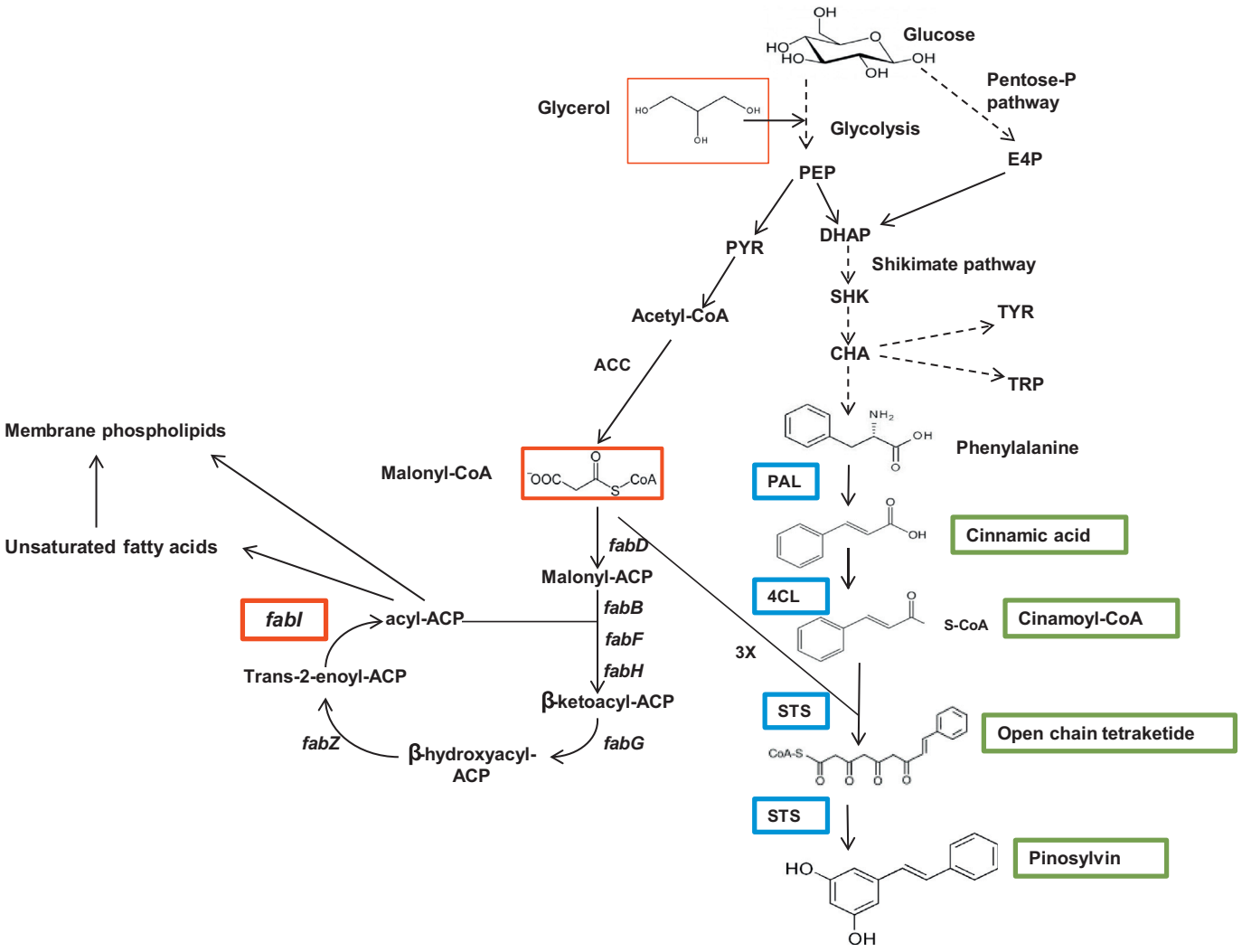

Fig. 1. Pinosylvin biosynthetic pathway. PAL, phenylalanine ammonia lyase, C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; STS, stilbene synthase; ACC, acetyl-CoA carboxylase, ACC, acetyl-CoA carboxylase; PEP, phosphoenolpyruvate; E4P, erithrose-4-phosphate; DHAP, dihydroxyacetone phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; SHK, shikimate; CHA, chorismate; *fabD*, gene encoding malonyl-CoA:acyl carrier protein (ACP) transacylase; *fabB*, β-ketoacyl-ACP synthase I; *fabF*, gene encoding β-ketoacyl-ACP synthase II; *fabH*, gene encoding β-ketoacyl-ACP synthase III; *fabG*, gene encoding NADPH-dependent β-ketoacyl-ACP reductase; *fabZ*, gene encoding β-hydroxyacyl-ACP dehydratase.

