Ferulic Acid production by metabolically engineered Escherichia coli

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Abstract

Ferulic acid (*p*-hydroxy-3-methoxycinnamic acid, FA) is a natural active substance present in plant cell walls, with antioxidant, anticancer, antithrombotic and other properties; it is widely used in medicine, food, and cosmetics areas. Production of FA by eco-friendly bioprocess is of great potential. In this study, FA was biosynthesized by metabolically engineered *Escherichia coli*. As the first step, the genes *tal* (encoding Tyrosine ammonia-lyase, *Rs*TAL) from *Rhodobacter sphaeroides*, *sam5* (encoding *p* - coumarate 3-hydroxylase, *Se*SAM5) from *Saccharothrix espanaensis* and *comt* (encoding Caffeic acid O-methytransferase, *Ta*CM) from *Triticum aestivum* were cloned in an operon on the pET plasmid backbone, *E. coli* strain containing this construction was proved to produce FA from L-tyrosine successfully, and confirmed the function of *Ta*CM as Caffeic acid O-methytransferase. Fermentation results revealed JM109(DE3) as more suitable host cell for FA production than BL21(DE3). After that the genes expression strength of FA pathway were optimized by tuning of promoter strength (T7 promoter or T5 promoter) and copy number (pBR322 ori or p15a ori), and the combination p15a-T5 works best. To further improve FA production, *E.coli* native *pntAB*, encoding pyridine nucleotide transhydrogenase, was selected from five NADPH regeneration genes to supplement redox cofactor NADPH for converting *p*-coumaric acid into caffeic acid in FA biosynthesis process. Sequentially, to further convert caffeic acid into FA, a non-native methionine kinase (MetK from *Streptomyces spectabilis*) was also over expressed. Based on the flask fermentation data which shows that the engineered *E. coli* strain produced 212 mg/L of FA with 11.8 mg/L caffeic acid residue, it could be concluded that it is the highest yield of FA achieved by *E.coli* K-12 strains reported to the best of our knowledge.

Introduction

FA is a ubiquitous phenolic acid naturally presents in plant cell walls, mainly cross linked with polysaccharides and lignin (Harris & Trethewey, 2009). It is the main effective component of *Angelica sinensis*, *chuanxiong*, *Ferula foetida* and other traditional Chinese medicine (Liang et al., 2018). FA is an antioxidant and act as a free radical scavenger for the plants. Its physiological functions like antimicrobial, anti-inflammatory, anticancer, anti-thrombotic and so on makes FA widely used in food, cosmetic and medicine areas (Sgarbossa et al., 2015; Wen & Ushio, 2017). Extracting FA from alkaline hydrolysis or ferulic acid esterase treated plant materials rich in FA, such as *Angelica sinensis, ferula, Ligusticum chuanxiong Hort., wheat* bran *and rice* bran, is the main source of medicinal or commercial FA, but it is hard to do the membrane separation in the following preparation and purification process and it costs high to treat the industrial waste.

Plant tissue culture is an effective way with higher yield than extraction directly from plant material, but pollution and browning are easy to occur in the process, so there is no industrial scale-up yet. Chemical synthesis of FA from vanillin by Wittig-Horner reaction or Knoevenagel reaction have short producing cycle, low cost, and large output, but the products are mixture of *trans*- and *cis*- ferulic acids, that makes it hard to carry out separation too.

Microbial approaches provide a promising alternative to chemical synthesis and extraction from plant sources. Products obtained through biotechnological procedures from natural substrates were considered as natural (Serra et al., 2005). With the approval of such products by the FDA and European legislation, many studies are focused on approaches based on biotechnological methods for production of flavors, fragrances, and pharmaceutical products (Fowler & Koffas, 2009; Goris et al., 2020; Luziatelli et al., 2019). There are many reports on FA related natural products like *p*-Coumaric acid, Caffeic acid, Vanillin, and curcuminoid heterologous biosynthesis (Braga & Faria, 2020; Jendresen et al., 2015; Rodrigues et al., 2015b), but very few researches on FA biosynthesis were reported in articles and patents. Overhage et al reported biotransformation of eugenol to FA by *vaoA* gene, encoding vanillyl alcohol oxidase, together with the genes *calA* and *calB*, encoding coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase, respectively. Resting cells f the corresponding recombinant strain *E. coli* XL1-Blue (pSK*vao*mP*calA*m*calB*) converted eugenol to FA (Overhage et al., 2003), the follow-up work mainly focuses on vanillin (Braga & Faria, 2020; Fleige et al., 2016; Ni et al., 2015). Choi et al reported FA production (7.1 ± 1.3 mg/L) by the sequential co-expression of the enzymes encoded by the Tyrosine ammonia-lyase and 4 -coumarate 3-hydroxylase genes (*tal*, *sam5*) from *S. espanaensis* and an O-methyltransferase gene (*comt*) from *Arabidopsis thaliana*. Tyrosine ammonia-lyase catalyzes nonoxidative deamination of the primary amino acid tyrosine to *p*-coumaric acid, which is converted into caffeic acid by a hydroxylation step at the 3-position of the benzyl ring by the *p*-coumarate 3-hydroxylase. Finally, FA is biosynthesized from caffeic acid by the enzyme caffeine O-methyltransferase. Under the action of these enzymes, tyrosine is transformed into ferulic acid sequentially (Choi et al., 2011). Kang et al pushed forward this work, they constructed a chromosomal engineered tyrosine over-producing *E.coli* C41(DE3) strain to exploring the potential of FA production from simple carbon sources, combined with *tal* gene codon optimization, the titer of FA increased greatly to 196 mg/L (Kang et al., 2012).

Besides the supplement of tyrosine, there are other aspects like pathway protein expression level tuning, redox cofactor and precursor supply in the heterologous biosynthesis of FA in *E.coli*.

The high copy number is generally desired for maximum gene expression; however, the metabolic burden effects that usually result from multiple plasmid copies could prove to be detrimental for maximum productivity in certain metabolic engineering applications. Similarly, choosing suitable promoter strength is also an effective way to obtain high yields. Using various promoters and gene copy-numbers combinations to modulate diverse expression level of upstream and downstream pathways of taxadiene synthesis succeeded in increasing the titer of taxadiene approximately 15,000-fold in an engineered *E.coli* strain (Ajikumar et al., 2010; Jones et al., 2000; Ni et al., 2015). It is convenient to tune the expression level of the pathway genes by different replicon origin and promoter strength combinations.

