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# A rapid throughput assay for screening (*R*)-2-(4-hydroxyphenoxy)propionic acid producing microbes

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ABSTRACT

(*R*)-2-(4-hydroxyphenoxy)propionic acid ((*R*)-HPOPA) is an important intermediate for the synthesis of optically pure aryloxyphenoxypropionic acid herbicides. Regioselective hydroxylation of (*R*)-2-phenoxypropionic acid ((*R*)-POPA) by microbes is one of the most useful methods for the industrial production of (*R*)-HPOPA. In this study, we designed and optimized a rapid throughput assay for screening (*R*)-HPOPA producing bacterial/fungal strains which can regioselectively hydroxylate (*R*)-POPA. (*R*)-HPOPA could react with 4-aminoantipyrine (4-AAP) in the presence of potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) to form indoxyl antipyrine, an orange-red chromophore, that can easily spectrophotometrically be determined at 550 nm. During the verification of the assay we observed an average recovery rate of between 97.3% and 104.5%. Apart from the rapid throughput, no obvious differences in detection (*R*)-HPOPA in the culture broth samples were found between our rapid throughput multiplate assay and a high-performance liquid chromatography method. Our optimized assay method is simple, rapid and accurate with high repeatability. It has the potential for high throughput screening (about 3000–5000 samples/day) of the (*R*)-HPOPA producing strains.

## 1. Introduction

Aryloxyphenoxypropionic acids, as reversible inhibitors of acetyl-CoA carboxylase, are a new class of herbicides (Fig. 1) that is widely applied as their ester derivatives in commercial herbicides for the selective elimination of most grass species from any non-grass crops (Liu et al., 2015; Xia et al., 2013). Some of aryloxyphenoxypropionic acid-type herbicides have chiral character, and generally, their *R*-(+) enantiomers shows great herbicidal activity and exhibits the desired effect (Ma et al., 2012; Wu et al., 2015).

(*R*)-2-(4-hydroxyphenoxy)propionic acid ((*R*)-HPOPA) is an important intermediate for the production of chiral aryloxyphenoxypropionic acid-type herbicides such as clod inafop-propargyl, cyhalofop-butyl, fenoxaprop-p-ethyl, metam ifop, quizalofop-p-ethyl, and quizalofop-p-tefuryl (Fig. 1). In general, there are three chemical methods for the preparation of (*R*)-HPOPA: (1) resolution of racemic HPOPA with alkaloids such as quinine, cinchonine, ephedrine, vauquline or chiral agents such as S-(−)-α-phenylethylamine, L-(+)-chloromethane, *R*-(+)-α-isopropyl-4-chlorobenzylamine (Jiang et al., 2000) and (2) asymmetric synthesis from L-lactic acids (Becker et al., 1984; Wu et al., 2015) or L-alanine (Weng et al., 2009). However, these methods are not always useful for industrial applications because of expensive reagents, problems with enantiopurity of product and/or low yields (Becker et al., 1984; Elango and Davenport, 1991; Watson, 1989; Wettling et al., 1999). Regioselective hydroxylation of (*R*)-2-phenoxypropionic acid ((*R*)-POPA) by microbes is a highly feasible method for the production of (*R*)-HPOPA (Fig. 2), which under mild conditions allows complete transformation of substrate into a single stereoisomeric product with a high yield and without byproduct formation (Beilen et al., 2003; Cooper et al., 1994; Dingler et al., 1996; Schmid et al., 2002).

Microbes which have the ability to efficiently hydroxylate (*R*)-POPA to (*R*)-HPOPA are uncommon and rare. It is important to screen new microbials or breed mutant strains with satisfactory catalysis performance and properties (Xue et al., 2011; Xue et al., 2015; Xue et al., 2017; Xu et al., 2018). Industries and chemists need new microbes with higher hydroxylation activity and desired characteristics, such as strong substrate tolerance and high stability. The traditional method utilized for screening microbes with hydroxylase activity is high performance liquid chromatography (HPLC) and several analytical HPLC methods

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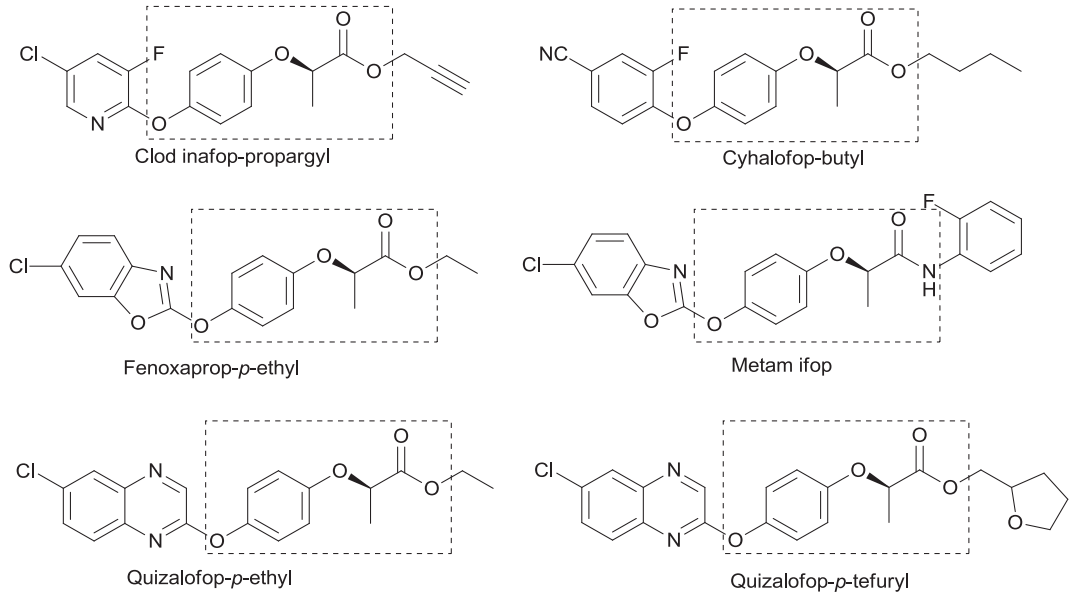


