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ORIGINAL PAPER

Biochemical characterization and high-level production of oxidized polyvinyl alcohol hydrolase from *Sphingopyxis* sp. 113P3 expressed in methylotrophic *Pichia pastoris*

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Abstract The *Sphingopyxis* sp. 113P3 gene *oph*, encoding oxidized polyvinyl alcohol hydrolase (OPH), was optimized with the preferred codons of *Pichia pastoris* and ligated into the pPIC9K vector behind the α-factor signal sequence. The vector was then transfected into *P. pastoris* GS115 and genomic integration was confirmed. Large-scale production of recombinant protein was performed by induction with 14.4 g/L methanol at 22 °C in a 3-L bioreactor. The maximal OPH activity obtained was 68.4 U/mL, which is the highest activity reported. The optimal pH and temperature of recombinant OPH were 8.0 and 45 °C, respectively. OPH activity was stable over a pH range of 5.0–8.5, and at a maximal temperature of 45 °C. The K_{cat}/K_m of recombinant OPH was 598 mM^{-1} s^{-1}, which was 4.27-fold higher than that of recombinant OPH derived from *Escherichia coli*. The improved catalytic efficiency of OPH expressed in recombinant *P. pastoris* makes it favorable for industrial applications.

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Introduction

Polyvinyl alcohol (PVA) has many desirable physicochemical properties, including high thermostability, emulsifiability, tensile strength, and adhesive strength [1, 2]. For these reasons, PVA is widely used for fiber coating and emulsion polymerization in the papermaking and textile industries [2]. However, since PVA degradation is difficult, large quantities of PVA are released directly into the environment.

PVA is traditionally degraded by exposure to high temperatures and strong oxidants, a process that consumes high levels of energy and causes heavy environmental pollution. Therefore, biodegradation by microbial enzymes would offer a significant advantage in terms of environmental protection [1]. To date, PVA biodegradation has been achieved successfully with symbiotic cultures and mixed microbial cultures [3–6]. However, in these cultures, the degradation of PVA by one symbiont is dependent on the provision of pyrroloquinoline quinone (PQQ) by the second symbiont. PQQ acts as a cofactor for PQQ-dependent PVA dehydrogenase (PVA-DH) [4]. In mixed microbial cultures, the PVA degradation process is complex [6, 7].

Biodegradation of PVA occurs in two steps: PVA is first oxidized to a beta-diketone by PQQ-dependent PVA-DH [8] or secondary alcohol oxidase [9], and then most of the oxidized PVA is cleaved by beta-diketone hydrolase or oxidized PVA hydrolase (OPH) into a methyl ketone and a carboxylic acid [10, 11]. A small amount of oxidized PVA can be hydrolyzed non-enzymatically [12].

The genes encoding PVA-degrading enzymes from *Pseudomonas* sp. VM15C [10, 13] and *Sphingopyxis* sp. 113P3 [11, 14] have been identified, and related enzymes show high similarity. Although the mechanisms by which PVA is biodegraded have been well-studied [1], relatively few studies have focused on developing methods to obtain high expression of PVA-degrading enzymes.

Pichia pastoris GS115, a methylotrophic yeast, can utilize methanol as the sole carbon source because of the strong inducing effect of methanol on the alcohol oxidase (AOX1) promoter [15]. *P. pastoris* offers many benefits as a host for heterologous gene expression, such as its ability to perform post-translational modifications and to secrete active molecules [16]. In addition, *P. pastoris* is easily cultured at high cell densities, and thus, large amounts of heterologous proteins can be obtained in the supernatant [17].

We previously reported the expression of the *Sphingopyxis* sp. 113P3 PVA-DH and OPH in *P. pastoris* [18] and *Escherichia coli* [19], respectively. However, the OPH yield in *E. coli* was low even under optimal conditions [19], indicating that *E. coli* may be not a suitable host for expression of this enzyme. Here, we report the expression of a codon-optimized *oph* gene from *Sphingopyxis* sp. 113P3 in *P. pastoris*. The biochemical and enzymatic properties of the enzyme expressed in *P. pastoris* were characterized and compared with the previously reported properties of *Sphingopyxis* sp. 113P3 OPH expressed in *E. coli* [19]. Finally, recombinant OPH was produced by high cell-density culture of *P. pastoris* in a 3-L bioreactor, which produced OPH with the highest activity ever reported.

