Induced effect of Ca$^{2+}$ and Al$^{3+}$ on chaetominine synthesis by *Aspergillus fumigatus* CY018 under submerged fermentation

Changqing Liu$^{1}$ | Tianwen Chen$^{2}$ | Jijie Chen$^{1}$ | Yuxuan Zhou$^{1}$ | Lina Deng$^{1}$ | Gongneng Feng$^{1}$ | Jian Gao$^{1}$ | Huixing Liang$^{1}$

$^{1}$ College of Marine and Biological Engineering, Yancheng Institute of Technology, Yancheng, Jiangsu, People’s Republic of China

$^{2}$ Economic and Trade Department, Yancheng Polytechnic College, Yancheng, Jiangsu, People’s Republic of China

**Abstract**

Chaetominine (CHA), an alkaloid with a biological activity obtained from *Aspergillus fumigatus* CY018, has strong anticancer activity against the human leukemia cells. However, its physiological and biochemical research is limited by CHA yield in the liquid-state fermentation, which is a problem that urgently needs effective biological solution. In this work, Ca$^{2+}$ and Al$^{3+}$ were found to have a strong promoting effect on CHA production after multiple metal ions screening. Then, the addition condition of Ca$^{2+}$ and Al$^{3+}$ was, respectively, optimized CHA production and dry cell weight. The intermediate metabolites were increased with coaddition of Ca$^{2+}$ and Al$^{3+}$. The activities of key enzymes of DAHPs, AroAs, and TrpCs in the CHA biosynthesis pathway were improved by 3.58-, 3.60-, and 3.34-fold, respectively. Meanwhile, the transcription level of *laeA*, *dahp*, *cs*, and *trpC* was upregulated by 3.22-, 12.65-, 5.58-, and 6.99-fold, respectively, by coaddition of Ca$^{2+}$ and Al$^{3+}$. Additionally, the fermentation strategy was successfully scaled up to a 5-L bioreactor, in which CHA production could attain 75.6 mg/L at 336 h. This work demonstrated that Ca$^{2+}$ and Al$^{3+}$ coaddition was an effective strategy for increasing CHA production, and the information obtained might be useful in the fermentation of filamentous fungi with the addition of metal ions.

**KEYWORDS**

aluminum ion, *Aspergillus fumigatus*, calcium ion, chaetominine, submerged fermentation

**Abbreviations:** CHA, Chaetominine; DCW, dry cell weight; DAHP, 3-deoxy-7-phosphoheptulonate; AroA, pentafunctional polypeptide; TrpC, anthranilate synthase; *cs*, chorismate synthase; *A. fumigatus*, *Aspergillus fumigatus*; PDA, potato dextrose agar; PDB, potato dextrose broth; ANOVA, analysis of variance; mM, mmol/L; EMP, Embden-Meyerhof-Parnas; SOD, superoxide dismutase; APX, ascorbate peroxidase; CAT, Catalase; PGM, phosphoglucomutase; PEP, phosphoenolpyruvate; DO, dissolved oxygen.

*Biotechnol Appl Biochem.* 2022;69:2733–2744. wileyonlinelibrary.com/journal/bab 2733
1 | INTRODUCTION

Aspergillus sp. is an endophytic fungus and has received considerable attention of researchers in recent years. Aspergillus fumigatus, an endophyte found in portunus, plants, and others, has an outstanding ability of synthesizing primary and secondary bioactive metabolites. The secondary metabolites biosynthesized by A. fumigatus have shown multiple biological activities for common human diseases. Fumigaclavine C was fermented by A. fumigatus CY018 through the two-stage culture process (oscillation and rest), which was an ergot alkaloid with anti-inflammatory and antipsychotic activity. The anti-tumor compound of Epothilone B could arrest cancer cells at the G2-M phase, the yield of which was improved with response surface methodology by A. fumigatus. Chaetominine (CHA) (Figure S1 in the Supporting Information), a quinazolinone alkaloid from the endophytic fungi of A. fumigatus CY018, was found to possess greater anticancer activity than 5-fluorouracil, which implied that CHA could be developed as an anticancer candidate in the future. Moreover, it is reported that CHA was able to inhibit the germination and growth of wheat and radish at low concentrations. However, the in-depth physiological and biochemical research of CHA has been seriously limited by the low yield in the submerged fermentation. Therefore, further enhancement of CHA production is urgently required considering its potential use in the field of medicine.

