aside from the improved catalytic performance when dealing with a high concentration of acetophenone, other features of M190V, such as a broad pH range (6.0 to 10.5), low optimal cosubstrate concentration (1% isopropanol), and a temperature optimum close to that of *E. coli* cells (35 °C), also contribute to its practical application as a whole-cell catalyst. In this study, we first designed a directed evolution means to engineer the enzyme and obtained the positive variant which has a high activity under high concentrations of acetophenone. After that, we optimized the catalytic performance of the variant to adapt to industrial applications.

Keywords: acetophenone tolerance; whole-cell catalyst; directed evolution; (*R*)-1-phenylethanol; short-chain dehydrogenase/reductase

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1. Introduction

The growing demand for chiral pharmaceuticals, flavors, agrochemicals, and functional materials in the past few decades have led to increasing interest in the production of optically pure enantiomers [1–3]. One of the most popular building blocks is the enantiomerically pure chiral alcohols, which are mainly prepared by chemical transformation or biotransformation at present [4]. Biological transformation is a sustainable and 'green' technique featured by remarkable stereoselectivity, mild reaction conditions, and environmental friendliness [5–7]. Furthermore, the theoretical yield for biocatalytic asymmetric reduction of prochiral ketones reaches 100% [8–10]. By increasing the size of the substrate-binding pocket and eliminating steric repulsion, the medium-chain dehydrogenase (MDR) from *Candida parapsilosis* was engineered to accept more space-demanding substrates with improved catalytic efficiency (Wang et al., 2014). The carbonyl reductase YueD derived from *Bacillus subtilis* was transformed by molecular docking and alanine screening technology, a

mutant V181A was screened out, and the conversion rate of m-bromoacetophenone was increased from 61% of the wild-type to 97% (Naeem, M et al., 2018). By providing a suitable substrate binding pocket for the enzyme starting from the configuration of the target product, efficient asymmetric reduction mutants of o-halogenated acetophenones, phenylacetone, aromatic ketone ester, and diarylketone were designed with >99% conversion and >98% ee (Su et al., 2019).

Recently, short-chain dehydrogenases/reductases(SDRs) have attracted increasing attention ascribed to their broad substrate spectrum, considerable thermostability, and tolerance against organic solvents when applied in the asymmetric reduction of carbonyl compounds [11–14]. Among these enzymes, EbSDR8 has been reported as a powerful biocatalyst for the stereoselective production of anti-Prelog alcohols [1]. The EbSDR8$^{G94A/L153I/Y188A/Y202M}$ was applied in the asymmetric bioreduction of 3-chloro-1-phenyl-1-propanoneto generate the important chiral drug intermediate (*R*)-3-Chloro-1-phenyl-1-propanol, which is widely used in the synthesis of antidepressant drugs and premature ejaculation drugs [15]. However, it shows limited tolerance to a high concentration of acetophenone. Therefore, improving the substrate tolerance is of significant importance for the industrialization of EbSDR8. Directed evolution has been extensively used as a potent tool for the modification of complex properties such as thermostability [16], organic solvent (toluene) tolerance [17], product (ethanol) tolerance [18], and substrate (acetate) tolerance [19]. However, a suitable and efficient high-throughput screening (HTS) method is a necessity.

In the present study, to facilitate the directed evolution of EbSDR8 for improved acetophenone tolerance, we developed an HTS method by adopting the chromogenic reaction depicted in Figure 1a. Acetophenone is catalyzed by EbSDR8 to form (*R*)-1-phenylethanol, accompanied by oxidation of NADH to NAD^+, and the hydrogen of NADH is meanwhile transferred by phenazine methosulfate (PMS) to thiazolyl blue tetrazolium bromide (MTT), generating the blue-violet formazan. The UV absorption of formazan is recorded at 575 nm, which can then be easily converted to NADH concentration, and finally, the concentration of (*R*)-1-phenylethanol formed can be calculated based on the NADH consumed [20]. Based on this method, the error-prone PCR library of EbSDR8 was transformed into *E. coli* BL21 and screened for positive mutants with improved performance at high acetophenone concentration. The underlying mechanism responsible for the enhanced acetophenone tolerance was then analyzed by molecular docking. Finally, the impacts of cosubstrate, pH, and temperature on the whole cell-catalyzed bioreduction of acetophenone using the mutant were investigated.