Materials and methods

Materials and bacterial strains

E. coli JM109, *P. pastoris* GS115, and the expression vector pPIC9K were purchased from Novagen (Madison, WI). PrimerSTAR® HS DNA Polymerase, the restriction enzymes *Sna*BI and *Not*I, EZ-10 Spin Column Plasmid Mini-Prep kits, agarose gel DNA purification kits, and T_4 DNA ligase were purchased from TaKaRa Biotechnology (Otsu, Japan). *p*-Nitrophenyl acetate (PNPA) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cloning and expression of the *Sphingopyxis* sp. 113P3 *oph* gene in *P. pastoris* GS115

The full-length *oph* gene (AB190288) from *Sphingopyxis* sp. 113P3 was optimized to the preferred codons of *P. pastoris* (http://www.kazusa.or.jp/codon) and synthesized by Sangon Biotech Co. (Shanghai, China). The pPICK9 plasmid, which contains a neomycin resistance gene for selection on G418 and an α-factor secretion signal sequence for protein export, was used for construction of the *oph* expression plasmid pPIC9K-OPH. The *oph* gene without its endogenous signal peptide was amplified by PCR using the forward primer 5′-ACGACATACGTAAAA TCCGAGTGGGCAT-3′ (*SnaB*I site underlined) and the reverse primer 5′-GTAATTGCGGCCGCTTACTTATAG TGATCAGTAAAACGAC-3′ (*Not*I site underlined). The amplification product was digested with *SnaB*I and *Not*I and ligated into pPIC9K digested with the same enzymes. The ligated mixture was transformed into *E. coli* JM109. The recombinant plasmid isolated from positive transformants was verified by DNA sequencing and restriction enzyme digestion.

To prepare competent cells, *P. pastoris* GS115 cells were cultured at 30 °C and harvested by centrifugation at 2,600×*g* for 5 min when the optical density at 600 nm (OD_{600}) reached 1.3–1.5. The cells were washed with ice-cold water and resuspended in an ice-cold solution of 1 M sorbitol, 100 mM EDTA, and 14 mM mercaptoethanol. pPIC9K-OPH was linearized with *Sac*I and electrically transformed into competent *P. pastoris* GS115 cells using a Gene Pulser (Bio-Rad, Hercules, CA, USA) set at voltage 1,500 V, capacitance 25 μF, and resistance 200 Ω. To confirm gene integration, *P. pastoris* genomic DNA was isolated and analyzed by PCR using α-factor and 3′ AOX1 primers.

For production of recombinant protein, positive *P. pastoris* GS115 transformants were cultured on a rotary shaker (200 rpm) at 30 °C in BMGY medium containing 13.4 g/L yeast nitrogen base, 4×10^{-4} g/L biotin, 20 g/L peptone, 10 g/L yeast extract, 10 g/L glycerol, and 100 mM potassium phosphate (pH 6.0). Cells were cultured for ~24 h and harvested by centrifugation at 3,000×*g* for 10 min when the OD_{600} reached 8. Cells were then inoculated into BMMY medium (identical to BMGY except glycerol was replaced with 4 g/L methanol) and cultured on a shaker at 200 rpm and 28 °C. Methanol was added to a final concentration of 4 g/L every 24 h. After induction for 112 h, the culture supernatant was collected by centrifugation at 10,000×*g* for 10 min at 4 °C. SDS-PAGE and OPH activity assays were performed on samples of supernatant as previously described [11].

OPH activity was measured at 37 °C for 1 min in a reaction mixture composed of 2 mM PNPA in 50 mM potassium citrate buffer, pH 6.0 [11]. The rate of nitrophenol formation was measured using a Shimadzu UV-2450 spectrophotometer (light path 0.5 cm, $\varepsilon = 540$ mol/L/cm; Shimadzu, Kyoto, Japan). One unit of OPH activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of PNPA per minute under the assay conditions [19].

