Synthetic and Systems Biotechnology 7 (2022) 1126–1132

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

A combined strategy for the overproduction of complex ergot alkaloid agroclavine

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ARTICLE INFO

Keywords:
Microbial cell factories
Cell-free systems
Overproduction
Agroclavine
Ergot alkaloids

ABSTRACT

Microbial cell factories (MCFs) and cell-free systems (CFSs) are generally considered as two unrelated approaches for the biosynthesis of biomolecules. In the current study, two systems were combined together for the overproduction of agroclavine (AC), a structurally complex ergot alkaloid. The whole biosynthetic pathway for AC was split into the early pathway and the late pathway at the point of the FAD-linked oxidoreductase EasE, which was reconstituted in an MCF (*Aspergillus nidulans*) and a four-enzyme CFS, respectively. The final titer of AC of this combined system is 1209 mg/L, which is the highest one that has been reported so far, to the best of our knowledge. The development of such a combined route could potentially avoid the limitations of both MCF and CFS systems, and boost the production of complex ergot alkaloids with polycyclic ring systems.

1. Introduction

Ergot alkaloids display diverse biological activities and are often found in fungus-infected grains or fungi-associated plants. There are about over 100 ergot-alkaloid type natural products, and currently, six FDA-approved ones are on the market, including treatments for parkinsonian syndrome and vasoconstrictors [1]. Ergot alkaloid agroclavine (AC, Fig. 1A) antagonizes depression that is induced by noradrenaline in the cerebral cortex of the rat [2] and was once used for ergot-based drug synthesis via the oxidation of the top methyl group [3]. Ergot alkaloids generally possess complex conjugated rings with several stereocenters (Fig. 1A), which led to their total synthesis being very difficult and not at all profitable [1]. The biosynthesis of ergot alkaloids requires tryptophan, Dimethylallyl pyrophosphate (DMAPP), and *S*-Adenosyl methionine (SAM) [5,16]. Tryptophan in fungus is synthesized by a shikimic acid pathway containing 6 enzymes and DMAPP is generated by the mevalonate pathway containing 7 enzymes, whereas the SAM regeneration cycle requires 3 enzymes (Fig. 1A). Starting from those precursors, three prenylated-tryptophan derivatives, dimethylallyltryptophan (DMAT), *N*-methyldimethylallyltryptophan (*N*–Me-DMAT), and prechanoclavine (PCC) are generated under the successive catalysis of prenyltransferase DmaW, methyltransferase EasF, and FAD-linked oxidoreductase EasE. Two additional enzymes chanoclavine synthase EasC and FAD-linked dehydrogenase EasD catalyze the biosynthesis of chanoclavine (CC) and chanoclavine-aldehyde (CCA), the two common tricyclic precursors for all ergot alkaloids. Finally, aldehyde dehydrogenase EasA and agroclavine dehydrogenase EasG complete the biosynthesis of AC. Overall, 22 enzymes are involved in the biosynthesis of AC in the native host (Fig. 1A). In conclusion, ergot alkaloids employ a large number of enzymes for the biosynthesis of

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2022.08.003
Received 25 June 2022; Received in revised form 10 August 2022; Accepted 16 August 2022
Available online 20 August 2022

precursors as well as tailoring modifications [4,5], which led to their construction of microbial cell factories (MCFs) and cell-free systems (CFSs) difficult.

MCFs and CFSs both have been developed to overproduce natural biomolecules, which are two alternative approaches of chemical synthesis as well as natural extractions. The titers of products like vitamins, amino acids, and a few natural products have reached the industrial standard by using environmentally friendly and sustainable MCFs [6,7]. Despite this, it remains problematic to obtain high titers for many complex natural products due to i) limited precursor supply, ii) uncontrollable native metabolic network, iii) inefficient product transportation caused by the cell membrane, and iv) low expression and low activity of foreign enzymes. Indeed, accompanied by the complexity of the biosynthetic pathways, the difficulties of the construction and engineering of MCFs for natural biomolecules become exponential growth. A recent work, which takes over 20-year works and invests millions of dollars, reached the industrial-scale production of artemisinic acid [8]. Whereas for many other biomolecules, including morphine [9], cannabidiols [10], and tropane alkaloids [11], their titer is still below 10 mg/L in the yeast, and further engineering is essential for their industrial application.

CFSs separate the enzyme synthesis from the following catalysis, and thus could effectively avoid the limitations caused by cell membranes, fermentations, and uncontrolled native metabolic networks in MCFs [12]. Benefitted from the absence of cell membranes, CFSs allow for easy manipulation and optimization [12]. In addition, CFSs can achieve higher product titers, faster reaction rates, and better tolerance of toxic precursors. There are two types of CFSs based on the methods of preparations: cell extract-based and purified enzyme-based [13,14]. However, commercially CFS for complex biomolecules is rare, until Merck recently reported the commercial development of a nine-enzyme cascade for the synthesis of islatravir [15]. CFSs generally require many enzymes, which could be logistically challenging for scaling. This combined with the expensive cofactors and starting materials, also makes CFSs difficult to bring costs down.

To avoid the limitations that arise from both MCFs and CFSs, and to take full advantage of both systems, we developed a combined system to synthesize clinically important and structurally complex AC in this study (Fig. 1B). We split the enzymes of its biosynthetic machinery into two sections, which were transformed into an MCF (*Aspergillus nidulans*) and a CFS system, respectively (Fig. 1B). The key to this designed MCF-CFS combined system is determining its splitting point for the construction of two systems. The combined system was demonstrated to efficiently synthesize AC, with the titer up to 1209 mg/L and an engineering period of up to 3 months. Thus, the combined MCF-CFS system represents a fast, robust as well as practical engineering methodology for laboratory use as well as industrial application.