**Fig. 1.** Representative of chiral aryloxyphenoxypropionic acid herbicides containing the structure of (*R*)-HPOPA.

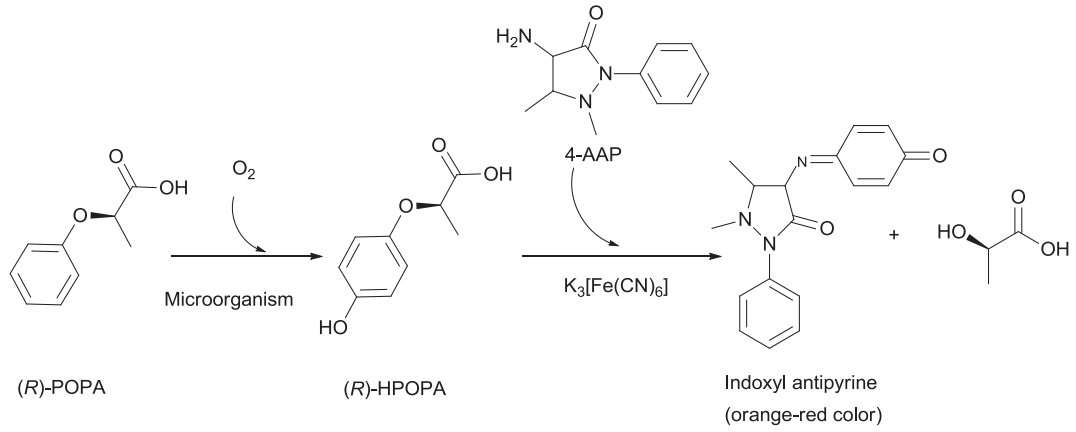


**Fig. 2.** Reaction of the color-developing of (*R*)-POPA with 4-AAP for monitoring the microbial hydroxylation of (*R*)-POPA to (*R*)-HPOPA. In the presence of $K_3[Fe(CN)_6]$, the produced (*R*)-HPOPA reacts with 4-AAP to form an orange-red dye (indoxyl antipyrine), which can be quickly quantified by spectrophotometric measurements.

for determining (*R*)-HPOPA have been reported (Kinne et al., 2008). However, these methods used for screening are expensive and time consuming. Therefore, it is important to develop a rapid screening method to identify new microbes with the desired activity and characteristics. Currently, there is no high throughput screening method for the identification of microbials that hydroxylate (*R*)-POPA to (*R*)-HPOPA, which makes the screening step rate limiting in production. High throughput screening technology is a highly efficient method widely applied in strain breeding, mutation analysis, drug testing, etc. (Lu et al., 2015; Qi et al., 2011; Tan et al., 2013; Tang et al., 2010; Wang et al., 2009; Zhu et al., 2014).

In this study, we designed and optimized a rapid throughput method for screening (*R*)-HPOPA producing bacterial/fungal strains which can regioselectively hydroxylate (*R*)-POPA. (*R*)-HPOPA in samples reacts with 4-aminoantipyrine (4-AAP) by adding potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) to form 1,5-dimethyl-4-((4-oxocyclohexa-2,5-dien-1-ylidene)amino)-2-phenylpyrazolidin-3-one, an orange-red compound (Fig. 2), which can be easily spectrophotometrically determined at 450–600 nm. This modification allows rapid detection of (*R*)-HPOPA over a broad concentration range and multiple sample can be analyzed in multiwell-plates combined with a microplate reader instead of the conventional single analysis HPLC methods. The microplate screening method has several advantages such as being rapid, accurate, sensitive, low cost, and exceptionally simple. Furthermore, our microplate adapted assay has the potential for high throughput screening (about 3000–5000 samples/day) of (*R*)-HPOPA producing strains.

## 2. Materials and methods

### 2.1. Chemicals and reagents

(*R*)-HPOPA and 4-AAP were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). (*R*)-POPA was provided by Shandong Rainbow Biotechnology Co., Ltd. (Jinan, China). Ultra-pure water was produced by Milli-pore Q Water Purification System (Millipore, MA, USA). (*R*)-POPA and (*R*)-HPOPA standard solution were prepared by dissolving 10 g of (*R*)-POPA and (*R*)-HPOPA each in 1.0 L ultra-pure water. The 4-AAP reagent contained 0.6 g/L 4-AAP and 18.0 mM $Na_2CO_3$. The pH of the 4-AAP reagent was adjusted to pH 10.0 using 2.0 M sodium hydroxide solution. The prepared light sensitive 4-AAP reagent was stored in a brown bottle. Other chemicals and reagents otherwise

demonstrated were of analytical grade from commercial sources.

### 2.2. Medium

The potato dextrose agar (PDA) solid screening medium in this study contained 4.0 g/L potato infusion (from 200 g infused potato), 20.0 g/L glucose, and 20.0 g/L agar under natural pH. The PDA liquid medium was used for seed growth. The fermentation medium for the microbial cultivation and (*R*)-HPOPA biosynthesis contained: 20.0 g/L glucose, 5.0 g/L yeast extract, 5.0 g/L $(NH_4)_2SO_4$, 3.6 g/L $K_2HPO_4$, 1.5 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4$, and 0.05 g/L $MnSO_4$. The pH of the medium was adjusted to 6.8 by 2.0 M sodium hydroxide solution.

### 2.3. Analytical microplate procedure for rapid throughput detection of (R)-HPOPA

Rapid throughput determination of (*R*)-HPOPA was performed based on the optical density (OD) value measured at a suitable wavelength on a SpectraMax M2 multifunction microplate reader (Molecular Device Company, USA). The color-developing reagent was prepared by adding 0.01–0.07 g $K_3[Fe(CN)_6]$ to 1.0 mL of 4-AAP reagent. Then, 100 μL of sample and 100 μL of color-developing reagent were added into each well in the microplate. The mixture was reacted at 25–50 °C for 15–45 min. All experiments were carried out in triplicate.

For a satisfied assay performance, the detection conditions of the rapid throughput screening method including the working wavelength, the $K_3[Fe(CN)_6]$ concentration, the reaction temperature, the reaction solution pH, and the reaction time was optimized.

A continuous wavelength scan from 470 to 800 nm by using microplate reader was performed on (*R*)-HPOPA and (*R*)-POPA standard solution after the addition of color-developing reagent. For the standards, 100 μL each of 2.0 g/L (*R*)-HPOPA, 4.0 g/L (*R*)-HPOPA, 2.0 g/L (*R*)-POPA, and 4.0 g/L (*R*)-POPA solution were added separately into a 96-well plate for color-developing reaction. The reaction time and temperature were set at 20 min and 30 °C.

For the standard curves, a (*R*)-HPOPA standard was dissolved in ultra-pure water to prepare serial standard solution with different concentrations. Then, the chromogenic reaction was conducted and the OD value of the reaction mixture was measured under the optimal conditions. The (*R*)-HPOPA concentration standard curve was established based on OD values and a standard curve equation was formulated. The significance of linear relationship between (*R*)-HPOPA concentration and the $OD_{550}$ value was evaluated through analysis of variance by *F*-test (Li and Hu, 2014). For the validation of the analytical procedure, two parameters including limit of detection (*LOD*) and limit of quantification (*LOQ*) were calculated (Eqs. (1)–(2)) to predict the sensitivity of method according to the International conference of Harmonisation (ICH) guidelines.

$$LOD = 3.3\,\sigma/S \quad (1)$$

$$LOQ = 10\,\sigma/S \quad (2)$$

where $\sigma$ represents the standard deviation of intercept; $S$ is the slope of the standard curve.

