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ORIGINAL PAPER

Expression and fermentation optimization of oxidized polyvinyl alcohol hydrolase in *E. coli*

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Abstract Oxidized polyvinyl alcohol (PVA) hydrolase (OPH) is a key enzyme in the degradation of PVA, suggesting that OPH has a great potential for application in textile desizing processes. In this study, the OPH gene from *Sphingopyxis* sp. 113P3 was modified, by artificial synthesis, for overexpression in *Escherichia coli*. The OPH gene, lacking the sequence encoding the original signal peptide, was inserted into pET-20b (+) expression vector, which was then used to transform *E. coli* BL21 (DE3). OPH expression was detected in culture medium in which the transformed *E. coli* BL21 (DE3) was grown. Nutritional and environmental conditions were investigated for improved production of OPH protein by the recombinant strain. The highest OPH activity measured was 47.54 U/mL and was reached after 84 h under optimal fermentation conditions; this level is 2.64-fold higher that obtained under sub-optimal conditions. The productivity of recombinant OPH reached 565.95 U/L/h. The effect of glycine on the secretion of recombinant OPH was examined by adding glycine to the culture medium to a final concentration of 200 mM. This concentration of glycine reduced the fermentation time by 24 h and increased the productivity of recombinant OPH to 733.17 U/L/h. Our results suggest that the recombinant strain reported here has great potential for use in industrial applications.

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Introduction

Polyvinyl alcohol (PVA), with its many desirable physical and chemical characteristics, is widely used in textile sizing, adhesives, fiber coating, and other applications. However, this extensive usage, particularly in the textile industry, has resulted in large amounts of PVA being discharged in the wastewater, causing environmental pollution due to the difficulty in degrading PVA.

The traditional process used to degrade PVA during the textile desizing stage involves the use of a strong base at a high temperature to break down the long chain structure of PVA. This process consumes large amounts of energy and water and may also damage the cotton fibers. In contrast, PVA degradation using microbial enzymes is an environmentally friendly approach that can save energy and reduce the problems caused by the purification treatment of sewage from the textile desizing stage. Many researchers have studied the biodegradation of PVA since 1936 [15], and several PVA-degrading microorganisms have been reported to date. Among these strains, *Pseudomonas* spp. are commonly researched species that can produce a variety of enzymes that degrade PVA [6, 10, 19, 24, 25, 28]. The PVA biodegradation process is generally divided into two steps: (1) adjacent hydroxyl groups are oxidized to form a β-diketone structure by PQQ-dependent PVA dehydrogenase (PVA-DH) [6, 8, 21] or secondary alcohol oxidase [5, 11, 19, 24, 27]; (2) the β-diketone is further hydrolyzed to a methyl ketone and a carboxylic acid by β-diketone hydrolase [18] and oxidized PVA hydrolase (OPH). OPH has been found to exhibit activity only towards oxidized

PVA, showing no activity towards monoketones and diketones [12, 22]. Oxidized PVA is unstable under some conditions, such as high temperature (>50°C), which can lead to degradation of the main chain structure of PVA, but only to a minor extent. However, oxidized PVA is easily hydrolyzed by OPH [11, 12, 17].

In recent decades, most studies have focused on the isolation of PVA-degrading strains and on understanding the mechanism of PVA degradation; however, the efficiency of PVA degradation by the isolated strains in these studies was relatively low. To enhance the production of the enzymes and PVA degradation, it is necessary to obtain increased the expression of PVA degradation enzymes using genetic engineering methods.

To date, only the sequences of genes encoding PVA degradation enzymes from *Pseudomonas* sp. VM15C [22, 23] and *Sphingopyxis* sp. 113P3 [7, 12, 13] have been registered in GenBank. PVA-DH and OPH from these two strains have similar enzymatic properties and their amino acid sequences show high similarities, 54 and 63%, respectively. OPH from *Pseudomonas* sp. VM15C has been intracellularly expressed in *Escherichia coli* with a low enzymatic activity, while OPH from *Sphingopyxis* sp. 113P3 was found to be expressed but without any enzymatic activity toward any of the substrates [11, 12, 22].

In this study, an *E. coli* strain was successfully constructed to achieve extracellular overexpression of recombinant OPH, thus improving the ability to produce OPH on an industrial scale.