Numerous effective biological strategies are suggested to improve the yield of secondary metabolites by a microorganism, such as medium component optimization, temperature regulation, oxygen concentration regulation, and others. Microbial growth and secondary metabolite biosynthesis were significantly regulated by metal ions as suggested in the literature. Luo et al. reported an effective strategy of the metal ions (Zn\(^{2+}\), Cu\(^{2+}\), and Mg\(^{2+}\)) coaddition could enhance the production of botrallin and TMC-264 by Hyalodendriella sp. Ponipodefi2. Dzurendova et al. determined the effects of varying levels of metal and phosphate ions on the growth and metabolism of Mucor circinelloides, demonstrating that Mg and Zn ions were essential for the growth and metabolic activity of M. circinelloides. The effects of different metal ions (Mg\(^{2+}\), Al\(^{3+}\), and Fe\(^{3+}\)) on the growth, lipid accumulation, and sedimentation of Scenedesmus sp. were explored by Kong et al., indicating that Fe\(^{3+}\) could promote the microalgal lipid biosynthesis and secretion in batch culture and the addition of Al\(^{3+}\) could enhance the sedimentation efficiency, whereas the addition of Al\(^{3+}\) had an inhibitory effect on the biomass of Scenedesmus sp. R-16 and lipid biosynthesis. It was evidently shown that the effect of metal ions would improve the biosynthesis of active metabolites by a microbe.

We also found that the addition of metal ions could change the synthesis of metabolites by A. fumigatus CY018 in the preliminary experiment. Therefore, the selection and optimization of metal ions on CHA production and biomass were investigated. Subsequently, the transcription level and enzyme activity of specific genes were analyzed by the addition of metal ions. The concentration of intermediate metabolites in the synthetic route of CHA was analyzed to demonstrate the regulatory mechanism of metal ions for CHA biosynthesis. Moreover, the additional strategy of metal ions was successfully reproduced in the 5-L bioreactor. This work would be useful in enhancing the production of target compounds in submerged fermentation.

2 | MATERIALS AND METHODS

2.1 | Microorganism

A. fumigatus CY018 as a filamentous fungus was kindly provided by the East China University of Science and Technology. The conidia of A. fumigatus CY018 were stored in 40% glycerin at –80°C in a refrigerator. The mycelia were activated on potato dextrose agar (PDA) medium and stored at 4°C in a refrigerator. A. fumigatus CY018 was transferred to a fresh medium every month.

2.2 | Fermentation conditions

The mycelia of A. fumigatus CY018 stored at 4°C in a refrigerator were first transferred and cultured on PDA at 28°C for 7 days in a biochemical incubator. An ager block (size of 1 cm\(^2\)) was cut from the PDA and transferred to a 500-mL shake flask with 200 mL potato dextrose broth, which was cultured as seed medium in a shaking incubator at 180 rpm, 28°C for 72 h. Subsequently, 7 mL of seed medium was transferred to a 250-mL Erlenmeyer flask with 50 mL fermentation medium, which was cultured in the shaking incubator at 180 rpm, 28°C for 384 h. The composition of the fermentation medium was as follows: 100 g/L sucrose, 5 g/L ammonium acetate, 2 g/L sodium tartrate, 2.4 g/L sodium glutamate, 1.2 g/L KH\(_2\)PO\(_4\), 0.84 g/L MgSO\(_4\)⋅7H\(_2\)O, 0.016 g/L FeSO\(_4\)⋅7H\(_2\)O.

2.3 | Selection and optimization of metal ions

The biomass and CHA production was detected with the addition of 0.5 and 1 mM of metal ions (AlCl\(_3\), CaCl\(_2\), CuCl\(_2\), MnCl\(_2\), and ZnCl\(_2\)) at 0 h of fermentation. These solutions of metal ions were sterilized in an autoclave at 121°C for 25 min. Whereafter, the addition concentration
and addition time of CaCl$_2$ and AlCl$_3$ were further optimized biomass and CHA production.

### 2.4 Metabolite analysis

After fermentation, the growth of *A. fumigatus* CY018 was measured by dry cell weight (DCW) with the total mycelium in the 250-mL Erlenmeyer flask. The fermented hypha was harvested by the suction filter device with the weighted filter paper. The filtered mycelium was dried until constant weight was obtained, and it was weighed with an analytical balance.

The determination of residual sugar in the fermentation broth was utilized by the revised anthrone sulfuric acid method. The sucrose standard curve was prepared by the experimental process of the phenol sulfuric acid method, which was used for the determination of residual sugar content in the fermentation broth. One milliliter of broth from the shake flask was taken to a mixed broth and centrifuged at 13,000 rpm and 4°C, in which the supernatant was diluted 100 times. Then, the diluent was measured at 485 nm by a spectrophotometer, and the absorbance value was used with the standard curve of the sucrose concentration to calculate the amount of residual sugar in the fermentation broth.