In order to verify whether the (*R*)-HPOPA can be quantitatively measured with this rapid throughput screening method, a known amount of (*R*)-HPOPA was added to blank samples before the reaction, and then the sample recovery was calculated from Eq. (3) described below.

A strain *Beauveria bassiana* CCTCC No: M 2018407 with a known (*R*)-HPOPA conversion rate was used as positive control, and a strain *Beauveria bassiana* ZJB16001-1 without (*R*)-HPOPA conversion capacity was used as negative control. The strains were inoculated into 250 mL shake flasks containing 50 mL fermentation medium with an inoculation size of 2% (v/v) and grew at 28 °C for 72 h. Then (*R*)-POPA (3.0 g/L) was added as substrate for the biotransformation for 7 d. The (*R*)-HPOPA concentration in the fermentation medium was determined. A known amount of (*R*)-HPOPA was added to the fermentation broth before the chromogenic reaction. The sample recovery was calculated by Eq. (3):

$$(R)-\text{HPOPA recovery}\,(\%) = (W_1 - W_2)/W_3 \times 100\% \quad (3)$$

where $W_1$ represents the measured (*R*)-HPOPA amount; $W_2$ is the analytical (*R*)-HPOPA amount in the sample; and $W_3$ is the amount of the added standard (*R*)-HPOPA. $W_1$, $W_2$, and $W_3$ were calculated by multiplying the values obtained by the (*R*)-HPOPA standard curve with the dilution factor.

### 2.4. Validation of assay: detection of (R)-HPOPA in fermentation broth samples using the rapid microplate assay

Fermentation broth samples were centrifuged at 5000 ×*g* for 20 min. The supernatant was transferred to the wells of a 96-well plate. Absorption at 550 nm was determined using the microplate reader. Then, (*R*)-HPOPA concentrations of fermentation broth samples were calculated from the standard curve equation.

### 2.5. Comparison of microplate assay: detection of (R)-HPOPA in fermentation broth samples using HPLC

The fermentation broth was centrifuged at 5000 ×*g* for 20 min and the supernatant was obtained. The concentration of (*R*)-HPOPA in the supernatant was diluted to < 1.0 g/L by the ultra-pure water. The supernatant was filtered by the 0.22 μm water filtration membrane. (*R*)-HPOPA amount in the supernatant was detected using HPLC on a Dionex's UltiMate 3000 instrument equipped with a diode array detector (DAD). For routine separations, the instrument was fitted with a $C_{18}$ phase column (4.6 × 250 mm, 5 μm). The column was eluted at 30 °C and 1.0 mL/min of mobile phase containing phosphoric acid solution (pH 2.0) / acetonitrile (3:2), for 10 min. The significance of the difference between rapid throughput method and HPLC was evaluated through analysis of variance by *F*-test (Li and Hu, 2014).

### 2.6. The rapid 96-well microplate screening assay for (R)-HPOPA

The rapid throughput screening method (Fig. 3) was constructed based on (*R*)-POPA tolerance and (*R*)-HPOPA concentration, for screening microbials from environmental samples. A PDA solid screening medium was used to obtain single microbial colonies that are tolerant to (*R*)-POPA concentrations > 5.0 g/L. Strains were inoculated into 96-well plates containing fermentation medium at 28 °C for 72 h. Then (*R*)-POPA (10.0 g/L) was added as substrate for the biotransformation. The biotransformation was allowed to continue for 7 days. The culture broth reaction mixtures in the microplate were centrifuged at 5000 ×*g* for 20 min. The broth supernatant was then transferred to corresponding wells of a clean 96-well microplate. The concentrations of (*R*)-HPOPA produced were detected using microplate reader.

### 2.7. Identification of the isolated strain

The total DNA from cells was extracted by a Fast DNA spin kit (ABigen, Beijing, China). The 18S rDNA gene was amplified using fungal universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR amplifications were performed in a 50 μL reaction volume that contained 1.5 μL of template, 0.5 μL of each primer, 6.5 μL of PCR Taqmix and 41 μL of $ddH_2O$. The PCR condition was as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The reactions were performed on a Mastercyle Gradient thermal cycler (Eppendorf, Shanghai, China). The PCR products were