Production of recombinant OPH in a 3-L bioreactor

Seed cultures were incubated in BMGY medium for 24 h and 100 mL was then inoculated into 900 mL basal salt medium containing 4.35 mL/L PTM1 trace element solution in a 3-L bioreactor (LiFlus GM BioTRON, Korea) [20]. The pH of the medium was adjusted to 5.5 with ammonium hydroxide.

Exponential glycerol feeding was used to achieve high cell-density before methanol induction, as previously reported [21]. The specific growth rate μ_{set} was set at 80 % of μ_{max} (0.176 h^{-1}), and the yield of cell to substrate $Y_{x/s}$ was 0.550 [22]. After the exponential glycerol-feeding period (0–8 h), the feeding rate was reset to 30 mL/L/h for 3 h. After glycerol exhaustion, the temperature was decreased from 30 to 22 °C and methanol (containing 12 mL/L PTM1) was added to the fermentor broth. The methanol concentration was controlled at 14.4 g/L by an online control station (FC2002, East China University of Science and Technology).

Purification and characterization of recombinant OPH

After 88 h of methanol induction in the fermentor, the culture broth was harvested and centrifuged at 10,000×*g* for 10 min at 4 °C. Ammonium sulfate was added to the supernatant to a final concentration of 1 M, and the mixture was filtered (0.22 μm) and concentrated using an Amicon Ultracentrifugal stirred cell with a 10,000 molecular weight cutoff membrane (Millipore, Bedford, MA, USA). A HiTrap Butyl Sepharose high-performance hydrophobic interaction chromatography column was pre-equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A (50 mM sodium phosphate, pH 7.0), and the concentrated supernatant was loaded onto the column. The column was washed with 10 bed volumes of 1 M $(NH_4)_2SO_4$ in buffer A, and protein was eluted with a reverse gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 3 mL/min. Fractions containing OPH were desalted with a HiTrap desalting column and the desalted fractions were loaded onto a HiTrap SP Sepharose fast-flow ion-exchange chromatography column pre-equilibrated with buffer A. The column was washed with 10 bed volumes of buffer A, and proteins were eluted with a gradient from 0 to 1 M NaCl in buffer A at a flow rate of 3 mL/min. The elution peak detected by measuring absorbance at 280 nm was collected and desalted for measurement of OPH activity. The final purified OPH preparation was used for biochemical and enzymatic characterization.

The effect of pH on OPH activity was evaluated by performing the assay at 37 °C in the following buffers (all 50 mM buffer): acetate, pH 5.0–6.0; phosphate, pH 6.0–7.0; and Tris–HCl, pH 7.0–9.0. The influence of temperature on OPH activity was evaluated in 50 mM Tris–HCl buffer (pH 8.0) at the indicated temperatures. The kinetic parameters were calculated under the conditions reported previously [11].

Results

Expression of the codon-optimized *Sphingopyxis* sp. 113P3 *oph* gene in *P. pastoris* GS115

To enhance the production of heterologous OPH in *P. pastoris*, we replaced several low-usage codons in the bacterial *oph* gene with *P. pastoris* high-usage codons [23]. The total G + C content was adjusted from 60.7 to 50.4 %, which is similar to the G + C content in the *P. pastoris* genome. The native and modified bacterial *oph* genes had 76.3 % sequence similarity. Positive *P. pastoris* transformants were selected by growth on yeast extract peptone dextrose plates containing 3 mg/mL G418. Genomic *P. pastoris* DNA was amplified by PCR with the appropriate primers, which yielded products corresponding to the predicted size of the *oph* gene plus the α-factor signal sequence, indicating successful plasmid integration into the yeast genome.

To confirm expression and to select a strain capable of producing high levels of recombinant protein, several *P. pastoris* transformants were cultured in shaking flasks and induced with methanol for 5 days. The highest OPH activity, 6.68 U/mL, was achieved by transformant No. 62, which was selected as the producer for further experiments. As shown in Fig. 1, the molecular weight of recombinant OPH was about 35 kDa, consistent with previous reports [11, 19].

Production of recombinant OPH in a 3-L fermentor

To scale up recombinant protein production, a high cell-density fed-batch culture was performed in a 3-L fermentor. The biomass reached 83.4 g/L during the glycerol-batch phase. The highest OPH activity, 68.4 U/mL, was reached after 88 h of induction by 14.4 g/L methanol (Fig. 2). Of note, this level of activity was tenfold higher than that obtained in small-scale shaking cultures. At 88 h, the biomass density was 151 g/L and the volumetric productivity of recombinant OPH was 777 U/L/h. Production of OPH was high during the primary induction phase, and this rapidly decreased afterwards (Fig. 2).