Materials and methods

Materials

The host strains *E. coli* JM109 and *E. coli* BL21 (DE3) and the expression vector pET-20b (+) were purchased from Novagen (Madison, WI). The PrimeSTAR HS DNA Polymerase, restriction enzymes (*Nco*I and *Eco*RI), T_4 DNA ligase, EZ-10 Spin Column Plasmid Mini-Preps kit, agarose gel DNA purification kit, and the cloning vector pMD18T- simple were supplied from TaKaRa Biotechnology (Otsu, Japan). *p*-Nitrophenyl acetate (PNPA) was purchased from Sigma–Aldrich (St. Louis, MO).

Modification of the OPH gene

To enhance the expression of OPH in *E. coli* [10, 14, 18], we modified the published *oph* sequence (AB190288) from *Sphingopyxis* sp. 113P3: 16 relative low-usage codons encoding leucine and arginine were replaced by high-usage codons from *E. coli*. The relative adaptiveness (RA) of *oph* was analyzed using the GCUA program (http://www.gcua.de; [20]). Emphasis was then placed on its leucine (Leu, L) codons, of which there are eight CTC (RA 26%), four TTG (RA 34%), two CTA (RA 16%), and one CTT (RA 39%). As shown in Fig. 1, these were replaced by CTG codons, which had the highest RA of 100%. In the same way, one codon, CGA (RA 30%), which is translated to arginine (Arg, R), was substituted by the CGT codon (RA 100%). The modified *oph* was used for OPH expression in *E. coli* BL21 (DE3). The disulfide bond prediction for OPH was performed by the Machine Learning and Neural Networks Group (http://disulfind.dsi.unifi.it/). The whole *oph* gene was synthesized by the Sangon Biotech Co. (Shanghai, China).

Construction of the expression vector pET20b (+)–OPH

The synthesized *oph* was used as a PCR template. The pair of primers designed for gene amplification of OPH without its endogenous signal peptide was 5′-CGAGCCATGGATA AATCCGAGTGGGCAT (*Nco*I underlined) and 5′-CGG GAATTCTTACTTATAGTGATCAGTAA (*Eco*RI underlined). The amplified gene was inserted downstream of the pelB signal peptide gene in the expression plasmid pET-20b (+), resulting in pET20b (+)–OPH. The pET20b (+)–OPH was then transformed into *E. coli* BL21 (DE3).

Expression of OPH in *E. coli* BL21 (DE3)

The *E. coli* BL21 (DE3) harboring the pET20b (+)–OPH expression vector was cultured on a rotary shaker (200 rpm) at 37°C in Luria–Bertani (LB) medium containing 5 g/L yeast extract (Angel Yeast, Hubei, China), 10 g/L peptone, 5 g/L NaCl, and 0.1 g/L AMP. After an overnight culture, 3% (v/v) of the seed culture was inoculated into Terrific Broth (TB) medium containing 5 g/L glycerol, 24 g/L yeast extract, 12 g/L peptone, 2.31 g/L KH_2PO_4, 16.43 g/L K_2HPO_4, and 0.1 g/L AMP and cultured at 37°C and 200 rpm until the OD at 600 nm reached 0.6–1. Isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.4 mM, and the temperature was shifted to 25°C. To confirm the expression of OPH in *E. coli*, both a culture of the recombinant strain without IPTG induction and a culture of *E. coli* harboring the pET-20b (+) with IPTG induction were used as controls.

The culture supernatant of the recombinant strain was obtained by centrifugation. The resulting cell pellets were thoroughly suspended in 50 mM potassium phosphate Buffer I (pH 7.0) and lysed on ice for 15 min by ultrasonic waves (sonication for 1 s and intermission for 2 s). The suspension was centrifuged at 10,000*g* for 5 min at 4°C. The precipitates were washed twice with Buffer I. The cell-free extract, cell precipitates, and culture supernatant that were obtained were subsequently used for sodium dodecyl

