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RESEARCH ARTICLE

Development of a biocatalytic cascade for synthesis of 2-oxo-4-(hydroxymethylphosphinyl) butyric acid in one pot

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ABSTRACT

2-Oxo-4-(hydroxymethylphosphinyl) butyric acid (PPO) is an important precursor compound for the broad-spectrum herbicide L-glufosinate (L-PPT). In this study, the gene of D-amino acid oxidase (DAAO) was cloned and expressed in *Escherichia coli*. By coupling exogenous catalase (CAT), a biocatalytic cascade was constructed for synthesis of PPO in one pot. The bioprocess was optimized on a 300 mL scale reaction by one factor at a time optimization. The conversion of this biocatalytic cascade achieved 46.8% towards 400 mM DL-PPT within 4 h. These results indicated that DAAO could be applied to the large-scale bioproduction of PPO and provide a promising route for the asymmetric synthesis of L-PPT by bio-enzymatic methods using PPO as the substrate.

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2-oxo-4-(hydroxymethyl-phosphinyl)butyric acid; D-amino acid oxidase; catalase; biocatalysis

1. Introduction

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyses the oxidative deamination of a wide range of D-amino acids to their corresponding imino acids with absolute stereospecificity, which immediately undergo non-enzymatic hydrolysis to the respective α-keto acids and ammonia (Pollegioni and Molla 2011; Han et al. 2015; Xue et al. 2018). With the presence of molecular oxygen, co-enzyme flavin adenine dinucleotide (FAD) is re-oxidized, while the electron-acceptor oxygen is reduced to hydrogen peroxide (H_2O_2) during the reaction (Scheme 1; Trost and Fischer 2002). The enzyme is widespread in nature, from microorganisms (Shimekake et al. 2019) to mammals (Murtas et al. 2019). To date, only a few DAAO have been obtained as a homogeneous preparation, such as *Pk*DAAO (Bakke and Kajiyama 2004) from pig kidney and *Rg*DAAO (Pollegioni et al. 2002) from yeast. The enzymes from microorganisms appear to be far more suitable catalysts for bioconversion (Zhu et al. 2011; Conti et al. 2015). *Rg*DAAO appears to be a suitable catalyst for industrial application due to its broader pH activity and a higher activity at saturating oxygen concentrations. Moreover, it has attracted more and more attention in the synthesis of α-keto acid and non-natural chiral amino acid, exhibiting favourable application prospect. Additionally, the tightly bound cofactor FAD, making additional FAD unnecessary during the biotransformation process, also favours its industrial applications in biotransformation (Hsieh et al. 2009).

Until now, the most important industrial application of DAAO is the production of 7-aminocephalosporanic acid (7-ACA), a key pharmaceutical intermediate for the synthesis of cephalosporin antibiotics (Pollegioni et al. 2004). Starting from 7-ACA, about 200 tons per year of semisynthetic cephalosporins are produced, demonstrating potent potential of DAAO in keto acid production. However, there is seldom report about DAAO in preparation of keto acids containing hetero atoms, such as 2-oxo-4-(hydroxymethylphosphinyl) butyric acid (PPO). PPO is a vital keto acid intermediate in the asymmetric synthesis of optically pure L-glufosinate (a low-toxicity, broad-spectrum, and safe organophosphorus herbicide) which exhibits twice the herbicidal activity of racemic glufosinate (Jin et al. 2019).Asymmetric amination of PPO, using transaminase or glutamate dehydrogenase as biocatalyst, to

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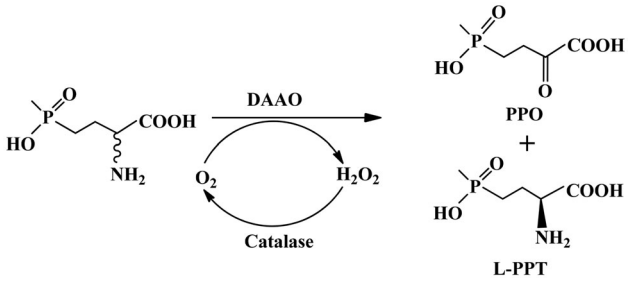