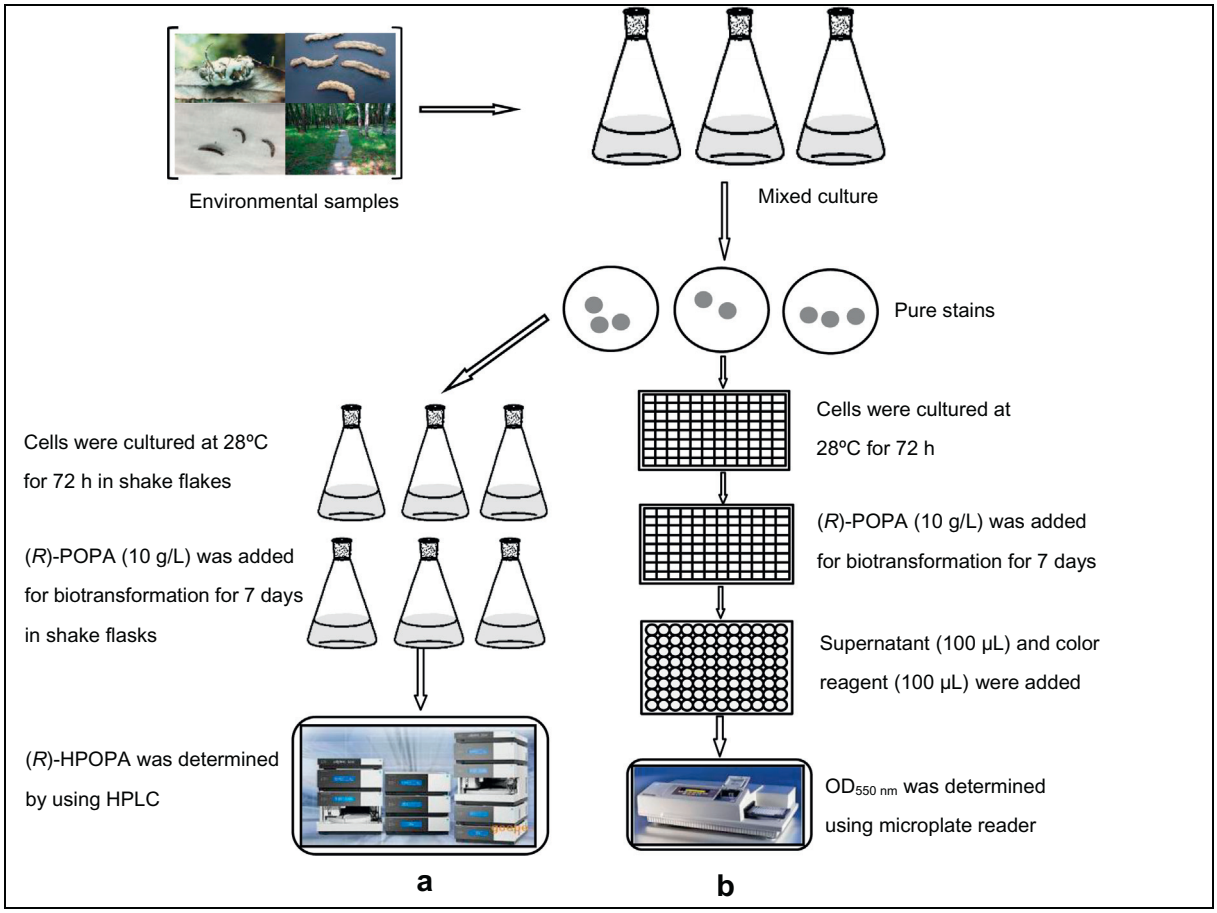


**Fig. 3.** Traditional method for screening the (*R*)-HPOPA producing strains (**a**), and rapid throughput method for screening (*R*)-HPOPA producing strains (**b**).

purified (DNA purification kit, ABigen, Beijing, China) and were sequenced (BioSune, Hangzhou, China). The 18S rDNA sequences were analyzed through the BLAST in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments and phylogenetic analyses were conducted using the Molecular Evolutionary Genetics Analysis (MEGA, Tokyo, Japan) software Version 5.0.

## 3. Results and discussion

### 3.1. Establishment of the rapid throughput screening procedure

#### 3.1.1. Determination of the working wavelength

In order to develop a spectrophotometric method for rapid throughput identification of (*R*)-HPOPA producing strains based on the color reaction, it is necessary to select the idea working wavelength. A continuous wavelength scan was measured using a multifunction microplate reader. The reaction mixtures with different (*R*)-POPA and (*R*)-HPOPA concentrations in 470–800 nm wavelength range were scanned, and absorption spectra are shown in Fig. 4. For the indoxyl antpyrine chromophore from the product (*R*)-HPOPA, the absorption increased with the decrease of the wavelength between 500 and 800 nm; for the substrate (*R*)-POPA, when the wavelength was higher than 500 nm, the absorption was nearly zero and maintained unchanged (Fig. 4a). The maximum absorption ratios of indoxyl antpyrine chromophore and substrate (*R*)-POPA for the same concentration of sample was detected within the range of 500–550 nm (Fig. 4b). We therefore selected 550 nm as the working wavelength in the following experiments.

#### 3.1.2. Optimal conditions for the color development

The effect of potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) on the absorption at 550 nm of (*R*)-HPOPA chromogenic reaction product, indoxyl antpyrine, was investigated to obtain the optimal $K_3[Fe(CN)_6]$ concentration for a quantitative reaction with (*R*)-HPOPA. Various concentrations of $K_3[Fe(CN)_6]$ over the range of 0.01–0.07 g/mL were examined. The color-developing reaction was performed at 30 °C for 20 min. Fig. 5a shows that as the concentrations of $K_3[Fe(CN)_6]$ was increased from 0.01 to 0.06 g/mL, the $A_{550}$ value increased from 0.22 to 0.55. When the concentrations of $K_3[Fe(CN)_6]$ was increased from 0.06 to 0.07 g/mL, $A_{550}$ values remained stable. Therefore, 0.06 g/mL $K_3[Fe(CN)_6]$ was recommended in the following test.

The result of optimizing the reaction temperature (from 25 to 50 °C) on the $A_{550}$ value is shown in Fig. 5b. The $A_{550}$ value increased when the reaction temperature was increased from 25 to 45 °C. When the temperature increased from 45 °C to 50 °C, $A_{550}$ values remained stable. The result showed that the optimal temperature for this reaction was 45 °C.

The influence of pH on the color development was tested at different pH values in the range of 7.0–11.0 at 45 °C for 20 min. The maximum $A_{550}$ value for the reaction was obtained at pH of 10.0 (Fig. 5c). When pH was higher than 10.0, the absorption at 550 nm decreased. Thus, a pH of 10.0 was considered to be the optimum pH.

The effect of the reaction time (from 15 to 45 min) on the $A_{550}$ values is shown in Fig. 5d. The $A_{550}$ values increased with reaction time with the maximum $A_{550}$ value obtained at 35 min, after which the absorption at 550 nm remained stable. Therefore, the optimal chromogenic reaction time was determined as 35 min.

#### 3.1.3. Standard curve and the detection limit

The (*R*)-HPOPA concentration standard curve (Fig. 6) was constructed based on assay with optimized reaction conditions ($K_3[Fe$

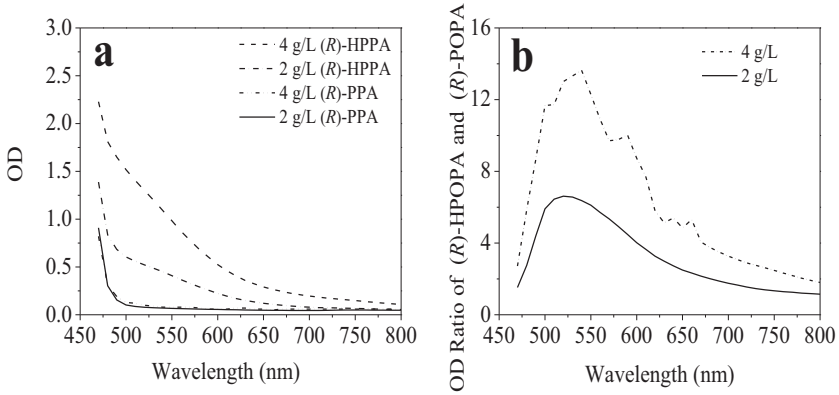


**Fig. 4.** Absorption spectrum of the chromophores which was formed by reacting 4-AAP in the presence of $K_3[Fe(CN)_6]$ with (*R*)-HPOPA or (*R*)-POPA (a) and the absorption ratio of (*R*)-HPOPA and (*R*)-POPA at the same concentration (b).