Approaches for generating microbial strains with the capacity of synthesizing pinosylvin are based on the expression of the complete or partial pinosylvin biosynthetic pathway, as well as other modifications that increase the availability of precursor molecules. A strain of *Streptomyces venezuelae* was generated for the production of stilbenes by expressing the gene encoding 4CL from *Streptomyces coelicolor* and a codon-optimized gene that codes for an STS from *Arachis hypogaea*. In cultures grown in a complex medium supplemented with 1.2 mM cinnamic acid, 0.6 mg/L of pinosylvin was produced [9].

A synthetic pinosylvin pathway was generated and evaluated in *Escherichia coli* by employing PAL, 4CL, and STS enzymes from several sources. The most suitable pathway configuration was found to consist of genes encoding PAL from *Petroselinum crispum*, 4CL from *S. coelicolor*, and STS from *Pinus strobus*. Directed protein evolution of *P. strobus* STS was performed to obtain two mutants that caused increased pinosylvin production when expressed with the rest of the biosynthetic pathway. An *E. coli* strain expressing such optimized pathway produced 70 mg/L of pinosylvin in the presence of cerulenin. When 3 mg/L of the precursor L-Phe was added to the culture medium, the pinosylvin titer increased to 91 mg/L [10]. Cerulenin is an inhibitor of fatty acid biosynthesis and reduces the consumption of malonyl-CoA by this pathway, thereby making this precursor available for stilbene biosynthesis [11]. However, the high cost of cerulenin (>$20/1 mg) would be a major drawback when considering employing it for industrial production.

In another report, a combinatorial approach was applied to generate *E. coli* strains that produce pinosylvin from glycerol and cinnamic acid. The genes encoding 4CL from *Arabidopsis thaliana* and STS from *Vitis vinifera* were expressed in a combinatorial fashion by employing several different ribosome binding site (RBS) sequences and plasmid origins of replication. Once the best combination of RBS and the origin of replication for pinosylvin production was identified, it was determined that *E. coli* Dh5α was the best producer among seven evaluated strains. To improve strain performance, a strategy to increase malonyl-CoA was evaluated. It consisted on employing clustered regularly interspaced short palindromic repeats interference (CRISPRi) to repress the expression of the genes *fabB*, *fabD*, and *fabF* encoding enzymes from the fatty acid biosynthesis pathway. It was determined that CRISPRi repression of *fabB*, *fabD,* and *fabF* resulted in 1.5-, 1.9-, and 0-fold increased production of pinosylvin, respectively. By employing a strain that has the inducible *fabD* repression, it was possible to transform 0.5 mM cinnamic acid to 47.5 mg/L (0.22 mM) pinosylvin [12].

As shown in the above examples, reduction in the consumption of malonyl-CoA by repression/inhibition of genes and enzymes from the fatty acid biosynthetic pathway results in increased stilbene production. Other approaches aimed at increasing malonyl-CoA availability have

been successfully applied to the production of compounds from the phenylpropanoid family. The malonate assimilation pathway from *Rhizobium trifolii* is composed of a malonyl-CoA synthetase and a malonate transport protein (*matB* and *matC*). Expression of these two genes in *E. coli* has been shown to increase (2S)-pinocembrin production when malonate is supplemented in the medium [13]. The enzyme acetyl-CoA carboxylase (Acc) catalyzes the synthesis of malonyl-CoA from acetyl-CoA. Overexpression of this enzyme from *Corynebacterium glutamicum* in *E. coli* from the strong T7 promoter in cultures performed at 25°C resulted in a threefold increase in malonyl-CoA concentration. To increase acetyl-CoA availability for malonyl-CoA synthesis, overexpression of acetyl-CoA synthetase (Acs) was combined with deletion of the genes *ackA-pta* and *adhE*, which encode acetate kinase, phosphotransacetylase and alcohol/aldehyde dehydrogenase. When tested in a strain that also overexpresses Acc from *C. glutamicum*, it was determined that malonyl-CoA concentration increased 15.7-fold [14].