Scheme 1 Preparation of PPO from DL-PPT by DAAO and catalase

generate L-PPT has the advantage of 100% maximum theoretical yield, and become a promising strategy for industrial production of L-PPT. Currently, the synthesis of PPO in industry is mainly through chemical methods, and there are no biological methods reported in related literature. In 1991, using 3-(methylethoxyphosphoryl)-propionic acid methyl ester as a starting material, Hoechst condensed it with diethyl oxalate, following by the hydrolysis, finally obtaining the target compound by the decrystallization with 65% total yield (Zeiss 1991). Serious environmental pollution, complex process and low yield are always critical problems in chemosynthesis of PPO (Baillie et al. 1983; Zeiss 1991). Considering strict stereoselectivity, mild reaction conditions, high recovery and simple separation and purification processes, deamination of racemic glufosinate by DAAO would be an ideal alternative due to its strict D-isomer enantioselectivity, retaining L-isomer with herbicidal activity.

H_2O_2, a byproduct of the aforementioned DAAO-catalysed reaction, is a disadvantageous factor in the production of keto acid, since the keto acid will be decarboxylated by H_2O_2 to form the corresponding carboxylic acid (Upadhya et al. 2000). The traces of catalase (E.C. 1.11.1.6) which converts H_2O_2 to water and molecular oxygen, can be seen in most DAAO-preparation processes (Seo et al. 2012; García-García et al. 2018), as catalase is a technical enzyme which is available in large quantities (Nadler et al. 1986). Moreover, in most cases, addition of free catalase is simpler and more economic than chemical methods to remove H_2O_2 (Han and Shin 2018).

Herein, a DAAO from *Rhodotorula gracilis* (*Rg*DAAO) was expressed in *E. coli*. A biocatalytic cascade was constructed for PPO production by exogenously adding catalase. After optimization of the catalase concentration, aeration flow rates and other reaction conditions, a maximum PPO yield of 46.8% and an *e.e.* value above 99% were obtained. This is the first report that racemic glufosinate converted to PPO by DAAO/CAT-combined cascade in one pot.

2. Materials and methods

2.1. Chemicals

DL-Glufosinate substrate (purity, >82.5%) and PPO (purity, >99%) were provided by Dezhou Lvba Fine Chemical Co., Ltd. (Dezhou, China). Plasmid Miniprep kits and DNA gel extraction kits were purchased from Axygen Scientific Inc (Hangzhou, China). Phanta Max Super-Fidelity DNA Polymerase used for mutagenesis was purchased from Vazyme (Nanjing, China). SDS-PAGE, Bis-Tris buffer and BCA protein assay kit were purchased from GenScript (Nanjing, China). Hydrogen peroxide (H_2O_2) assay kit was purchased from Solarbio. Unless otherwise stated, all other chemicals were of analytical grade and commercially available.

2.2. Fed batch fermentation production of DAAO in 5 L fermenter

DAAO gene was synthesized and cloned into the first ORF frame of pCDFduet-1 vector. The recombinant strain containing *Rg*DAAO was cultured in Luria–Bertani (LB) medium containing streptomycin (50 μg/mL) at 37 °C for 12 hours. A 1% (v/v) cell culture was then inoculated into a 500 mL shake flask containing Terrific Broth (TB) medium (100 mL). The cells were cultivated at 37 °C and 180 rpm for 6 h as a seed liquid to inoculate the 3 L batch medium (yeast extract 12 g/L, peptone 15 g/L, NaCl 10 g/L, glycerine 12 g/L, $(NH_4)_2SO_4$ 5 g/L, K_2HPO_4 1.36 g/L, $KH_2PO_4.3H_2O$ 2.28 g/L, $MgSO_4.7H_2O$ 0.375 g/L) for fed-batch fermentation in a 5 L fermenter (Winpact thermometer system fermenter, New York, USA) cultured under the following conditions: 37 °C, 500 rpm agitation, 1.6 vvm aeration, pH 7.0 (kept constant by adding 50% (v/v) aqueous $NH_3{\cdot}H_2O$ and 50% (v/v) aqueous H_3PO_4). When the optical density (OD_{600}) value reached 8–10, lactose (10 g/L) was added, and the culture was induced at 28 °C for 12 h. Recombinant cells were harvested by centrifugation at 8,000 rpm and 4 °C for 10 min. All assays were performed in triplicate.