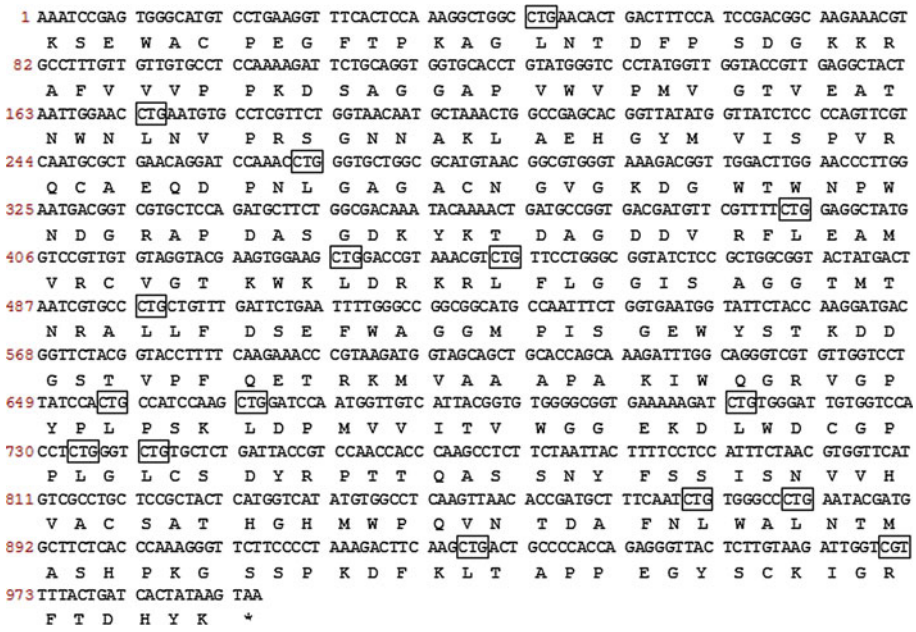

Fig. 1 The nucleotide sequence and the deduced amino acid sequence of oxidized polyvinyl alcohol (PVA) hydrolase (OPH). The modified codons are *boxed*. The stop codon is marked with an *asterisk*

sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and for the enzymatic activity assays.

Optimization of fermentation conditions

To enhance the expression of OPH, the effects of different concentrations of the yeast extract (nitrogen source; 40, 50, 60, 70, 80, 90, 100 g/L), the initial pH of the TB medium (6, 6.5, 7, 7.5, 8, 8.5, 9), and the addition of glycine (50, 100, 150, 200, and 250 mM) were investigated separately. The recombinant *E. coli* carrying the pET20b (+)–OPH expression vector was cultured overnight in LB medium and then inoculated into the modified TB medium and cultured at 25°C for 84 h. The culture supernatant was collected by centrifugation and used for the OPH activity assay.

OPH activity analysis

Enzymatic activity was analyzed in a reaction mixture (2 mM PNPA, 50 mM potassium citrate buffer, pH 6.0) with a pre-incubation at 37°C for 1 min [12]. The formation rate of nitrophenol was measured using a Shimadzu UV-2450 spectrophotometer (light path 0.5 cm, $\varepsilon =$ 540.16 mol/L/cm; Shimadzu, Kyoto, Japan). One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol PNPA per minute under the assay conditions.

SDS–PAGE analysis

The samples collected above were prepared in denaturing buffer at 100°C for 10 min, and 10 μL of each pretreated sample was loaded onto a gel composed of 5 and 12% polyacrylamide for concentration and separation, respectively. Protein bands were visualized in gels by staining with Coomassie Brilliant Blue R [29].

Results and discussion

Expression of OPH in *E. coli* BL21 (DE3)

To express OPH in *E. coli*, we constructed the pET20b (+)–OPH expression vector and transformed it into *E. coli* BL21 (see Materials and methods). As shown in Fig. 2a, *E. coli* harboring the pET20b (+)–OPH vector produced a distinct band of OPH after IPTG induction, having a molecular weight of approximately 35 kDa, which is similar to that reported previously [13]. However, most of the OPH was expressed as in an insoluble form, and a minimal amount was detected in the culture supernatant (OPH activity 3.11 U/mL). When the recombinant *E. coli* was cultured without IPTG induction, a majority of the OPH was secreted into the culture supernatant, with an activity of 16.34 U/mL being measured, although the total expression of OPH decreased (Fig. 2b). Unexpectedly, small amounts of IPTG (0.03 mM) decreased the secretion of OPH by the recombinant strain. In addition, all cultivations achieved a low expression of the target protein and low enzymatic activity in the cell-free extract. It has been reported that the reducing environment of the cytoplasm of *E. coli* prevents the formation of disulfide bonds in recombinant proteins that might lead to the incorrect folding of proteins [1, 3, 4, 14]. OPH was predicted to have four disulfide bonds. Therefore, secretion of the recombinant protein may promote the correct folding of OPH.