Purification and characterization of recombinant OPH

Samples collected at each stage of OPH purification were concentrated and analyzed by SDS-PAGE (Fig. 1) and

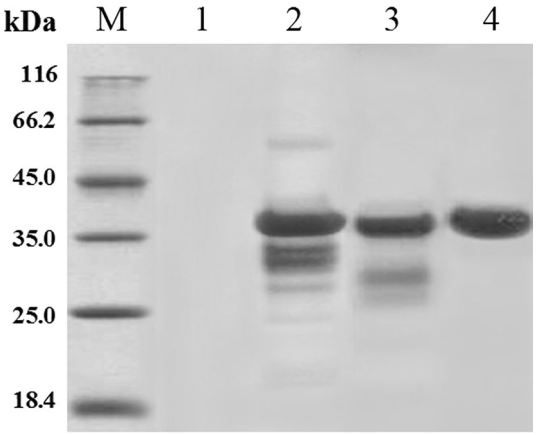

Fig. 1 Each purification step of OPH. *Lane M* protein molecular weight standard; *lane 1* pPIC9K empty vector (control); *lane 2* culture supernatant after 88 h induction of methanol in the 3-L fermentor, 20 μg total protein; *lane 3* fractions pass through the HiTrap Butyl Sepharose HP column with buffer A (50 mM sodium phosphate, pH 7.0), 20 μg total protein; *lane 4* fractions pass through the HiTrap SP Sepharose FF column with 1 M NaCl in buffer A, 20 μg total protein

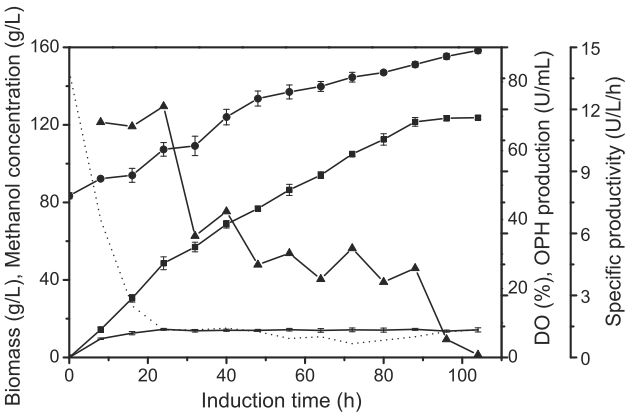

Fig. 2 Time courses of OPH production and cell growth in the 3-L fermentor and profiles of fermentation parameters including biomass (*circle*), OPH production (*square*), dissolved oxygen (*dotted line*), methanol concentration (*solid line*), and OPH productivity (*triangle*)

enzymatic assays (Fig. 3). The optimal pH for OPH activity was 8.0, and the enzyme was stable between pH 5.0 and pH 8.5, retaining >90 % of the original activity after 12 h of incubation. Beyond this pH range, the time to 50 % inactivation of OPH (activity half-life) was less than 1 h. The optimal temperature for OPH activity was 45 °C, with higher temperatures resulting in a dramatic decrease in activity. The half-life activity of OPH was 5 h at 47.5 °C. OPH activity was unaffected by 12 h incubation at temperatures below 45 °C. From the Arrhenius equation, $\ln(k) = \ln(A) + (-Ea/RT)$, we calculated the activation energy (E_a) of OPH to be 335 ± 12 kcal/mol (Fig. 3b, insert).