2. Material and methods

2.1. General materials and methods

The authentic compounds prechanoclavine and agroclavine were purified and characterized, which are stored in our laboratory. All vectors and strains, including those of *Aspergillus nidulans* for the overproduction of PCC, used in this study were stored in our laboratory. A Bio-Red MicroPulser (Bio-Red 1652100, USA) was used to perform Electroporation. The analytical grade of chemicals and solvents were

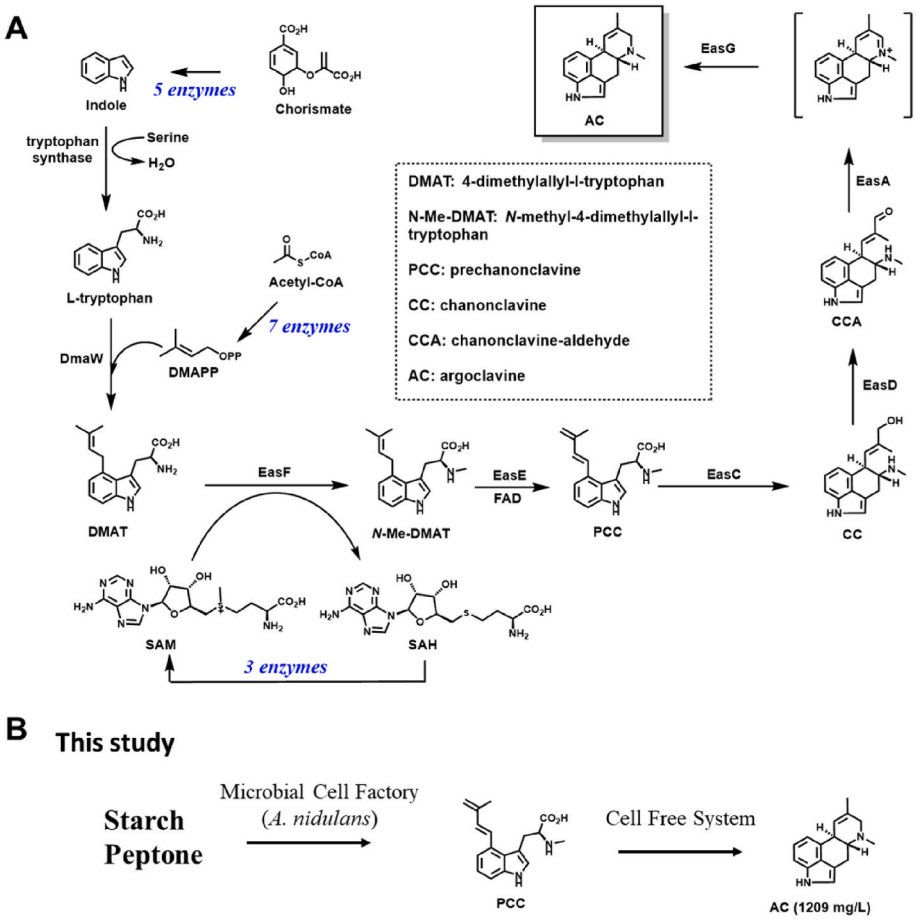

Fig. 1. The biosynthetic pathway of AC. (A) The biosynthetic pathway of AC in the native hosts. (B) the combined system designed for overproduction of AC in the current study.

purchased and used in this study. Milli-Q water was used to prepare all buffers and solutions. DNA sequencing was performed by Tsingke Biotechnologies (Beijing, China). Primers were synthesized by Synbio Technologies (Beijing, China). Restriction enzymes and Q5 DNA polymerase from New England Biolabs (USA) were used in this study. *C. purpurea* 3.1003, *A. fumigatus* 3.772, *A. niger* 3.1454, *A. oryzae* 3.334, and *A. terreus* 3.15736 were purchased from China General Microbiological Culture Collection Center (CGMCC). AGILENT-1200HPLC/6520QTOFMS system (Agilent, USA) using C18 analytical column (Ultimate XB-C18 100 × 2.1 mm, particle size 3 μm; Welch) was used to perform liquid chromatograph-mass spectrometer (LC-MS) analysis. HPLC grade Acetonitrile was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gene synthesis was performed at Genscript Technology Co., Ltd. (Nanjing, China). A Shimadzu LC-2030C 3D Plus system (Shimadzu, Japan) using C18 analytical column (Gemini 250 × 4.6 mm, particle size 5 μm; Phenomenex) was used to perform high performance liquid chromatography (HPLC) analysis.

