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Biosynthesis and genetic engineering of phenazine-1-carboxylic acid in *Pseudomonas chlororaphis* Lzh-T5

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Phenazine-1-carboxylic acid (PCA) is a biologically active substance with the ability to prevent and control crop diseases. It was certified as a pesticide by the Ministry of Agriculture of China in 2011 and was named "Shenzimycin." Lzh-T5 is a *Pseudomonas chlororaphis* strain found in the rhizosphere of tomatoes. This strain can produce only 230mg/L of PCA. We used LDA-4, which produces the phenazine synthetic intermediate trans-2,3-dihydro-3-hydroxyanthranilic acid in high amounts, as the starting strain. By restoring *phzF* and knocking out *phzO*, we achieved PCA accumulation. Moreover, PCA production was enhanced after knocking out negative regulators, enhancing the shikimate pathway, and performing fed-batch fermentation, thus resulting in the production of 10,653mg/L of PCA. It suggested that *P. chlororaphis* Lzh-T5 has the potential to become an efficiency cell factory of biologically active substances.

KEYWORDS

phenazine-1-carboxylic acid, *Pseudomonas chlororaphis*, genetic engineering, non-scar deletion, fed-batch fermentation

1. Introduction

Rice, one of the world's major crops, occupies an important position in the global food supply chain (Molina et al., 2011; Zhao et al., 2022). Worldwide, rice is often affected by serious diseases and insect pests. Although chemical pesticides, such as tricyclazole and epoxiconazole, are often used to control these pests and diseases, they cause environmental and food pollution (Padovani et al., 2006; Yan et al., 2015; Garcia-Jaramillo et al., 2016; Meng et al., 2018; Medina et al., 2021; Sefiloglu et al., 2021; Li H. et al., 2022). Therefore, there are increasing restrictions on the use of chemical pesticides. This has resulted in biological pesticides becoming a good alternative (Sharma et al., 2020; Ahmad et al., 2021). In this context, phenazine-1-carboxylic acid (PCA), a type of biological pesticide, has been registered and certified as a Chinese pesticide and is named "Shenzimycin" owing to its good efficacy against diseases (Jin et al., 2015; Karmegham et al., 2020; Li et al., 2021; Sun et al., 2021).

Currently, PCA is primarily produced by the strain *Pseudomonas aeruginosa*, despite it being an opportunistic pathogen and a major harmful bacterium present in hospitals (Du et al., 2015; Walker and Moore, 2015; Zhou et al., 2016; Petitjean et al., 2021). Compared with *P. aeruginosa*,

P. chlororaphis not only contains the phenazine-synthesizing gene cluster p*hzABCDEFG* but it is also a safe strain and is more suitable as an engineered strain for the production of phenazine derivatives (Liu et al., 2016; Li et al., 2020).

Lzh-T5 is a *P. chlororaphis* strain normally found in the rhizosphere of tomatoes (Li et al., 2018). In addition to *phzABCDEFG*, it contains the phenazine modification gene *phzO*. PCA and 2-OH-PHZ can be detected in the fermentation product of the strain; therefore, it shows the potential to efficiently produce PCA (Li et al., 2018; Liu et al., 2021).

The shikimate pathway is a key metabolism pathway that is widely present in organisms, including *P. chlororaphis* (Liu et al., 2016; Li et al., 2020). A series of substances, such as coenzyme Q, aromatic amino acids, and phenazines, can be produced through the shikimate pathway (Talapatra and Talapatra, 2005; Liu et al., 2016). Trans-2,3-dihydro-3-hydroxyanthranilic acid (DHHA) is also an accumulation product of the shikimate pathway truncated in *P. chlororaphis*. Our previous study revealed that *P. chlororaphis* Lzh-T5 can produce DHHA (Liu et al., 2021).

In this study, we used a derivative strain of *P. chlororaphis* Lzh-T5, LDA-4, which possess high DHHA production capacity, as the starting strain and achieved PCA accumulation as a single product through genetic engineering. Gene expression and feed-bath strategies were used to increase the PCA production by the strain, resulting in an engineered strain that produced 10.65 g/L of PCA.

