Efficient production of α-keto acids by immobilized E. coli-pETduet-1-PmiLAAO in a jacketed packed-bed reactor

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α-keto acids are compounds of primary interest for the fine chemical, pharmaceutical and agrochemical sectors. L-amino acid oxidases as an efficient tool are used for α-keto acids preparation in this study. Firstly, an L-amino acid oxidase (PmiLAAO) from Proteus mirabilis was discovered by data mining. Secondly, by gene expression vector screening, pETDuet-1-PmiLAAO activity improved by 130%, as compared to the pET20b-PmiLAAO. PmiLAAO production was increased to 9.8 U ml⁻¹ by optimized expression condition (OD600 = 0.65, 0.45 mmol l⁻¹ IPTG, 20 h of induction). Furthermore, The PmiLAAO was stable in the pH range of 4.0–9.0 and in the temperature range of 10–40°C; the optimal pH and temperature of recombinant PmiLAAO were 6.5 and 37°C, respectively. Afterwards, in order to simplify product separation process, E. coli-pETduet-1-PmiLAAO was immobilized in Ca-alginate beads. Continuous production of 2-oxo-3-phenylpropanoic acid was conducted in a packed-bed reactor via immobilized E. coli-pETduet-1-PmiLAAO. Significantly, 29.66 g l⁻¹ 2-oxo-3-phenylpropanoic acid with a substrate conversion rate of 99.5% was achieved by correspondingly increasing the residence time (25 h). This method holds the potential to be used for efficiently producing pure α-keto acids.
1. Introduction

The α-keto acid is a compound containing both a carboxyl group and a ketone group in a molecule involved in the TCA cycle (tricarboxylic acid cycle, also well known as citric acid cycle), decomposition of amino acids and significant in biological systems [1]. α-keto acids and their derivatives (α-hydroxy acids) as a building block have been applied to chemical synthesis, dietary supplements, chemical industries and pharmaceutical (such as ibuprofen and naproxen, (S)-camptothecin, (S)-oxybutynin) [2]. For instance, d-phenylglycine was used for the preparation of HCV NS5A inhibitor [3] and β-lactam antibiotics (ampicillin and cephalaxin) with a consumption of several thousand tons per year [4]. Especially, 4-fluoro-d-phenylglycine and 4-chloro-d-phenylglycine have been used for h5-HT1D receptor agonist [5] and macrocyclic hedgehog pathway inhibitor preparation [6], respectively. However, α-keto acids as a kind of unstable compound are difficult to synthesize, and are easily decarboxylated, decarbonylated and oxidized. Three different approaches have been developed for α-keto acids preparation: (i) chemical synthesis: complex raw materials, expensive catalysts and complicated processes; (ii) microbial fermentation: low yield, purification process is complex and costly and (iii) biotransformation. Biotransformation with high performance has been regarded as the core of green chemistry.

Biotransformation as a well-suited method has been applied to prepare α-keto acids and chiral rare amino acids, in which enzymes as ‘green’ biocatalysts have been used to produce fine chemicals and pharmaceuticals [7,8]. Amino acid oxidase (EC 1.4.3.2), also called amino acid deaminase (AAD), including l-amino acid oxidase (l-AAO) and d-amino acid oxidase (d-AAO), is a type of flavoenzyme with a broad spectrum of oxidase activity [9]. α-amino groups of amino acids can be removed by AAD in the presence of oxygen and produce α-keto acids and ammonia [10]. AAO can be produced by a variety of microorganisms, including Trichoderma viride, Pseudomonas, Proteus mirabilis, Penicillium, Aspergillus niger and Aerobacter aerogenes [11–13]. In past years, the d-amino acid oxidase (d-AAO) has been applied to prepare l-amino acids from racemic mixtures [14]. In this study, we are trying to obtain an efficient biocatalyst for α-keto acid production. To obtain high-yield, high-purity α-keto acids to meet the needs of industrial production, an amino acid oxidase from Proteus mirabilis was selected by data mining and screening. Moreover, the amino acid oxidase genes have been cloned and the production of the LAAO was optimized by cultivation conditions. In addition, the production of α-keto acid has been enhanced by immobilization of E. coli-pETduet-1-PmiLAAO in Ca-alginate beads and reaction in a packed bed reactor (scheme 1). This method holds the potential to be used for efficient production of pure α-keto acids.