2.3. Biosynthesis of PPO

To develop a more convenient and economical process, whole cells were used as catalysts in the reaction. The collected cells were suspended in deionized water. The initial reaction mixture exists in two reaction systems: (i) DL-PPT (150 mM) and catalase (193 U/mL) made up to a final volume of 30 mL in a 50 mL shake flask. This reaction was used to optimize the temperature and pH conditions. (ii) DL-PPT (400 mM)

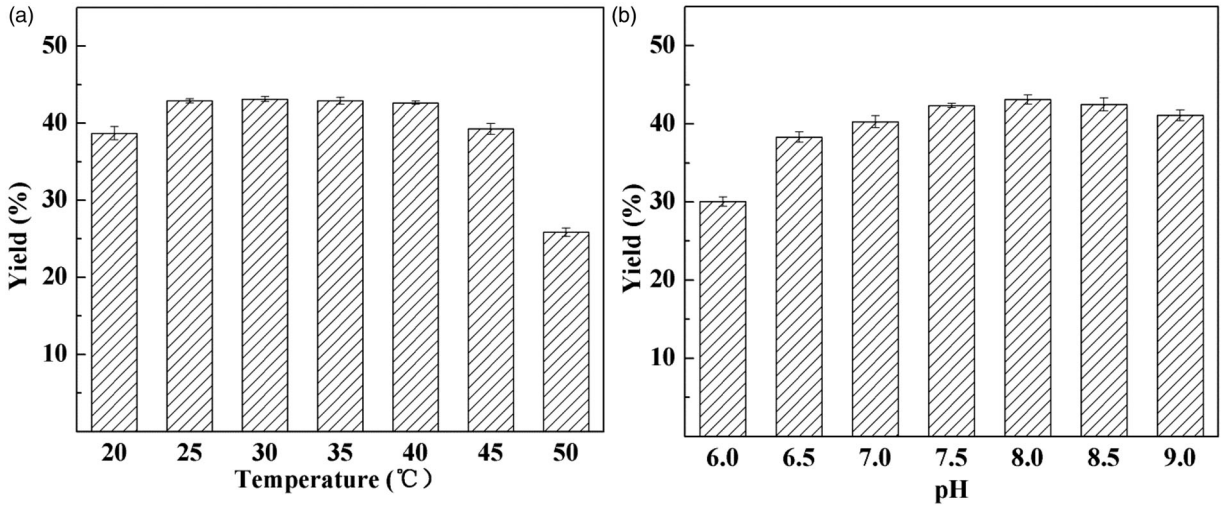

Figure 1. Effect of reaction temperature (a) and pH (b) on the yield of PPO.

and catalase (1926 U/mL) with an aeration flow rate of 0.8 L/min, made up to a final volume of 300 mL in a 1 L bioreactor with 1‰(v/v) antifoam added, conditions such as aeration flow rate and the addition of catalase were optimized. The wet whole cells containing DAAO in both the above reactions had concentrations of 20 g/L, and the pH of the initial reaction was 8.0 by titration with $NH_3{\cdot}H_2O$, reactions were performed with magnetic stirring at 500 rpm in a water bath at 30 °C. The experiments were conducted in triplicate. Samples were withdrawn from the reaction mixture at different time points, and 6 M HCl (4 μL) was added to 200 μL of the sample to terminate the reaction, followed by the addition of 6 M NaOH (4 μL) before HPLC analysis.