2. Materials and methods

2.1. Strains and bacterial culture

Different *Escherichia coli* and *P. chlororaphis* strains were used in this study (Table 1). *E. coli* is primarily used for gene knockout vector construction and biparental hybridization, whereas *P. chlororaphis* is primarily used for the construction of engineered strains producing phenazine derivatives. *E. coli* was cultured in LB medium at 37°C, and *P. chlororaphis* was cultured in King's B medium at 28°C. The composition of these media has been described in our previous study (Li et al., 2020). Ampicillin and kanamycin sulfate were used as antibiotics at concentrations of 100 and 50 μg/ml, respectively.

2.2. Genetic engineering

The plasmids and primers used in this study are presented in Table 1 and Supplementary Table S1, respectively. *phzF* was restored into the genome of the LDA-4 strain through gene insertion. First, the primers for *phzF* insertion were designed according to the genome sequence of *P. chlororaphis* Lzh-T5 (Supplementary Table S1). Subsequently, *phzF* and its upstream and downstream fragment phzFUD was amplified through PCR using phzF-F1/phzF-R2 as primers and the Lzh-T5 genome as the template.

The restriction enzymes EcoRI and XbaI were used to digest the phzFUD fragment and the pK18mobSacB plasmid, respectively. After recovery through agarose gel electrophoresis, the recombinant plasmid pK18-phzFUD was obtained using T4 ligase. This plasmid was transformed into *E. coli* S17-1 (λ), resulting in the recombinant strains *E. coli* S17-1 (λ) and *P. chlororaphis* LDA-4; these strains were cultured respectively, and the pK18-phzFUD plasmid was introduced into pseudo-LDA-4 by biparental hybridization. The plasmid and the bacterial genome were double-exchanged on a King's B medium plate containing 10% sucrose, and the *phzF*-restored strain was obtained through PCR.

Genes such as *rsmE*, *parS*, and *lon* have been knocked out from the genome of *P. chlororaphis* either individually or together, as described in our previous studies (Liu et al., 2016; Li et al., 2020).

2.3. Quantitative RT-PCR

Quantitative RT-PCR was performed to detect the transcriptional changes of related genes in different *P. chlororaphis* strains as described in our previous study (Liu et al., 2021). *rpoD*, a housekeeping gene of *P. chlororaphis*, was selected as the internal reference gene. The mRNA fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Fed-batch fermentation of strains

To maximize the efficacy of the engineered strains, we added the high-yielding strains LDPCA-6 to a 5-L fermenter to perform fed-batch fermentation with specific fermentation parameters according to our previous research (Li et al., 2020).

2.5. Separation, preparation, and detection of phenazine samples

PCA must be extracted from the fermentation broth and then detected and quantified by high-performance liquid chromatography (HPLC). We first extracted PCA from the fermentation broth using ethyl acetate and subjected it to reversed-phase C18 column (Agilent Technologies, 5 μm, 4.6*250 mm). Both 2-OH-PHZ and PCN are phenazine derivatives and exhibit similar material properties; the specific extraction and detection steps of these samples are described in our previous studies (Liu et al., 2016; Li et al., 2020).

3. Results

3.1. Restoration of *phzF* in LDA-4 resulted in the accumulation of phenazines

Lzh-T5 is a *P. chlororaphis* strain selected from the rhizosphere of tomato. It is known that its genome contains the key *phzABCDEFG* gene cluster for the synthesis of phenazine derivatives. The fermentation broth of this strain in KB would turn to orange (which is the color of 2-hydroxyphenazine), with the potential to produce phenazine derivatives. In our previous research, we blocked the biosynthesis of phenazine by knocking out *phzF*, which led to the accumulation of DHHA, an important chemical intermediate. We strengthened the synthetic pathway of DHHA via genetic engineering and obtained the engineered strain LDA-4. The yield of DHHA using LDA-4 reached 5.52 g/L in the shake flask (Liu et al., 2021). In the present study, we used LDA-4 to obtain high yield of PCA. DHHA accumulation occurred in LDA-4 owing to the knockout of *phzF* in the phenazine synthesis pathway (Figure 1). We restored the function of *phzF* in the

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TABLE 1 Strains and plasmids used in this study.

