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Enhanced activity of *yqh*D oxidoreductase in synthesis of 1,3-propanediol by error-prone PCR

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Abstract

*yqh*D oxidoreductase was determined to be an NADP-dependent dehydrogenase, and was more active toward 3-HPA when compared to 1,3-propanediol oxidoreductase. To further improve enzyme activity towards 3-hydroxypropionaldehyde (3-HPA), error-prone PCR was implemented to mutant *yqh*D gene. Two mutants, D99QN147H and Q202A with increased catalytic and affinity efficiency, were obtained after one round of error-prone polymerase chain reaction. And the catalytic efficiency of the mutant D99QN147H was up to 4-fold greater than the wild enzyme ($0.0375\ min^{-1}\ mM^{-1}$ vs. $0.0078\ min^{-1}\ mM^{-1}$). The recombined strain containing pET28*yqh*D D99QN147H yielded 28 $g\ L^{-1}$ of 1,3-propanediol in the fed-batch LB cultures (1 L volume) with an initial 3-HPA concentration of 40 $g\ L^{-1}$, which was higher than the 21 and 17 $g\ L^{-1}$ of 1,3-propanediol from the mutant Q202A and the wild-type, respectively. Except for propionaldehyde, the optimal mutant D99QN147H also exhibited higher activity on a range of substituted aldehydes than the wild-type.
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Keywords: *yqh*D oxidoreductase; Error-prone PCR; Catalytic efficiency; 1,3-Propanediol

1. Introduction

1,3-Propanediol (1,3-PD) is a key industrial chemical, applied mainly for the synthesis of polypropylene glycol terephthalate (PPT) with highly desired properties for large volume markets. In nature, PD is produced by the fermentation of glycerol. At present, 1,3-PD is manufactured commercially through carbonylation of ethylene oxide or hydration of acrolein. However, these two methods both require non-regenerative petroleum as the basic material, coupled with toxic and hazardous intermediates in the production process. Comparatively, biosynthesis of 1,3-PD has received increasing attention due to its environmental and resource advantages. A large number of bacteria including *Citrobacter* [1], *Clostridium* [2], *Enterobacter* [3], *Klebsiella* [4], and *Lactobacillus* species can produce 1,3-PD under anaerobic growth on glycerol, and as such, the current focus is on improving biological production of 1,3-PD by various methods including fermentation optimization of the natural glycerol-utilizing process and construction of genetically engineered bacterial strains. Utilizing genes from natural strains of bacteria that produce 1,3-PD from glycerol, metabolic engineering has enabled the development of a recombinant strain that utilizes the lower cost feedstock D-glucose [5–9]. For example, DuPont has integrated four genes into a single *Escherichia coli* microorganism [5,6], including the glycerol-3-phosphate dehydrogenase (*DAR*1) and glyceride 3-phosphate dehydrogenase (*GPP*2) genes obtained from *Saccharomyces cerevisiae* to provide glycerol [9], and the glycerol

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dehydratases (*dhaB*) and 1,3-PD oxidoreductase (*dha*T) genes obtained from *Klebsiella pneumoniae* to enable the conversion of glycerol to 1,3-PD [7–10]. Using this approach, the yield of 1,3-PD could reach 50.1 g L^{-1}, a much higher yield than the natural strain.

Interestingly, a previously uncharacterized oxidoreductase (termed puritative oxidreductease in GenBank) encoded by the puritative oxidreductease (*yqh*D) gene from *E. coli* was found to substitute 1,3-PD oxidoreductase (*Dha*T) from *K. pneumonia*, and was thus considered a 1,3-PD oxidoreductase isoenzyme [11]. The industrial preference for *yqh*D over the more obvious *dha*T results from fed-batch fermentation studies where strains utilizing *yqh*D produced 1,3-PD titers of approximately 130 g L^{-1}; such high titers could not be obtained in identical strains utilizing *dha*T nor in glycerol-fed fermentation using natural 1,3-PD-producing organisms. It has been proven that the 1,3-PD yield only can reach 0.41 g L^{-1} under the same conditions as when the *yqh*D gene encoding puritative oxidreductease was knocked off. Therefore, the *yqh*D gene is considered to play a pivotal role in the fermentation of 1,3-PD.