2.4. Analytical methods

DL-PPT was derivatized at 30 °C for 5 minutes with derivative reagent [2.01 g *o*-phthalaldehyde and 2.43 g *N*-acetyl-L-cysteine dissolved in 1 L boric acid buffer (100 mM, pH 9.8)]. Then the concentration and excess (*e.e.*) of DL-PPT were determined by fluorescence detector (UltiMate FLD-3100, E_x 340 nm and E_m 450 nm) of high performance liquid chromatography (HPLC, ThermoFisher UltiMate 3000) on a C18 column (Unitary C18, 5 μm, 100 A, 4.6 mm × 250 mm) at a flow rate of 1.0 mL/min. The column temperature was maintained at 35 °C. Each sample (10 μL) was eluted with 50 mM ammonium acetate (pH 5.7) , containing 10% methanol as mobile phase. The retention time of D-PPT was 13.6 min (Lv et al. 2019). The calculation formula of *e.e.* value is $e.e. = \frac{C_{L-PPT} - C_{D-PPT}}{C_{L-PPT} + C_{D-PPT}} \times 100\%$, $C_{L\text{-PPT}}$ and $C_{D\text{-PPT}}$ are measured by the peak area.

The concentration of PPO was determined by Diode Array Detector (UltiMate DAD-3000, 232 nm) of the same HPLC instrument and column with the same flow rate. The mobile phase contained 50 mM ammonium dihydrogen phosphate (pH 3.8) buffer, 0.1% tetrabutyl ammonium bromide and 12% acetonitrile. The column temperature was maintained at 40 °C. The retention time of PPO was 9.4 min.

3. Results and discussion

3.1. Effect of temperature and pH on DAAO-mediated chiral resolution of racemic glufosinate

Reaction temperature has a profound effect on enzyme activity (Thakur et al. 2019). Therefore, the influence of temperature on reaction was studied between 20 and 50 °C. As shown in Figure 1(a), the DAAO exhibits absolute enantioselectivity (>99% *e.e.*) under all reaction temperature. In the range of 25–40 °C, the yield of PPO reached 43.1%, indicating that the enzyme had excellent temperature adaptability. Above and below this range, a decreased PPO yield was observed. A sharp decline in PPO yield was observed above 50 °C. Therefore, 30 °C was selected as the optimal reaction temperature for further study.

The effect of pH on the enzymatic reaction was also investigated in the pH range of 5.0–9.0 using two different buffers, K_2HPO_4-KH_2PO_4 (pH 6.0–8.0) and Tris-HCl (pH 8.5–9.0), with the same concentrations of 150 mM. As shown in Figure 1(b), the PPO yield was greater than 42% in the pH range of 7.5–8.5. The highest PPO yield was obtained at pH 8.0. When the pH was lower than 6.5 or higher than 8.5, the PPO yield decreased, indicating that these pH conditions are harmful to the enzyme stability and activity. It is