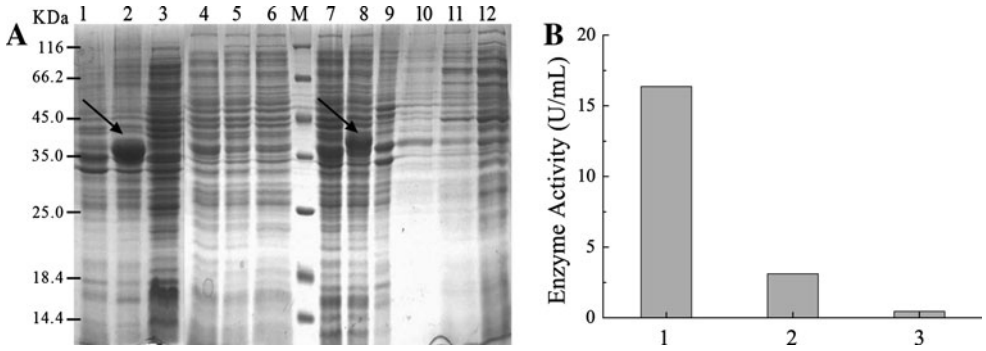

Fig. 2 Expression of the OPH gene in *Escherichia coli* BL21 (DE3). **a** Sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) analysis. *Lanes*: *1*, *4*, *7*, *10 E. coli* BL21 (DE3) harboring the pET20b (+)–OPH expression vector without induction (OPH^+IPTG^-), *2*, *5*, *8*, *11 E. coli* BL21 (DE3) harboring the pET20b (+)–OPH vector with induction (OPH^+IPTG^+), *3*, *6*, *9*, *12 E. coli* BL21 (DE3) harboring the pET-20b (+) vector with induction (OPH^-IPTG^+). *Lanes*: *1–3* Cell precipitates, *4–6* cell-free extract, *M* protein molecular weight standard, *7–9* total cellular protein. *10–12* culture supernatant. **b** OPH activity examination: *1*, *2*, *3* Enzymatic activity of culture supernatant in OPH^+IPTG^-, OPH^+IPTG^+ and OPH^-IPTG^+, respectively. Induction was initiated by the addition of IPTG to a final concentration of 0.4 mM. *IPTG* Isopropyl *β*-D-thiogalactopyranoside

Optimization of yeast extract concentration and initial pH

To improve the production of the recombinant OPH, we tested the effect of the concentration of yeast extract on OPH expression. As shown in Fig. 3a, both the cell density of the recombinant strain and the extracellular OPH activity improved with increasing yeast extract concentration. OPH activity did not increase further once the concentration of yeast extract in the medium was >70 g/L, with increasing concentrations of nitrogen resource (>70 g/L) only resulting in increased cell growth of *E. coli*. Therefore, we chose 70 g/L as the optimal yeast extract concentration under current fermentation conditions. A study of the effect of the initial medium pH on OPH expression revealed that the cell density reached its highest value at pH 8.5, whereas maximum OPH activity (47.54 U/mL) in the culture supernatant was obtained at pH 9.0 (Fig. 3b). One possible explanations is that a weakly basic environment removes the inhibition of cell growth caused by acetate accumulation during *E. coli* cultivation and therefore enhances cell growth and improves production of the recombinant protein [2].

Following optimization of the medium using the parameters determined during our study, the enzymatic activity of OPH reached 47.54 U/mL, which is 2.64-fold higher than that achieved prior to optimization with the recombinant strain induced at 25°C.

Improvement of OPH expression by the addition of glycine

Glycine has been reported to improve the secretion of protein from *E. coli* through enhancement of cell membrane permeability [9, 16, 26]. To further improve the production of enzymatically active OPH, we therefore added different final concentrations of glycine to the culture medium at 24 h after culture initiation (the middle of the logarithmic phase, $OD_{600} = 13$). As shown in Fig. 4, a high concentration of glycine (not exceeding 200 mM) led to increased extracellular active OPH but inhibited the cell growth of *E. coli*. Notably, the addition of 200 mM of glycine to the medium shortened the fermentation time by 24 h and increased the productivity of OPH from 565.95 (0 mM glycine) to 733.17 U/L/h.