2. Material and methods

2.1. Construction of recombinant strain

The corresponding LAAO genes were synthesized by the Sangon (Shanghai, China). The LAAO genes were then amplified by polymerase chain reaction (PCR) techniques and the used primers were shown as follow: PmiLAAO-F (5'-ATGACATTCCAAGGAGAAAGCTAC-3') and PmiLAAO-R (5'-ACCTTC TTAAAACGATCCAAACT-3'); RoLAAO-F (5'-ATGGCATTCACACGTAGATCTTTCA-3') and RoLAAO-R (5'-TCAGGCTTCCTGGGCCACG-3') [5]; DrLAAO-F (5'-AGTCTTCAAGCCAATAAG-3') and DrLAAO-R (5'-GGGACATAGCTCCTAGAAT-3') [6]. Then the amplified genes were ligated into plasmid pACYCduet-1, pETDuet-1, PRSFduet-1, PCDFduet-1 and pET20b (Novagen, Germany), respectively, then verified by plasmid PCR and DNA sequencing. The recombinant plasmids were transformed into E. coli BL21 (DE3) for expression. The positive clones were cultivated in LB medium containing ampicillin (100 μg ml⁻¹) at 37°C, 200 r.p.m. Subsequently, recombinant E. coli BL21 (DE3) induced by supplementing of 0.45 mM IPTG for further 20 h at 16°C while the OD600nm was 0.6 approximately.

2.2. Purification of PmiLAAO

After fermentation, the cells were collected by centrifugation at 6000 r.p.m. for 20 min. The cells were washed twice with 0.2 M phosphate buffer (PBS, pH 7.2) and suspended in 0.2 M PBS (pH 7.2). The cells were homogenized by ultrasonic cell disruptor (power 300 W, ultrasound 4 s, pause 8 s, total 30 min) under ice-cooling. The supernatant was obtained by centrifugation at 8000 r.p.m. for 10 min.
In order to remove those proteins with a molecular mass below 30 kDa, the supernatant was filtered by ultrafiltration with an Amicon Ultra-15 30 K device (Millipore, USA). The Ni(II)-NTA agarose matrix (QUIAGEN), a metal chelation chromatography, was used for further purification of the concentrate containing recombinant PmiLAAO.

2.3. Measurement of the amino acid oxidase activity

The AAO activity was determined at pH 7.5 and 30°C with L-Phe as substrate according to the method of Sacchi et al. [15]. The initial production rate of hydrogen peroxide (H₂O₂) with a coupled peroxidase assay was measured. Briefly, the crude extracts of the L-AAO 100 μl were added with 100 μl testing solution (1 mmol l⁻¹ o-dianisidine (o-DNS), 20 mmol l⁻¹ L-Phe and 20 U ml⁻¹ horseradish peroxidase (HRP), in 0.2 mol l⁻¹ dipotassium pyrophosphate buffer, pH 7.5) cultivated 10 min at 30°C. The time course of the absorbance change at 440 nm was recorded by using UV/Vis spectrophotometer. The amount of enzyme required to consume 1 μmol of L-Phe per minute under the described conditions has been defined as one unit of LAAO activity.

2.4. The PmiLAAO characterization

The optimum pH for PmiLAAO was determined in the range of 4–11. pH-stability was investigated at different pH under 30°C. The optimum temperature of PmiLAAO was determined in the range of 10–70°C. Thermo-stability was investigated at different temperatures in the 0.2 M phosphate buffer (PBS, pH 6.5). The residual activity of the samples was measured as described above.