Strains and plasmids	Relevant gene type	Reference/source
Strains		
Escherichia coli DH5α	*E. coli* F$^-$Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_k^- m_k^-) *phoA supE44 thi*$^{-1}$ *gyrA96* relA1	Lab stock
Escherichia coli S17-1(λpir)	res$^-$ pro mod$^+$ integrated copy of RP4, mob$^+$, used for incorporating constructs into *P. chlororaphis*	Lab stock
Pseudomonas chlororaphis Lzh-T5	*P. chlororaphis* Lzh-T5 wild-type strain	Lab stock
Pseudomonas chlororaphis LDA-4	*phzF, pykF, psrA* and *rpeA* in-frame deletion mutant of Lzh-T5	Lab stock
Pseudomonas chlororaphis LDA-4-phzF	*phzF* in-frame insertion mutant of *P. chlororaphis* LDA-4	This study
Pseudomonas chlororaphis LDPCA-1	*phzO* in-frame deletion mutant of *P. chlororaphis* LDA-4-phzF	This study
Pseudomonas chlororaphis LDPCA-2	*rsmE* in-frame deletion mutant of *P. chlororaphis* LDPCA-1	This study
Pseudomonas chlororaphis LDPCA-3	*parS* in-frame deletion mutant of *P. chlororaphis* LDPCA-2	This study
Pseudomonas chlororaphis LDPCA-4	*lon* in-frame deletion mutant of *P. chlororaphis* LDPCA-3	This study
Pseudomonas chlororaphis LDPCA-5	*ppsA* in-frame insertion mutant of *P. chlororaphis* LDPCA-4	This study
Pseudomonas chlororaphis LDPCA-6	*tktA* in-frame insertion mutant of *P. chlororaphis* LDPCA-5	This study
Plasmids		
pEASY-Blunt	Blunt vector for gene coloning, Ap^r, Kan^r	Lab stock
pEASY-Blunt-tktA	PCR cloning amplification vector for gene *tktA*	This study
pEASY-Blunt-ppsA	PCR cloning amplification vector for gene *ppsA*	This study
pK18mobsacB	Broad-host-range gene replacement vector, *sacB*, Kan^r	Lab stock
pK18-phzF	pK18mobsacB containing *phzF* flanking region	This study
pK18-phzO	pK18mobsacB containing *phzO* flanking region	This study
pK18-rsmE	pK18mobsacB containing *rsmE* flanking region	This study
pK18-parS	pK18mobsacB containing *parS* flanking region	This study
pK18-lon	pK18mobsacB containing *lon* flanking region	This study
pK18-ppsA	pK18mobsacB containing *ppsA* flanking region	This study
pK18-tktA	pK18mobsacB containing *tktA* flanking region	This study

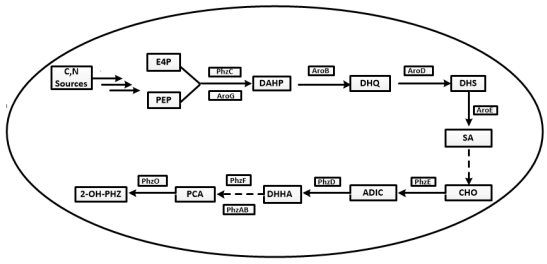

FIGURE 1
The synthetic pathway of phenazine-1-carboxylic acid and its derivatives in *Pseudomonas chlororaphis*. E4P, erythrose 4-phosphate; PEP, phosphoenolpyruvic acid; DAHP, 3-deoxy-darabinoheptulosonate-7-phosphate; DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; SA, shikimic acid; CHO, chorismate; ADIC, 2-amino-4-deoxy branched acid; DHHA, Trans-2,3-dihydro-3-hydroxyanthranilic acid; PCA, Phenazine-1-carboxylic acid; 2-OH-PHZ, 2-hydroxyphenazine.