The concentration of CHA was analyzed by HPLC with a C18 column (4.6 mm × 250 mm, 5 μm, Agilent ZORBAX Eclipse XDB-C18) under 226 nm, and the method is described in detail by Liu et al.$^{14}$ The HPLC condition was followed: the mobile phase was acetonitrile/water (40:60, v/v); the flow rate was 1 mL/min; column temperature was 25°C; sample injection volume was 20 μL. The concentration of CHA was measured in comparison with the standard, which was isolated and purified in the laboratory.

The detection of organic acids (pyruvate, DAHP, chorismate, and tryptophan) during the CHA biosynthetic pathway was utilized by HPLC with the Agilent Hi-plex ligand exchange column (7.7 mm × 3000 mm, 8 μm) under 338 nm, in which the detector was a UV detector. The HPLC condition for organic acids was followed: the mobile phase was 0.01% trifluoroacetate; the flow rate was 1 mL/min; column temperature was 25°C; sample injection volume was 20 μL. The concentration of organic acids was measured in comparison with the standard.

### 2.5 qRT-PCR for key genes

One milliliter of broth from the shake flask was taken from a mixed broth and centrifuged at 13,000 rpm and 4°C, in which the supernatant was carefully removed. The fungus was ground and broken by a mortar with liquid nitrogen in the asepsis room. Then, 200 μL of double distilled water was utilized to dissolve the extract, which was used to analyze the bioactivity and transcription level of a key gene at taken points. The TRIzol solution was utilized to extract the total RNA of *A. fumigatus* CY018 at a sampling point of fermentation. The extracted solution was used for the RNA concentration using a spectrophotometer at A260/280. The method of Li et al.$^{15}$ was utilized to detect the transcription level of *laeA*, *dahp*, *cs*, *trpC*, and *actin* with coaddition of Ca$^{2+}$ and Al$^{3+}$. The extracted RNA and the Premix Ex Taq II Kit (Takara) were used for reverse transcription. The Premix Ex Taq TM II (Takara) was utilized to determine transcriptional levels of key genes according to the manufacturer’s procedure. The sequences of primer pairs for PCR amplification and qRT-PCR assay are displayed in Tables 1 and 2.

The CFX96 real-time PCR detection system was used for the qRT-PCR experiment, and the test kit was SYBR Premix Ex Taq II Kit. The transcriptional level of the measured genes was corrected by the conserved gene Actin as a normalized internal standard. The process of qRT-PCR followed as first pre-denatured at 95°C for 5 min, and amplification occurred in two steps: 5 s at 95°C for denaturing, 30 s at 55°C for annealing and 60 s at 72°C for extension for 40 cycles. The CFX Manager software was used to calculate standard curves, CT values, and detect the transcriptional level of samples.

### 2.6 Biochemical analysis of key enzymes

One milliliter of broth from the shake flask was taken to a mixed broth and centrifuged at 13,000 rpm and 4°C, in which the supernatant was carefully removed. The precipitated thallus was broken by a mortar with liquid nitrogen in the asepsis room. Then, 200 μL of double distilled water was utilized to dissolve the extract, which was used for analysis of bioactivity of a key enzyme in the shikimate pathway. The bioactivity of DAHP synthase (DAHPs) was analyzed as described by Liu et al.$^{16}$ A substrate solution and 100 μL of treated crude enzyme solution were incubated at 37°C for 10 min. Subsequently, the mixture was added with 0.2 mL of 10% trichloroacetic acid and then centrifuged at 13,000 rpm for 5 min. The 0.25-mL mixed solution was added with HIO$_4^-$, NaAsO$_3^-$, thiobarbital for the reaction, and the final solution was centrifuged fast and determined with a spectrophotometer at 549 nm by measuring the decrease of PEP.

The AroA synthase (AroAs) was measured at 28°C in 50-μL reaction volume mixtures (50 mM of Hepes buffer (pH 7.0), 1 mM of shikimate-3-phosphate, 1 mM of PEP, and 0.4 μg of the purified cell disruption). The detection
TABLE 1  Sequences of primer pairs for PCR amplification

| Target gene | Primer name  | Primer sequence (5′–3′) |
|-------------|--------------|------------------------|
| laeA        | laeA-forward | CCAAGCTTATGTTTCTCAACGGGCAGGGCGGA |
|             | laeA-reverse | CGCGGATCCTCATTGCGAGGATTTTC |
| Dahp        | dahp-forward | CCAAGCTTATGTTGAGCCCAGACCTCCACAG |
|             | dahp-reverse | CGCGGATCCTCAAGACCATGGGAGCTTTTG |
| Cs          | cs-forward   | CCAAGCTTATGTTGACGAGTCGCTCAG |
|             | cs-reverse   | CGCGGATCCTCAAGACCATGGGAGCTTTTG |
| trpC        | trpC-forward | CCAAGCTTATGTTGACGAGTCGCTCAG |
|             | trpC-reverse | CGCGGATCCTCATTGCGAGGATTTTC |