2.2. Plasmid construction and protein purification

The cDNAs of EasC, EasD, EasA, and EasG from different fungal strains were synthesized for the heterologous protein expression, which were inserted into the site NdeI/XholI of pET28a by the Gibson method to generate the corresponding plasmids. The resultant plasmids were transformed into the competent cells of *E. coli* BL21(DE3) and plated on LB solid mediumwith 50 mg/mL kanamycin at 37 °C overnight. The correct transformant was cultured in a 10 mL LB liquid medium containing 50 mg/mL kanamycin at 37 °C and 220 rpm overnight for 24 h, which is used a seed medium and transferred into 2-L LB liquid medium. Isopropyl-β-D-thiogalactoside (IPTG, 0.1 mM) was used for inducing protein expression. The bacteria were concentrated and suspended in Tris–HCl buffer, which were sonicated using an ultrasonic processor (SX-605D, Henglong instrument Co. Ltd., Changzhou, China). The bacterial lysate was centrifuged using a Thermo Scientific Sorvall ST 16R centrifuge (Thermo Scientific, Germany), and the obtained supernatant was mixed with nickel resin using a 5 mL HisTrap™HP (GE Healthcare Life Science, Uppsala, Sweden). The mixture was subjected into a column, which was then washed using 3 column volume of buffer B (Tris–HCl 20 mM, NaCl 300 mM, imidazole 40 mM, glycerin 10%). The buffer C (Tris–HCl 20 mM, NaCl 300 mM, imidazole 250 mM, and glycerin 10%) was used to elute the bonded protein. The obtained pure protein was concentrated, which was then subjected to buffer exchange using 30 kDa Amicon Ultra (Merck Millipore). Bradford assay was used to determine the protein concentration using bovine serum albumin (BSA) as a standard.

2.3. Enzyme assays

Biochemical assays with the substrate PCC were performed in a 50 μL or 2 mL system, containing different concentrations of enzymes and cofactors, under different conditions. All the assays were quenched with methanol, which were then mixed and centrifuged at 12,000 g for 5 min. A 0.22-μm microfilter was used to filter the obtained supernatant, which was subjected to HPLC and LC-MS for further analyses.

2.4. HPLC and LC-MS analysis

Shimadzu LC-2030C 3D Plus system (Shimadzu, Japan) with C18 columns (Gemini 250 × 4.6 mm, particle size 5 μm; Phenomenex) and a diode array detector was used for product analyses. A routine program of a linear gradient of 90%–0% H_2O (v/v, 0.1% formic acid)-acetonitrile (MeCN)− in 25 min followed by 100% acetonitrile (v/v, 0.1% formic acid) for 5 min with a flow rate of 1.0 mL/min was used for all samples. A 10 μL of injection volume was for all samples. An AGILENT1200HPLC/6520QTOFMS system (Agilent, USA) with C18 analytical columns (Ultimate XB-C18 100 × 2.1 mm, particle size 3 μm; Welch) was used for LC–MS analysis, and its mass spectrometry was performed under the positive ion mode. A routine procedure was used with a flow rate of 0.3 mL/min, which was a linear gradient of 10–100% acetonitrile (MeCN)− H_2O (v/v, 0.1% formic acid) in 12 min followed by 100% MeCN (v/v, 0.1% formic acid) for 3 min. All samples were dissolved in MeOH (chromatographic grade) and a 1.0 μL sample was used for injection.

2.5. Culture, fed-batch fermentation of the strains, and metabolite extraction

If there is no specific notation, each *A. nidulans* strain was cultured in 50 mL liquid CD-ST medium (20 g/L starch; 20 g/L tryptone; 50 mL/L 20 × nitrate salts; 1 mL/L trace elements; adjust pH to 6.5 with 1 M KOH) in 250-mL flasks at 37 °C and 250 rpm for 3 days. To measure the starch concentration in the fed-batch fermentation, a commercial kit (Boxbio, Gansu Province, China) using the glucose oxygenation method was used. A 200 μL of fermentation broth were sampled and diluted 25 times, which was then subjected to an EnSpire microplate reader (PerkinElmer, USA) for measuring the optical density at 620 nm (OD620). A standard calibration curve was built for the calculation of the starch. The culture for fed-batch fermentation was performed in 250 mL CD-ST medium in 2000-mL flasks. The concentrations of starch and the titer of m-cresol were measured at intervals of 24 h by sampling 10 mL culture broth. The five-fold concentrated CD-ST medium was used to supplement the exhausted starch as well as other nutrition. The fed-batch mode was terminated until the concentration of starch was steady. Standard deviation of the product titers was calculated based on that each experiment was repeated three times.

The broth and hyphae were separated by filtration after fermentation. The hyphae were dissolved in acetone, which was then subjected to ultrasonic sonication for 20 min. The obtained broth was extracted using equal-volume ethyl acetate for three times. The crude extract was obtained by evaporating all organic phases to dryness, which was further dissolved with MeOH. The methanol-dissolved extract was filtered using a 0.22-μm filter, which was then subjected for HPLC and LC-MS analysis.

2.6. Measurement of dry cell weight (DCW)

To obtain mycelia, we use the Buchner funnel to filter the fermentation broth. The obtained mycelia were then dried in an oven at 65 °C to a constant weight, which is weighted using an electronic balance. From the relevant changes between fermentation time and DCW, we can obtain the growth curves of the strains.

3. Results

3.1. Determining the splitting point of the combined MCF-CFS system

To design a combined MCF-CFS system, we evaluated the application of either MCF or CFS system solitarily for the overproduction of ergot alkaloids. For the CFS system, i) our recent studies demonstrated the effort of purification of soluble EasE from *Escherichia coli*, *S. cerevisiae* or *Trichoderma reesei* was not successful [17]. In the current study, we also synthesized the cDNA of EasE from *A. japonicus*. However, further protein expression in *E. coli*, *S. cerevisiae,* or *Trichoderma reesei* failed. When we expressed *easE* from *A. japonicus* in *E. coli*, only insoluble protein could be detected, suggesting EasE most likely could not be correctly fold in *E. coli*. Further expression of *easE* from *A. japonicus* in *S. cerevisiae* only resulted in the yield of trace amount protein, which is not isolable. Final attempt that used the host *Trichoderma reesei* gave no trace of soluble or insoluble protein. One possible explanation for this is that EasE needs oxidative folding in ER catalyzed by the disulfide bridge formation enzymes [18], which is absent in the heterologous hosts. This combined with the fact that the whole pathway requires 22 enzymes makes the CFS overproduction of AC challenging.