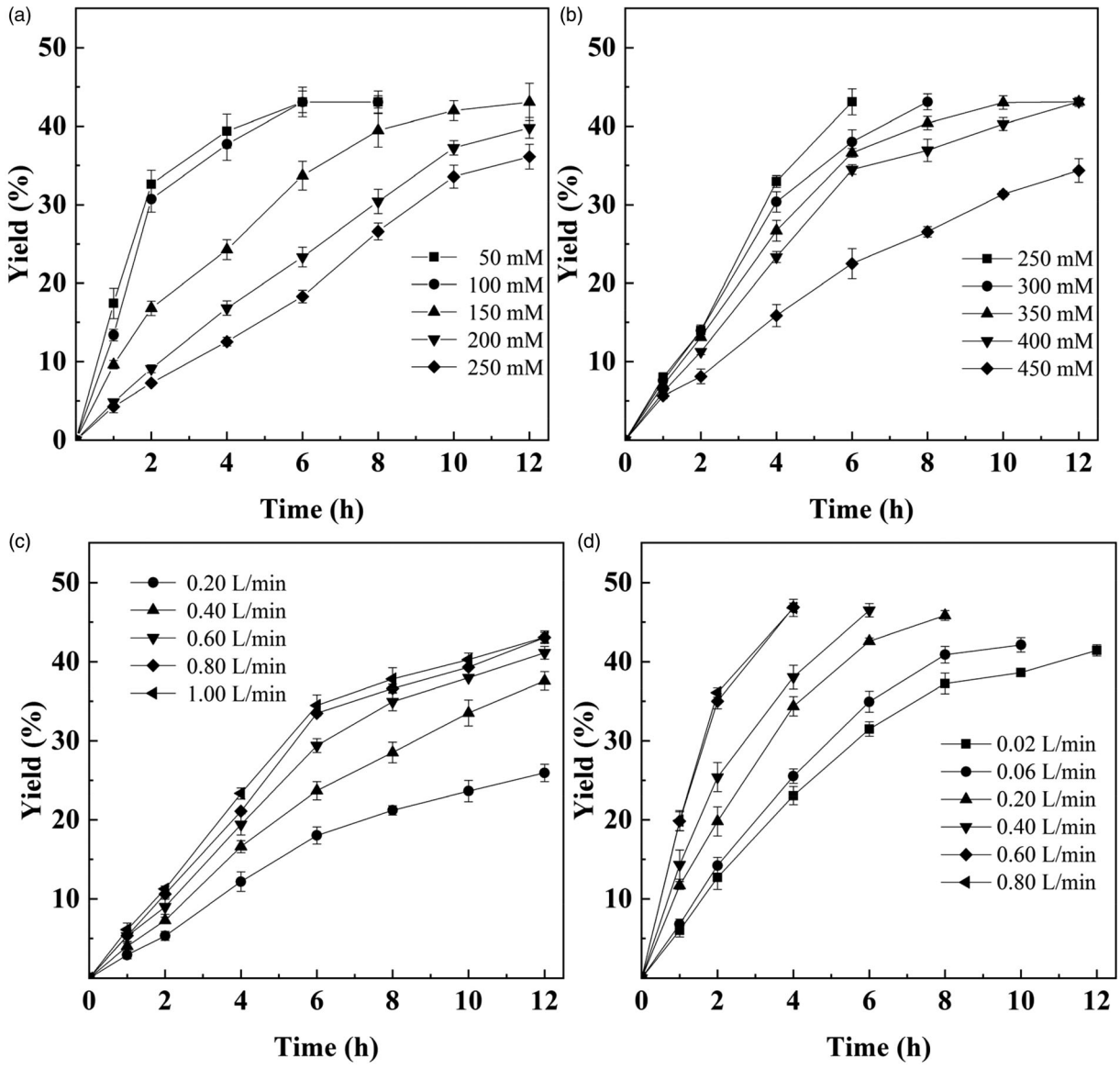

Figure 2. Time courses of the catalytic reaction under different conditions. Effect of DL-PPT on the yield of PPO in 50 mL (a) and 1 L reactor (b); Effect of flow rate of air (c) and pure oxygen (d) on the yield of PPO.

reported that the stereoselectivity of DAAO is strict and is not related to the reaction pH. Furthermore, when the pH was adjusted to 8.0 with aqueous ammonia, the same yield was obtained as in the phosphate buffer (pH 8.0). Combining the above results, and considering the subsequent separation and purification, and cascade reaction with transaminase or glutamate dehydrogenase to synthesize L-PPT, ammonia was used in subsequent experiments to adjust the reaction system to pH 8.0.