genome of LDA-4 through genetic engineering to obtain the strain LDA-4-phzF. To confirm whether *phzF* could indeed play a role, we performed RT-PCR to verify its expression level in different strains. Our results revealed increased expression of *phzF* in LDA-4-phzF compared with that in LDA-4 (Figure 2A). At 28°C, the colony of the strain turned red (Figure 3). HPLC revealed three phenazine derivative peaks in LDA-4-phzF (Figure 4). Compared with the wild-type strain Lzh-T5, the yield of phenazines was increased to 3560.4 mg/L in LDA-4-phzF (Figures 5, 6).

3.2. Knockout of *phzO* resulted in PCA accumulation in the strain

After genome sequencing, the phenazine-related gene *phzO* was detected in *P. chlororaphis* Lzh-T5 (Li et al., 2018). The PhzO protein encoded by *phzO* is a phenazine-modified protein that catalyzes PCA conversion into 2-OH-PCA, which then spontaneously converts into 2-OH-PHZ (Chen et al., 2014). Therefore, both Lzh-T5 and its derivative strain LDA-4-phzF produce three phenazine derivatives, PCA, 2-OH-PCA, and 2-OH-PHZ (Liu et al., 2021). In this study, we knocked out *phzO* from the genome of the LDA-4-phzF strain using traceless knockout technology to obtain the LDPCA-1 strain. The metabolites of LDPCA-1 were extracted and detected using HPLC. Only PCA was accumulated in LDPCA-1 (Figure 4), with a yield of 4,863 mg/L, which was 20.9 times higher than that in the wild-type strain Lzh-T5 (Figures 5A, 6).

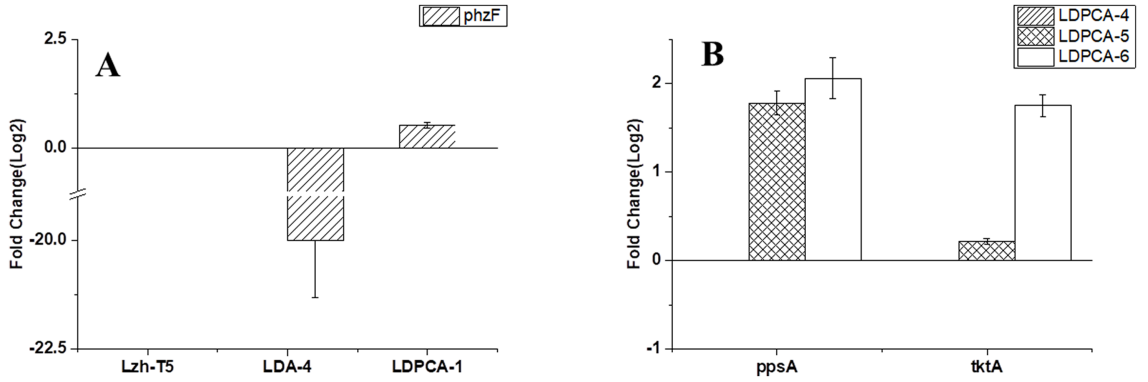

FIGURE 2
Transcriptional validation of different genes using Quantitative RT-PCR. **(A)** Transcriptional validation of phzF in different strains. **(B)** Transcriptional validation of tktA and ppsA in different strains. The data represent the means ± SD for three independent cultures.

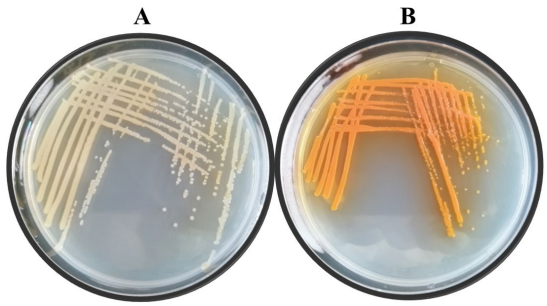

FIGURE 3
Colony morphology of different strains. **(A)** *Pseudomonas chlororaphis* LDA-4. **(B)** *Pseudomonas chlororaphis* LDA-4-phzF.