On the other hand, our study about the overproduction of ergot alkaloids using MCF (*A. nidulans*) indicated that accompanied by the growing numbers of biosynthetic enzymes reconstituted into the system, the titers of final ergot alkaloids significantly decreased concurrently. For example, the titer of AC is less than 25% of that of its precursor PCC, the MCF of which required four fewer enzymes [19]. We also attempted to produce rugulovasine, another well-known ergot alkaloid, in *A. nidulans*, however, no product could be detected from the transformant with all required enzymes (data was not shown). This is consistent with recent research about overproducing plant biomolecules in yeast, which gave significantly higher titers for certain precursors than those of the final products [9–11].

Taken together, we selected the FAD-linked oxidoreductase EasE as the splitting point (Fig. 1). The enzymes of the early pathway before EasE (including EasE) were reconstituted into an MCF to overproduce PCC, followed by a CFS containing the enzymes of the late pathway to overproduce AC by reacting with PCC (Fig. 1B). The designed MCF for overproducing PCC involves the enzymes for the biosynthesis of DMAPP, SAM, and tryptophan, as well as the first three tailoring enzymes EasE, EasF, and DmaW (Fig. 1). The following CFS for converting PCC to AC contains the rest four enzymes EasA, EasD, EasG, and EasC (Fig. 1). The MCF for overproducing PCC based on *A. nidulans* is already successfully constructed, which gave a tier of ~300 mg/L, and fermentation optimization may further increase its production.

3.2. Overproduction of PCC using the MCF (A.nidulans) system

Based on the native biosynthetic pathways for tryptophan, DMAPP, and SAM in a heterologous host, only three more tailoring enzymes DmaW, EasF, and EasE are required for the biosynthesis of PCC. An *A. nidulans* PCC-producing strain was constructed in our recent study [19]. In this strain, there are two copies of DmaW, EasF, and EasE, and the precursor biosynthetic enzymes, including *N*-terminal truncated 3-hydroxy-3-methylglu-taryl CoA reductase (tHMGR), SAM synthetase (SamS), and Trp syn-thase (TrpS), were individually overexpressed. In the current study, we set up to conduct the fed-batch fermentation of this PCC-producing strain in the bioreactor. Before fed-batch fermentation, we firstly determined the starch consumption, growth curve, and PCC production, respectively, which were cultured in 2 L shake-flasks containing 400 mL liquid CD-ST medium. It shows that i) 20 g/L of starch was exhausted after 36-h fermentation (Fig. 2A); ii) the strains had the maximum biomass after 84-h fermentation (Fig. 2B); iii) the maximum titers of PCC were obtained after 108-h fermentation (Fig. 2C), which is 341.8 mg/L, about 15% higher than that of normal fermentation (298.2 mg/L) in this study. The titer of PCC decreased significantly in the late-stage fermentation (Fig. 2C), possibly due to its decomposition in the acidic broth that is generated by the long-time fermentation. A notable decrease was also observed for fungal wet weight after 72 h' culture in the normal fermentation (Fig. 2C). A possible explanation for the this is the autocytolysis of the dead fungal cells, which could be generated as the nutrients are exhausted in the normal fermentation.

Subsequently, the fed-batch fermentation of the PCC-producing strain was carried out in duplicate in 7.5 L bioreactors, and we used the data of shake-flask fermentation as guidance. After 36 h' culturing, the initial 20 g/L of starch was exhausted, as identified by shake-flasks fermentation. Then we tried the fed-batch strategy by feeding a two-fold concentrated CD-ST medium at a flow rate of 8.0 mL/h. The titers of PCC were determined at 12-h intervals and the final titers of PCC reached 2050 mg/L after 120-h fermentation (Fig. S5), which is 5-fold more than that of flash-flask fermentation. Similar to the flash-flask fermentation, the titer of PCC begins to decrease after it reaches the peak of productivity (Fig. S5)

3.3. Determining the optimal reaction parameters for the CFS system

An initial CFS system (named CFS A1) for the generation of AC using the purified enzymes of EasC from *A. fumigatus* ($EasC_{af}$), EasD from *A. fumigatus* ($EasD_{af}$), EasA from *Clavieps purpurea* ($EasA_{cp}$), and EasG from *A. fumigatus* ($EasG_{af}$), which are all generated by *E. coli* expression (Fig. 3A and Figs. S1–S4), was constructed. The CFS A1 contains 2 mM PCC, 10 mM EasC, 10 mM EasD, 20 mM $EasA_{cp}$, 10 mM EasG, 4 mM NADPH, 1 mM NAD^+ (Fig. 3A). When the reaction was firstly carried out in a 50 μL system, around 60% of 2 mM PCC is transformed to AC in 1 h (Fig. 3A). The optimal buffer and reaction temperature for the CFS A1 was determined, which are Tris-HCL (pH = 7.5) and 20 °C–30 °C, respectively (Fig. 3B and C). Interestingly, the CFS A1 is still highly efficient in the production of AC at a very high temperature of 70 °C (Fig. 3B), suggesting the enzymes in the CFS A1 system are quite stable and are prone to engineering in the future.