2.5. Immobilization of E. coli-PmiLAAO

E. coli-PmiLAAO entrapment in calcium alginate beads. Sodium alginate aqueous solution (2.5%) was mixed with E. coli-PmiLAAO whole-cell suspension (30 mg cells ml⁻¹) with a ratio of 1:1 (v/v). After that, in order to prepare Ca-alginate beads, the mixture was dropped into 0.3 mol l⁻¹ CaCl₂ solution using a syringe. Preparation of cell-carrageenan beads and gelatin beads were completed according to the Ca-alginate beads.

Cell cross-linking with glutaraldehyde was conducted as follows: E. coli-PmiLAAO whole-cell was suspended in 0.1 M PBS buffer (pH = 7.0) at a concentration of 25 g l⁻¹. Subsequently, 2.5% (v/v) glutaraldehyde was mixed with E. coli-PmiLAAO whole-cell suspension. To obtain a cross-linked preparation, the mixtures were incubated at room temperature (25°C) for 60 min. The cell cross-linked E. coli was obtained and the supernatant containing excess glutaraldehyde was removed by centrifuging for 10 min. Moreover, preparation of polyacrylamide gel beads was completed according to the protocol by Skryabin & Koshcheenko [16].
2.6. Preparation of α-keto acids from L-amino acid by whole-cell biocatalyst

The α-keto acids preparation reaction was shown as follows: 1 g l\(^{-1}\) L-phenylalanine or its derivative, whole-cell biocatalyst 2 g l\(^{-1}\) (wet cell weight, w/v), 0.2 mol l\(^{-1}\) phosphate buffer (PBS, pH 6.5) at 37\(\degr\)C, 200 r.p.m. The reactions were stopped by centrifugation at 8000 r.p.m. for 15 min. Then, the supernatant was withdrawn and the α-keto acids content was measured by high-performance liquid chromatography (HPLC) \[4\]; briefly, HPLC equipped with an AminexHPX-87H column at 35\(\degr\)C with the injection volume of 10\(\mu\)l. The mobile phase was 5 mmol l\(^{-1}\) H\(_2\)SO\(_4\), and the flow rate was 0.6 ml min\(^{-1}\).

3. Results and discussion

3.1. Discovery of L-amino acid oxidase

In order to construct a library of L-amino acid oxidases (LAAO) for α-keto acid production, the amino acid sequence of \(Pmi\)LAAO, \(No\)LAAO and \(Mg\)LAAO as a query were used for a pBLAST search. Twelve proteins possibly having L-amino acid oxidase activity from various microorganisms were selected by data mining. In addition, the Km value of the amino acid oxidases towards L-phenylalanine has been investigated (table 1). However, many of LAAO gene sequences with lower Km value (including \(Nc\)LAAO, \(Pre\)LAAO, \(Th\)LAAO, \(Hs\)LAAO and \(Cat\)LAAO) failed to be accessed from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). \(No\)LAAO, \(Cr\)LAAO, \(Cad\)LAAO, \(Mg\)LAAO, \(Pmi\)LAAO, \(Ro\)LAAO and \(Dr\)LAAO with available sequence data were selected as the candidates. Afterwards, pET20b-\(No\)LAAO, pET20b-\(Cr\)LAAO, pET20b-\(Cad\)LAAO, pET20b-\(Mg\)LAAO, pET20b-\(Pmi\)LAAO, pET20b-\(Dr\)LAAO and pET20b-\(Ro\)LAAO were constructed and transformed into the expression host \(E.\ coli\) BL21 (DE3), subsequently. Moreover, the amino acid oxidase activity of the recombinant strains has been tested. Unfortunately, the \(No\)LAAO, \(Cr\)LAAO, \(Cad\)LAAO, \(Mg\)LAAO and \(Dr\)LAAO showed no amino acid oxidase activity. Of those, the amino acid oxidase activity of \(E.\ coli\) BL21 (pET20b-\(Pmi\)LAAO) was measured and found to be 1.1 U ml\(^{-1}\) and 1.8 U ml\(^{-1}\), respectively. Therefore, the \(E.\ coli\) BL21 (pET20b-\(Pmi\)LAAO) showing the highest activity was selected for further study instead of the pET20b-\(Ro\)LAAO (figure 1).