3.2. Effect of oxygen for DAAO catalysed oxidation

Oxidases require oxygen, which in biotechnology traditionally is supplied by bubbling air through the reactor (Toftgaard Pedersen et al. 2015). Firstly, the oxidative reaction was performed in the 50 mL shake flask, When the reaction substrate was only 150 mM, the aeration had little effect on the reaction, and the conversion could be completely achieved without addition of air (Figure 2(a)). The practical concentration of O_2 (aq) is quiet low (data not shown), indicating the effect of O_2 on the reaction process is not being exhibited since the O2 mass transfer was limited. Previous study has demonstrated that the mass transfer capability of O_2 is limited by selected equipment and the maximum oxygen transfer rate in an industrial scale bioreactor is typically around 100 mmol/L/h (Charles 1985; Toftgaard Pedersen et al. 2015; Chapman et al. 2018). Subsequently, the reaction was performed in the 1 L stirred-tank reactor since

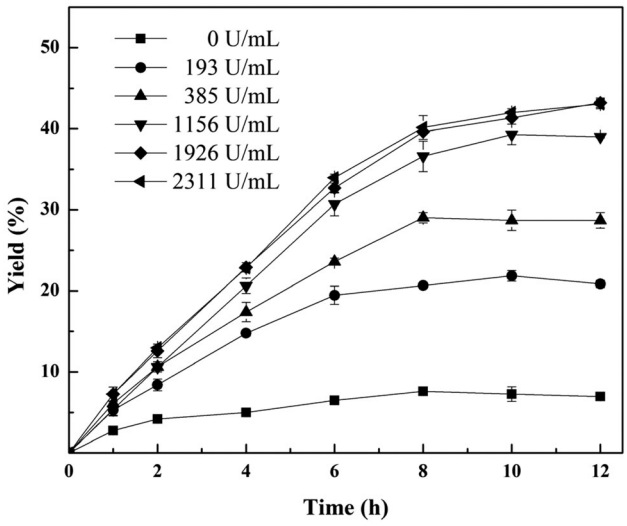

Figure 3. Effect of catalase concentration on the yield of PPO.

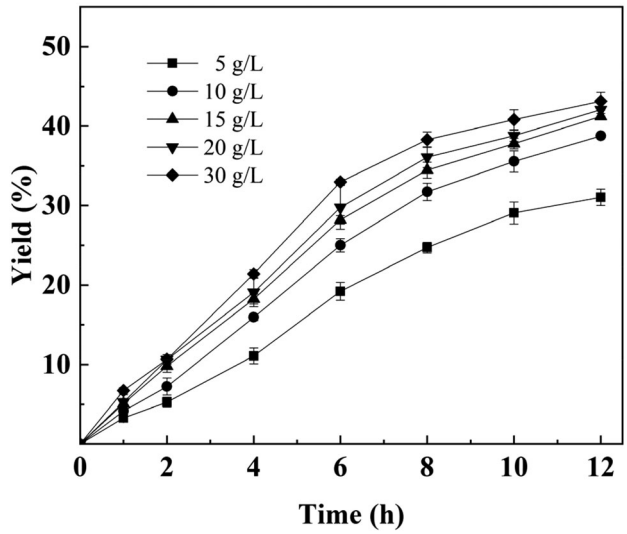

Figure 4. Effect of biocatalyst concentration on the yield of PPO.

the propeller caused more stronger force for $O_2(g) \rightarrow O_2(aq)$. The corresponding results are shown in Figure 2(c), when the air flow rate was 0.8 L/min, the maximum substrate concentration was increased from 150 mM to 400 mM gradually, representing a 1.67-fold increase compared with that of the 50 mL reaction system (Figure 2(b)). At the maximum substrate concentration, the yield of PPO improved from 30% to 43.1% after 12 h gradually, with the flow rate of air increased from 0 L/min to 0.8 L/min (Figure 2(c)). In addition, the catalytic rate was slightly accelerated at the beginning of the reaction (from 12.2 mM/h to 22.4 mM/h).

To further improve the catalytic efficiency, or in other words, relieve the limit of O_2, pure oxygen with different flow rate was introduced to the reactor. The catalytic time was shortened and the maximum PPO yield of 46.8% was achieved at 4 h (Figure 2(d)). Compared with 0.8 L/min air, 0.6 L/min pure oxygen increases the maximum yield of PPO from 43.1% to 46.8%. However, from an applied point of view, it is optional to introduce 0.8 L/min of air into the catalytic system for the reaction.