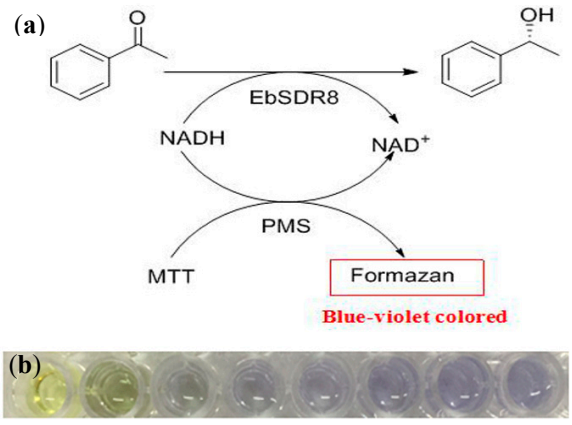

Figure 1. *Cont.*

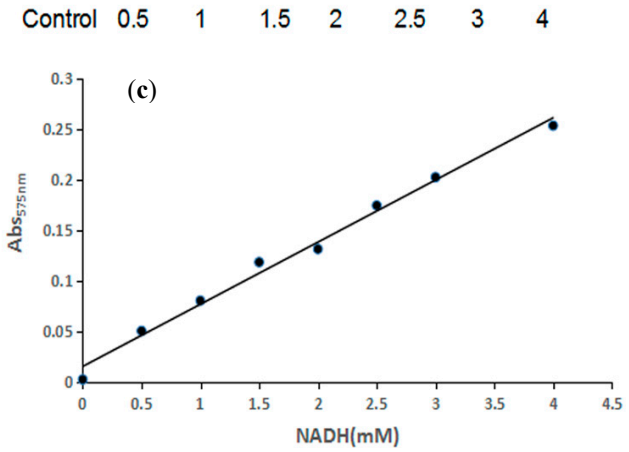

Figure 1. Development of the HTS method. (**a**). Principle of the HTS method. MTT, thiazolyl blue tetrazolium bromide; PMS, phenazine methosulfate. (**b**). The color variance of reaction mixture containing different concentrations of NADH. (**c**). The linear range of the measurement.

2. Results and Discussion

2.1. High-Throughput Screening Method and Activity Assay of the Mutants

To verify the linearity between the NADH concentration and the color variance, so as to validate the HTS method developed, NADH (0–4 mM) with different concentrations was added to the reaction system (Figure 1b). The results showed that the color turned blue-violet with increasing NADH amount, and the change of color was visible to the naked eye. Furthermore, the blue-violet formazan was quantified by recording the absorbance at 575 nm (Abs_{575}) [20] of the reaction systems in Figure 1b with a microplate reader. The linear range of the measurement outcome in accordance with NADH is shown in Figure 1c ($y = 0.0615x + 0.0148$, $R^2 = 0.99$). Therefore, if the value of Abs_{575} is within the linear range, we can compute the NADH amount using this standard curve. Since the bioreduction of acetophenone consumes NADH, a lower NADH amount indicates higher catalytic activity.

Based on the HTS method, directed evolution of EbSDR8 was conducted, and screening of about 10,000 mutants resulted in a positive mutant, M190V. Subsequent saturation mutagenesis with M190V as a template generated two mutants, M190F and M190W, with higher specific activity than the wild-type but not as high as that of M190V (Figure 2). Therefore, M190V was chosen for further study. As shown in Figure 2, the mutant M190V demonstrated 74.8% activity improvement in the reduction of 200 mM acetophenone as compared with the wild-type. This is consistent with the kinetic results in Table 1. The K_m value of the mutant M190V was 8% lower than that of the wild-type (4.57 vs. 4.95 mM), whereas the catalytic efficiency (k_{cat}/K_m) of M190V was 70% higher than that of the wild-type (870 vs. 511 s^{-1} M^{-1}). Therefore, the improved specific activity of the mutant can be commendably explained by these kinetics.