Various approaches have been evaluated to generate and improve pinosylvin production strains, including increasing malonyl-CoA availability. Even though these strategies have a positive effect regarding the increase in precursor availability, such modifications can have negative effects on strain growth capacity or process cost. For this reason, it is desirable to explore alternative approaches that improve pinosylvin production without compromising strain physiology.

The gene *fabI* encodes enoyl-acyl carrier protein reductase, an enzyme that has a key role in fatty acid biosynthesis. To study the role of this enzyme on malonyl-CoA metabolism, an *E. coli* strain that has a thermosensitive FabI mutant has been characterized. It was determined that growth of this strain at a nonpermissive temperature of 42°C resulted in a 30% increase in the malonyl-CoA pool [15]. It should also be noted that *fabI* is an essential gene; therefore, it cannot be deleted in *E. coli*. Based on this information, we decided to explore an approach based on reducing the amount of enoyl-acyl carrier protein reductase to increase the malonyl-CoA pool. The strategy consisted on deleting the -35 promoter sequence of gene *fabI*, expecting a reduction in its transcript level. This strategy would avoid the undesired stress responses caused by increasing the culture temperature to 42°C, if a thermosensitive FabI mutant was used [16].

In this work, we constructed an *E. coli* strain that can convert cinnamic acid to pinosylvin and showed the feasibility of reducing the expression level of the gene *fabI*, encoding the enzyme enoyl-acyl carrier protein reductase, by employing a recombineering strategy to delete the -35 and upstream promoter regions. An engineered strain with such modification was found to produce 52.67 mg/L pinosylvin, a level 1.5-fold higher compared to an isogenic wild type strain without reduction in the expression level of *fabI*.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strain W3110 was employed in this study [17] (Table 1). The genes 4CL(M) from *S. coelicolor* A3 (2) (Sc4CL(M)) and STS from *V. vinifera* (VvSTS) were synthesized with codon optimization for *E. coli* by Life Technologies [18]. The plasmid pTrc-Sc4CL(M)-VvSTS expresses the synthetic genes described above from the Trc promoter.

2.2. Construction of plasmid pTrc-Sc4CL(M)-VvSTS

A 1325-bp DNA fragment containing the gene VvSTS was obtained from the plasmid pTrc-Sc4CL-VvSTS [16] by digestion with the restriction enzymes HindIII/KpnI and gel-purified. The plasmid pTrc-Sc4CL(M)-AhSTS was digested with the restriction enzymes HindIII/KpnI, and the 5694-bp DNA fragment containing the gene Trc promoter, the gene coding for the Sc4CL(M), ampicillin resistance cassette, and plasmid origin of replication was gel-purified and ligated with the 1325-bp fragment. The resultant plasmid pTrc-Sc4CL(M)-VvSTS was verified by restriction and sequence analyses.

Table 1
Strains and plasmids used in this study.

	Characteristics	Reference
Strain		
W3110	*E. coli* F⁻, λ⁻, INV (rrnD⁻ rrnE)1	ATCC 27325
W3110Δ-35*fabI*::Cm	W3110 with a deletion of 258 bp upstream of the *fabI* gene, starting at the -28 position.	This work
Plasmid		
pTrc-Sc4CL (M)-VvSTS	Synthetic codon-optimized genes encoding mutant version of 4CL from *S. coelicolor* A2 and STS from *Vitis vinifera* cloned in the expression vector pTrc99A.	[18]