3.3. Enhancement of PCA production by knockout of negative regulatory factors

To enhance the production of PCA, several widely occurring negative regulator genes in *P. chlororaphis* were selected for knockout. We knocked out the negative regulatory factor *rsmE* from the genome of LDPCA-1 to obtain LDPCA-2 strain. HPLC revealed that PCA production by this strain increased to 5362.1 mg/L (Figures 5A, 6). Knockout of *parS*, another negative regulatory factor in the genome of LDPCA-2, resulted in the strain LDPCA-3, which presented with an increase in PCA yield to 6042.3 mg/L (Figures 5A, 6). The engineered strain LDPCA-4 was obtained by knocking out *lon*, and the PCA yield of this strain increased to 6562.3 mg/L (Figures 5A, 6).

3.4. Increasing PCA production by enhancing the shikimate pathway

Phenazine derivatives in *Pseudomonas* spp. are synthesized through the shikimate pathway. Enhancing this phenazine synthesis pathway can help promote PCA production. The shikimate pathway is an important metabolic pathway in *Pseudomonas* spp., which can not only synthesize phenazines but is also the lead pathway for coenzyme Q and aromatic amino acid synthesis (Talapatra and Talapatra, 2005; Liu et al., 2016). Phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) are the direct synthetic precursors of the shikimate pathway (Talapatra and Talapatra, 2005). Enhancing PEP and E4P supply can enhance the shikimate pathway (Rodriguez et al., 2014). Therefore, we selected *ppsA* and *tktA*, genes that can improve E4P and PEP production (Hu et al., 2017; Li et al., 2020). Accordingly, *ppsA* was integrated at the *lon* position of LDPCA-4 via homologous integration to obtain the recombinant strain LDPCA-5. Next, *tktA* was integrated at the *parS* position of the LDPCA-5 strain to obtain the recombinant strain LDPCA-6. RT-PCR revealed that the expression levels of *tktA* and *ppsA* in LDPCA-6 and LDPCA-5 were higher than those in LDPCA-4 (Figure 2B), suggesting that the genes integrated into the genome are transcribed and play a role in enhancing the shikimate pathway. HPLC detection revealed that the PCA production of LDPCA-5 and LDPCA-6 was increased, reaching 7091.4 and 7963.4 mg/L, respectively (Figures 5B, 6).

3.5. Enhancing PCA production by fed-batch fermentation

Pseudomonas spp. are aerobic bacteria; therefore, shake flask fermentation often limits the maximum efficiency of bacteria owing to insufficient oxygen supply. Moreover, after a period of growth, the efficiency of bacteria is limited due to lack of nutrients. In this study, we used the fed-batch method to ferment the engineered strain to improve PCA production by the bacterial strain. The fed-batch experiments were conducted in a 5-L fermenter after activating LDPCA-6 in a shake flask. PCA was extracted from the fermentation broth during the fermentation process. HPLC detection revealed that the bacterial growth reached a stable stage at 48 h after inoculation, and PCA production continued to increase for 60 h. The maximum yield was 10,653.1 mg/L, and the DCW yield was 2286.6 mg/g (Figures 6, 7).

4. Discussion

Phenazine derivatives are a class of biologically active substances that can be naturally produced by the *Pseudomonas* and