3.3. Effect of H_2O_2 and protection by catalase in the reaction

H_2O_2 is a byproduct of the DAAO-catalysed oxidative deamination of D-amino acids. Previous studies have demonstrated that it not only decarboxylates oxidized keto acids to form a carboxylic acid with one carbon less, but also inactivates DAAO by oxidizing cysteine and serine residues of the enzyme (Fernández-Lafuente et al. 1999; Trost and Fischer 2002). Removing H_2O_2 by catalase would generate more dissolved oxygen to facilitate reaction, preventing PPO being degraded and protecting the activity of the enzyme. As shown in Figure 3, D-PPT was completely converted with 193 U/mL catalase. Hydrogen peroxide generated in the reaction was not effectively removed and PPO was oxidized to 3-(hydroxymethylphosphinyl) propionic acid (MPP), resulting in a low PPO yield. Interestingly, the PPO yield achieved 43.1% with 1926 U/mL catalase adding, but 50% yield could not be achieved with addition of more catalase. Furthermore, we found the concentration of H_2O_2 in the reaction mixture is quite low (about 0.5 mM), while the concentration in the whole cells of the reaction solution is very high (about 5.8 mM). We concluded that much more catalase was not helpful for removing H_2O_2 thoroughly and some PPO was oxidized in the whole cells, leading to the loss of total yield. Thus, 1926 U/mL catalase was selected as the optimal adding amount.

3.4. Effect of biocatalyst concentration on catalytic reaction

Insufficient addition of biocatalyst will cause slow conversion of substrate, while excessive biocatalyst concentration might affect gas transmission. To further improve the catalytic performance of DAAO, the whole cell catalyst concentration was optimized. As shown in the Figure 4, biocatalyst concentration below 20 g/L could not convert substrate completely, and the product yield increased with increasing whole cells concentration. But when the concentration of the biocatalyst was higher than 20 g/L, the PPO yield remained basically unchanged. Therefore, the optimal DAAO whole cells concentration was selected at 20 g/L.

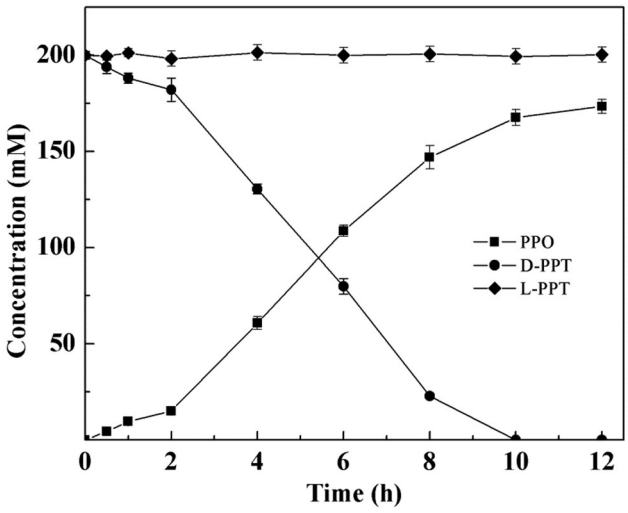

Figure 5. Time course of recombinant cell catalytic synthesis of PPO.

3.5. Large-scale preparation of PPO under optimal conditions

To further evaluate the feasibility of the bioprocess for production of PPO for practical application, a 3 L scale reaction system for the oxidization of DL-PPT under the above-mentioned conditions was performed in a 5 L stirred-tank reactor. The initial reaction mixture contained DL-PPT (400 mM), recombinant wet whole cells harbouring DAAO (20 g/L), and catalase (1926 U/mL), with aeration of 0.8 L/min. The final volume was made up to 3 L in a 5 L bioreactor. The reaction mixture was agitated at a speed of 500 rpm and a temperature of 30 °C. The reaction profile is shown in Figure 5, indicating that the total yield and efficiency were not affected by scaling up. As expected, a conversion of >49.9% was reached, and a product yield of 43.1% was obtained after 12 h. To date, using racemic glufosinate as a substrate to produce PPO by bioenzymatic method is the method with the highest yield, indicating that this biocatalytic method is the method of choice for industrial production of PPO.