In the FA biosynthetic pathway, *p* -coumarate 3-hydroxylase, SAM5, catalyzes *p*-Coumaric acid into Caffeic acid and consumes NADPH (Nicotinamide adenine dinucleotide phosphate) at the same time. NADPH regeneration rate is crucial to maximizing reactions catalyzed by such enzymes, which is of enormous importance for the synthesis of industrially valuable compounds such as carotenoids, polymers, antibiotics, and so on. Quantities of previous studies showed that increasing NADPH

regeneration rates could increase both pathway productivities and product yields. Based on rationally engineered heterologous Entner-Doudoroff pathway that could increase the NADPH regeneration rate in *E. coli* MG1655, the terpenoid titer of carotenoid biosynthesis pathway was increased by 97% (Martinez et al., 2008; Ng et al., 2015). The pentose phosphate pathway (PPP), the tricarboxylic acid (TCA) cycle and the transhydrogenase system constitute the main sources of NADPH (Sauer et al., 2004). The NADPH/ $NADP^+$ ratio in *E.coli* is enhanced in varying degrees by over-expressing several NADPH regeneration genes. During fucosyllactoses synthesis, NADPH is involved in key substrate - guanosine 5'-diphosphate (GDP)-L-fucose formation. Intracellular redox regeneration pathways including such genes were engineered to further enhance the production of 2- Fucosyllactoses, and 3- Fucosyllactoses (Huang et al., 2017). These genes are promising to be effective in FA heterologous biosynthetic strain.

Meanwhile, the reaction catalysis by the caffeic acid *O*-methyltransferase, COMT, consumes *S*-Adenosyl-l-methionine (SAM), which is a ubiquitous intracellular methyl donor involved in a great number of reactions (Han et al., 2016). Increasing intracellular SAM level is proposed to be a generally useful tool for improving the production of natural products (Wang et al., 2007). Methionine adenosyltransferase /Sadenosylmethionine synthetase catalyzes the formation of SAM from L-methionine and ATP, and it has been found in *E.coli*, baker's yeast, *Mycobacterium smegmatis*, rat liver, bovine brain, and *Saccharomyces cerevisiae* (Han et al., 2016). Methionine adenosyltransferase is also worth trying in FA heterologous biosynthetic strain.

In this study, we engineered *E.coli* for efficient FA production. Firstly, we constructed *tal-sam5-comt* cassette using enzymes from three different species. The copy number and promoter strength of FA biosynthetic pathway gene expression cassette was tuned to set heterologous gene expression at proper level. To further increase the yield by supplying redox cofactor NADPH and precursor SAM, five NADPH regeneration enzymes and one SAM formation enzyme were tested in this system. Finally, the flask fermentation production of FA increases to a high titer.

Materials And Methods

Bacterial strains and plasmids and chemicals

E. coli DH10B was used for plasmid construction. *E. coli* JM109(DE3) was used for the expression of heterologous genes and the production of FA. pET vectors and pCL1920-T7 were used to express multiple genes. Authentic chemical FA standards, *p*-Coumaric acid and caffeic acid were purchased from Yuanye (Shanghai). All restriction enzymes and DNA ligase were purchased from New England Biolabs (Shanghai). One-step-directed cloning kit was purchased from Novoprotein (Shanghai). The polymerase chain reaction (PCR) primers are synthesized from Sangon Biotech (Shanghai) company (Table S1). The *E. coli* strains and plasmids used in this study are listed in Table 1.

Biosynthetic pathway construction and assembly

Restriction enzyme digestions, transformations, PCR, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and other molecular biology techniques were carried out by the standard method. The primers used are listed in Table S1. Cells were grown in Luria-Bertani (LB) medium containing appropriate antibiotics at 37 °C. The working concentration of antibiotics was ampicillin (100 mg/L), apramycin (100 mg/L), Kanamycin (50 mg/L), and streptomycin (50 mg/L).

To construct the plasmid harboring FA biosynthetic pathway, pET21d was used as a backbone plasmid. Tyrosine ammonia-lyase, encoded by *tal* from *Rhodobacter sphaeroides* (Genbank No. CP033447.1) was in lab stocked plasmid pET21d-*tal*. *p*-Coumaric acid 3-hydroxylase, encoded by *sam5* from *Saccharothrix espanaensis* (Genbank No. HE804045.1), and caffeic acid *O*-methyltransferase, encoded by *comt* from *Triticum aestivum* (Genbank No. EF413031.1), were codon optimized and synthesized by Genscript Company and cloned into pET21a as pET21a-*sam5* and pET21a-*comt*. Nucleotide sequences of genes used in this study are given in Table S2. The whole pathway was sequentially assembled by the strategy like *BioBrick* Assembly method™ using a pair of isocaudarner (*Xba*I/*Spe*I). The *Xba*I/*Hin*dIII excised DNA fragment from pET21a-*sam5* was inserted into *Spe*I/*Hin*dIII sites of pET21d-tal to obtain plasmid pET21d-*tal-sam5*. Then the *Xba*I/*Hin*dIII excised DNA fragment from pET21a-*comt* was sequentially inserted into *Spe*I/*Hin*dIII sites of pET21d-*tal-sam5* to generate plasmid pET21d-*tal-sam5-comt*.

To construct plasmid pHJ345 (pBR322 ori, Apr resistance, T5 promoter) with backbone pBR322-T5, we substituted T7 promoter in plasmid pJF25 (Wang et al., 2013) (pBR322 ori, Apr resistance, T7 promoter) with T5 promoter sequence from pQE30 (colE1 ori, Amp resistance, T5 promoter) by infusion method using two PCR products: using pJF25 as template and primers pro-VF/pro-VR, to obtain a 5.3 kb DNA fragment as vector fragment, using pQE30 as template and primers pro-T5-in-F/pro-T5-in-R, to obtain a 110 bp DNA fragment as insert fragment.

Similarly, to construct plasmid pHJ352(p15a ori, Apr resistance, T7 promoter) with backbone p15a-T7, we substituted pBR322 ori in plasmid pJF25(pBR322 ori, Apr resistance, T7 promoter) with p15a ori sequence from pACYC184 (p15a ori) by infusion method using two PCR products: using pJF25 as template and primers ori-VF/ori-VR, to obtain a 3.3 kb DNA fragment as vector fragment, and using pACYC184 as template and primers ori-p15a-inF/ori-p15a-inR, to obtain a 913bp DNA fragment as insert fragment.

Plasmid pHJ354(p15a ori, Apr resistance, T5 promoter) with backbone p15a-T7 was constructed by infusion method using two PCR products: using pHJ352 as template and primers pro-VF/pro-VR, to obtain a 4.3 kb DNA fragment as vector fragment, and using pQE30 as template and primers ori-p15a-inF/ori-p15a-inR, obtain a 110bp DNA fragment as insert fragment.

FA biosynthesis pathways with different backbones, pBR322-T7-*tal-sam5-comt*, pBR322-T5-*tal-sam5-comt*, p15a-T7-*tal-sam5-comt* and p15a-*T5-tal-sam5-comt* were constructed by T4 ligase using *Xba*I/*Hin*dIII excised DNA fragment from pET21d-*tal-sam5-comt* as insert, and *Xba*I/*Hin*dIII excised DNA fragments from pJF25, pHJ345, pHJ352, pHJ354 as vector fragments respectively.

We modified low copy plasmid pCL1920 (G.Lerner & Inouye, 1990) to get pCL1920-T7 to co-express NADPH regeneration system with FA biosynthetic pathway. pCL1920-T7 is constructed by infusion method using two PCR products: using pCL1920 as template and primers pCL1920VF/pCL1920VR to obtain a 4.0 kb DNA fragment as vector fragment, and using pET28a as template and primers T7 operator F/ T7 operator R to obtain a 1.9 kb DNA fragment as insert fragment.