Unfortunately, only a few studies on the function of the *yqh*D gene produced from *E. coli* are available in GenBank. In a study by Nakamura and Whited, in contrast to *dha*T, *yqh*D was considered to utilize NADPH rather than NADH, and the differences in the cofactor reduced/oxidized ratios likely contributed to the higher titer, which resulted in more 3-HPA deoxidized to 1,3-PD [12]. Using the single wavelength anomalous diffraction method at the Pt edge, the structure of *yqh*D at 2.0 Å resolution was reported as an NADP-dependent dehydrogenase, a result confirmed by activity measurements with several alcohols, and *yqh*D was defined as an alcohol dehydrogenase (ADH) with preference for alcohols longer than C_3 [13]. Further, as well as being involved in catalyzing the *in vitro* reduction of acetaldehyde, malondialdehyde, propanaldehyde, butanaldehyde, and acrolein in a NADPH-dependent reaction, *yqh*D was also recently shown to be part of a glutathione-independent response mechanism to lipid peroxidation [14].

Although some progress in the biotransformation of 1,3-PD has been achieved [11,15,16], and DuPont has declared to set up a commercial unit of 1,3-PD manufacture through fermentation in the presence of microbes, new approaches for economic biological production of 1,3-PD are required. Over the past decade, the use of directed evolution for enhancing enzyme activity and stability, and even for creating new catalytic function, has been exploited [17–19], resulting in many evolved enzymes with improved or new functions [20–22]. However, as yet there are no reports on directed evolution applied to *yqh*D; thus, considering the significant role of *yqh*D in glycerol-fed fermentation, the aim of the present study was to further increase its 3-HPA-deoxidizing activity by error-prone PCR in order to generate more efficient 1,3-PD production.

2. Materials and methods

2.1. Enzymes and reagents

Restriction endonucleases, *Taq* DNA polymerase and T_4 DNA ligase were purchased from Takara Biotech Co. Ltd. (Dalian, China). NADPH was obtained from Roche. All chemicals were of analytical grade or higher quality and purchased from Biocolor (Shanghai, China).

2.2. Strains and plasmids

The *E. coli* strain Novablue (DE3) was used as the host for expression of proteins. Plasmid pET28a(+)-*yqh*D encoding *yqh*D oxidoreductase gene was prepared as described previously [23].

2.3. Mutant library construction by error-prone PCR

The *yqh*D gene of *E. coli* K12 was used as the template for the construction of mutant library. An error-prone PCR was conducted with a set of primers, N-terminal primer NheI: 5′-GCAGCTAGCTAGGAGGCCATA ACTATGAACAAC-3′ and C-terminal primer BamHI: 5′-CATGGATCCTGTCATGATTTTCGCCCAGTTGG GTC-3′.

The mutagenic buffer (50 μl) was prepared, which contained 12 pmol of unbalanced dNTPs, 20 pmol of each primer, about 1 ng of template DNA and 0.025 mM $MnCl_2$. Initial template denaturation was programmed for 10 min at 95 °C. The PCR profile was 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C for 50 cycles, and a final 72 °C extension step for 10 min. The resulting genes were purified and digested with NheI and BamHI, and then subcloned into an expression vector pET28. The transformed *E. coli* novablue with these constructs was spread on Luria–Bertani (LB) plate containing kanamycine (30 μg ml^{-1}) grown overnight at 37 °C.

2.4. Screening of positive clones and sequence analysis

The clones were screened for the conversion of NADPH to NADP during *yqh*D oxidoreductase catalyzes 3-HPA into 1,3-PD in 96-well plate format. The clones were grown in 200 μl of LB media containing 30 μg ml^{-1} kanamycine. The plate was inoculated with 4 μl/well of the inoculated cells from the master plate and incubated for 20 h at 37 °C (250 r/min and 85% relative humidity). The 20 h cultures were subcultured into 2.2 ml deep well plates containing 500 μl/well of LB media with 30 μg ml^{-1} kanamycine and grown at 37 °C (200 r/min and 85% relative humidity). Cells grown at the OD_{578} 0.7–1.0 to were induced with 0.4 mM IPTG to express proteins and were incubated under the same conditions for 12 h. The cells were collected by centrifugation at 3500 r/min for 30 min, treated with lysozyme (1 mg ml^{-1}) and DNAse (1 μg ml^{-1}), and then frozen and thawed. The crude cell extracts were obtained

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by centrifugation at 4000 r/min for 30 min and the supernatant was used for the activity assay in the active screening. About 300 μl supernatant aliquots were transferred into new 96-well plates for screening, and 3-HPA (200 μM) and *b*-mercaptoethanol (1 μM) were added to the lysate in the presence of 5 μM $ZnCl_2$, and the plates were incubated for 10 min at room temperature before 20 μl NADPH (500 μM) solution was added. NADPH consumption rate was measured for 3 min at room temperature at 340 nm and calculated using the equation: $A = \varepsilon_M bc_{NADPH}$ ($\varepsilon_M = 6200 \text{ mol L}^{-1} \text{ cm}^{-1}$). The background signal originating from the NADPH consumption without addition of the substrate served as a negative control.