2.3. Chromosomal deletion of the -35 and upstream promoter regions of the gene fabI

The transcriptional start site of the gene *fabI* has been experimentally determined by My et al. [19]. Based on this information, the -10 and -35 promoter sequences were localized. For the deletion of the -35 and upstream regions of the *fabI* promoter, the primers employed were -35fabI-F (5'TGCTGGAGAATATTCTGCAGGAACTGAACCGCCGGTCACCCTCTCCCTGATGTGTAGGCTGGAGCTGCTTC3') and -35fabI-R (5'GTAAACAGTACGAACAGATAAACGGTTATTATAATCAACCTGGCTGTGAGCATATGAATATCCTCCTTAG3'). The 5′ end of the primer -35fabI-F has 50 bases of homology to the 32′ terminal region of the gene *ycjD* up to the stop codon and 20 bases of homology to the P1 sequence of the plasmid pKD3 for amplification of FRT sequence and the chloramphenicol (Cm) resistance cassette [18]. The 5′ end of the primer -35fabI-R includes 50 bases of homology to the 5′ region of the gene fabI up to the -28 position of the promoter region and 20 bases of homology to the P2 sequence of the plasmid pKD3. The PCR reaction was performed with standard buffer conditions, Pfu DNA polymerase, one cycle of initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 60 s, and extension at 68°C for 4 min for a total volume of 50 μL. The PCR product was gel-purified and digested with the restriction enzyme DpnI. The purified PCR DNA from the restriction reaction mixture was purified and electroporated into the strain W3110 that was previously transformed with the plasmid pKD46 [20]. Candidate mutant strains were screened by plating them in Luria–Bertani (LB) solid medium with Cm at a concentration of 30 μg/mL. Colonies displaying Cm resistance were screened by performing PCR with the primers c2-F (5'GATCTTCCGTCACAGGTAGG3') [18] and fabII1-R (5´TACGGATCGGACCAGCAGAG3´) and sequencing the amplified product. A colony displaying the expected chromosomal deletion was named W3110Δ-35fabI::Cm.

2.4. Culture media and growth conditions

Solid and liquid LB media were used. The antibiotics carbenicillin (Cb; 100 μg/mL) and Cm (30 μg/mL) were used for strain selection. Cultures for pinosylvin production were grown in LB medium supplemented with glycerol 10 g/L. The inoculum for pinosylvin production was started with an overnight 3-mL culture in LB medium supplemented with Cb 100 μg/mL in glass tubes at 37°C and 300 rpm for 12 h. This culture was used to inoculate 50 mL of LB medium supplemented with cinnamic acid 3 mM and Cb 100 μg/mL present in 250-mL baffled shake flasks. The initial O.D.600 of the culture was 0.1, and when it reached an O.D.600 of 0.8, isopropyl-β-d-thiogalactopyranoside was added at a final concentration of 0.5 mM. After 20 h, a sample was taken for pinosylvin quantification. All cultures were performed in triplicate.

2.5. RNA extraction, cDNA synthesis, and RT-qPCR analysis

Samples from shaked flask cultures of the strains W3110/pTrc-Sc4CL(M)-VvSTS and W3110Δ-35fabI::Cm/pTrc-Sc4CL(M)-VvSTS were collected for RNA extraction at the exponential phase. RNA was extracted using hot phenol equilibrated with water, and cDNA synthesis was performed with the RevertAid H First Strand cDNA Synthesis kit (Fermentas, USA) and a mixture of specific DNA primers, as previously reported by Flores et al. [21]. The qPCR experiments were performed with the ABI Prism 7300 Real-Time PCR System (Applied Biosystems, USA) using Maxima SYBRGreen PCR Master Mix (Fermentas, USA) and reaction conditions described previously [21]. The quantification technique used to compare data was the 2-ΔΔCT method [22], and the results were normalized using the *ihfB* gene as an internal control. The qPCR experiments complied with the MIQE guidelines for publication of quantitative real-time PCR experiments [23]. RNA extraction and cDNA synthesis reactions were performed from three independent cultivations for each strain.

2.6. Analytical methods

Cell growth was monitored by measuring optical density at 600 nm (O.D.600) in a spectrophotometer (Beckman DU-70; Fullerton, CA, USA). Dry cell weight (DCW) was calculated by multiplying the absorbance at 600 nm with a previously determined coefficient factor of 0.37 g/L [24]. Pinosylvin concentration was determined using an HPLC system (Agilent 1100 System, Agilent Technologies, Palo Alto, CA, USA), with a reverse-phase column (Phenomenex Synergi Hydro RP C18, 150 × 4.6 mm, 4 μm, Phenomenex, Torrance, CA, USA) and employing photodiode array detection. Samples were eluted using a gradient in which the mobile phase A was 0.1% TFA in water and the mobile phase B was 0.1% TFA in methanol. Gradient started at 20% B, followed by linear increases to 40% in 5 min, then to 80% in 5 min and then maintained for 3 min. Flow rate was 0.5 mL. Pinosylvin was detected by diode array at $\lambda = 306$ nm. Pinosylvin (98% purity) used for preparing the standard curve was purchased from Sigma-Aldrich.