The genes *zwf, gnd, icd and pntAB* were PCR amplified from the genomic DNA of *E.coli* MG1655 and cloned into the pET vectors. After that the restriction enzyme digested fragments were inserted into corresponding restriction enzyme digested pCL1920-T7 fragments to generate pCL1920-T7-*zwf*, pCL1920-T7-*gnd*, pCL1920-T7-*icd*, pCL1920-T7-*pntAB*, respectively. Gene *gapN* from *Clostridium. acetobutylicum* ATCC 824 (Genbank No. AE001437.1) was directly cloned into pCL1920-T7 to generate pCL1920-T7-*gapN* using infusion method. Gene *metK* from *Streptomyces spectabilis* (Genbank No. WP_144002349.1) was codon optimized and synthesized by Genscript Company and cloned into pET21a, were constructed by T4 ligase using *Xba*I/*Hin*dIII excised DNA fragment from pET21a-*metK* as insert, and *Xba*I/*Hin*dIII excised DNA fragment from pCL1920-T7 as vector fragments, generating pCL1920-T7-*metK*. All constructs were further verified by Sanger sequencing (Sangon Biotech).

Flask fermentation

The clones transformed with related plasmid combinations were picked into LB medium at 37 °C, 250 rpm for 12 h as flask fermentation seeds. 500μL of overnight LB culture was inoculated into 10 mL of M9Y medium supplemented with 2% (*v/v*) glycerol and 1 g/L L-tyrosine, and appropriate antibiotics. M9Y medium is modified M9 minimal salt medium containing 1 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 2 mmol $MgSO_4$, 0.1 mmol $CaCl_2$, 0.5 g yeast extract and 1 mL Trace elements (0.03 g/L H_3BO_3, 1 g/L thiamine, 0.94 g/L $ZnCl_2$, 0.5 g/L $CoCl_2$, 0.38 g/L $CuCl_2$, 1.6 g/L $MnCl_2$, and 3.6 g/L $FeCl_2$) per liter. IPTG was added to the cultures to a final concentration of 0.1 mM, and cultures were transferred to a shaker at 28 °C, 250 rpm for 3 to 5 days. Samples were collected at intervals of 24 h when needed to monitor the OD_{600} and product concentration by HPLC (High-performance liquid chromatography). All the experiments were conducted in triplicate.

Analytical methods

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600} nm) with an UV-1200 spectrophotometer (APADA).

The products FA, *p*-Coumaric acid, and caffeic acid in culture were extracted twice by an equivalent volume of ethyl acetate. The supernatant was collected and evaporated to dryness and dissolved in an equal volume methanol. The samples were analyzed by high-performance liquid chromatography on an Ultimate 3000 system (Thermo Scientific, USA) using a C18 column (Silgreen HPLC column 250×4.6 mm; particle size, 5 μm) and a DAD detector at 310 nm. Compounds were separated by water (containing 0.1% acetic acid, mobile phase B) and methanol (containing 0.1% acetic acid, mobile phase C) at a flow rate of

1.0 mL/min and column temperature 30°C under the following conditions: 80% B + 20% C in 0-2 min, 50% B + 50% C in 15 min, 80% B + 20% C in 17 min, 80% B + 20% C in 20 min. The retention time of FA, *p*-Coumaric acid, and caffeic acid were 17.318 min, 16.5 min, and 12.99 min, respectively. The compounds were quantified based on calibration curves of various concentrations of standards using peak area.

Accession numbers

The GenBank accession numbers for the nucleotide sequence of the codon-optimized *tal, sam5, comt* and *metK* genes are MW403919, MW403920, MW403921 and MW403922, respectively.

Results

Construction of a heterologous FA biosynthetic pathway

To construct FA biosynthetic pathway (Fig. 1), the genes *tal*, *sam5* and *comt* that encodes tyrosine ammonia-lyase, *p*-coumarate 3-hydroxylase, and caffeic *O*-methyltransferase from *Rhodobacter sphaeroides*, *Saccharothrix espanaensis,* and *Triticum aestivum* respectively were codon-optimized, synthesized, and assembled into pET plasmid as one operon under the control of T7 promoter, getting plasmid pBR322-T7-*tal-sam5-comt*. The construction was introduced to *E. coli* BL21(DE3) and JM109(DE3) separately, and the recombinant *E. coli* were fermented in Terrific Broth (TB) medium with additional 1 g/L L-tyrosine and 2% (*v/v*) glycerol at 28°C 250 rpm for 3 days. We got the final ferulic acid product with little *p*-coumaric acid residual in the fermentation broth. FA, caffeic acid and *p*-Coumaric acid were detected in fermentation broth in HPLC analysis, and the retention time was identical to that of authentic FA and caffeic acid (Fig. 2 a). The data showed that using *E.coli* JM109 (DE3) as host cell produced significantly more FA than using BL21(DE3) as host cell (60 mg/L *vs* 14 mg/L , Fig. 2 b). So *E.coli* JM109(DE3) was chosen as the host cell for FA production in the following study.

The SDS-PAGE results showed high level protein expression of these genes in *E. coli* JM109(DE3), the expression level of gene *tal*, *sam5* and *comt* was very high under T7 promoter control at tested temperature (37°C, 28°C, 22°C), the protein solubility was improved when temperature was lower for gene *tal* and *sam5*, gene *comt* was soluble expressed at 3 tested temperature (Fig. S1 a, b, c). In order to alleviate the expression burden, we exchanged the promoter controlling biosynthesis pathway into T5 promoter, the expression level of each gene was lower but solubility was improved under T5 promoter control at tested temperature (28°C) compared with T7 promoter, as showed in Fig. S1 d. The new construction with *tal*, *sam5* and *comt* expression cassette under T5 promoter control was introduced to *E. coli* JM109 (DE3), resulting as the strain T5FA. Subsequently, the recombinant *E. coli* T5FA was fermented in M9Y medium supplemented with 2% (*v/v*) glycerol and 1 g/L L-tyrosine, which is recommended by another study about vanillin (Ni et al., 2015). The time course showed the FA and caffeic acid production increased quickly in the first two days, and reached the platform period at 48 hours, which was 130 mg/L and 33 mg/L, respectively. The production of *p*-Coumaric acid was lowest and decreased in the first two days and almost undetectable after 48 hours (Fig. 2 c).

Pathway optimization by copy number and promoter strength tuning

As mentioned above, gene expression fine tuning could impact greatly on the final production, here, we replaced the medium copy number replicon pBR322 of FA biosynthesis pathway with the lower copy number replicon p15a. Combined with promoter T7 or T5, we got FA biosynthesis pathway in plasmid backbones pBR322-T5, pBR322-T7, p15a-T5 and p15a-T7 (Fig. 3), which were expected to present different gene expression level. Flask fermentation was carried out at the same condition as previous mentioned and the result showed that strain p15aT5 with FA biosynthesis pathway in plasmid p15a-T5-*tal-sam5-comt* works best (Fig. 4). FA production of strain p15aT5 reached 180 mg/L which is significantly higher than other strains. And there is little caffeic acid residual (~5 mg/L) in the fermentation, compared with strain T5FA, in which FA and caffeic acid production was 130 mg/L and 33 mg/L, respectively.