3.2. Preparation of the recombinant \(Pmi\)LAAO

Different expression vector (pETDuet-1, pCDFduet-1, pET20b, pACYCduet-1 and pRSFduet-1) were selected for \(Pmi\)LAAO genes expression and were subjected to LAAO activity test. Figure 2a shows

| no. | amino acid oxidases (LAAO)\(^b\) | microorganism | Km (mM)\(^a\) |
|-----|---------------------------------|--------------|--------------|
| 1   | RoLAAO                          | Rhodococcus opacus | 0.022        |
| 2   | NoLAAO                          | Neurospora crassa | 0.16         |
| 3   | PreLAAO                         | Providencia rettgeri | 3.1         |
| 4   | DrLAAO                          | Daboia russelli | 0.0665       |
| 5   | ThLAAO                          | Trichoderma harzianum | 11.73      |
| 6   | NoLAAO                          | Naja oxiana     | 0.051        |
| 7   | CrLAAO                          | Calloselasma rhodostoma | 0.05       |
| 8   | CadLAAO                         | Crotalus adamanteus | 0.03782     |
| 9   | CatLAAO                         | Crotalus atrax   | 0.036        |
| 10  | MgLAAO                          | Meleagris gallopavo | 3.50       |
| 11  | HsLAAO                          | Hebeloma cylindersporum | 2.20    |
| 12  | PmiLAAO                         | Proteus mirabilis | 22.0        |

\(^a\)Data were collected from Enzyme Database-BRENDA (https://www.brenda-enzymes.org/).
\(^b\)Data were obtained by pBLAST (https://www.ncbi.nlm.nih.gov/gene).
that the LAAO activity reached the maximum value (3.5 U ml$^{-1}$) when the PmiLAAO was carried by pETDuet-1 vector. The LAAO activity of pETDuet-1-PmiLAAO was increased by 130% comparing to the pET20b-PmiLAAO. The optimal induction time was at mid-log phase (OD$_{600nm}$ ≈ 0.65) (figure 2b). The optimal IPTG concentration was 0.45 mmol l$^{-1}$. Moreover, the maximum PmiLAAO amino acid oxidase activity (9.8 U ml$^{-1}$) was observed after 20 h of induction (figure 2c,d).
The recombinant PmiLAAO was concentrated by ultrafiltration and purified by affinity chromatography (table 2). The specific activity of PmiLAAO was up to 84.75 \( \pm \) 3.43 mU mg\(^{-1}\) with a purification fold of 17.92 by HisTrap affinity chromatography. In addition, the results show that the optimal pH and temperature of recombinant PmiLAAO were 6.5 and 37°C, respectively. The PmiLAAO was stable in the pH range of 4.0–9.0 and in the temperature range of 10–40°C (figure 3a, b).

3.3. Enhancing the catalytic efficiency of PmiLAAO by immobilization

In order to enhance the \( \alpha \)-keto acids production and simplify product separation process, different carriers were used to immobilize the recombinant E. coli-pET duet-1-PmiLAAO. The result showed that the method of glutaraldehyde crosslinking and carrageenan entrapment, gelatin entrapment or polyacrylamide gel entrapment was not suitable for the recombinant E. coli whole-cell immobilization. Less than 50% of PmiLAAO activity remained after cross-linking with glutaraldehyde, entrapment in carrageenan or entrapment in gelatin and immobilization in polyacrylamide. On the contrary, E. coli-pETduet-1-PmiLAAO immobilized in Ca-alginate beads remained 87% activity (figure 4a). The immobilized cells further demonstrated improved efficacy by retaining 60% activity even in the seventh reuse cycle (figure 4b).