DNA sequence analyses were performed on both strands using a synthetic or universal primer purchased from Biocolor (Shanghai, China).

2.5. Enzyme purification

Escherichia coli cells were grown in 250 ml of LB broth and induced with 0.4 mM IPTG for 2.5–3 h when the OD_{600} reached about 0.45. The collected cells by centrifugation were suspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and disrupted by ultrasonification. After centrifugation at 14,000 r/min for 30 min, the soluble fraction was loaded onto an affinity resin column, Ni–NTA (Qiagen), pre-equilibrated with the cell lysis buffer. The column was completely washed with the same buffer and then eluted with a buffer containing 250 mM imidazole. Aliquots of each eluted fraction were subjected to SDS–PAGE and enzyme assay.

2.6. Determination of kinetic parameters

The assay was carried out in preparations containing 50 mM Tris–HCl buffer (pH 7.7), 1 mM EDTA, 1 mM *b*-mercaptoethanol, in the presence of 5 mM $ZnCl_2$. The wild-type or mutated *yqh*D enzyme (1.5 mM) was pre-incubated with discrete dilution of 3-HPA (0–150 mM) or other substrate solutions, and the reaction was started by the addition of NADPH (50 mM) [13]. The decrease of absorption at 340 nm, due to the consumption of NADPH, was read at 40-s interval for 3 min at room temperature. All values were measured in triplicates. The Michaelis–Menten parameters were determined by standard methods.

2.7. Determination 3-HPA and 1,3-PD concentration

Concentrations of 3-HPA and 1,3-PD were determined by GC [24]. The instrument for GC was equipped with a flame ionization detector, and a 2 m × Φ3 m stainless-steel chromatographic column packed with macromolecule microsphere GDX2401 (110 MS) (made in China). The injector temperature and detector temperature were both set at 250 °C .The column temperature was fixed at 220 °C. Nitrogen was used as the carrier gas at a flow rate of 1.0 ml min^{-1} with the split ratio of 1:100.

3. Results

3.1. Construction of a random mutant library

To further improve *yqh*D enzyme activity, the method of random mutagenesis was applied to the complete *yqh*D oxidoreductase. The *yqh*D gene was selected as a template for the round of random mutagenesis. After one round of random mutagenesis, mutant libraries were produced by error-prone PCR with different mutagenic factors. From the iterative screenings of a total of approximate 7500 produced clones, eight candidates exhibited 1.25- to 4.6-fold higher enzyme activity when compared to the wild enzyme (Table 1). The greatest improvement in activity was that of clone H6, which had a 4.6-fold higher activity.

The eight clones were subjected to sequence analysis. Results revealed that only five of eight clones were mutated by error-prone PCR. Four mutants acquired one additional amino acid substitution of either I152S, T266P, G21L, or Q202A, and one mutant acquired the additional amino acid substitutions (D99Q and N147H). All of mutations in the five mutants are shown in Fig. 1.

3.2. Kinetic assay of mutant enzyme toward 3-HPA

Five potentially highly active variants were detected and further characterized. After protein expression, purification, and the calculation of specific activity, only two mutants had greater activity than the wild enzyme (Table 2). It should be due to the errors brought by the details of a screening procedure, such as the influence of the host cell background and the growth medium, etc.

The K_m values of the two mutants towards 3-HPA and the V_{max} extrapolated after non-linear regression of the experimental points can be seen in Table 2. Even though the K_{cat} values of the two new mutants were still small ($\leqslant 0.3 \text{ min}^{-1}$) and the affinity for the 3-HPA substrate were poor (K_m values $\geqslant$ 8 mM), the two mutants D99QN147H

Table 1
Apparent activity of 1,3-PD formation in wild and potential mutant clones

	Average activity[a]	sd[b]	Fold improvement
WT	1.24	0.012	1
B6	1.98	0.015	1.6
C7	2.23	0.014	1.8
C12	3.89	0.017	3.14
E2	2.47	0.020	1.99
F1	3.56	0.018	2.87
H8	1.55	0.015	1.25
H6	5.70	0.041	4.6
G10	3.22	0.028	2.6

[a] Apparent activity = $\mu\text{mol min}^{-1} \text{ ml}^{-1}$ in whole cells. Activities are normalized to the optical density of cell suspensions.
[b] Standard deviation.