OPH enzyme activity was strongly inhibited by incubation with 1 mM phenylmethanesulfonyl fluoride (PMSF),

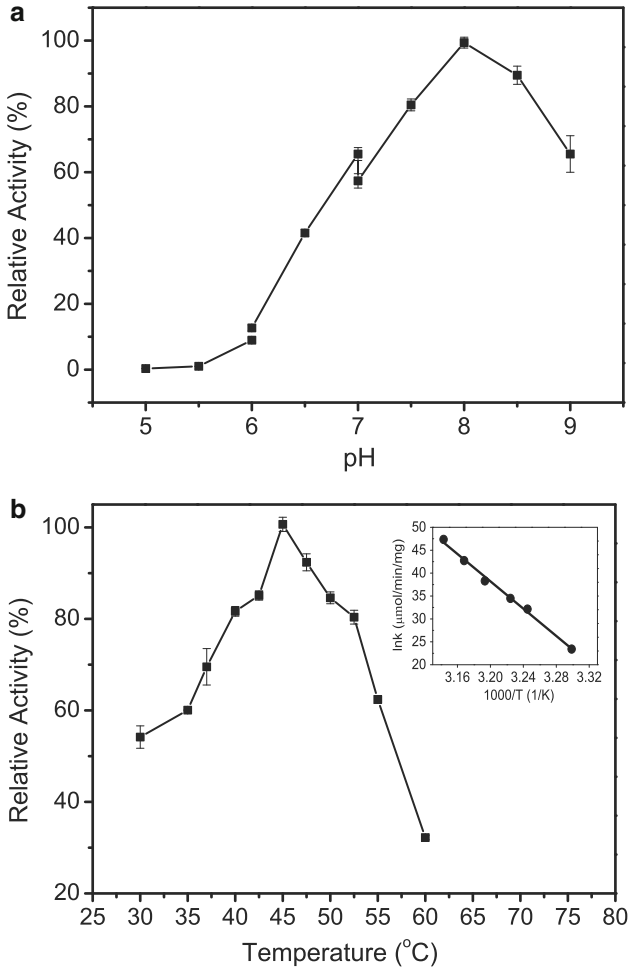

Fig. 3 pH and temperature optimum of recombinant OPH. **a** Effect of pH on OPH activity. Experiments were performed at 37 °C and the various pH values in 50 mM buffer. **b** Effect of temperature on OPH activity. *Error bars* standard deviation of three determinations. The *inset* presents Arrhenius plot of activation reaction rate against the reciprocal of absolute temperature (*T*)

Hg^{2+}, Cr^{2+}, or Fe^{2+}, and slightly inhibited by 1 mM Ca^{2+}, Ba^{2+}, Sn^{2+}, Cu^{2+}, or Co^{2+}. In contrast, 1 mM Mn^{2+} had a slight stimulatory effect on OPH activity (Fig. 4). Recombinant OPH hydrolyzed PNPA with a K_m of 0.97 mM and K_{cat}/K_m of 598 $mM^{-1}s^{-1}$ (Table 1).

Discussion

Many studies have shown that production of heterologous proteins in *P. pastoris* can be significantly increased by replacing rarely used codons in the target gene with codons more frequently used by *P. pastoris* [24]. The total G + C content of the construct is also an important factor for protein production, a slight A + T bias has been reported to affect mRNA secondary structure and improve protein production in *P. pastoris* [23]. Here, we found that

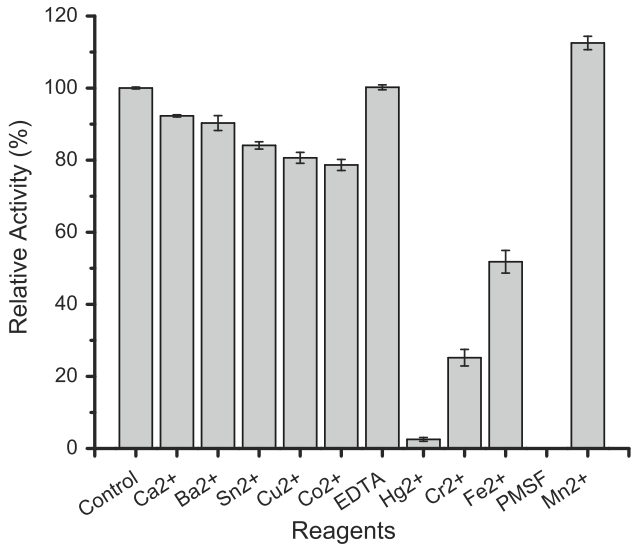

Fig. 4 Effect of reagents on the OPH activity. The recombinant OPH was preincubated with 1-mM metal ions, metal chelator, or PMSF at 37 °C for 5 min, and then assayed for OPH activity against PNPA. *Error bars* standard deviation of three determinations

optimization of the *oph* sequence with yeast-favored codons resulted in a high level of recombinant OPH protein expression (2.30 g/L) in *P. pastoris.*