3.4. Whole-cell transformation of \( \alpha \)-keto acids in packed-bed reactor

The ability of the immobilized whole-cell E. coli-pETduet-1-PmiLAAO on resolution of racemate mixtures (\( \tau \)-/L-Phe) has been investigated. The greater than 97.5% enantiomeric excess (ee) of (R)-2-amino-2-phenylpropanoic acid (\( \tau \)-Phe) and 49.5% yield of 2-oxo-3-phenylpropanoic acid were observed (figure 5a, b), respectively. Moreover, for the immobilized whole-cell E. coli-pETduet-1-PmiLAAO, the optimum substrate concentration was 10.0 g l\(^{-1}\). The conversion was decreased, while the substrate concentration was higher than 10.0 g l\(^{-1}\) (figure 5c). The conversion of \( \tau \)-Phe was up to 98%, while the whole-cell biocatalyst loading was 40 g l\(^{-1}\) (figure 5d). In addition, the changes of conversion were not significant by further increasing the whole-cell biocatalyst loading. After conditional optimization, 10.0 g l\(^{-1}\) \( \tau \)-phenylalanine was almost completely transformed to 2-oxo-3-phenylpropanoic acid with a conversion of 99.5% (figure 5e).

It was shown that the conversion did not increase along with the increasing substrate concentration (figure 5c), which indicated the immobilized E. coli-pETduet-1-PmiLAAO caused substrate inhibition. In

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**Table 2. Purification of PmiLAAO.** Note: data are presented as the mean ± s.d. with triple independent measurements.

| steps          | total protein (mg) | total activity (mU) | specific activity (mU/mg) | yield (%) | purification fold |
|----------------|--------------------|---------------------|--------------------------|-----------|--------------------|
| cell lysate (5 l) | 1880 ± 45          | 8900 ± 323          | 4.73 ± 0.166              | 100       | 1.00               |
| ultrafiltration  | 432 ± 16           | 6846 ± 240          | 15.83 ± 0.55              | 76.92     | 3.33               |
| HisTrap         | 21.5 ± 0.63        | 1822 ± 110          | 84.75 ± 3.43              | 20.47     | 17.92              |

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Figure 3. Optimal pH and pH stability of \( \tau \)-AAD (a). Optimal temperature and thermo stability of \( \tau \)-AAD (b). (a) (filled diamonds) pH, (open diamonds) pH stability. (b) (filled squares) temperature, (open squares) thermostability.
Figure 4. Immobilization of the whole-cell recombinant E. coli (a). Reusability of immobilized whole cells (b). Remaining activity = \((\text{PmiLAAO activity before immobilization} - \text{PmiLAAO activity after immobilization}) / \text{PmiLAAO activity before immobilization}\) × 100%.

(a) 1 1a 1b

| substrate | product | time (h) | lb (yield %) | 1a (ee %) |
|-----------|---------|----------|--------------|-----------|
| L-Phe     | 2-oxo-3-phenylpropanoic acid | 0.6 | 98.5 |
| D-Phe     | 2-oxo-3-phenylpropanoic acid | 0.5 | 49.5 |

Figure 5. Preparation of chiral amino acids and α-keto acids by immobilized E. coli-pETduet-1-PmiLAAO with a 4 mM substrate (a). Racemic resolution of 2-amino-2-phenylpropanoic acid by PmiLAAO (b). Solutions of 16 mM (2.64 g l\(^{-1}\)) (ω/)-phenylalanine with 5 g l\(^{-1}\) (wet cell weight, WCW) immobilized E. coli-pETduet-1-PmiLAAO in 200 mM phosphate buffer (pH = 6.5) were incubated at 37°C with gentle shaking. Samples were withdrawn regularly, and the product were analysed by HPLC. Effect of L-phenylalanine concentration on α-keto acids production by immobilized E. coli-pETduet-1-PmiLAAO (c); effect of the whole-cell biocatalyst loading on α-keto acids production (d); time curve of 2-oxo-3-phenylpropanoic acid production from L-phenylalanine (10 g l\(^{-1}\)) by immobilized E. coli-pETduet-1-PmiLAAO(E).
In order to enhance the 2-oxo-3-phenylpropanoic acid production, immobilized \textit{E. coli}\,-pETduet-1-\textit{PmiLAAO} was filled into the jacketed tubular reactor (figure 6). Along with the increasing flow rate, the substrate conversion decreased and the space–time yield increased while the L-phenylalanine concentration was 5 g l\(^{-1}\) (table 3). Although the conversion was close to 100\%, the lowest space–time yield of 0.2 g l h\(^{-1}\) was obtained at a residence time of 25 h (5 g l\(^{-1}\) L-phenylalanine). In order to maintain a high substrate conversion rate, the residence time (12.50 h) and flow rate (20 ml h\(^{-1}\)) was selected for further study.