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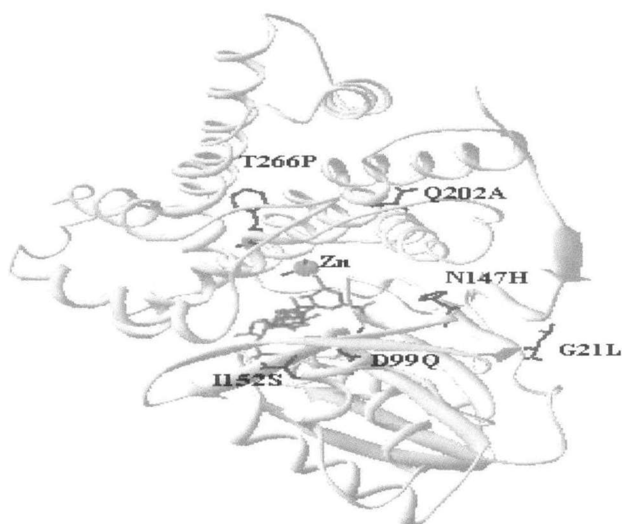

Fig. 1. Model of *yqh*D oxidoreductase with mutation sites. The Zn atom is displayed as a sphere.

Table 2
Comparison of kinetic parameters among the mutants and wild-type enzymes

	K_m (mM)	K_{cat} (min^{-1})	V_{max} (min^{-1} mM)	K_{cat}/K_m (min^{-1} mM^{-1})
WT	17 ± 2	0.133 ± 0.01	0.2 ± 0.01	0.0078
D99QN147H	8 ± 0.8	0.3 ± 0.02	0.45 ± 0.02	0.0375
Q202A	11 ± 1.2	0.22 ± 0.02	0.33 ± 0.02	0.02

and Q202A exhibited an increase in catalytic and affinity efficiency compared to the wild-type enzyme, with the catalytic efficiency of the D99QN147H mutant up to 4-fold greater than the parent enzyme (0.0375 min^{-1} mM^{-1} vs. 0.0078 min^{-1} mM^{-1}), indicating that the mutant enzymes were a better match for the 3-HPA substrate.

Biotransformation of 3-HPA into 1,3-PD by whole cells of *E. coli* strains novablue pET28*yqh*DD99QN147H, Q202A, and wild-type strain were examined under the same fermentation conditions (Fig. 2). Cultures were grown in LB media with 3-HPA. Fed-batch cultures of 1 L volume with an initial 3-HPA concentration of 40 g L^{-1} and utilizing either the mutant *yqh*D oxidoreductase D99QN147H, mutant *yqh*D oxidoreductase Q202A, or the wild-type (these were purified from cell-free culture broth by extraction) yielded 28 g L^{-1}, 21 g L^{-1}, or 17 g L^{-1} of 1,3-PD, respectively; the yield was highest in using the *yqh*D oxidoreductase D99QN147H mutant. The ability of the mutants to produce 1,3-PD in whole cell fermentation is important as the improvement in 1,3-PD yield produced by the two mutants *in vivo* equals their increased enzyme catalytic efficiency *in vitro*.

3.3. Kinetic assays of mutant enzyme toward other aldehydes

The activity of the optimal mutant D99QN147H was examined on a range of substituted aldehydes (Figs. 3 and 4). Results showed that *yqh*D oxidoreductase and its mutant D99QN147H can also catalyze other aldehydes besides 3-HPA, although 3-HPA was found to be the most appropriate substrate for *yqh*D oxidoreductase in this study, suggesting that *yqh*D oxidoreductase is indeed a 1,3-PD oxidoreductase isoenzyme. As for propionaldehyde, the mutant D99QN147H exhibited a higher activity than wild-type enzyme. However, no particular trend was observed in relation to the length of aldehyde chain.

4. Discussion

To date, there are relatively few studies on the application of random mutagenesis to enzymes in the glycerol metabolic pathway. Due to the fact that *yqh*D oxidoreductase, but not 1,3-PD oxidoreductase, can deoxidize 3-HPA in the presence of NADPH in the glycerol metabolic pathway [11], and plays the more important role in the fermentation of 1,3-PD than 1,3-PD oxidoreductase, in the present study we implemented directed evolution to enhance the catalytic properties of *yqh*D oxidoreductase, as a 1,3-PD oxidoreductase (*dha*T) isoenzyme, in deoxidizing 3-HPA into 1,3-PD. Using random mutagenesis with a high through-put screening system, two variants with a higher activity toward 3-HPA than wild-type were identified, and they had acquired mutations.