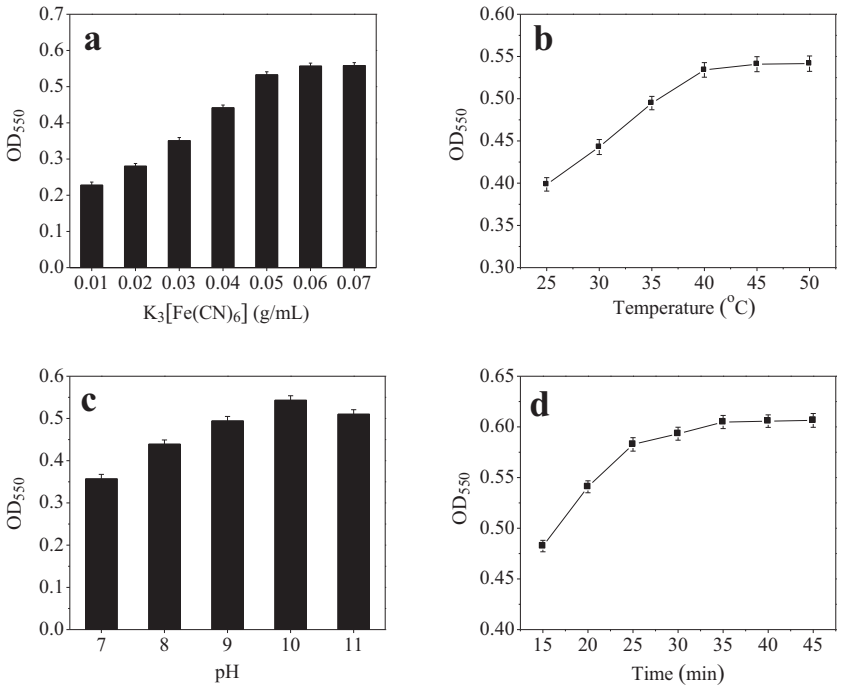


**Fig. 5.** Optimization of chromogenic reaction showing the effect on color development of (a) $K_3[Fe(CN)_6]$ concentration (2.0 g/L (*R*)-HPOPA, 0.6 g/L 4-AAP, 30 °C, pH 10.0, 20 min); (b) temperature (2.0 g/L (*R*)-HPOPA, 0.6 g/L 4-AAP, 0.06 g/mL $K_3[Fe(CN)_6]$, pH 10.0, 20 min); (c) pH (2.0 g/L (*R*)-HPOPA, 0.6 g/L 4-AAP, 0.06 g/mL $K_3[Fe(CN)_6]$, 45 °C, 20 min); and (d) reaction time (2.0 g/L (*R*)-HPOPA, 0.6 g/L 4-AAP, 0.06 g/mL $K_3[Fe(CN)_6]$, 45 °C, pH 10.0). All experiments were conducted in triplite.

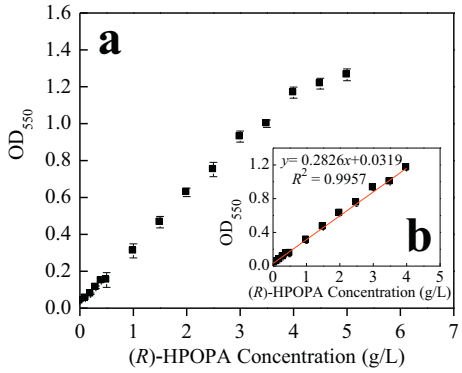


**Fig. 6.** The correlation between (*R*)-HPOPA concentration and the $OD_{550}$ value in rapid throughput screening system.

$(CN)_6]$ 0.06 g/mL, 45 °C, pH 10, and 35 min). The color reaction from yellow to brown were measured at 550 nm over a (*R*)-HPOPA concentration range. Linear regression of the linear part of the cure yielded the fitted equation as $A_{550} = 0.2826$ [(*R*)-HPOPA] +0.0319 ($R^2 = 0.9957$). The mean of the slope and standard deviation of response were obtained by plotting three standard curves. The LOD and LOQ were calculated to be 0.372 ± 0.028 g/L and 1.071 ± 0.046 g/L, indicating a good sensitivity of the proposed method. The result of variance analysis (Table 1) showed that $F > F_{0.01}$ (1, 11), meaning that there has very significant linear relationship between (*R*)-HPOPA concentration and the $A_{550}$ value within the range of 0–4.0 g/L. The maximum residual was 0.177 g/L (Fig. 7a). The correlation with HPLC standard curve was shown in Fig. 7b.

**Table 1**
The variance analysis of (*R*)-HPOPA standard curve (*F*-test).

| Source of variance | Sum of squares (*SS*) | Degrees of freedom (*df*) | Mean square (*MS*) | *F |
|---|---|---|---|---|
| Regression | 1.943 | 1 | 1.943 | 5198.95 |
| Residual | 0.0041 | *n*-2 (11) | 0.00037 | |
| Total | 1.947 | *n*-1 (12) | | |

*n* = number total of observations; $^*F_{0.01}$ (1, 11) = 9.65, for 99% of significance level.

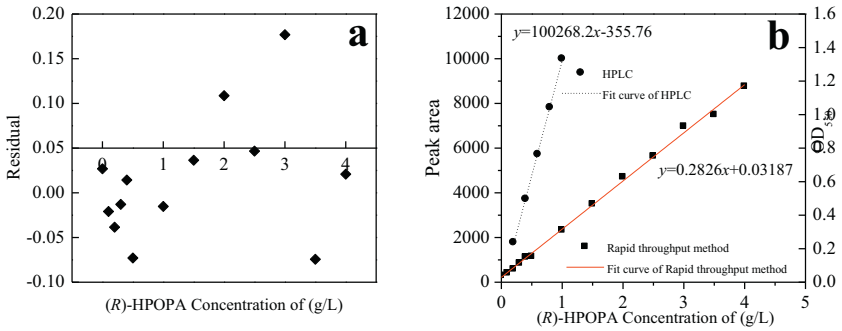


**Fig. 7.** The residuals of (*R*)-HPOPA concentration standard curve within 0–4.0 g/L (a) and the correlation with (*R*)-HPOPA concentration standard curve of in HPLC analysis (b).

**Table 2**
The recovery results of (*R*)-HPOPA ($n$ = 8).

| Sample | Analytical value ($W_2$) (g/L) | Added amount ($W_3$) (g/L) | Measure value ($W_1$) (g/L) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| 1 | 0 | 1.00 | 1.02 ± 0.05 | 102 ± 5 | 1.2 |
| 2 | 0 | 1.50 | 1.46 ± 0.08 | 97.3 ± 5.3 | 0.8 |
| 3 | 0 | 2.00 | 1.98 ± 0.06 | 99 ± 3 | 1.5 |
| [a]1 | 2.62 ± 0.07 | 1.00 | 3.62 ± 0.09 | 99.7 ± 9.2 | 1.4 |
| [a]2 | 2.51 ± 0.08 | 1.00 | 3.46 ± 0.08 | 98.3 ± 2.3 | 0.7 |
| [a]3 | 2.89 ± 0.09 | 2.00 | 4.91 ± 0.03 | 100.8 ± 2.8 | 1.2 |
| [b]1 | 0 | 1.00 | 1.04 ± 0.04 | 103.2 ± 4.4 | 1.5 |
| [b]2 | 0 | 1.50 | 1.53 ± 0.04 | 102.5 ± 3.5 | 1.3 |
| [b]3 | 0 | 2.00 | 2.09 ± 0.07 | 104.5 ± 7.3 | 1.9 |

(*a*), positive control; (*b*), negative control.