Improve the FA production by NADPH regeneration enzyme

The transformation of *p*-Coumaric acid into caffeic acid in FA biosynthesis pathway is catalyzed by NADPH consuming *p* -coumarate 3-hydroxylase (SAM5) (Fig. 1). To increase NADPH availability for FA production, genes encoding NADPH regeneration enzymes (Fig. 5), *zwf*, *gnd*, *icd*, *pntAB* from *E.coli* MG1655 and *gap*N (GenBsank No. AE001437.1) form *Clostridium. acetobutylicum* ATCC 824 were cloned into the low-copy vector pCL1920-T7 (~5 copies per cell) and introduced into JM109(DE3) together with FA biosynthesis pathway separately.

The result showed that the strain T5FA + *pntAB* co-expressing *pntAB* gene with FA biosynthesis pathway could increase the production significantly compared with the control strain T5FA + pCL1920-T7. The production was 192±9.6 mg/L FA and 30 ±10.0 mg/L caffeic acid residue *vs* 140±7.7 mg/L FA and 30 ±14.8 mg/L caffeic acid residue respectively.

Interestingly, FA production of the control strain *E. coli* T5FA+pCL1920-T7 (140 mg/L FA and 30 mg/L caffeic acid, Fig. 6) was slightly higher than strain *E. coli* T5FA (130 mg/L FA and 33 mg/L caffeic acid, Fig.2 c), which carries only FA biosynthesis pathway plasmid. Production of other candidates (*icd, zwf, gnd, gapN*) was higher than strain T5FA but lower than the control strain co-transfected with the empty plasmid pCL1920-T7, which carries spectinomycin resistance gene.

Improve the FA production by SAM synthetase

To increase SAM availability for FA production, the non-native *metK* from *Streptomyces spectabilis* (Genbank No. WP_144002349.1) was cloned and fused downstream of *pntAB*, forming a bicistronic structure with *pntAB* under the control of T7 promoter on the low-copy vector pCL1920-T7 (~5 copies per cell), and introduced into JM109(DE3) together with FA biosynthesis pathway p15a-T5-*tal-sam5-comt*. The fermentation results showed that the strain p15aT5 + *pntAB+metK* co-expressing *pntAB* gene and *metK* gene with FA biosynthesis pathway p15aT5-*tal-sam5-comt* could increase the production significantly compared with the control strain p15aT5 + pCL1920-T7, the production was 212.5 ± 11.4

mg/L FA and 11.9 ±0.5 mg/L caffeic acid *vs* 178.3±1.8 mg/L FA and 15.4 ±0.6 mg/L; But no significant difference with strain p15aT5 + *pntAB* (producing 207.3 ± 2.9 mg/L FA and 13.0 ± 0.7 mg/L caffeic acid) (Fig. 7). While adding L-metionine during fermentation process decreased FA production surprisingly (Table S3).

Discussion

Construction of a heterologous FA biosynthetic pathway

The exogenous genes *tal* (Genbank No. CP033447.1), *sam5* (Genbank No. HE804045.1) and *comt* (Genbank No. EF413031.1) comprising FA biosynthetic pathway were chosen based on previous work (Berner et al., 2006; Ma & Xu, 2008; Watts et al., 2004). Jendresen et al reported high efficiency Tyrosine Ammonia-Lyases (TAL) from diverse origins enable enhanced production of aromatic compounds in bacteria and *Saccharomyces cerevisiae* (Jendresen et al., 2015). The *tal* gene (Genbank No. CP033447.1) is stored in our lab and the fermentation of FA biosynthesis pathway using BL21(DE3) as host cell in TB showed *p*-coumaric acid accumulation (Fig. 2), and this *tal* worked well in our previous work of *de Novo* biosynthesis of resveratrol *in E.coli* (Wang et al., 2014). Rodrigues et al reported compare of *p* -coumarate 3-hydroxylase from *Saccharothrix espanaensis* (*sam5*) and *Rhodopseudomonas palustris* (CYP199A2) converting *p*-coumaric acid into caffeic acid from tyrosine in *E. coli*, and found CYP199A2 worked better, but it needs two redox partners, which may complicate the system (Haslinger & Prather, 2020; Rodrigues et al., 2015a). COMT from *A. thaliana* (GenBank No. AY062837) has previously been used to catalyst caffeic acid to FA in several reports (Choi et al., 2011; Heo et al., 2017); here we tried COMT from wheat *Triticum aestivum* L. *cv*. H4564 (Genbank No. EF413031.1), which is named TaCM in original literature, it is proposed to methylate phenol substrates containing aldehyde, flavonoid and CoA moieties; and it may have broad substrate preferences, not only involves in converting caffeic acid to ferulic acid and 5-hydroxyferulic acid to sinapic acid, but also in the conversion of caffeoyl-CoA to feruloyl-CoA, and 5-hydroxyFeruloyl-CoA to sinapoyl-CoA (Ma & Xu, 2008). Interestingly, we note that protein sequence of TaCM is 100% identity with TaOMT2 (Genbank No. DQ223971), which showed 100% activity specifically to tricetin in substrate preference test against a number of phenolic compounds, and proposed to accept caffeic acid and 5- hydoxy-ferulic acid as substrates (Zhou et al., 2009; Zhou et al., 2006). Here we used it in FA biosynthesis pathway and certificated its activity of catalyst caffeic acid to FA. The caffeic acid *O*-methyltransferase function of TaCM /TaOMT2 was confirmation for the first time as far as we know.

Well, strain BL21(DE3) is a more effective expression host compared with strain JM109(DE3), for BL21(DE3) is a low acetate producer at aerobic high cell density culture, and JM109(DE3) is a high acetate producer (Shiloach et al., 1996). Acetate accumulation would reduce growth rate and recombinant protein synthesis (Noronha et al., 2000). Besides, in Kang's work, *E. coli* C43(DE3) (mutant strain of *E. coli* BL21(DE3)) was engineered to tyrosine over-producing host for artificial biosynthesis of phenylpropanoic acids (Kang et al., 2012). While in Huang et al's work, *E.coli* BW25113 (mutant strain of *E. coli* K-12) was used to biosynthesis caffeic acid (Huang et al., 2013; Lin & Yan, 2012). Here after

construction the heterologous biosynthetic pathway under T7 promoter control on PET plasmid, the fermentation test was carried out in host cell *E. coli* JM109(DE3) and BL21(DE3), the results showed it could convert L-tyrosine into FA successfully, with a small amount of caffeic acid or *p*-Coumaric acid residual (Fig. 2a). Interestingly, FA yields is higher when using strain JM109(DE3) as host cell than BL21(DE3) in our test, so JM109(DE3) was chosen as heterologous expression host cell for the following research.