3. Results and discussion

3.1. Construction of an E. coli strain with chromosomal deletion of the -35 and upstream promoter regions of the gene fabI

A recombineering strategy was employed to delete 258 bp upstream of the *fabI* gene, starting at the -28 position, thus including the -35 promoter sequence [19,20] (Fig. 2). The resulting strain was named W3110Δ-35*fabI*::Cm. To determine whether such modification had an effect on growth capacity, the strains W3110Δ-35*fabI*::Cm and W3110 were grown using several culture medium compositions. When grown in LB medium, the strains W3110 and W3110Δ-35*fabI*::Cm displayed μ values of 1.32 ± 0.017 and 1.03 ± 0.010 h^{-1}, respectively. In M9 medium + glucose 10 g/L, the strains W3110 and W3110Δ-35*fabI*::Cm showed μ values of 0.49 ± 0.001 and 0.50 ± 0.001 h^{-1}, respectively. A 22% reduction in specific growth rate of the mutant strain compared to that of the wild type was observed in LB medium. By contrast, no difference in μ values was detected when these two strains were grown in the M9 minimal medium. An explanation for these results is that both strains are considered to grow faster on LB medium; therefore, to sustain such growth rate, a higher cellular demand for fatty acid synthesis is required. Under these conditions, a reduction in the expression of *fabI* gene would cause a reduction in the level of enoyl-acyl carrier protein reductase, thus limiting fatty acid biosynthetic capacity and growth.

3.2. Construction and characterization of E. coli strains for the production of pinosylvin

To generate *E. coli* strains to produce pinosylvin from cinnamic acid, it is necessary to express genes that encode the 4CL and STS enzymes. In this work, we utilized a synthetic gene that encodes a mutant version of 4-coumaroyl-CoA ligase from *S. coelicolor* A3 (2) having a replacement of amino acid residue alanine at position 294 for a glycine residue (A294G) [25]. This mutant version of 4CL (Ss4CL(M)) displays threefold higher catalytic activity toward cinnamic acid compared to the wild-type enzyme [25]. A synthetic gene encoding STS from *V. vinifera* (VvSTS) was also used in this study because this enzyme has been shown to employ cinnamoyl-CoA as the substrate [12,18]. Genes encoding Ss4CL(M) and VvSTS were cloned as an operon under transcriptional control of the Trc promoter to generate plasmid pTrc-Sc4CL(M)-VvSTS. The strains W3110 and W3110Δ-35*fabI*::Cm were transformed with this plasmid to generate W3110/pTrc-Sc4CL(M)-VvSTS and W3110Δ-35*fabI*::Cm/pTrc-Sc4CL(M)-VvSTS. To determine the effect of deleting the -35 and upstream promoter regions of the gene *fabI* on its transcript level in the production strains, RT-qPCR was performed with total mRNA extracted from W3110/pTrc-Sc4CL(M)-VvSTS and W3110Δ-35fabI::Cm/pTrc-Sc4CL(M)-VvSTS grown in LB + glycerol 10 g/L + 3 mM cinnamic acid. These experiments showed a 60% reduction in *fabI* transcript level in mutant strain W3110Δ-35fabI::Cm/pTrc-Sc4CL(M)-VvSTS compared to W3110/pTrc-Sc4CL(M)-VvST.

Both the -35 and -10 promoter motifs are required to allow recognition by the RNA polymerase carrying sigma 70. However, the -10 sequence is recognized as the most highly conserved and essential