Exponential glycerol feeding is a highly efficient method to obtain maximal biomass and alleviate growth inhibition due to excessive glycerol accumulation [21]. The cell concentration at the end of the glycerol-feeding phase is an important parameter for optimal recombinant protein production [17]. The temperature during the methanol-induction phase has also been shown to be important for heterologous protein expression by *P. pastoris* [25]. Wang et al. [26] reported that low temperatures can decrease cell death and proteolysis. Therefore, we induced OPH production at the 22 °C in this study.

Methanol metabolism by *P. pastoris* generates toxic metabolites such as hydrogen peroxide, formic acid, and formaldehyde, which can decrease cell viability and expression efficiency [27]. In addition, Wang et al. have demonstrated that under oxygen-limited conditions, specific heterologous protein production per biomass (Qx) decreases with increasing cell density at the same agitation speed and aeration rate, especially when the cell density rises above 75 g/L [27]. We observed an increase in biomass from 85.1 to 157 g/L, which may have led to oxygen insufficiency. This is the likely explanation for the decrease in OPH productivity during the methanol-induction phase (Fig. 2).

In our previous work, we expressed *oph* in *E. coli* [19]. The mature OPH protein contained eight cysteine residues that were predicted to form four disulfide bonds. However, the reducing environment of *E. coli* cytoplasm is not optimal for formation of disulfide bonds [16], and we found that recombinant proteins expressed in a cell-free *E. coli* extract either formed inclusion bodies or did not fold correctly. In contrast, the intracellular environment of *P. pastoris* allows correct formation of disulfide bonds in heterologous proteins and, in the presence of a recombinant α-factor signal peptide, efficient secretion of the mature protein [15, 16, 24]. This could be why the K_{cat}/K_m of recombinant OPH from *P. pastoris* was 4.27-fold higher than that of recombinant OPH from *E. coli*.

Recombinant OPH proteins expressed from *P. pastoris* and *E. coli* exhibited the same temperature and pH optima (Table 1). However, as an expression host, *P. pastoris* was superior to *E. coli* in both the activity of expressed OPH (1.44-fold higher) and volumetric productivity (1.37-fold higher) [19]. Moreover, the specific activity of OPH produced by *P. pastoris* reached 143 U/mg, which was 3.23-fold higher than that of the enzyme expressed by *E. coli*.

Table 1 The recovery of purifying recombinant OPH from *P. pastoris* culture supernatant

Purification step	Total volume (mL)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Supernatant	1,000	2,300	143	100	1.00
Ultrafiltration	333	1,750	148	78.7	1.03
HiTrap Butyl Sepharose HP	320	805	201	49.2	1.41
HiTrap SP Sepharose FF	231	487	239	35.4	1.67

Table 2 Comparison of the recombinant OPH from *Escherichia coli* and *Pichia pastoris*

	Temperature (°C)		pH		Kinetic parameters		
	Optimal	Stability range	Optimal	Stability range	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}s^{-1}$)
Recombinant OPH from *E. coli* [19]	47.5	<45	8.0	5.5–8.5	1.4	196	140
Recombinant OPH from *P. pastoris*	45	<45	8.0	5.0–8.5	0.97	580	598

The increased production and specific activity of OPH also made it easier to purify from the supernatant of *P. pastoris* (Table 2).

We found that recombinant OPH activity was abolished by PMSF, suggesting that the enzyme belongs to the serine hydrolase family. Consistent with this, the active site of OPH contains a catalytic Ser^{156}-His^{279}-Asp^{237} triad characteristic of the serine hydrolase family [28].

In conclusion, this is the first report of high-level production of recombinant OPH in *P. pastoris*, and the highest OPH activity ever reported. The culture process for recombinant *P. pastoris* is easily scaled up, and the highly efficient secretion makes it economically feasible to purify expressed enzyme from the supernatant. More importantly, the improved catalytic efficiency of recombinant OPH makes it an attractive alternative for industrial applications.

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