To increase the FA production, we firstly analyzed single protein expression under T7 promoter control by decreasing temperature form 37°C to 22°C. SDS-PAGE result showed that TAL and SAM5 are mostly in the form of inclusion body, the solubility was slightly improved at lower temperature (Fig S1 a, b, c). In the next step, we changed the promoter T7 into a reduced strength promoter T5, and protein expression was changed great. Even the total protein expression level decreased, the solubility was improved, especially TAL and SAM5. Based on this, we replaced the T7 promoter of FA expression cassette by T5 promoter, and carried out the fermentation using a simple M9Y medium, FA production increased to 130 mg/L, was even higher than previous (Fig. 2c). This result suggests that FA yield could be optimized by tuning the gene expression to a proper level.

Conclusion

In this work, we constructed the FA biosynthesis pathway by introducing three heterologous genes from three different species, and the function of TaCM as caffeic acid *O*-methyltransferase was verified for the first time. FA production was tested in two *E.coli* strain JM109(DE3) and BL21(DE3), and JM109(DE3) as host cell yields more FA than BL21(DE3). Tuning down the promoter strength of the expression cassette significantly increased final FA production, so we continued to optimize pathway expressing level by combinations of different replicon and promoter strength and increased FA production. To further increase the production, *E.coli* endogenous NADPH regeneration gene *pntAB* and heterologous SAM formation enzyme *SsmetK* were used to improve key precursor NADPH and SAM supplying, finally we increased the production of FA from 130 mg/L to 212 mg/L. Further work needs to do to realize the superposition effect of NADPH and SAM supplement, and the host cell could be engineered to be tyrosine over-producing strain or chromosome integration of the biosynthesis pathway in order to reduce the cost of fermentation. Adjusting medium components and strategies that can modulate the pathway metabolites flux more refined may unlock greater production potential of the host cells, especially BL21(DE3), which is widely used as heterologous expression host. Our work offers an inspiration for other research of natural product biosynthesis engineering.

Abbreviations

FA: *p*-hydroxy-3-methoxycinnamic acid; NADPH: Nicotinamide adenine dinucleotide phosphate; SAM: S-Adenosyl-l-methionine; HPLC: High-performance liquid chromatography.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

HJL and YW conceived the project and revised the manuscript. HJL and YZ performed most of the experiment work, including the construction of the plasmids, fermentation and production detection; JS performed part of the plasmid's construction at the beginning of this project; HLL established the detection method of *trans*- ferulic acid, *p*-coumaric acid and caffeic acid. HJL wrote the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to publish this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 The *E. coli* strains and plasmids used in this study

Strain and plasmid	Description	Source
JM109(DE3)	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14- [F' traD36 proAB+ laclq lacZΔM15] hsdR17(rK-mK+) + λ(DE3)	Lab stock
DH10B	F-mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15ΔlacX74 recA1endA1 araD139 (ara, leu)7697 galU galK-rpsL nupG	Lab stock
pET21d-tal	pET21d derived, T7 Prom-tal-T7 Term	Lab stock
pET21a-sam5	pET21a derived, T7 Prom-sam5-T7 Term	This study
pET21a-comt	pET21a derived, T7 Prom-comt-T7 Term	This study
pET21d-tal-sam5	pBR322 ori, Amp resistance, T7 Prom-tal-sam5-T7 Term	This study
pJF25	pBR322 ori, Apr resistance, T7 Promoter	(Wang et al. 2013)
pHJ345	pBR322 ori, Apr resistance, T5 Promoter	This study
pHJ352	P15a ori, Apr resistance, T7 Promoter	This study
pHJ354	P15a ori, Apr resistance, T5 Promoter	This study
pBR322-T7-tal-sam5-comt	pBR322 ori, Apr resistance, T7 Prom-tal-sam5-comt-T7 Term	This study
pBR322-T5-tal-sam5-comt	pBR322 ori, Apr resistance, T5 Prom-tal-sam5-comt-T7 Term	This study
p15a-T7-tal-sam5-comt	P15a ori, Apr resistance, T7 Prom-tal-sam5-comt-T7 Term	This study
p15a-T5-tal-sam5-comt	P15a ori, Apr resistance, T5 Prom-tal-sam5-comt-T7 Term	This study
pCL1920	pSC101 ori, spectinomycin resistance, cloning vector	(G.Lerner, C. 1990)
pCL1920-T7	pSC101 ori, spectinomycin resistance, T7 Prom-MCS-T7 Term	Lab stock
pCL1920-T7 -Ecicd	pSC101 ori, spectinomycin resistance, T7 Prom- Ecicd -T7 Term	This study
pCL1920-T7 -Ecgnd	pSC101 ori, spectinomycin resistance, T7 Prom- Ecgnd -T7 Term	This study

pCL1920-T7 - Eczwf	pSC101 ori, spectinomycin resistance, T7 Prom- Eczwf -T7 Term	This study
pCL1920-T7 - EcpntAB	pSC101 ori, spectinomycin resistance, T7 Prom- EcpntAB -T7 Term	This study
pCL1920-T7 - CagapN	pSC101 ori, spectinomycin resistance, T7 Prom- CagapN -T7 Term	This study

Figures

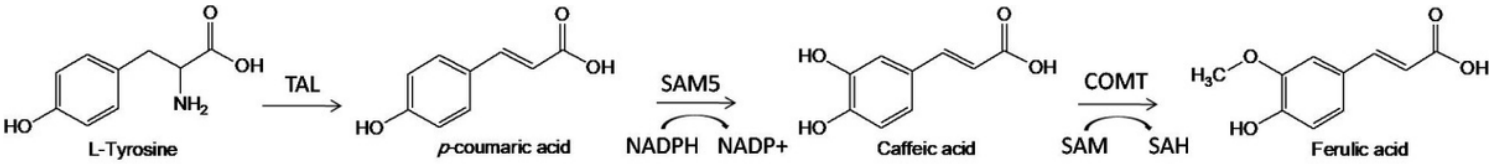

Figure 1

Biosynthesis pathway of FA in E.coli Tyrosine ammonia-lyase (TAL) catalyzes nonoxidative deamination of the primary amino acid tyrosine to p-coumaric acid. p-Coumaric acid is converted into caffeic acid by a hydroxylation step at the 3-position of the benzyl ring by the p-coumarate 3-hydroxylase (sam5), FA is biosynthesized from caffeic acid by the enzyme caffeate O-methyltransferase.