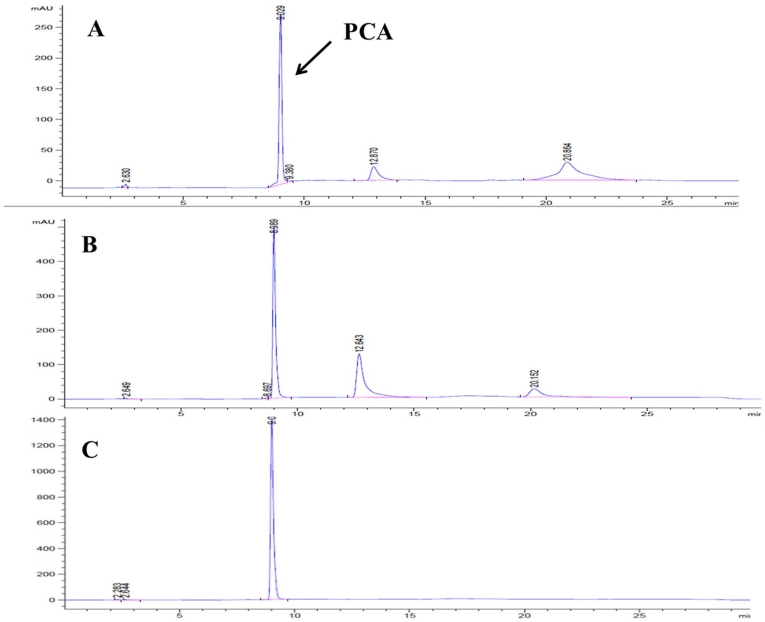

FIGURE 4
HPLC detection of fermentation broth of different strains. **(A)** *Pseudomonas chlororaphis* Lzh-T5. **(B)** *Pseudomonas chlororaphis* LDPCA-1. **(C)** *Pseudomonas chlororaphis* LDPCA-1.

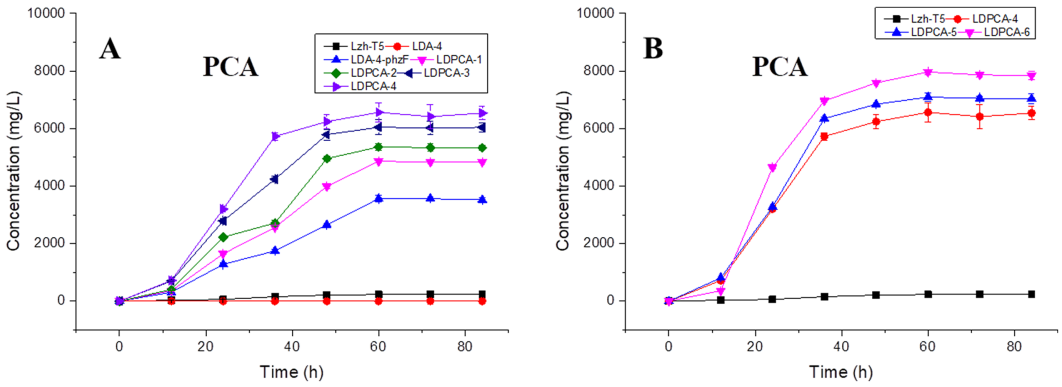

FIGURE 5
Effects of genetic modifications on PCA production. **(A)** PCA production of non-scar deletion strains. **(B)** PCA production of gene over-expression strains. The data represent the means ± SD for three independent cultures.

Streptomyces spp. (Chin et al., 2000; Laursen and Nielsen, 2004; Mavrodi et al., 2006; Lugtenberg and Kamilova, 2009; Bilal et al., 2017). In this study, we focused on PCA production by *Pseudomonas* spp.

The synthesis steps of PCA by *Pseudomonas* are as follows. First PEP and E4P are converted to shikimate via the shikimate pathway. Then, shikimate is converted to PCA via enzymes encoded by the conserved phenazine synthesis gene cluster *phzABCDEFG* (Figure 1).

Different phenazine modification genes are present in *Pseudomonas* spp. that convert PCA into different phenazine derivatives, such as *phzO*, which converts PCA into 2-hydroxyphenazine; *phzH*, which converts PCA into phenazine-1-carboxamide; *phzS*, which converts PCA into 1-hydroxyphenazine; and *phzS* and *phzM*, which act simultaneously to convert PCA into pyocyanin (Chin et al., 2001; Mavrodi et al., 2001; Parsons et al., 2007; Greenhagen et al., 2008; Du et al., 2013).