Table 1. Apparent kinetic parameters of the wild-type and the mutant M190V.

Mutants	*K*m (mM)	*K*cat (s^{-1})	*K*cat/*K*m ($S^{-1}M^{-1}$)
Wild-type	4.95	2.53	511
M190V	4.57	3.98	870

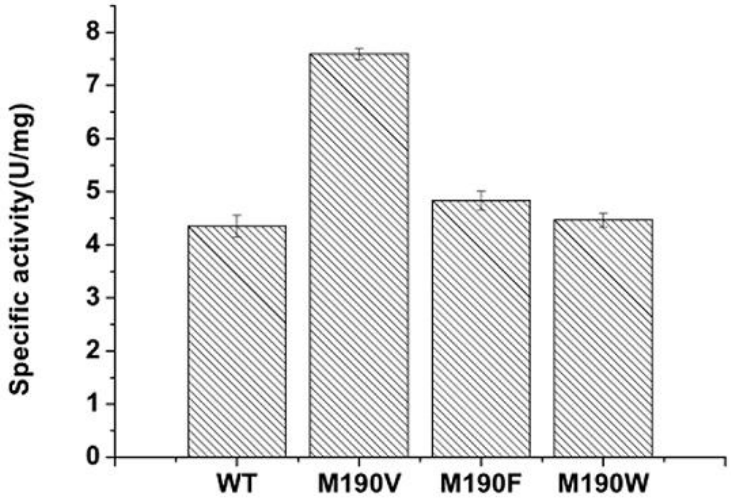

Figure 2. The specific activity of the wild-type and the mutants of M190X measured with purified proteins. Reaction conditions: 500 μL reaction media consisting of sodium phosphate buffer (100 mM, pH 7.5) and 50 μL pure protein, 5 mM NADH, 1% isopropanol, 200 mM of acetophenone, 30 °C, and 10 min. Error bars indicate the standard deviation.

2.2. Comparative Structure Analysis of the Mutant M190V and the Wild-Type

To gain insight into the molecular basis for the activity enhancement in acetophenone reduction, molecular dynamics simulations were conducted. The substrate-enzyme complexes of acetophenone and wild-type EbSDR8 or the variant M190V were employed as representative models (Figure 3a,b). On the basis of the suggested catalytic mechanism of SDR, the C4 atom in the nicotinamide ring of NADH and the hydroxy group of Tyr156 donate their hydrogen atoms to the carbon and oxygen atoms of the carbonyl group of the substrate acetophenone, respectively [21,22]. Moreover, the orientation of acetophenone ensures that NADH delivers its hydride to the Si face of acetophenone to produce (*R*)-1-phenylethanol, following antiPrelog's rule (Figure 3c). Compared with the wild-type, the M190V mutation in the variant resulted in altered protein flexibility, giving the coenzyme more space to select the appropriate conformation, which was conducive to the binding and release of the coenzyme, and thus improved the acetophenone tolerance. Compared with rational design, one of the greatest benefits of directed evolution is the ability to find important amino acids that are not predictable based on the known protein structure or reaction mechanism [23].