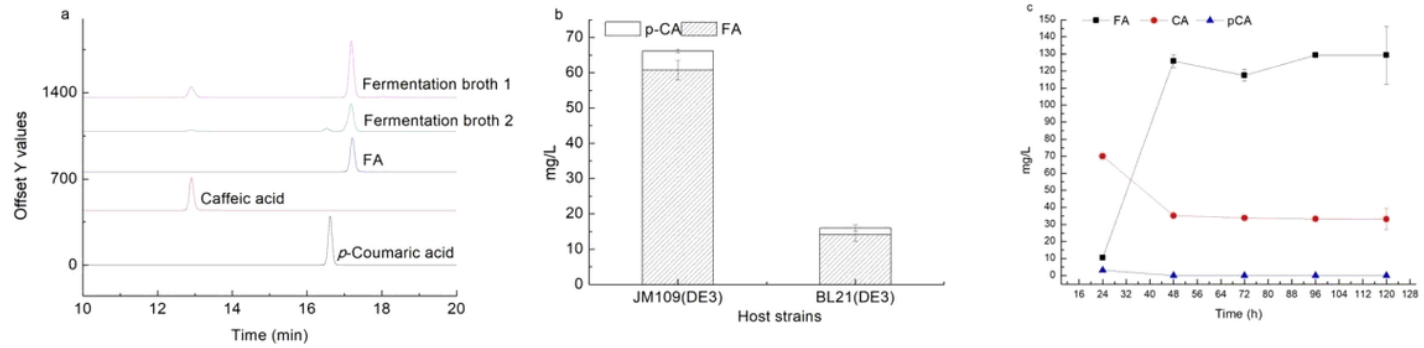

Figure 2

FA heterologous production in E.coli (a) HPLC profiles of fermentation products and the FA, p-coumaric acid and caffeic acid standards. (b). Testing FA Production by strain JM109(DE3) and BL21(DE3). pBR322-T7-tal-sam5-comt was transformed into competent cells of the above two host strains. The fermentation was carried out in Terrific Broth (TB) medium with additional 1 g/L L-tyrosine and 2% (v/v) glycerol at 28°C 250rpm for 3 days. p-CA, p-coumaric acid. FA, trans- Ferulic acid. (c). Time course of FA production from L-tyrosine by E. coli JM109(DE3) T5FA in M9Ymedium supplemented with 20 2% (v/v) glycerol and 1 g/L L-tyrosine at 28°C 250rpm for 5 days.