Lzh-T5 is a strain of *P. chlororaphis* commonly found in the rhizosphere of tomatoes in Dezhou, China. Using genome sequencing, this strain was found to contain the typical phenazine synthesis gene cluster *phzABCDEFG* and a phenazine modification gene *phzO*. After fermentation, both PCA 2-OH-PCA and 2-OH-PHZ were present in the fermentation broth. We knocked out *phzO* using a traceless knockout method, which cut off the conversion of PCA into 2-OH-PHZ. After fermentation, HPLC detection revealed that the fermentation broth could accumulate PCA alone (Figure 4).

The wild strain *Pseudomonas* Lzh-T5 can produce PCA, although the yield is very low at only 220 mg/L. In our preliminary work, we used Lzh-T5 to produce DHHA through genetic engineering and

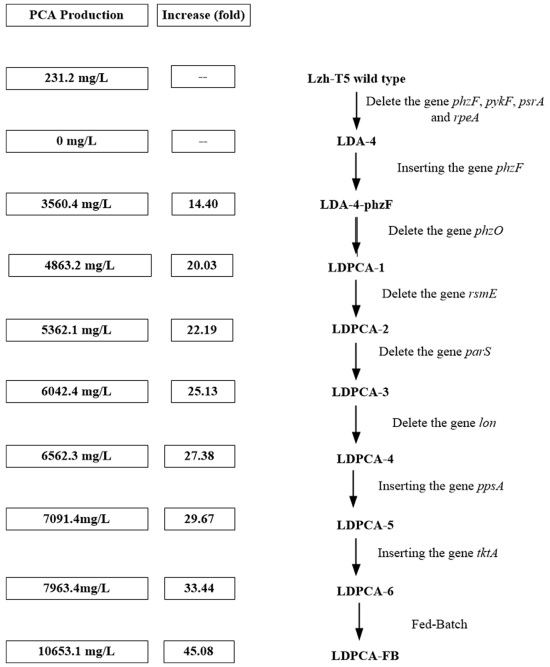

FIGURE 6
A summary of PCA production improve via genetic engineering operations in *Pseudomonas chlororaphis*.

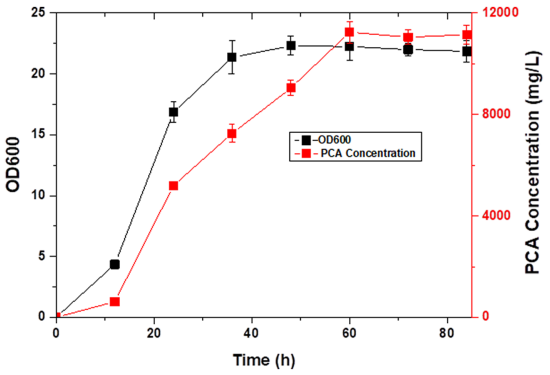

FIGURE 7
PCA production in LDPCA-6 during fed-batch fermentation.

obtained a yield of 11 g/L (Liu et al., 2021). DHHA is the synthetic precursor of phenazine derivatives in *Pseudomonas* and was synthesized by *Pseudomonas* after *phzF* knockout (Liu et al., 2021). Because of the metabolic modification of the DHHA-producing strain in this study, its metabolic flux in the shikimate pathway was stronger than that in the wild-type strain Lzh-T5. In this study, we selected the high DHHA-producing strain LDA-4 to produce PCA by restoring *phzF*.