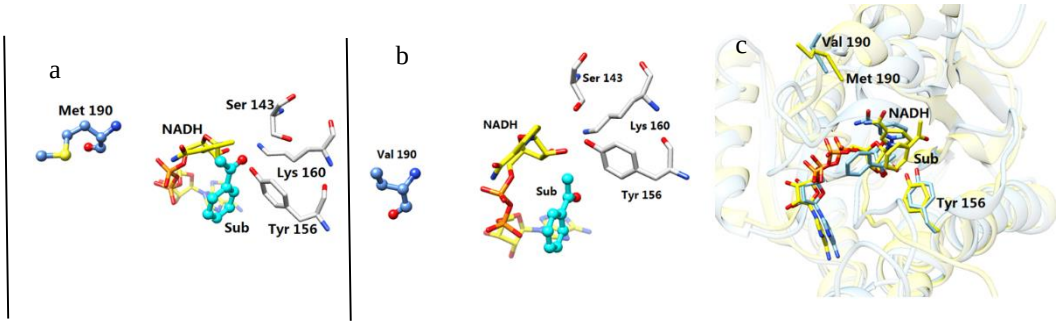

Figure 3. Wild-type EbSDR8-acetophenone complex models. (**a**). Complex models of acetophenone and wild-type EbSDR8. (**b**). Variant M190V-acetophenone complex models. (**c**). Binding pose of the complex models of acetophenone and the wild-type EbSDR8 or variant M190V. The wild-type EbSDR8 complex is shown in gold ribbons, while the variant M190V complex is shown in cyan ribbons.

2.3. Process Optimization of the M190V Whole-Cell-Catalyzed Asymmetric Reduction of Acetophenone

The whole-cell stereoselective biological reduction system consists of two parallel reactions, the asymmetric reduction of carbonyl compounds catalyzed by reductase accompanied by oxidation of reduced cofactors, and the regeneration of the reduced cofactor through the dissimilatory metabolism of a cosubstrate [24]. In such a cofactor regeneration recycling system, the cosubstrate is of significant importance and is essential for the sequential proceeding of biocatalytic reduction [25,26]. Isopropanol has been identified as a suitable cosubstrate in our previous work [1]. In order to find out the ideal isopropanol concentration in the M190V-catalyzed high-concentration acetophenone system, the influence of isopropanol concentration on the asymmetric reduction of acetophenone was subsequently tested. As illustrated in Figure 4a, when adding 1% isopropanol to the reaction system, the conversion increased from 9.73% to 23.66%, manifesting the effective utilization of isopropanol for cofactor recycling by the recombinant whole-cell system. However, further elevation of isopropanol concentration (up to 6%) negatively influenced the acetophenone conversion. Nevertheless, the conversion was still higher than the cosubstrate-free control when the isopropanol concentration was not higher than 5%.

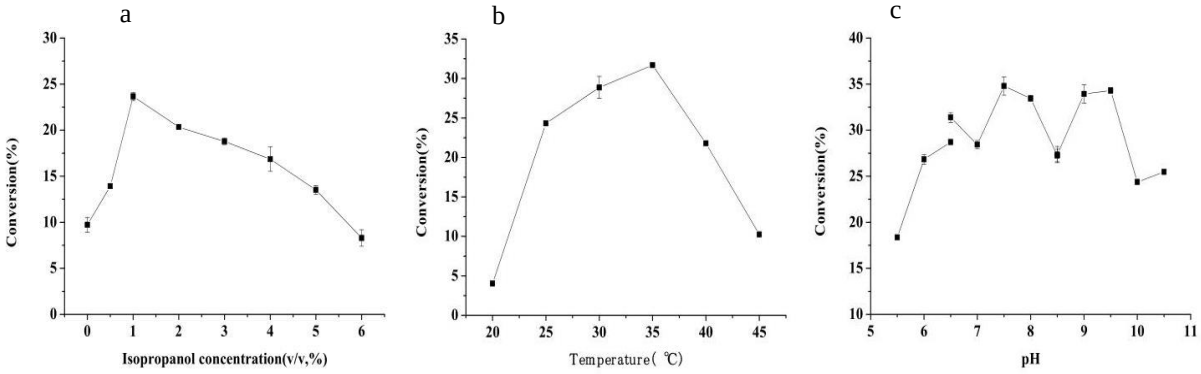

Figure 4. Characterization of EbSDR8 mutant M190V. Reaction condition: isopropanol, 0.025 g wet resting cells, 200 mM of acetophenone, and 500 μL reaction mixture. Error bars indicate the standard deviations. (**a**). The effect of isopropanol concentration (0–6%) on the M190V whole-cell-catalyzed asymmetric reduction of acetophenone. (**b**). The effect of temperature (20–45 °C) on the M190V whole-cell-catalyzed asymmetric reduction of acetophenone. (**c**). The effect of buffer pH (citrate buffer, 5.5–6.5; Sodium phosphate buffer, 6.5–8.5; glycine-NaOH buffer, 8.5–10.5) on the M190V whole-cell-catalyzed asymmetric reduction.