$EasA_{cp}$, an Old Yellow Enzyme homolog, acts as an isomerase in the biosynthesis of AC and converts chanoclavine aldehyde (CCA) to an imine intermediate, which is catalyzed by imine reductase EasG to generate AC (Fig. 1A, [20]). Recent studies show that thiol compounds can be used for replacing EasA to achieve a higher conversion rate [21]. Different thiol compounds including GSH, 2-mercaptoethanol, and dithiothreitol were selected to incubate with $EasC_{af}$, $EasD_{af}$, and $EasG_{af}$ instead of $EasA_{cp}$ to generate CFSs A2-A4, respectively. However, in contrast to the results given in the literature, all new CFSs showed a significant decrease in the production of AC with large amounts of remaining PCC (Fig. 3D), which suggested $EasA_{cp}$ is the superior catalyst in our study.

EasC is a catalase that catalyzes the key oxidative cyclization to generate CC from PCC [17]. To improve the titer as well as the scalability for the CFS, different EasC cDNAs from *A. japonicus*, *C. fusiformis*, *Periglandula ipomoeae*, *C. purpurea*, and *C. paspali* were synthesized and all introduced into *E. coli* for protein expression (Fig. S1) and the obtained proteins were used to replace $EasC_{af}$ in the CFS A1 to generate CFSs A5-A9. Our results indicated that all the new CFSs produce lower titers of AC compared to that of CFS A1 (Fig. 3E), although CFS A6 harboring EasC from *C. fusiformis* gave relative high titers among five

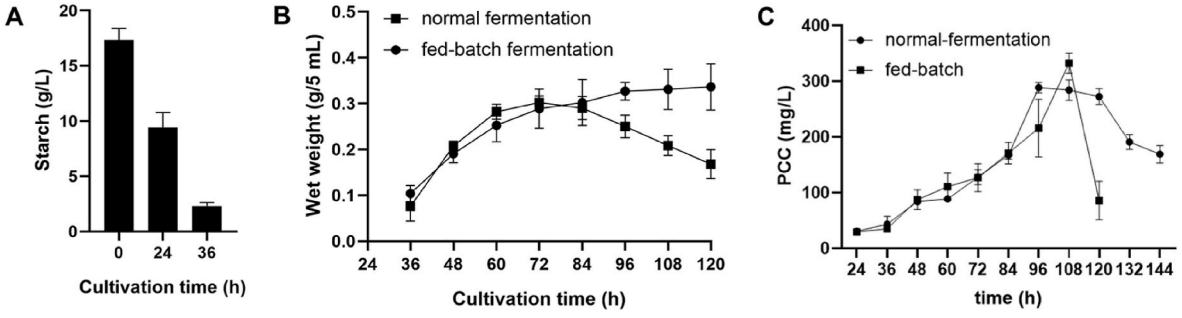

Fig. 2. Determination of the starch consumption, growth curve, and production of PCC in shake-flasks fermentation. (A) The starch consumption of PCC producing strain. (B) The growth curves of PCC producing strain. (C) The time-course titers of PCC producing strains.