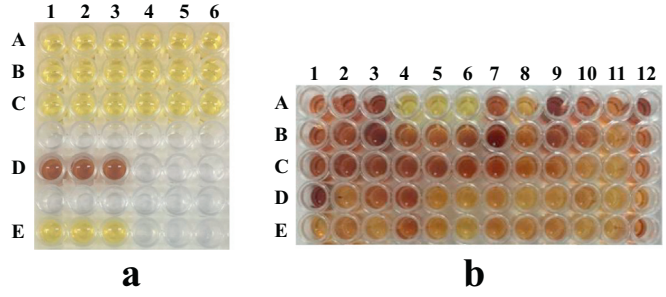


**Fig. 8.** An example of a microplate screen of (*R*)-HPOPA producing strains using our optimized procedure with 4-AAP and $K_3[Fe(CN)_6]$. The color reaction from yellow to brown in the microplate wells indicated that the corresponding cultures of strains grown in 96-well plate contains the desired catalytic activity for hydroxylation of (*R*)-POPA to (*R*)-HPOPA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
(a) The chromogenic reaction of blank control, positive control and negative control: A1-C1 were blank control with 0.0 g/L (*R*)-POPA, A2-C2 were blank control with 2.0 g/L (*R*)-POPA, A3-C3 were blank control with 4.0 g/L (*R*)-POPA, A4-C4 were blank control with 6.0 g/L (*R*)-POPA, A5-C5 were blank control with 8.0 g/L (*R*)-POPA, A6-C6 were blank control with 10.0 g/L (*R*)-POPA, D1-D3 were positive control, E1-E3 were negative control; (b), the chromogenic reaction of samples, dark color in the microplate indicated that the corresponding strains possess high catalytic activity.

### 3.1.4. Verification of assay

Recovery studies were conducted to verify the practical feasibility of the method. Experiments were carried out and mean recovery values were calculated. Table 2 showed that the mean recovery values were between 97.3 and 104.5% with RSD < 2.0%, suggesting that it is desired to apply this rapid throughput screening method for quantification of (*R*)-HPOPA concentrations.

**Table 3**
The detection results of (*R*)-HPOPA biosynthesis with different methods.

| Strain No. | DCW (g/L) | (*R*)-HPOPA titer[a] (g/L) | (*R*)-HPOPA titer[b] (g/L) | Conversion[c] (%) |
|---|---|---|---|---|
| 1 | 7.75 ± 0.34 | 6.98 ± 0.21 | 7.06 ± 0.23 | 64.31 ± 2.10 |
| 2 | 8.07 ± 0.11 | 5.78 ± 0.13 | 5.77 ± 0.24 | 52.63 ± 2.19 |
| 3 | 8.86 ± 0.15 | 6.42 ± 0.22 | 6.67 ± 0.31 | 60.84 ± 2.83 |
| 4 | 8.66 ± 0.13 | 9.68 ± 0.35 | 9.59 ± 0.38 | 87.48 ± 3.47 |
| 5 | 8.56 ± 0.22 | 8.74 ± 0.25 | 8.64 ± 0.31 | 78.81 ± 2.83 |
| 6 | 7.82 ± 0.19 | 7.05 ± 0.23 | 7.07 ± 0.33 | 64.49 ± 3.01 |
| 7 | 8.79 ± 0.26 | 8.46 ± 0.32 | 8.52 ± 0.41 | 77.72 ± 3.74 |

(*a*), detected with rapid throughput method; (*b*), detected with HPLC; (*c*), determined based on the HPLC detection and expressed as mol/mol.

### 3.2. Strains screening based on the rapid throughput screening procedure

Most of the screened (*R*)-HPOPA producing microbial strains do not necessarily produce hydroxylase(s), therefore different strains need to be isolated as single colonies. About 5000 single colonies tolerant to (*R*)-POPA of 5.0 g/L were obtained through cultivation on PDA screening plate. In the traditional screening procedure, the selection of (*R*)-HPOPA producing strains is random, blind, labour intensive with low efficiency. By contrast, the rapid throughput screening method enabled us to identify the ideal strains efficiently. The chromogenic reaction results of some tested strains were shown in Fig. 8. The lighter color represented there were no or few product (*R*)-HPOPA biosynthesized; while the darker color implied an existence of relatively higher concentration of (*R*)-HPOPA. Through this rapid throughput screening method, > 40 strains with catalytic activity have been obtained, including seven strains with a high conversion rate (Table 3). Especially a strain of No. 4, whose conversion rate reached 95.9% (g/g, the mole conversion was 87.48%) at the substrate concentration of 10 g/L, the chromatographic spectrum in HPLC analysis was presented in Fig. 9, representing the highest level in the isolated strains, were obtained and named as ZJB16002. Furthermore, the results of variance analysis (Table 4) showed that our rapid throughput screening method was comparable with the HPLC method which directly analyzed (*R*)-HPOPA concentrations with a $F < F_{0.05}$ (1, 6). Through the high throughput 96-well microplate test, > 800 conversion samples were tested within 1 h, and the HPLC detection required about 1300 h, so the screening efficiency was greatly improved.

### 3.3. 18S rDNA gene sequence analysis and identification of the isolated strain

The isolated strain ZJB16002 with ideal (*R*)-HPOPA producing characteristics was identified as a member of the *Beauveria* genus based on the 18S rDNA sequence analysis. Phylogenetic relationships between *Beauveria* sp. strain ZJB16002 and other species of the *Beauveria* genus were constructed using neighbor-joining algorithm according to their