Although the conversion was decreased along with the increasing substrate concentration while the L-phenylalanine concentration was 5 g l\(^{-1}\), higher productivity could be enhanced by increasing the substrate concentration. Significantly, the maximal space–time yield of 0.2 g l h\(^{-1}\) was achieved at a residence time of 12.5 h (30 g l\(^{-1}\) L-phenylalanine). A concentration of 29.66 g l\(^{-1}\) 2-oxo-3-phenylpropanoic acid with a substrate conversion rate of 99.5\% was achieved by correspondingly increasing the residence time (25 h) under fixed substrate concentration of 30 g l\(^{-1}\).

Table 3. Continuous production of 2-oxo-3-phenylpropanoic acid from L-phenylalanine. Note: the aqueous phase containing an amount of unreacted substrate (L-phenylalanine) obtained from product was used to react again.

| L-phenylalanine con. (g l\(^{-1}\)) | flow rate (ml h\(^{-1}\)) | residence time (h) | 2-oxo-3-phenylpropanoic acid con. (g l\(^{-1}\)) | conversion (%) | space–time yield (g l h\(^{-1}\)) |
|---------------------------------|-----------------|-----------------|---------------------------------|---------------|-----------------|
| 5                               | 10              | 25.00           | 4.96                            | 99.9          | 0.20            |
|                                 | 20              | 12.50           | 4.92                            | 99            | 0.39            |
|                                 | 40              | 6.25            | 4.40                            | 88.5          | 0.70            |
|                                 | 60              | 4.17            | 3.62                            | 72.8          | 0.87            |
|                                 | 80              | 3.13            | 3.23                            | 65            | 1.03            |
|                                 | 100             | 2.50            | 2.65                            | 53.4          | 1.06            |
| 10                              | 20              | 12.50           | 9.16                            | 92.2          | 0.73            |
| 20                              | 20              | 12.5            | 15.40                           | 77.5          | 1.23            |
| 30                              | 20              | 12.5            | 18.72                           | 62.8          | 1.50            |
| 40                              | 20              | 12.5            | 17.69                           | 44.5          | 1.42            |
| 50                              | 20              | 12.5            | 13.02                           | 26.2          | 1.04            |
| 60                              | 20              | 12.5            | 7.87                            | 13.2          | 0.63            |
| 30                              | 20              | 25              | 29.66                           | 99.5          | 1.19            |

Figure 6. Continuous biosynthesis of 2-oxo-3-phenylpropanoic acid from \(\alpha\)-Phe by immobilized whole-cell of \textit{E. coli}\,-pETduet-1-\textit{PmiLAAO} in packed-bed reactor. The pH of the aqueous phase containing unreacted amino acids (substrate) had been adjusted to 6.5 before recycling.
4. Discussion

In conclusion, an l-amino acid oxidase (PmiLAAO) from Proteus mirabilis has been cloned and expressed in E. coli. pETDuet-1-PmiLAAO activity improved by expression vector screening and expression condition optimization (OD$_{600}$ = 0.65, 0.45 mmol l$^{-1}$ IPTG, 20 h of induction). Afterwards, E. coli-pETduet-1-PmiLAAO was immobilized in Ca-alginate beads to simplify product separation process. Therefore, continuous production of 2-oxo-3-phenylpropanoic acid was conducted in a packed-bed reactor via immobilized E. coli-pETduet-1-PmiLAAO. Significantly, 99.5% substrate conversion rate was achieved by correspondingly increasing the residence time.