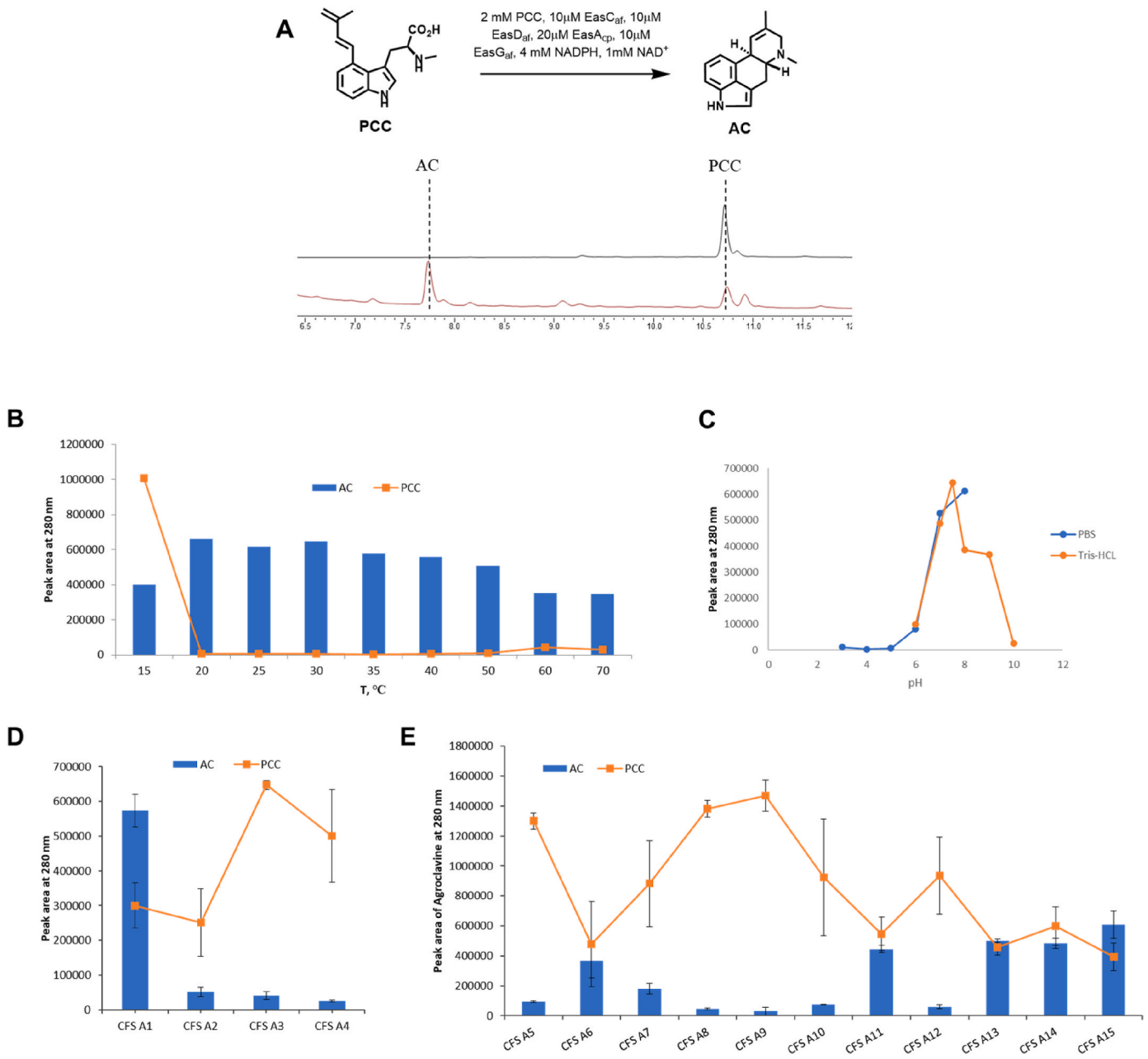

Fig. 3. CFSs designed for converting PCC to AC. (A) Construction of CFS A1 and HPLC analysis of its product profile. (B) Production of AC of the CFS A1 conducted under different temperatures. (C) Production of AC of the CFS A1 conducted with two buffer solutions (PBS and Tris-HCL) in different pH values. (D) Production of AC of the CFSs A1-A4. In the CFSs A2-A4, GSH, 2-mercaptoethanol, and dithiothreitol was used to replace EasA$_{cp}$ in the CFS A1, respectively. (E) Production of AC of the CFSs A1 and A5-A14, which contain different orthologs of EasC and EasG. For Fig. 3D and E, the assays were repeated three times, and the error bars represent the standard deviation (n = 3).

new CFSs, with the biggest peak area at 280 nm for the produced AC.

The function of EasG is to reduce the proposed cyclized iminium product generated by EasA [4,5]. Similarly, proteins of various alleles of *easG* from *Paenibacillus* sp., *Diplodia corticola*, *Madurella mycetomatis*, *Colletotrichum chlorophyte*, and *Valsa mali* var. *pyri* were all purified and used to replace EasG$_{af}$ in the CFS A1 to generate CFSs A10-A14, respectively (Fig. 3E and Fig. S2). The new CFSs A11, A13, and A14 harboring EasGs from *Paenibacillus* sp., *Madurella mycetomatis*, *Colletotrichum chlorophyte*, respectively, showed high productivity (Fig. 3E). However, the titers of all new CFSs are still lower than that of CFS A1. Overall, EasC$_{af}$ and EasG$_{af}$ were selected for the following engineering studies.

3.4. Engineering the CFS systems for transforming PCC to AC

With all the optimized conditions in hand, then we engineered the CFS A1 by individually titrating each reaction enzyme while keeping the remaining ones constant. However, individually increasing a single enzyme in the CFSs could not substantially enhance the production of AC (data not shown). Next, all cofactors and enzymes were simultaneously enhanced to increase the production of AC in the CFSs. We constructed a new CFS A15 by improving the concentrations of all the enzymes and cofactors to 2.5-fold of those in the CFS A1. The titer of AC in CFS A15 was significantly improved when treated with 5 mM PCC, which is all transformed to AC, with a markedly increased peak area for AC at 280 nm (Fig. 4A, CFS A15: 5 mM). The reaction was nearly

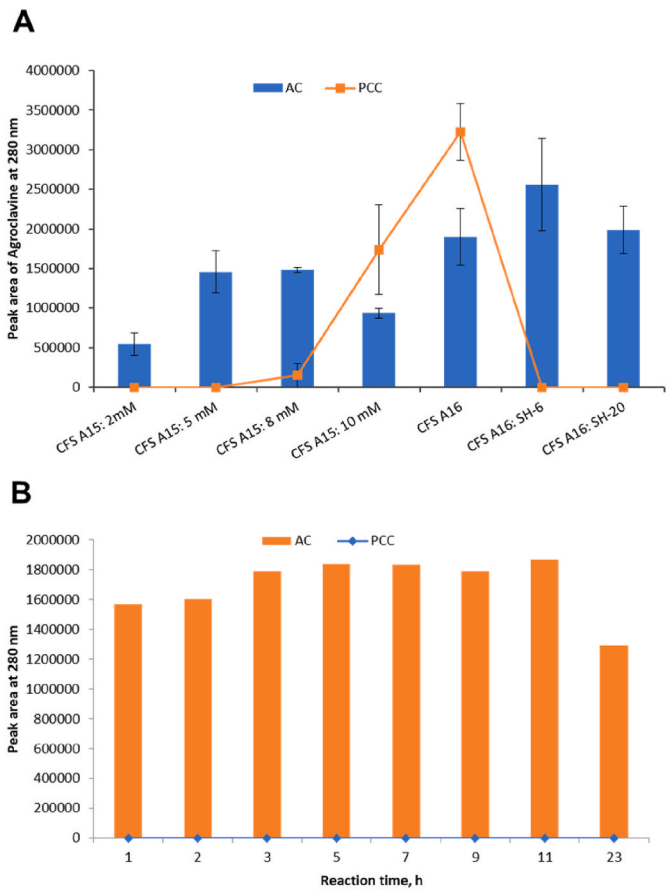

Fig. 4. Engineering the CFSs for the overproduction of AC. (A) AC-production of CFS A15 with different substrate concentrations, and AC-production of CFS A16 under different reaction conditions. (B) time-course study of AC production of the CFS A15 with 5 mM substrate. For Fig. 4A, the assay was repeated three times, and the error bars represent the standard deviation (n = 3).

completed in 1 h and the titer was not significantly improved when the reaction lasted more than 1 h (Fig. 4B).