TABLE 2  Sequences of primer pairs for quantitative real-time RT-PCR (qRT-PCR) assay

| Target gene | Primer name  | Primer sequence (5′–3′) |
|-------------|--------------|------------------------|
| laeA        | laeA-forward | TTCTTTCGAGCTGCCGTCAA |
|             | laeA-reverse | TCCATGGTATGTCCGTGCT |
| Dahp        | dahp-forward | GACAGGACAATGCCGTAGC |
|             | dahp-reverse | TCAGGACAGGTATGATTAGCG |
| Cs          | cs-forward   | CCCCCGAACAAATGAATCGC |
|             | cs-reverse   | GGTACTTCTCGCAATGGCT |
| trpC        | trpC-forward | AGGTCGACGTCTTTGCAGT |
|             | trpC-reverse | AGTCACCTTCAACGGAGGAG |
| actin       | actin-forward | TCCGAGACCTCAACGGAGGAG |
|             | actin-reverse | ATGGGACACGTAGTGAC |

enzyme activity of AroAs was determined by following the method of Liu and Cao. TrpC synthase (TrpCs) was measured through the reaction of indole and l-serine with purified cell disruption, following the modified method as described by Zhao et al.

2.7  Scale-up experiments

The experiments of lab-scale fermentation process were performed in a 5-L stirred bioreactor as mentioned by Liu et al., in which was coadded with Ca$^{2+}$ and Al$^{3+}$ as the optimized concentration for 120 h. Samples were taken at an interval of 48 h.

2.8  Statistical analysis

All data obtained in the shake flask and 5-L bioreactor are the mean of experiments in triplicate. The statistical significance of differences in optimization of metal ions and detection of parameters (intermediate metabolites, enzyme activity, and transcription level of key enzymes) was evaluated using a one-way analysis of variance (ANOVA) and Duncan’s multiple range tests in SPSS version 16.0. A value of $p < 0.05$ was considered statistically significant.

3  RESULTS

3.1  Effects of multiple metal ions for biomass and CHA production

The strategy of metal ions addition was usually beneficial to the growth of microbe and the biosynthesis of active metabolites. In the present work, the different content (0.5 and 1 mM) of five metal ions (Al$^{3+}$, Ca$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$) were individually tested for selecting the appropriate metal ion for CHA production and biomass of *A. fumigatus* in Figure 1. The effect of 0.5 mM Al$^{3+}$ could effectively enhance the CHA production (52.3 mg/L), which was 1.5 times that of the control. The higher concentration (1 mM) of Al$^{3+}$-promoted CHA synthesis was less than the low concentration. The production of CHA was achieved, respectively, 55.31 and 40.18 mg/L by the addition of 0.5 and 1 mM Ca$^{2+}$. It was found that the addition of Cu$^{2+}$ and Al$^{3+}$ could increase CHA production, but it had no obvious effect on the biomass of *A. fumigatus*. The effect of Cu$^{2+}$ on CHA production was decreased with the increase in the concentration, meanwhile the effect of Cu$^{2+}$ on biomass was increased. A similar result was shown by the effect of Mn$^{2+}$, in which the inhibition effect was stronger than that of Cu$^{2+}$. The effect of Zn$^{2+}$ completely inhibited CHA biosynthesis and decreased the biomass of *A. fumigatus*. In
comparison with the effects of five metal ions, the low concentration of Ca$^{2+}$ and Al$^{3+}$ could significantly improve the CHA production, which indicated that Ca$^{2+}$ and Al$^{3+}$ were dominant metal ions for the CHA biosynthesis.