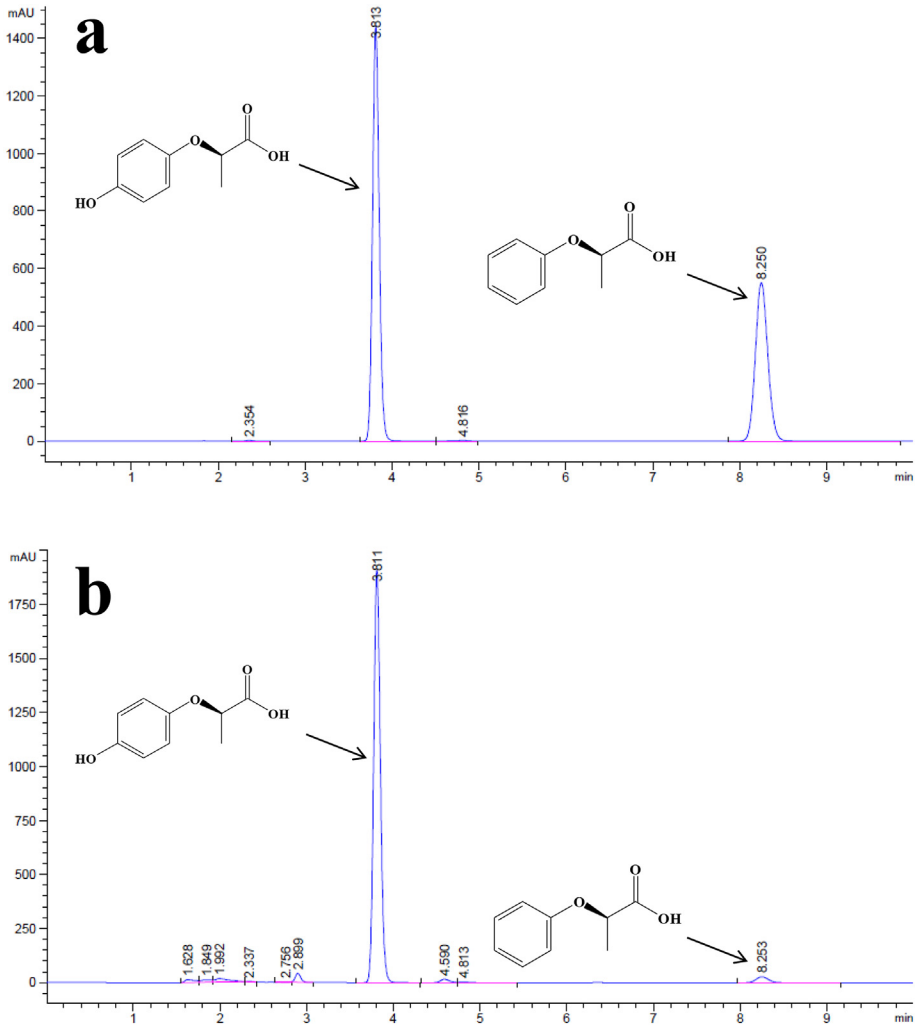


**Fig. 9.** The chromatographic profiles of HPLC analysis. (a) Standard (*R*)-HPOPA (0.8 g/L) (3.81 min) and (*R*)-POPA (0.8 g/L) (8.35 min); (b) The supernatant of fermentation broth of strain NO. 4 (diluted 10 times).

**Table 4**
The variance analysis of the data from rapid throughput method and HPLC (*F*-test).

| Source of variance | Sum of squares (*SS*) | Degrees of freedom (*df*) | Mean square (*MS*) | *$F$ |
|---|---|---|---|---|
| Treatments | 0.0032 | *n*-1 (1) | 0.0032 | 0.0017 |
| Residual | 22.43 | *n*-*r* (6) | 1.87 | |
| Total | 22.433 | *n*-1 (7) | | |

*r* = number of different methods; *n* = number total of observations; *$F_{0.05}$ (1, 6) = 4.75, for 95% of significance level.

18S rDNA gene sequences (Fig. 10). The *Isaria javancia* CBS 134.22 was selected as the out group. The closest genetic distance from strain ZJB16002 was *Beauveria bassiana* IMI 356817 with a confidence of 80%.

## 4. Conclusions

This work demonstrated that (*R*)-HPOPA can react with 4-AAP with the existence of potassium ferricyanide and result in a formation of orange-red dye, indoxyl antipyrine, based on which, a rapid throughput screening method for (*R*)-HPOPA biosynthetic strains was developed. The advantage of this method is the ability to quickly measure the concentration of the (*R*)-HPOPA product in a transformed microbial culture sample. For the conventional HPLC analysis method, the analysis time per sample is considerably longer and the sample processing capacity is limited. In this study, the concentration of (*R*)-HPOPA in the mixed component of the transformed sample was obtained through measuring the absorption at 550 nm of (*R*)-HPOPA chromogenic products. Compared with HPLC, the method does not need to separate samples. Using the 96-well microplate screening, the operation time is greatly shortened. The amount of the samples screened every day can reach 3000-5000, which approaches the requirement of a high throughput assay. This assay has been applied to not only the selection of (*R*)-HPOPA producing strain, but also the strain breeding, and mutation analysis, which greatly promoted the development of (*R*)-HPOPA production by microbes.

## Disclosure statement

The authors declare that there is no conflict of interest in this study.

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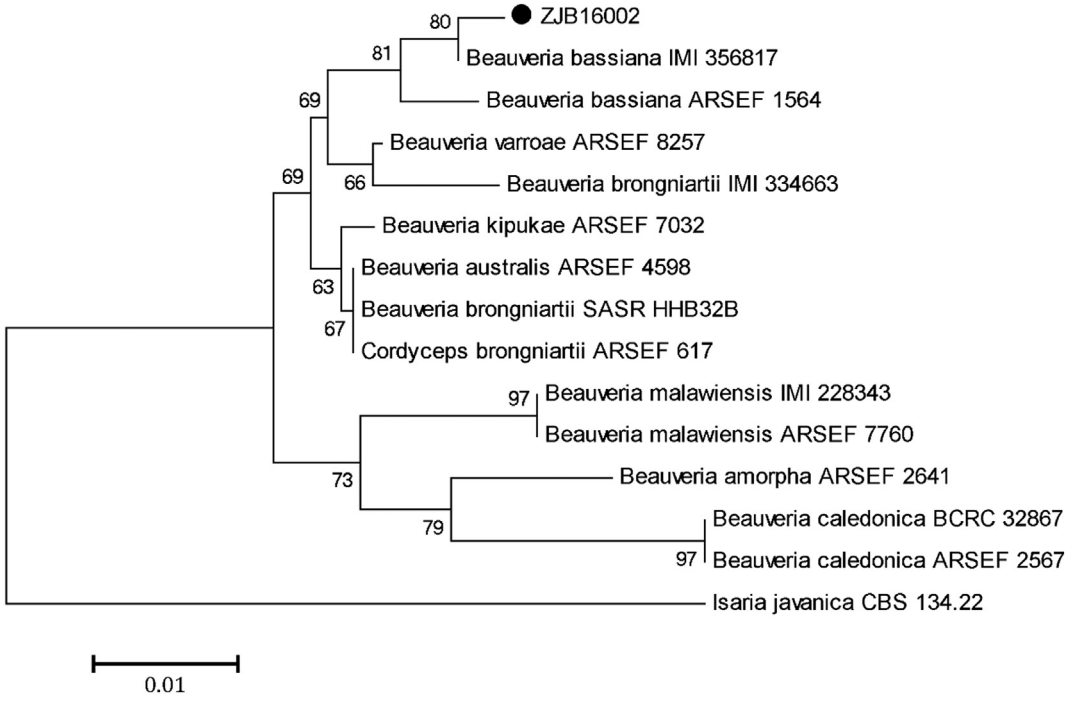


**Fig. 10.** Phylogenetic tree constructed by the neighbor-joining method based on 18S rDNA sequences, showing the position of strain ZJB16002 and representatives of some related strains. *Isaria javancia* CBS 134.22 was used as an out group. Bootstrap values were 1000 replicates, in which ≥50% were reported near the corresponding nodes. The scale bar indicates the percentage of genetic distance.