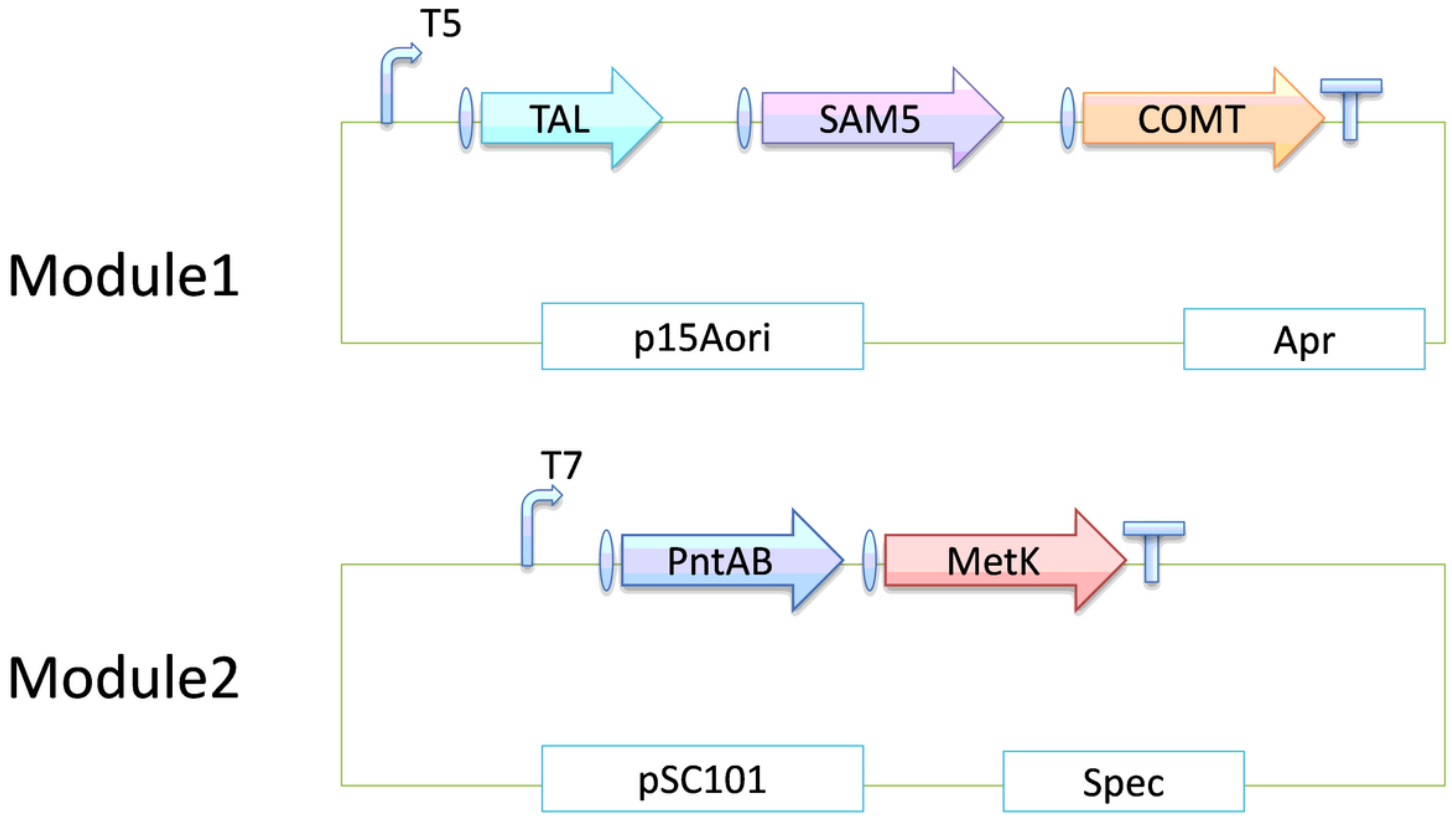

Figure 3

Schematic representation of biosynthetic pathway

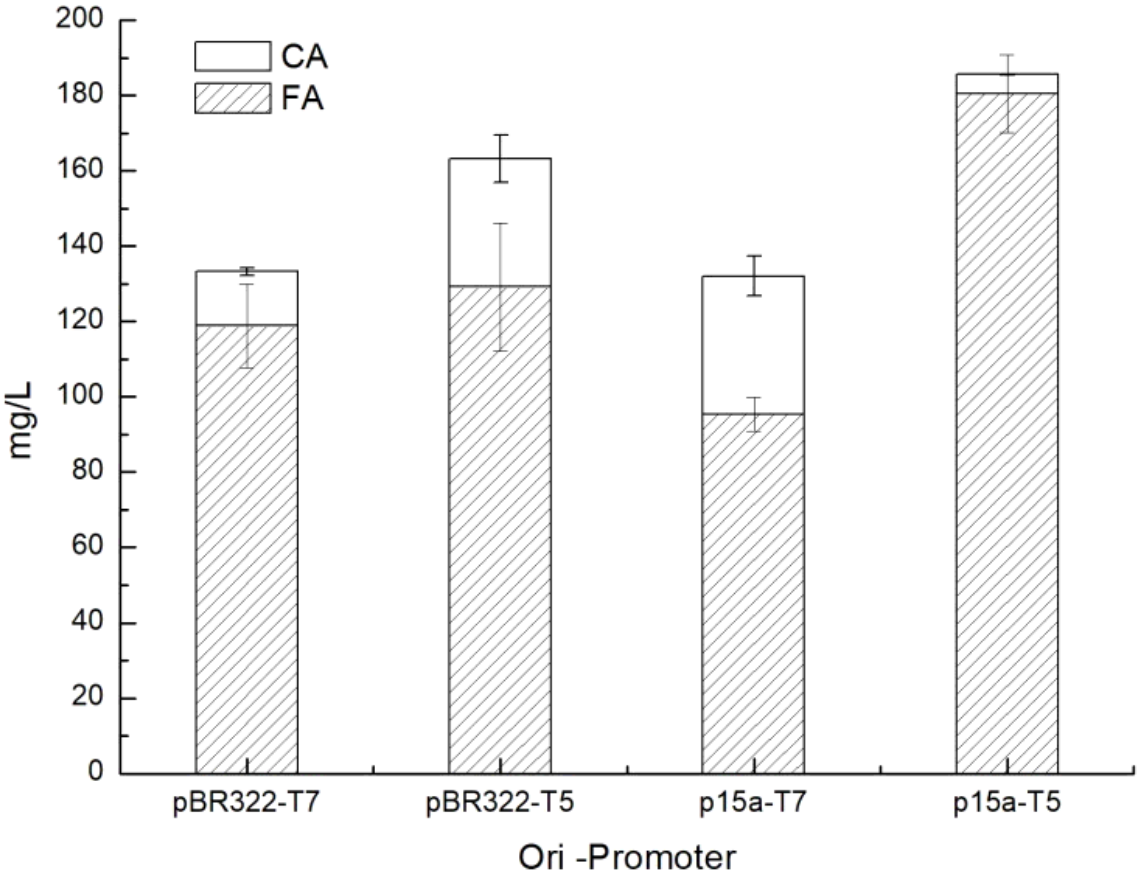

Figure 4

Effect of combination of plasmid copy number (pBR322, p15a) and promoter strength (T7 promoter, T5 promoter) on FA production in host cell JM109(DE3). The fermentation was carried out in M9Ymedium supplemented with 2% (v/v) glycerol and 1 g/L L-tyrosine at 28°C 250rpm for 5 days.

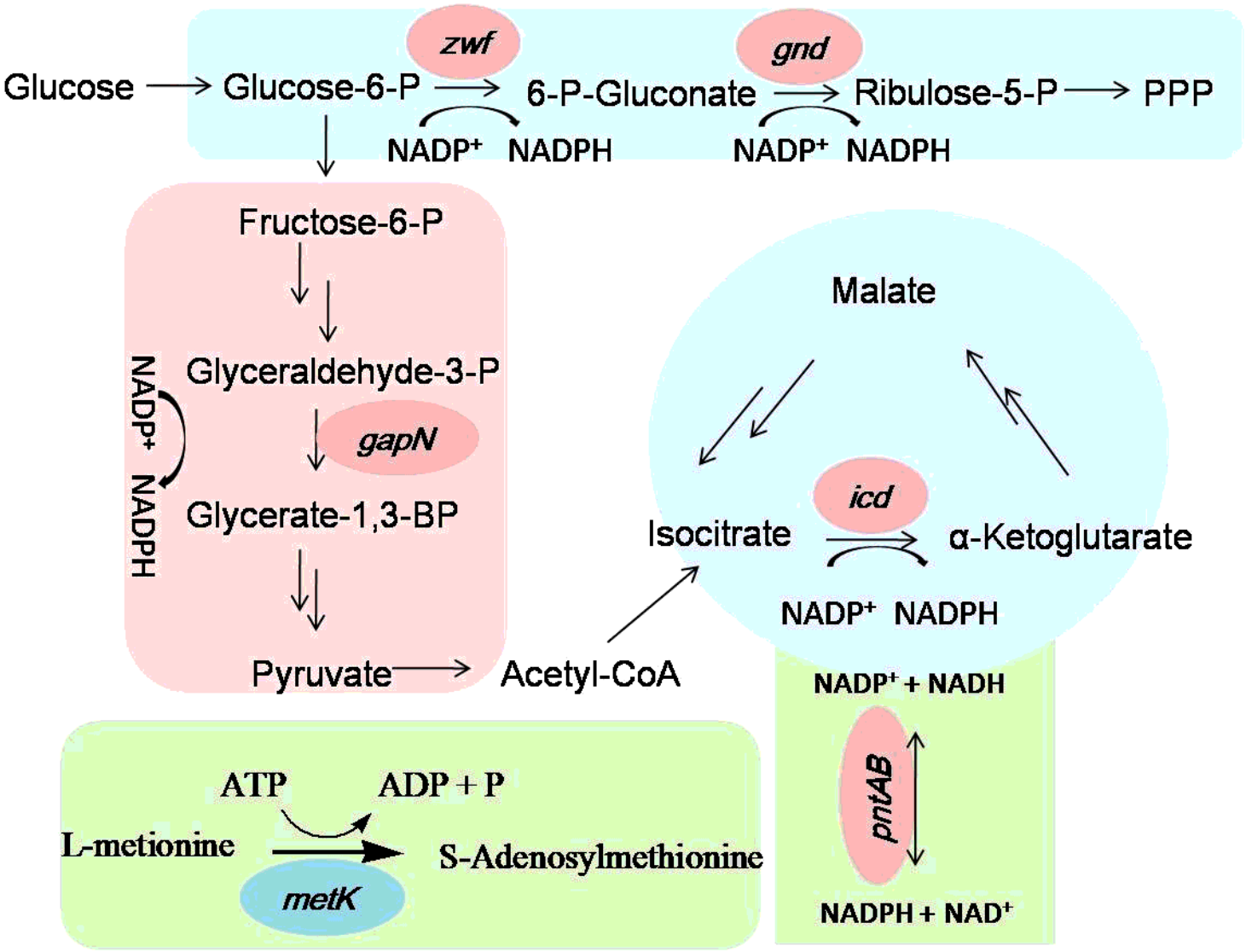

Figure 5

NADPH and SAM regenerating enzymes used for FA production.