When keeping the concentrations of all the enzymes and cofactors in CFS A15 unchanged, we further increased the substrate concentration to 8 mM, which only slightly improved the titer of AC (Fig. 4A, CFS A15: 8 mM). In addition, treating CFS A15 with 10 mM PCC gave an even lower production with a large amount of remaining substrate (Fig. 4A, CFS A15: 10 mM), probably due to the high portion of DMSO in the PCC solution that reduces the activities of enzymes. Thus, we used the substrate concentration of 8 mM for all further optimizations.

We further improve the titer of AC by the construction of a new CFS A16, which contains 3.5-fold of enzymes and cofactors compared to the CFS A1 (Table 1). The new CFS A16 showed a 30% improvement in the AC-production compared to CFS A15 (Fig. 4A, CFS A16). Finally, the reactions of CFS A15 were proceeded in the shaker at 220 rpm for 6 h and 20 h, respectively. Indeed, the incubation of CFS A16 in the 6-h shaking led to a significantly higher titer (Fig. 4A, CFS A16: SH-6H), which is nearly five-fold of that of CFS A1, whereas the reaction in 20-h shaking didn't generate notable improvement (Fig. 4A, CFS A16: SH-12H). Using the calibration curve of AC that we established previously [19], the production of AC of CFS A16 in the 6-h shaking condition is defined as 1209 mg/L, which is the highest titer that has been reported so far, to the best of our knowledge, with an overall conversion rate of 63%.

4. Discussion

The combined system established in this study seems to be superior compared to either the MCF system or the CFS system. The non-accessible soluble protein of EasE as well as the expensive cofactors, substrates, and a large number of enzymes required make the overproduction of ergot alkaloids via the CFS system difficult to achieve. In accord with this, no such studies have been reported for ergot alkaloids or AC (Fig. 5). On the other hand, the highest production of AC using the MCF systems is up to 79 mg/L (Fig. 5), which is not profitable for industrial production. To overcome those dilemmas, a combined MCF (*A. nidulans*)-CFS system was developed for the overproduction of AC in the current study, which led to the highest titer (1209 mg/L, Fig. 5) in the literature to the best of our knowledge.

Currently, over half of the ergot alkaloids were still produced by a field-production mode, while the ergot alkaloids isolated from ergot fermentation can be impure because of the abundance of similar metabolites. These difficulties are exacerbated for ones with a low natural abundance, such as chanoclavine and AC. Thus, developing a new mode to synthesize ergot alkaloids could be beneficial for the production of ergot-based pharmaceuticals. We apply a *A. nidulans*-based MCF to overproduce PCC at the titer of 2050 mg/L in the bioreactor, followed by the CFS system based on a four-enzyme cascade to overproduce the tetracyclic ergot alkaloid AC at the titer of 1209 mg/L. Future optimization of this combined system would further improve the AC production and accelerate the industrial production of this clinically important natural product. The concept of this new combined MCF-CFS system, with picking a suitable splitting point as the key step, could be further applied for the biosynthesis of more structurally complex natural products and pharmaceutical molecules, which are currently inaccessible through either the MCF or CFS systems.

CRediT authorship contribution statement

Zhi-Pu Yu: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Chunyan An:** Conceptualization,

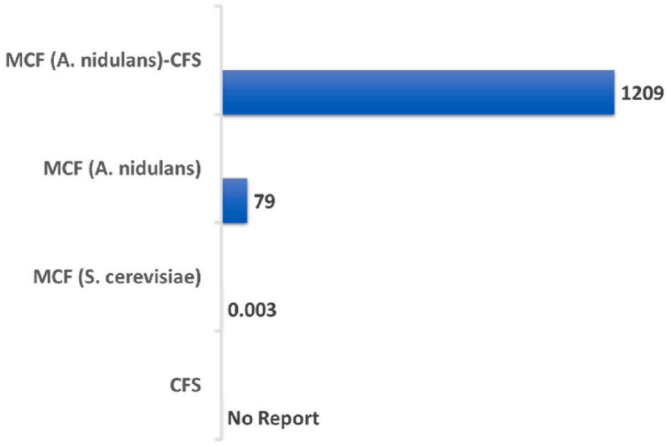

Fig. 5. The overall titers (mg/L) of AC generated from different strategies.

Table 1
The optimal reaction parameters of CFSs.

PCC	EasC	EasD	EasA	EasG	NADPH	$NADP^+$	Buffer	Shaking Speed	Time
8 mM	35 μM	35 μM	70 μM	35 μM	14 mM	3.5 mM	Tris-HCL, pH = 7.5	220 rpm	6 h

Methodology. **Yongpeng Yao:** Methodology, Investigation, Funding acquisition. **Chang-Yun Wang:** Writing – review & editing. **Zhoutong Sun:** Supervision. **Chengsen Cui:** Supervision. **Ling Liu:** Supervision, Project administration, Writing – review & editing. **Shu-Shan Gao:** Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Acknowledgments

This study was supported by the National Key Research and Development Program of China (grant nos. 2021YFC2100600, 2019YFA0905100 and 2018YFA0901600), the National Natural Science Foundation of China (grant nos. 31872614, 32022002, 21977113), the Youth Scientists Innovation Promotion Association of CAS (2019090) to S.S.G., Innovative Cross Team project of Chinese Academy of Sciences, CAS (grant no. JCTD-2019-06), Shandong Provincial Natural Science Foundation (Major Basic Research Projects) (grant no. ZR2019ZD18). We would like to thank Prof. Ping Zhu and Dr. Jingjing Chen for kindly providing the cDNA library of *C. purpurea*.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.08.003.