## References


Becker, R., Oeser, H.G., Acker, R.D., 1984. Preparation of 2-(Hydroxyphenoxy)-Carboxylates. (US4489207, 1984-12-18).

Beilen, J.B., Duetz, W.A., Schmid, A., Witholt, B., 2003. Practical issues in the application of oxygenases. Trends Biotechnol. 21, 170–177.

Cooper, B., Ladner, W., Hauer, B., Siegel, H., 1994. Preparation of 2-(4-Hydroxyphenoxy) Propionic Acid by Fermentation. (US5296363A, 1994-3-22).

Dingler, C., Ladner, W., Krei, G.A., Cooper, B., Hauer, B., 1996. Preparation of (*R*)-2-(4-hydroxyphenoxy) propionic acid by biotransformation. Pestic. Sci. 46, 33–35.

Elango, V., Davenport, K.G., 1991. Synthesis of 2-(4-Hydroxyphenoxy) Alkanoic Acid Esters. (US5008439, 1991-4-16).

Jiang, M.G., Yang, H., Chen, R.D., Liu, Y.P., Bo, L.Y., Lou, Y.L., Zhang, S.M., Zhang, D.Y., 2000. Study on optical isomers of aryl-oxy-propionic acid herbicides. J. Nanjing Agric. Uni. 97–100.

Kinne, M., Ullrich, R., Hammel, K.E., Scheibner, K., Hofrichter, M., 2008. Regioselective preparation of (*R*)-2-(4-hydroxyphenoxy)propionic acid with a fungal peroxygenase. Tetrahedron Lett. 49, 5950–5953.

Li, Y.Y., Hu, C.R., 2014. Experiment Design and Data Processing, 2th ed. Chemical Industry Press, Beijing.

Liu, Q.X., Liu, A.P., Hu, A.X., Huang, M.Z., Ou, X.M., Zhou, H.F., 2015. Progress on 2-(4-Aryloxyphenoxy) Propionic Acid Derivatives. Agrochemicals 54, 551–558.

Lu, Z.Y., Guo, X.J., Li, H., Huang, Z.Z., Lin, K.F., Liu, Y.D., 2015. High-throughput screening for a moderately halophilic phenol-degrading strain and its salt tolerance response. Int. J. Mol. Sci. 16, 11834–11848.

Ma, B., Xu, M., Zheng, X.Q., Lin, C.M., 2012. Enantioseparation of five aryloxyphenoxypropionic herbicides by supercritical fluid chromatography. Agrocheincals 51, 193–196.

Qi, X., Zhang, Y., Tu, R., Lin, Y., Li, X., Wang, Q., 2011. High-throughput screening and characterization of xylose-utilizing, ethanol-tolerant thermophilic bacteria for bioethanol production. J. Appl. Microbiol. 110, 1584–1591.

Schmid, A., Hollmann, F., Park, J.B., Buhler, B., 2002. The use of enzymes in the chemical industry in Europe. Curr. Opin. Biotechnol. 13, 359–366.

Tan, J., Chu, J., Hao, Y.Y., Guo, Y.X., Zhuang, Y.P., Zhang, S.L., 2013. High-throughput system for screening of cephalosporin C high-yield strain by 48-deep-well microtiter plates. Appl. Biochem. Biotechnol. 169, 1683–1695.

Tang, L.X., Li, Y., Wang, X., 2010. A high-throughput colorimetric assay for screening halohydrin dehalogenase saturation mutagenesis libraries. J. Biotechnol. 147, 164–168.

Wang, Y.Z., Li, H.F., Wang, J.H., Chen, X., U, D.S., Zhou, B.H., 2009. A method of high throughput screen of catechol-producing strain. Biotechnology 19, 48–51.

Watson, K.G., 1989. Process for the Synthesis of Phenoxyalkane Derivatives. (US4837355, 1989-6-6).

Weng, J.Q., Gao, Z.J., Xu, B., Ding, C.R., Tan, C.X., 2009. Asymmetrical synthesis of *R*-(+)-2-(4-hydroxyphenoxy) propionic acid. J. Zhejiang Univ. Technol. 37 (362–365, 371).

Wettling, T., Dimmler, M., Hupfeld, B., Henkelmann, J., Henklemann, J., 1999. Preparation of 2-(4-Hydroxyphenoxy) Propionates. (US5886209, 1999-3-23).

Wu, C.L., Liu, K.L., Ma, D.L., 2015. Technical improvement for synthesizing *R*-(+)-2-(4-hydroxyphenoxy)-propionic acid. Guangzhou Chem. Ind. Technol. 43, 92–93, 166.

Xia, X.D., Ma, H.J., Xu, M.H., Zang, Z.J., Li, J.H., 2013. Research progress on molecular mechanism of resistant weeds to aryloxyphenoxypropionate herbicides. Chin. J. Pesticide Sci. 15, 609–614.

Xu, Z., Cai, T., Xiong, N., Zou, S.P., Xue, Y.P., Zheng, Y.G., 2018. Engineering the residues on "a" surface and C-terminal region to improve thermostability of nitrilase. Enzyme Microb. Technol. 113, 52–58.

Xue, Y.P., Xu, S.Z., Liu, Z.Q., Zheng, Y.G., Shen, Y.C., 2011. Enantioselective biocatalytic hydrolysis of (R,S)-mandelonitrile for production of (R)-(−)-mandelic acid by a newly isolated mutant strain. J. Ind. Microbiol. Biotechnol. 38, 337–345.

Xue, Y.P., Shi, C.C., Xu, Z., Jiao, B., Liu, Z.Q., Huang, J.F., Zheng, Y.G., Shen, Y.C., 2015. Design of nitrilases with superior activity and enantioselectivity towards sterically hindered nitrile by protein engineering. Adv. Synth. Catal. 357, 1741–1750.

Xue, Y.P., Jiao, B., Hua, D.E., Cheng, F., Liu, Z.Q., Zheng, Y.G., 2017. Improving catalytic performance of an arylacetonitrilase by semirational engineering. Bioprocess Biosyst. Eng. 40, 1565–1572.

Zhu, Z.X., Jin, J., Xue, N.N., Song, X.Y., Chen, X.G., 2014. Development and validation of high-throughput screening assays for poly(ADP-ribose)polymerase-2 inhibitors. Anal. Biochem. 449, 188–194.