Biocatalysis has been regarded as an efficient and economical method for preparation of $\alpha$-keto acids and optically chiral amino acids under mild conditions. However, most biotransformations are short of ideal catalyst to meet the requirement of industrial application [17–19]. Enzymes with high selectivity and specific activity are an important prerequisite for industrial application. In the previous study, $\alpha$-keto acids, key intermediates for the preparation of $\alpha$-amino acids and optically active $\alpha$-hydroxyl carboxylic acids had been prepared by amino acid oxidases, however, only a few biocatalysts were available. Therefore, in order to obtain an usable and efficient $\alpha$-keto acids production and simplify product separation process, E. coli-pETduet1-PmiLAAO whole cell was used to prepare the $\alpha$-keto acids, the conversion of the 10 g l$^{-1}$ L-phenylalanine was up to 99.5% with a working time of 80 min. The catalytic performance of E. coli-pETduet1-PmiLAAO whole cell is

| 2-oxo-3-phenylpropanoic acid con. (g l$^{-1}$) | conversion (%) | space–time yield (g l h$^{-1}$) | ref. |
|----------------------------------------------|----------------|-------------------------------|------|
| chemical synthesis                           | —              | 50 (yield)                    |      |
| fermentation                                 | 1.054          | 80                            | 0.0337 | [22] |
| $\alpha$-amino acid oxidase from porcine kidney | 0.23        | 55                            | 0.038 | [24] |
| coimmobilized $\alpha$-amino acid oxidase/catalase | 3.304   | 50                            | 1.802 | [25] |
| PmiLAAO                                      | 0.75           | 75                            | 0.1   | this study |
| immobilized whole-cell                       | 1.31           | 99.8                          | 0.66  | this study |
| immobilized whole-cell + packed-bed reactor  | 29.66          | 99.5                          | 1.19  | this study |
| pure enzyme                                  | 2.6            | 86.7                          | 1.04  | [21] |
| whole-cell                                   | 3.3            | 82.5                          | 0.55  | [21] |
| LAAO-D165 K/F263 M/L336 M + substrate feeding | 22.8          | 68                            | 2.85  | [4] |

Table 4. Comparison of 2-oxo-3-phenylpropanoic acid production efficiency. Note: dash indicates that it is not stated in the text.
higher than l-amino acid oxidase (l-AAO) from Rhodococcus opacus, which has been used for conversion of L-4-chlorophenylalanine with a low activity [21]. Afterwards, E. coli-pETduet1-PmiLAAO holds the potential to be used for industrial preparation of α-keto acids. Comparing the report of Ying Hou [4], the production of 2-oxo-3-phenylpropanoic acid was increased by 23.12% with a conversion of 99.5%, however, the space–time yield was reduced 58.24% (table 4).

Although the production of α-keto acids could be increased by increasing the substrate concentration, the substrate conversion was inefficient [26]. The substrate conversion hindering the α-keto acids preparation as a key factor has been observed in this study. Substrate conversion reduced along with the increasing substrate concentration was observed in this study. In order to further increase the yield of α-keto acids, we are trying our best to eliminate the product inhibition and enhance the substrate affinity by means of protein engineering.

Data accessibility. All data are included in the article. We have conducted our experiments systematically and reported their experimental procedure clearly in the experimental section and provided all the necessary data in the results and discussion section in the main manuscript.

Authors’ contributions. Z.L., P.L. and L.W. developed the ideas. L.W., G.W. and P.L. carried out the measurements and participated in data analysis. L.W. and X.G. wrote the manuscript. All authors commented, reviewed and gave final approval for publication.

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