The reaction temperature is another key factor in biocatalysis [27]. As illustrated in Figure 4b, when the temperature rose from 20 °C to 35 °C, the conversion dramatically increased from 4.03% to 31.69%, but with further rising of the temperature to 45 °C, the conversion rate decreased. Nevertheless, the conversion of the acetophenone was maintained above 10.24% at a temperature as high as 45 °C, indicating the good thermostability of M190V.

In addition, variation of pH has been extensively shown to affect the activity of the biocatalyst [27]. To determine the optimal pH for the acetophenone asymmetric bioreduction system using M190V, a range of buffers was used, with pH varying from 5.5 to 10.5. As illustrated in Figure 4c, when the buffer pH increased from 5.5 to 6, the conversion of acetophenone was obviously improved, and relatively high conversions (24.38–34.79%) were obtained over a pH range of 6.5–10.5. The highest conversion was recorded in sodium phosphate buffer (pH 7.5). The considerable capability of M190V whole cells in catalyzing the reduction of acetophenone to (*R*)-1-phenylethanol over such a wide pH range (5.5–10.5) is of significance for its practical application. In fact, biocatalysts with such extraordinary pH adaptability are still few [28,29].

2.4. Time Course Study on the M190V Whole-Cell-Catalyzed Asymmetric Reduction of Acetophenone

To determine the maximum conversion that could be achieved by the asymmetric bioreduction of acetophenone using M190V whole cells, a time course study was conducted. As illustrated in Figure 5, under the optimal reaction condition, the conversion reached 87.52% by 135 min, whereas the conversion of the wild-type under the same condition was only 68.65% by the end (Figure 5). This result further demonstrated the improved catalytic performance of M190V at a high concentration of acetophenone.

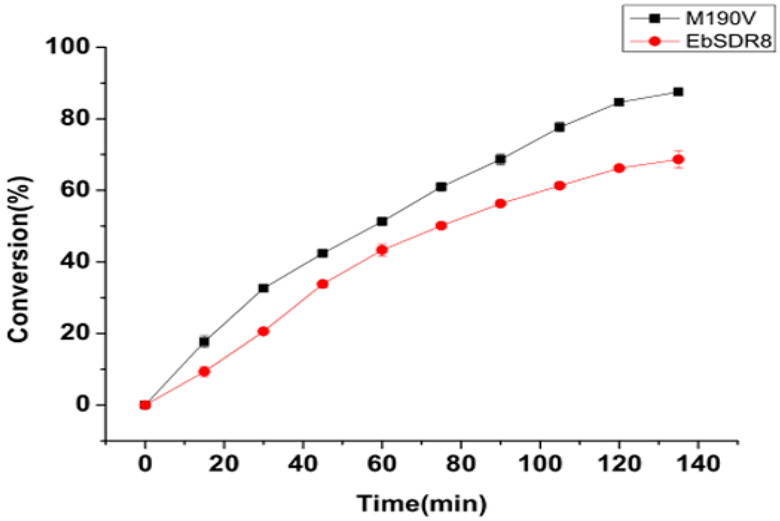

Figure 5. Time courses of M190V and wild-type EbSDR8 whole-cell-catalyzed asymmetric reduction of acetophenone. Reaction condition: 1% isopropanol, 0.05 g wet resting cells, and 200 mM of acetophenone in 500 μL reaction mixture, 35 °C. The conversion of wild-type EbSDR8 is shown in red circles, while that of the variant M190V is shown in black squares. Error bars indicate the standard deviations.

3. Materials and Methods

3.1. Materials

The short-chain dehydrogenase EbSDR8 was deposited in the native laboratory [1]. The plasmid pET30a (Novagen) was used as an expression vector, and *E. coli* strain BL21 (DE3) was employed as the expression host. *EasyTaq* DNA polymerase was purchased from TransGen Biotech Ltd. (Beijing, China). Restriction endonucleases (*BamH*I and *Xho*I) and T4 DNA ligase were purchased from TaKaRa (Dalian, China). Primers were synthesized in Sangon Biotech Ltd. Chemical reagents used in the experiments were all analytical grade and obtained from local companies.