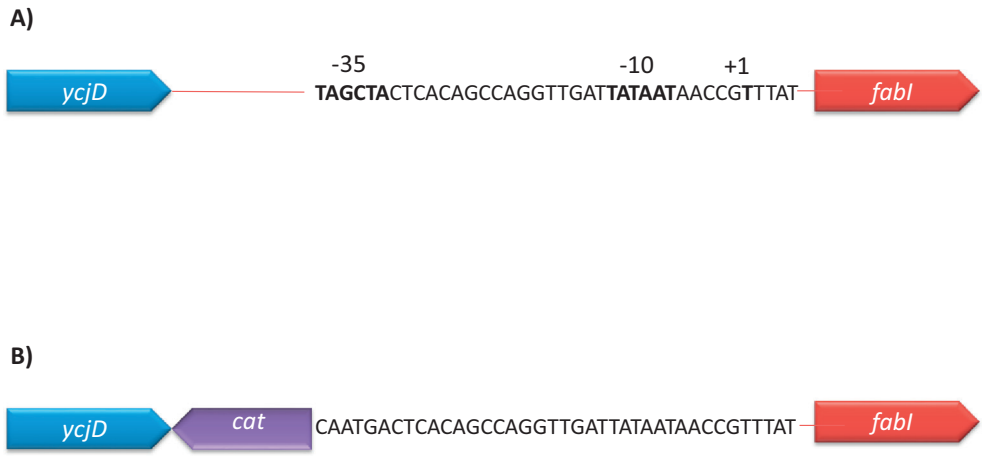

Fig. 2. Deletion of *fabI* -35 and upstream region. Chromosomal content of the gene *fabI* in W3110 A) and W3110Δ-35fabI::Cm B). The transcription start site for the gene *fabI* is indicated with +1 and the promoter hexamer sequences with -10 and -35.

promoter motif [26,27]. The -10 promoter sequence is particularly important because its recognition drives the initial promoter opening, which is an essential step in transcription initiation [28]. In the gene *fabI*, the -10 promoter motif has the sequence TATAAT, which is identical to the *E. coli* consensus (Fig. 2). By contrast, the -35 motif sequence TAGCTA shows low similarity to the consensus (TTGACA) [27]. Therefore, in this study, the -35 motif of the gene *fabI* was eliminated, expecting to have a negative effect on transcription initiation activity but without the complete elimination of promoter activity.

For the production of pinosylvin, these strains were grown in shake flask cultures in LB medium with glycerol 10 g/L and cinnamic acid 3 mM (0.44 g/L). Under these conditions, μ values of 0.63 ± 0.001 and 0.51 ± 0.001 h^{-1} were determined for the strains W3110 and W3110Δ-35*fabI*::Cm, respectively. After 23.5 h of culture time, the biomass concentration was 4.17 ± 0.09 g/L for W3110/pTrc-Sc4CL(M)-VvSTS. The strain W3110Δ-35*fabI*::Cm/pTrc-Sc4CL(M)-VvSTS reached 3.51 ± 0.07 g/L of biomass after 24 h. In these cultures, the final pinosylvin titers corresponded to 34.89 ± 4.81 and 52.67 ± 2.95 mg/L for W3110/pTrc-Sc4CL(M)-VvSTS and W3110Δ-35*fabI*::Cm/pTrc-Sc4CL(M)-VvSTS, respectively. Fig. 3 shows an HPLC chromatogram including standards for pinosylvin (panel A) and cinnamic acid (panel B), as well as a sample from a production culture (panel C), where the substrate cinnamic acid and the produced