References

[1] Liu H, Jia Y. Ergot alkaloids: synthetic approaches to lysergic acid and clavine alkaloids. Nat Prod Rep 2017;34(4):411–32.
[2] Gross JD, Gross M. Genetic analysis of an Escherichia coli strain with a mutation affecting DNA polymerase. Nature 1969;224(5225):1166–8.
[3] Křen V, Brěmek J, Flieger M, Kozová J, Malinka Z, Řeháček Z. Bioconversion of agroclavine by free and immobilized Claviceps fusiformis cells. Enzym Microb Technol 1989;11:685–91.
[4] Jakubczyk D, Cheng JZ, O'Connor SE. Biosynthesis of the ergot alkaloids. Nat Prod Rep 2014;31(10):1328–38.
[5] Gerhards N, Neubauer L, Tudzynski P, Li S-M. Biosynthetic pathways of ergot alkaloids. Toxins 2014;6(12):3281–95.
[6] Ramzi AB. Engineering of microbial cell factories for omics-guided production of medically important biomolecules. In: Microbial cell factories engineering for production of biomolecules; 2021. p. 393–406.
[7] Gallego-Jara J, Terol GL, Martinez RAS, Diaz MC, de Diego Puente T. Engineering of microbial cell factories for production of plant-based natural products. In: Microbial cell factories engineering for production of biomolecules; 2021. p. 381–92.
[8] Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Fleck M, Bajad S, Dang G, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 2013;496(7446):528–32.
[9] Galanie S, Thodey K, Trenchard IJ, Filsinger Interrante M, Smolke CD. Complete biosynthesis of opioids in yeast. Science 2015;349(6252):1095–100.
[10] Luo X, Reiter MA, d'Espaux L, Wong J, Denby CM, Lechner A, Zhang Y, Grzybowski AT, Harth S, Lin W, Lee H, Yu C, Shin J, Deng K, Benites VT, Wang G, Baidoo EEK, Chen Y, Dev I, Petzold CJ, Keasling JD. Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. Nature 2019;567(7746):123–6.
[11] Srinivasan P, Smolke CD. Biosynthesis of medicinal tropane alkaloids in yeast. Nature 2020;585(7826):614–9.
[12] Lu Y, Lu Y. Cell-free synthetic biology: engineering in an open world. Synth Syst Biotechnol 2017;2(1):23–7.
[13] Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, Ueda T. Cell-free translation reconstituted with purified components. Nat Biotechnol 2001;19(8):751–5.
[14] Rollin JA, Tam TK, Zhang YHP. New biotechnology paradigm: cell-free biosystems for biomanufacturing. Green Chem 2013;15(7):1708–19.
[15] Huffman MA, Fryszkowska A, Campos KR, Canada KA, Devine PN, Forstater JH, Grosser ST, Halsey HM, Hughes GJ, Jo J, Joyce LA, Kolev JN, Maloney KM, Mann BF, Marshall NM, McLaughlin M, Moore JC, Murphy GS, Nawrat CC, Patel NR, Robaire SA, Truppo MD, Whittaker AM, Xu Y, Yang H, Alvizo O, Borra-Garske M, Duan D, Liang J, Nazor J, Novick S, Rodriguez-Granillo A, Sherer EC, Verma D, Xiao L. Design of an in vitro biocatalytic cascade for the manufacture of islatravir. Science 2019;366(6470):1255–9.
[16] Jakubczyk D, Cheng JZ, O'Connor SE. Biosynthesis of the ergot alkaloids. Nat Prod Rep 2014;31(10):1328–38.
[17] Yao Y, An C, Evans D, Liu W, Wang W, Wei G, Ding N, Houk KN, Gao S-S. Catalase involved in oxidative cyclization of the tetracyclic Ergoline of fungal ergot alkaloids. J Am Chem Soc 2019;141(44):17517–21.
[18] Nielsen CAF, Folly C, Hatsch A, Molt A, Schroeder H, O'Connor SE, Naesby M. The important ergot alkaloid intermediate chanoclavine-I produced in the yeast Saccharomyces cerevisiae by the combined action of EasC and EasE from Aspergillus japonicus. Microb Cell Factories 2014;13:95/1–95/11.
[19] Yao Y, Wang W, Shi W, Yan R, Zhang J, Wei G, Liu L, Che Y, An C, Gao S-S. Overproduction of medicinal ergot alkaloids based on a fungal platform. Metab Eng 2022;69:198–208.
[20] Cheng JZ, Coyle CM, Panaccione DG, O'Connor SE. Controlling a structural branch point in ergot alkaloid biosynthesis. J Am Chem Soc 2010;132(37):12835–7.
[21] Matuschek M, Wallwey C, Xie X, Li S-M. New insights into ergot alkaloid biosynthesis in Claviceps purpurea: an agroclavine synthase EasG catalyses, via a non-enzymatic adduct with reduced glutathione, the conversion of chanoclavine-I aldehyde to agroclavine. Org Biomol Chem 2011;9(11):4328–35.