3.2. Error-Prone PCR and Construction of Mutant Library

The error-prone PCR reaction mixture (50 μL) was made up of 10 × *EasyTaq* buffer (5 μL), dNTPs (2.5 mM, 4 μL), ddH_2O (30 μL), template plasmid (1 μL), forward primer (5′-GCTGAGGATCCATGTCAATATTAAAAGATAAGGTAGC-3′) (10 μM, 2 μL), reverse primer (5′-GCATCCTCGAGTTAAACTGCTGTATATCCTCCATC-3′) (10 μM, 2 μL), Mn^{2+} (1 mM, 5 μL), and *EasyTaq* DNA polymerase (5 U/μL, 1 μL). The PCR program was as follows: a denaturation step of 3 min at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 1 min elongation at 72 °C. After double digestion with *BamHI* and *XhoI,* the error-prone PCR product was ligated with the expression vector pET30a and then transformed into *E. coli* BL21 (DE3) competent cells. Finally, the cells were cultured overnight at 37 °C on LB agar plates (containing 50 μg/mL kanamycin), creating the EbSDR8 mutant library.

3.3. High-Throughput Screening

The single colonies of EbSDR8 mutants were selected into conical deep 96-well plates containing 400 μL LB media supplemented with 50 μg/mL of kanamycin. Then, the 96-well plates were shaken at 200 rpm and 37 °C for about 16 h. Subsequently, 10 μL of each culture were transferred into a new 96 deep-well plate, with each well containing 500 μL LB media supplemented with 50 μg/mL of kanamycin. The daughter plates were incubated for 2 h in the same condition as above. In order to induce enzyme expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to obtain a final concentration of 0.1 mM. After 6 h incubation at 26 °C, cells were harvested by centrifugation at 4000 rpm for 15 min. Then, cells were lysed at 37 °C after being supplemented with 250 μL lysis buffer (100 mM Na_2HPO_4-NaH_2PO_4 buffer, 5 mM $MgCl_2$, 250 mg/mL lysozyme, pH 7.5). After 2 h, the cells were pelleted by centrifugation at 4000 rpm for 15 min, and 70 μL of the supernatant was transferred to 96-well microplates. Then, 10 μL of 20 mM NADH and 10 μL of 2 M acetophenone were added into each well of the microplate, followed by 10 min incubation at 30 °C, and the addition of 10 μL of color agent (4.2 mM MTT, 16.6 mM PMS, and the MTT:PMS = 1:2). Then, the UV absorption was determined at 575 nm using a microtiter plate reader [20]. Finally, the variants giving lower absorbance than the wild-type were selected for further verification.

3.4. Enzyme Activity Assay and Kinetic Characterization

The crude enzyme samples of the wild-type and positive mutants were prepared by cell lysis, as described above in Section 2.3., and then purified with a Ni^{2+}-charged His-tag affinity column (GE Healthcare) following the manufacturer's instructions. The concentrations of the protein were measured using the Bradford method [30] with BSA as the protein standard. The amount of enzyme catalyzing the oxidation of 0.3 mM NADH per minute under measurement conditions was defined by one unit of enzyme activity. Enzyme kinetic parameters were obtained using different acetophenone concentrations, and the constants were computed from the Lineweaver–Burk double-reciprocal plot [31].

3.5. Characterization of EbSDR8 Mutant

Using acetophenone as substrate, the catalytic properties of EbSDR8 mutant were analyzed. The biotransformations were carried out at 220 rpm, 30 °C. In total, 200 mM acetophenone, Na_2HPO_4-NaH_2PO_4 buffer (100 mM, pH7.5), and 0.025 mg of EbSDR8 mutant in a total volume of 500 μL constituted the standard reaction mixture. Samples were extracted with ethylacetate for about 2 min, then dried over the organic layer using anhydrous Na_2SO_4. Eventually, the conversion was measured by chiral gas chromatography(GC) analyses. In order to ensure the accuracy of the results, all experiments were conducted in triplicate.