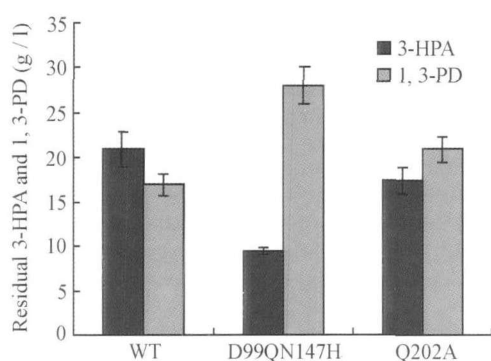

Fig. 2. Concentrations of residual 3-HPA and product of 1,3-PD in biosynthesis of 1,3-PD with recombined strains containing *yqh*D oxidoreductase or wild-type mutants under the same fermentation conditions. Fed-batch cultures of 1 L volume with an initial 3-HPA concentration of 40 g L^{-1}.

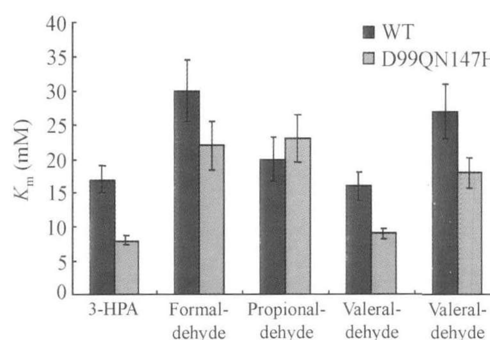

Fig. 3. K_m of *yqh*D oxidoreductases toward some aldehydes. The reaction contained 0.6 μM *yqh*D oxidoreductase from the mutant D99QN147H and wild enzyme, and 0.2 mM NADPH.

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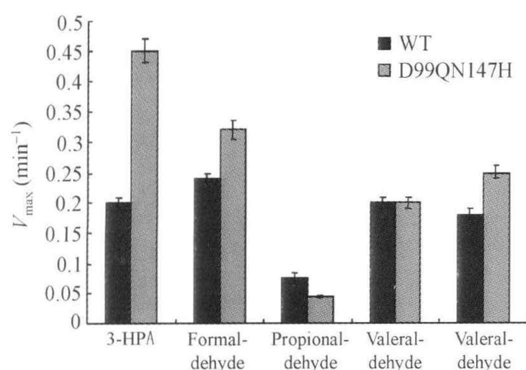

Fig. 4. V_{max} of *yqh*D oxidoreductases toward some aldehydes. The reaction contained 0.6 μM *yqh*D oxidoreductase from the mutant D99QN147H and wild enzyme, and 0.2 mM NADPH.

The *yqh*D oxidoreductase with the active site containing a Zn atom has been shown to catalyze many alcohols, aldehydes, and even some sugars under anaerobic conditions, and it is a dimer of 2 × 387 residues [13]. In the present study, according to the modeling (Fig. 1) the mutations in the D99QN147H mutant were located close to the active domain in the 3-D structure. These replacements significantly improved K_{cat}/K_m (up to 4-fold) and the affinity ability (up to more than 2-fold). The functional amide group of glutamine, an amide derivative of the acidic amino acid glutamate, is highly polar. In this mutant D99QN147H, the hydrogen-bonding capability of glutamine may have had a significant effect on enzyme activity. Trudy and James described that when histidines at appropriate positions are located on or near to the active domain, they also play an important role in the catalytic activity of numerous enzymes, although histidine is a weak base, the residuals of which act as a buffer [25]. In the present study, Asn147 substituted by His may have improved enzyme activity. Further experiments are required to validate the function of positions 99 and 147.

The position 202 is buried into the 3-D structure of *yqh*D oxidoreductase, and is a little far away from the active domain. Nevertheless, the introduction of alanine at position 202 also had a positive effect on the catalytic efficiency of *yqh*D oxidoreductase (0.02 min^{-1} mM^{-1}), suggesting that an amino acid in position 202 plays a valuable role in the relationship between the structure and function of *yqh*D oxidoreductase. Glutamine is a neutral polar amino acid. In contrast, alanine is a non-polar, hydrophobic amino acid and has a different effect on substrate interaction which resulted in a change in the overall dynamics of the molecule, and thus, influenced the enzyme activity.

We expect that further improvement of *yqh*D oxidoreductase is possible, as the initial 3-HPA concentration of 40 g L^{-1} in the biotransformation only formed 28 g L^{-1} of 1,3-PD when utilizing the best mutant D99QN147H. Further improvements are desirable to lower the cost of 1,3-PD.

In conclusion, we demonstrated that the catalytic efficacy of *yqh*D oxidoreductase can be improved by directed evolution. Small-scale conversion in the whole cell using the evolved enzyme exhibited a higher performance than the wild-type enzyme, suggesting that the variant might be effectively used as a novel biocatalyst for the production of 1,3-PD.

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