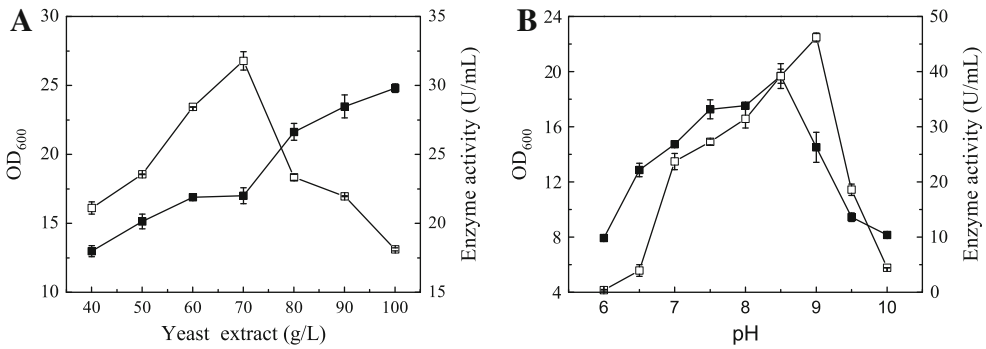

Fig. 3 Effect of the concentration of yeast extract (**a**) and the initial pH of the medium (**b**) on cell growth (*filled squares*) and enzymatic activity in the culture supernatant (*open squares*) of the recombinant *E. coli* BL21 (DE3). *Error bars* Standard deviation of three determinations

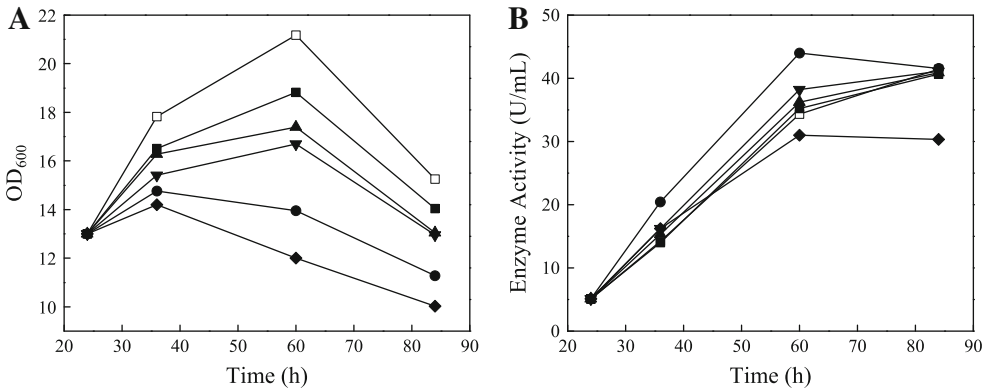

Fig. 4 Effect of glycine on cell growth (**a**) and enzymatic activity in the culture supernatant (**b**) of the recombinant *E. coli* BL21 (DE3). *Open square* 0 mM glycine, *filled square* 50 mM glycine, *filled triangle* 100 mM glycine, *inverted, filled triangle* 150 mM glycine, *closed circle* 200 mM glycine, *closed diamond* 250 mM glycine

Although attempts have been made to express OPH in *E. coli*, the activity of its enzyme has not been reported earlier [12, 22]. The recombinant *E. coli* constructed in this study had a relatively high expression yield and productivity of recombinant OPH. The fermentation process is free of IPTG, and industrial materials are used as a nitrogen resource for cell growth. These properties suggest that this process is feasible for the industrial production of recombinant OPH.

Conclusions

In this research, *E. coli* BL21 (DE3) harboring the pET20b (+)–OPH expression vector was constructed to produce biologically active OPH. This active OPH was successfully secreted by the recombinant strain. Optimization of cultivation conditions resulted in 47.54 U/mL of OPH and 565.95 U/L/h (or 733.17 U/L/h in the presence of 200 mM glycine) of productivity.

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