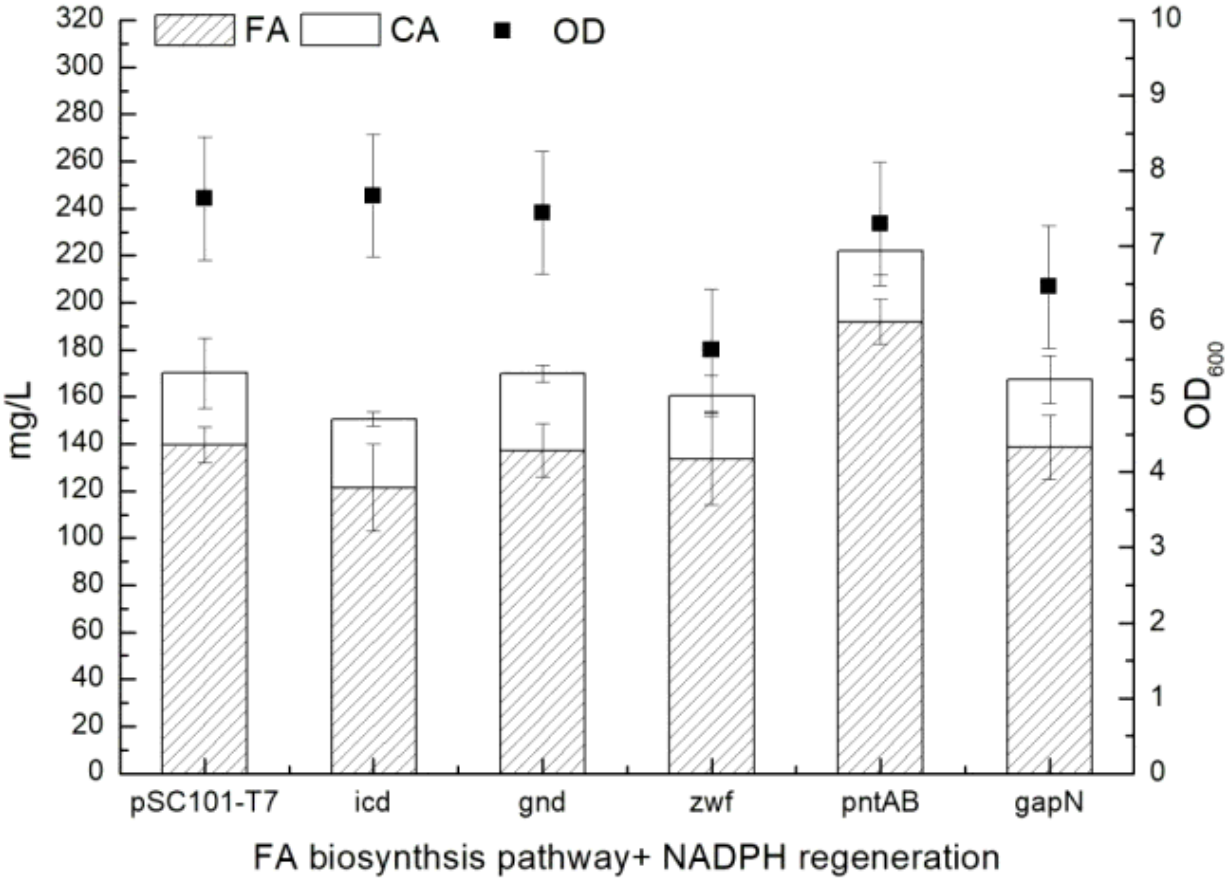

Figure 6

Compare of FA biosynthesis strains co-expressed with NADPH regenerating enzymes pSC101-T7 refer to FA biosynthesis pathway pBR322-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7 in host cell JM109(DE3); icd refer to FA biosynthesis pathway pBR322-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7-Ecicd in host cell JM109(DE3), gnd refer to FA biosynthesis pathway pBR322-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7- Ecgnd in host cell JM109(DE3), zwf refer to FA biosynthesis pathway pBR322-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7- Eczwf in host cell JM109(DE3), pntAB refer to FA biosynthesis pathway pBR322-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7- EcpntAB in host cell JM109(DE3), gapN refer to FA biosynthesis pathway pBR322-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7- CagapN in host cell JM109(DE3). The fermentation was carried out in M9Y medium supplemented with 2% (v/v) glycerol and 1 g/L L-tyrosine at 28°C 250rpm for 5 days.

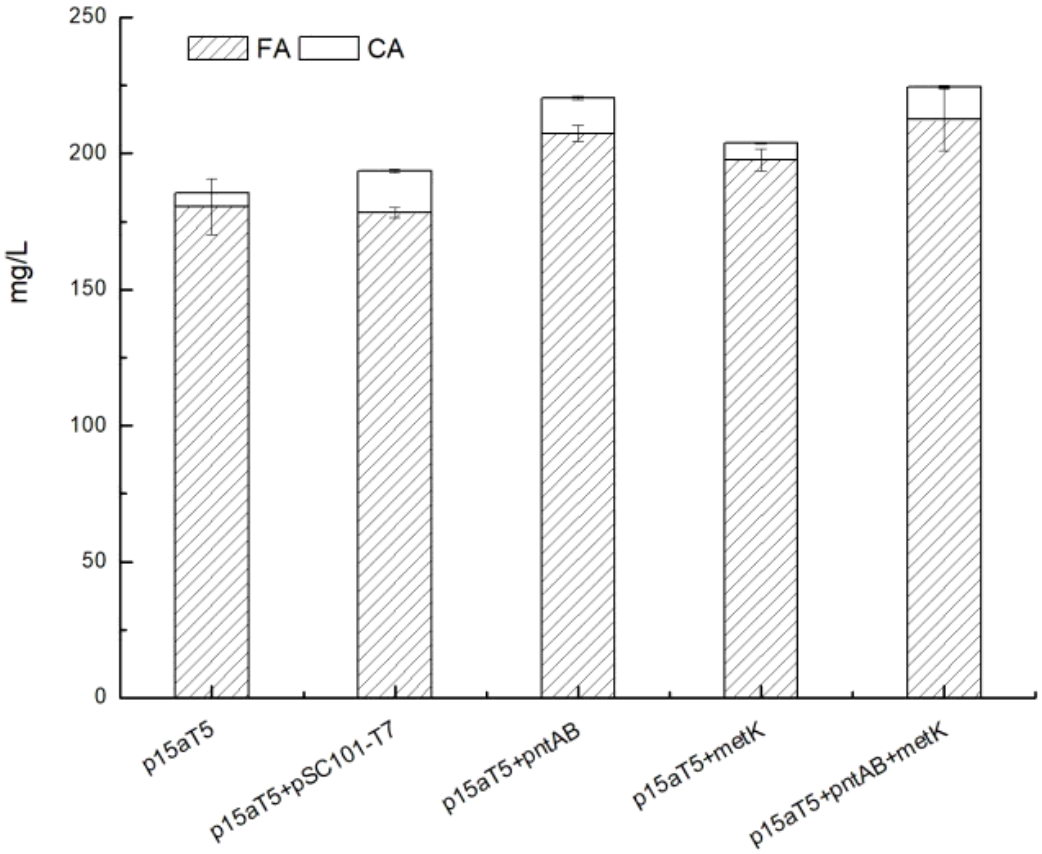

Figure 7

Effect of combination of NADPH regenerating enzyme gntAB with S-Adenosyl-l-methionine synthetase metK in FA biosynthesis strain p15a-T5 refer to FA biosynthesis pathway p15a-T5-tal-sam5-comt in host cell JM109(DE3); p15a-T5+pSC101-T7 refer to FA biosynthesis pathway p15a-T5-tal-sam5-comt co-expressed with plasmid pCL1920-T7 in host cell JM109(DE3); p15a-T5+pntAB refer to FA biosynthesis pathway p15a-T5-tal-sam5-comt co-expressed with plasmid pCL1920-pntAB in host cell JM109(DE3); p15a-T5+metK refer to FA biosynthesis pathway p15a-T5-tal-sam5-comt co-expressed with plasmid pCL1920-metK in host cell JM109(DE3); p15a-T5+pntAB+metK refer to FA biosynthesis pathway p15a-T5-tal-sam5-comt co-expressed with plasmid pCL1920-pntAB-metK in host cell JM109(DE3); The fermentation was carried out in M9Y medium supplemented with 2% (v/v) glycerol and 1 g/L L-tyrosine at 28°C 250rpm for 5 days.

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