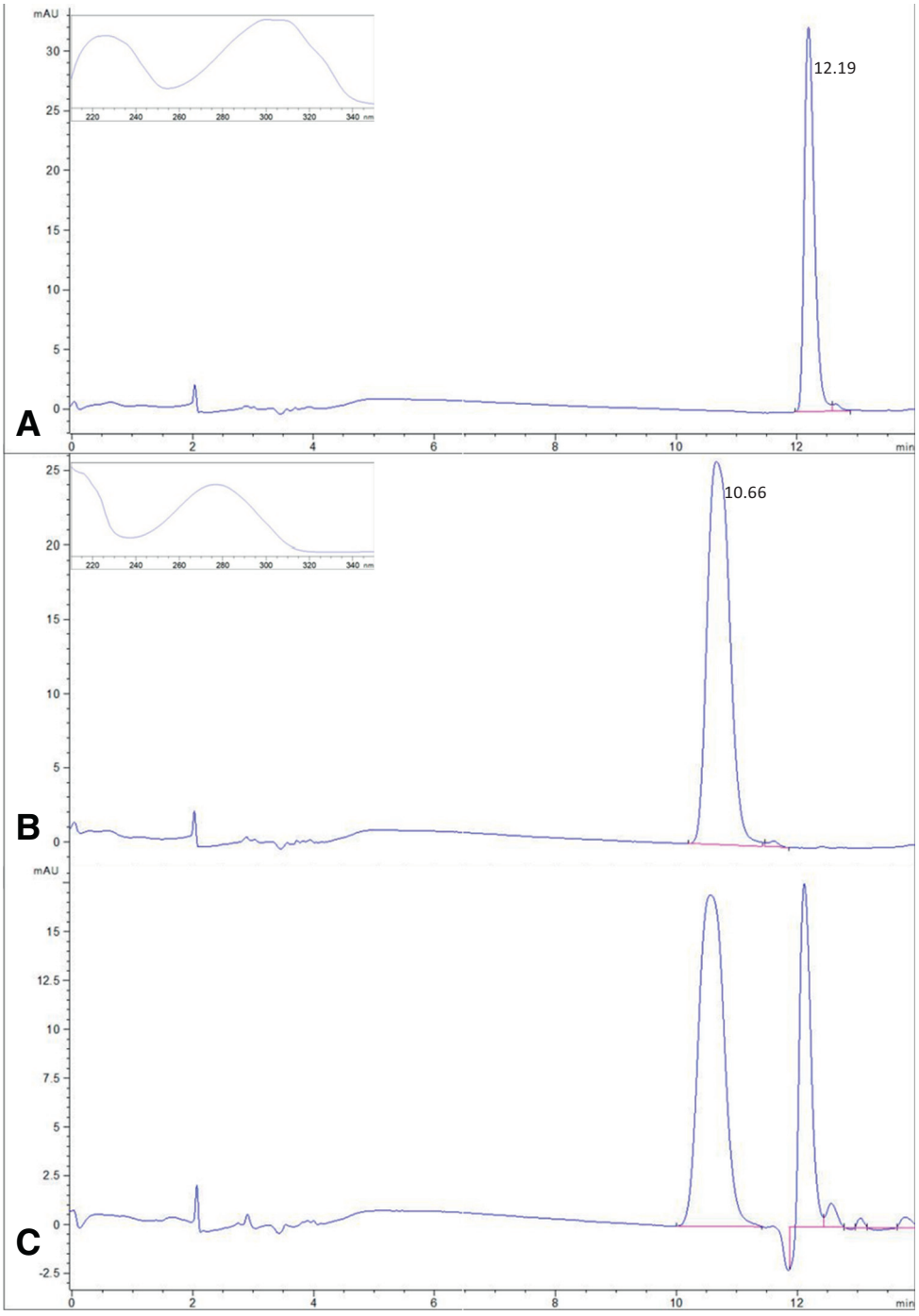

Fig. 3. Chromatograms and associated diode array detection spectra of A: Pinosylvin standard. B: Cinnamic acid standard. C: Production sample at the end point of culture. To confirm peak identity in C, in addition to retention times, diode array detection spectrum was compared to that of the corresponding standards.

pinosylvin were detected and quantified. The calculated yields of pinosylvin produced by unit of biomass ($Y_{pinosylvin/biomass}$) corresponded to 8.37 and 15 mg pinosylvin/g biomass for W3110/pTrc-Sc4CL(M)-VvSTS and W3110Δ-35*fabI*::Cm/pTrc-Sc4CL(M)-VvSTS, respectively.

4. Conclusion

We report here the construction and characterization of *E. coli* strains for the production of the stilbene pinosylvin from glycerol and cinnamic acid. An approach based on the reduction in the expression of the *fabI* gene was evaluated. A deletion of the *fabI* promoter region resulted in a reduction in transcript level and an increase in pinosylvin production. These results strongly suggest an increased availability of the precursor malonyl-CoA for stilbene biosynthesis in the mutant strain. Based on these results, further efforts toward strain improvement can focus on exploring methods to modulate *fabI* expression by employing synthetic promoters or regulatory circuits with the aim of determining the optimal level of FabI for improving the synthesis of stilbenes and other compounds that use malonyl-CoA as precursor.

Ethical approval

This article does not include any studies with human participants or animals performed by any of the authors.

Informed consent

This article does not involve any informed consent.

Conflict of interest

The authors declare that they have no conflict of interest.

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