The influence of isopropanol concentration on the asymmetric reduction of acetophenone was investigated with an isopropanol concentration varying from 0–6%. The effects of reaction temperature and pH were determined in a temperature range of 20 °C to 45 °C and a pH range of 5.5–10.5 (100 mM buffers: citrate buffer pH 5.5–6.5, sodium phosphate buffer pH 6.5–8.5, glycine-NaOH buffer pH 8.5–10.5).

3.6. Analytical Methods

Quantification of acetophenone and 1-phenylethanol was performed on a GC-9790 gas chromatography system (Fuli, Wenling, China) equipped with an FID detector using nitrogen as the carrier gas. The injector and detector temperature were both set to 240 °C. Acetophenone and 1-phenylethanol were separated by a chiral capillary column Cyclodex-B (Agilent, Santa Clara, CA, USA, 30 m × 0.32 mm, 0.25 μm film thickness) at 120 °C, and the retention times were as follows: acetophenone (4.86 min), (*R*)-1-phenylethanol (7.04 min), and (*S*)-1-phenylethanol (7.41 min).

3.7. Molecular Simulation

The processor used was Intel(R)Core(TM)i5-7300HQ CPU @2.50 GHz. The display adapter was the NVIDIA GeForce GTX 1050. The wild-type structure of short-chain dehydrogenase/reductase EbSDR8 was obtained by homology modeling (MODELER) [32], and the modeling of the mutant was performed using Swiss-Pdb Viewer 4.03. The receptor model was built using MGLTools 1.5.6, and the ligand model was constructed using ChemOffice 2014. The docking tests of the wild-type and variant towards acetophenone were conducted with AutoDock Vina 1.1.2. During the process of molecular docking, the center is the binding site of small molecule ligands in the tripartite structure of the protein. The Grid Box size was set to 60, 60, and 60, the grid interval was the default value of 0.375 Å, and the other parameters were set to default. Using the parameter files generated by Autogrid, the default settings of the genetic algorithm were selected, and 300 configurations were run for selection. The optimal protein-substrate docking concomplex was selected based on the catalytic mechanism and conformational energy.

4. Conclusions

In this paper, we established a colorimetric HTS method for acetophenone bioreduction and used this method to facilitate the directed evolution of the short-chain dehydrogenase/reductase EbSDR8. A positive mutant, M190V, with a 74.8% improvement in specific activity towards 200 mM acetophenone was obtained. Aside from the enhanced acetophenone tolerance, M190V was also shown to have many other qualities regarding its practical application as a whole-cell biocatalyst, including a broad pH range, moderate reaction temperature, and low optimal cosubstrate concentration. A comparative molecular simulation study of the mutant and the wild-type indicated that the enhanced acetophenone tolerance could probably be attributed to the improved binding and release of coenzyme. Furthermore, directed evolution is proven to be helpful in creating mutants that are not predictable by the rational design of protein, and is therefore particularly suitable for modification of complex enzymatic properties such as substrate/product tolerance and environmental tolerance. However, mutants that may have more advantages in this random screening method were not detected, so they still need to be studied in the future. In future industrial production, attention should be paid to the catalytic capacity under the condition of a high concentration of chiral ketone, and the carbon source, nitrogen source, pH, inoculation capacity, fermentation temperature, fermentation speed, and fermentation cycle should still be optimized, so as to improve the expression of the enzyme protein to improve the catalytic activity of the unit cell.

Author Contributions: S.Y., H.Z. and L.Y. conceived of the study and its design, developed a chromogenic reaction-based high-throughput screening method, participated in employed directed evolution to enhance the acetophenone tolerance of EbSDR8 research, and drafted the manuscript. H.Z., B.W. and H.Y. carried out the directed evolution to enhance the acetophenone tolerance of EbSDR8, performed the statistical analysis, and helped to draft the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethics Approval and Consent to Participate: Not applicable.

Abbreviations

SDRs, short-chain dehydrogenases/reductases; PMS, phenazine methosulfate; MTT, thiazolyl blue tetrazolium bromide; HTS, High throughput screening; IPTG, isopropyl β-D-1-thiogalactopyranoside; GC, gas chromatography.

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