Two-component signal transduction (TCST) systems are a class of regulatory systems that are widely present in bacteria and help bacteria adapt to changes in the surrounding environment. According to previous reports, *Pseudomonas* spp. contains a large number of TCST systems, such as the GacS/GacA and rpeA/rpeB systems (Bejerano-Sagie and Xavier, 2007; Peng et al., 2018). The GacS/GacA system is a TCST system that has been previously identified in *Pseudomonas* spp. Studies have reported that the GacS/GacA system affects the production of phenazines through intracellular small RNAs and negatively regulates the production of phenazines through *rsmE* (Liu et al., 2016; Li et al., 2020). We found *rsmE* to be present in the Lzh-T5 genome, and, after knocking out *rsmE* in the LDPCA-1 strain genome, PCA production increased from 4863.2 mg/L to 5362.1 mg/L. Compared with the GacS/GacA system, the parS/parR system has recently been discovered. Studies have also reported that the parS/parR system can regulate phenazine production in *Pseudomonas* (Liu et al., 2016; Peng et al., 2018). We obtained the strain LDPCA-3 by knocking out *parS* in the LDPCA-2 genome; moreover, HPLC revealed increased PCA production (Figures 5, 6). In addition to the TCST systems that can affect the production of phenazines, some enzymes can indirectly regulate phenazine synthesis through the TCST system. Reportedly, the protease LON has been reported to be a negative regulatory factor of phenazine derivatives (Laskowska et al., 1996; Whistler et al., 2000; Wang et al., 2013; Takeuchi et al., 2014; Liu et al., 2016). And LON protease affects the production of 2-hydroxyphenazine by affecting the stability of GacA protein (Liu et al., 2016; Li et al., 2020). After detection, LON was found in *P. chlororaphis* Lzh-T5. Our results indicate that LON negatively regulates PCA production (Figures 5, 6).

In *Pseudomonas* spp., the shikimate pathway is the leading pathway for phenazine synthesis. This pathway uses PEP and E4P as direct synthetic substrates (Talapatra and Talapatra, 2005). Previous research has shown that the expression of PEP synthase (encoded by *ppsA*) and transketolase (encoded by *tktA*) can increase the PEP and E4P pools in *Pseudomonas* cells, enhancing the shikimate pathway (Liu et al., 2016). In some previous studies, genes such as *ppsA* and *tktA* were overexpressed to promote the synthesis of phenazines, achieving good yields (Liu et al., 2016; Hu et al., 2017; Li et al., 2020). However, these studies generally used plasmids as vectors for overexpression. Plasmids are generally confirmed by antibiotics to ensure their stable existence in bacteria and are easily lost in the absence of antibiotics. Furthermore, when multiple genes are expressed simultaneously, the sequence order will affect the expression effect of different genes (Juminaga et al., 2012; Liu et al., 2016). These problems are not encountered when the overexpressed gene is introduced into the genome of the host bacterium. In this study, we used the principle of homologous recombination to introduce *ppsA* and *tktA* into the genome of *Pseudomonas* for over expression. The results of RT-PCR and PCA detection showed that the introduced *ppsA* and *tktA* promoted the shikimate pathway for PCA production (Figure 2).

PCA is a secondary metabolite of *Pseudomonas* spp. and needs to be produced during the growth of *Pseudomonas* spp. (Li S. et al., 2022; Olyaei and Sadeghpour, 2022). The growth and development of bacteria can be generally divided into the lag, logarithmic growth, and extinction phases. PCA production is more favorable with *Pseudomonas* in a stable state (Liu et al., 2016; Yue et al., 2018). Compared with batch fermentation, the fed-batch fermentation medium has rich ingredients, stable oxygen supply, and relatively constant pH value, which are more conducive to the rapid reproduction of microorganisms and the production of metabolites (Li et al., 2020). Therefore, the fed-batch process is the preferred fermentation mode. Moreover, PCA produced in this study is a secondary metabolite of *Pseudomonas*, and a large amount of

accumulation will lead to feedback inhibition in the bacteria. The problems of substrate inhibition and catabolite inhibition can be overcome in the fed-batch process by the continuous addition of new medium. This significantly increases the yield of the target product. In this study, we added the PCA high-yielding strain LDPCA-6 to a 5-L fermenter for fed-batch fermentation. The HPLC results showed that the bacterial growth reached a stable stage at 60 h after inoculation, and the production of PCA continued to increase for 72 h. The maximum yield was 10,653.1 mg/L, which was 33.8% higher than that using batch culture.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

LL and KL conceived, designed the experiments, and drafted the manuscript. KL, ZL, XL, YX, and YC performed the experiments. KL, RW, LL, and PL analyzed the data. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1186052/full#supplementary-material

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