3.6. Discussion

PPO is a vital keto acid containing phosphorus atom, but biological or chemical strategies for synthesis of PPO have seldom been reported. In this work, we successfully constructed a recombinant *E. coli* harbouring *Rg*DAAO and used it to produce PPO.

Firstly, we investigated pH and temperature profiles of *Rg*DAAO. To our surprise, the *Rg*DAAO exhibited excellent stability in range of pH 7.5–8.5 and 25–40 °C, extending the great applied potential in PPO synthesis. The enzyme was probably denatured at 50 °C which is the preferred temperature in industry. Mild pH profiles may be a favourable characterization for industrial application.

Oxidation reactions are notorious types of reaction where O_2 is always a bottleneck (Umhau et al. 2000; Pollegioni et al. 2007; Rosini et al. 2011). In the 50 mL flask shake, the initial rate was significantly limited by the depletion of oxygen. When the reaction was scaled up to 1 L stirred-tank reactor, initial rate and catalytic performance were improved with addition of air, while the feeding of pure oxygen further verified the effect of O_2. We found the maximum PPO yield was obtained at 4 h when pure oxygen was introduced into the reactor. The shortened reaction time also contributed to an increase in yield, as only a small part of H_2O_2 was generated and less PPO was consumed. The higher oxygen transfer rate and dissolved oxygen concentration (decomposition of H_2O_2) potentially increased both the catalytic efficiency and biocatalyst yield. Nevertheless, the benefit from applying pure oxygen has to be weighed against the product value and added cost from purifying.

Although numerous efforts were made, the yield of PPO was still unable to reach 50%. The byproduct of oxidation of D-PPT, H_2O_2, probably decomposed PPO to MPP, and the chemical structure of DAAO was possibly affected (Grey et al. 2007). Hence, catalase is highly desirable to keep the concentration of hydrogen peroxide, formed as a byproduct, at a minimum to avoid enzyme deactivation and product degradation. In this work, we dramatically enhanced the yield of PPO through feeding of catalase, however, the maximum yield was reached up to 46.8% with 49.9% conversion, indicating the formation of byproduct MPP under the interference by H_2O_2 in this reaction was inevitable, and the H_2O_2 could not be completely removed. How to effectively remove H_2O_2 is a major challenge in the DAAO-catalysed keto acid production (Volpato et al. 2010). Finding a catalase with lower K_m value and co-expression with DAAO in single cell would be worthwhile.

From an applied point of view, exorbitant biocatalysts concentration will cause additional cost of solid–-liquid separation and superfluous waste. 20 g/L wet cell was enough to achieve maximum yield. Furthermore, whole cells are easy to operate, do not need to go through steps such as crushing and purification, and can be immobilized to recycle easily.

After optimization of pH, temperature, biocatalyst concentration, oxygen, and catalase, the reaction was further scaled up to 3 L with 400 mM substrate. 43.1% yield was obtained after 12 h which is the same with

1 L reaction system, suggesting the great potential of DAAO for application.

4. Conclusions

In summary, we have successfully constructed recombinant *E. coli* strains expressing DAAO for the production of PPO. The ventilation flow rate and amount of exogenous catalase addition were the rate-determining factors. Introducing air was more cost-effective, although the effect of introducing pure oxygen was better. Consequently, a certain flow rate of air must be introduced into the reaction system and an appropriate amount of catalase must be added to promote the reaction process. More importantly, this is the highest reported yield of D, L-PPT produced by biocatalytic production of PPO, laying the foundation for industrial applications.

Disclosure statement

No potential conflict of interest was reported by the authors.

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