3.2 Optimization of addition time and concentration by dominant metal ions

For further enhancement of CHA production, the addition concentration and time of Ca$^{2+}$ and Al$^{3+}$ were, respectively, investigated, which was based on selection results of metal ions. The different concentrations (0, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7 mM) of Ca$^{2+}$ were added and analyzed about CHA production and biomass of *A. fumigatus* in the Figure 2A. The tendency of CHA production was gradually rose and then slowly declined with the increasing concentration of Ca$^{2+}$. When the content of Ca$^{2+}$ was 0.5 mM, the CHA production reached the maximum value (58.8 mg/L), which was ascertained as the optimized concentration. However, the effect of the Ca$^{2+}$ concentration was not obvious on the biomass of *A. fumigatus*, in which biomass of 0.5 and 0.55 mM Ca$^{2+}$ was relatively lower than others. After the addition concentration of Ca$^{2+}$ was confirmed, the addition time (0, 72, 120, 168, 216, 264, 312 h) of Ca$^{2+}$ was subsequently studied and the results are shown in Figure 2B. The CHA production was sharply increased and reached the peak (62.74 mg/L, at 120 h) during 0–120 h, then became constant. The biomass of *A. fumigatus* had an analogous tendency at optimization of the concentration; which was that the tendency of biomass was not correspond to that of CHA production. At this point, the optimized addition concentration and time of Ca$^{2+}$, respectively, were determined as 0.5 mM and 120 h.

The analogous experimental methods were utilized to research the effect of the Al$^{3+}$ concentration and time on the CHA production and biomass of *A. fumigatus*. Similarly, the test concentration of Al$^{3+}$ was designed as 0, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7 mM, and the results are displayed in Figure 3A. The concentration of Al$^{3+}$ was increased slowly from 0 to 0.45 mM, then remained constant from 0.45 to 0.6 mM, and decreased slowly from 0.6 to 0.7 mM. When the content of Al$^{3+}$ was 0.5 and 0.55 mM, the CHA production was, respectively, 49.45 and 49.15 mg/L. The effect of the addition concentration of Al$^{3+}$ on biomass was weak, the trend of which was relatively constant. The appropriate addition concentration of Al$^{3+}$ was defined as 0.5 mM, then the addition time of Al$^{3+}$ was determined. The addition time of Al$^{3+}$ was set at 0, 72, 120, 168, 216, 264, 312 h, and the results are shown in Figure 3B. The effect of addition time of Al$^{3+}$ was increased quickly
and then decreased slowly, in which the maximum value of the CHA production was 50.43 mg/L at 120 h. Therefore, the optimized conditions of Ca$^{2+}$ and Al$^{3+}$ addition were determined, which was added with 0.5 mM Ca$^{2+}$ and Al$^{3+}$ at 120 h.

### 3.3 Intermediate metabolites response to metal ions addition

The addition condition of Ca$^{2+}$ and Al$^{3+}$ was already optimized (0.5 mM, 120 h), then the change in the trend of intermediate metabolites in the synthesis pathway of CHA was studied by exploring the mechanism of metal ions addition. Pyruvate was rapidly formed at the early stage of fermentation (24–96 h) and slowly consumed at the late stage of fermentation, in which the concentration of pyruvate after coaddition of Ca$^{2+}$ and Al$^{3+}$ was lower than the control as shown in Figure 4A. Before 120 h of fermentation, the change and concentration of DAHP were similar to both the experimental group and control group. The production of DAHP with the addition of metal ions was higher than the control from 168 to 312 h (Figure 4B). The tendency of the chorismate biosynthesis was similar to that of DAHP, in which the biosynthetic capacity of chorismate was also higher than the control after coaddition of Ca$^{2+}$ and Al$^{3+}$ (Figure 4C). The concentration of tryptophan with coaddition of Ca$^{2+}$ and Al$^{3+}$ was fast increased from 0 to 216 h and then gradually decreased from 216 to 360 h, in which the produced tryptophan was far more than the control during 120–384 h. Clearly, the addition of Ca$^{2+}$ and Al$^{3+}$ was beneficial to the biosynthesis of intermediate metabolites (DAHP, chorismite, and tryptophan) in the CHA biosynthetic pathway.

### 3.4 Enzyme expression response to metal ions addition

After investigating intermediate metabolites with coaddition of Ca$^{2+}$ and Al$^{3+}$, the activities of key enzymes (DAHPs, AroA, and TrpC) in the CHA biosynthetic pathway were studied. The key enzymes played an important role in the CHA production with the addition of metal ions. DAHPs was a key enzyme in the shikimate pathway, which was also an important enzyme in the CHA biosynthetic pathway. The activity of DAHPs with coaddition of Ca$^{2+}$ and Al$^{3+}$ was increased first and then decreased, which was all higher than the control at every sampling time. DAHPs activity was significantly improved 3.58-fold than control at 216 h (Figure 5A). AroAs induced the synthesis of 3-dehydroquinic acid, which was an important node of the CHA biosynthetic pathway. The activity of AroAs in the trend of AroAs activity was analogous to DAHPs as shown in Figure 5B, and the AroAs activity was improved with different degrees. TrpCs induced indole glycerol phosphate to synthesize tryptophan, which was a rate-limiting enzyme in the CHA biosynthetic pathway. The coaddition of Ca$^{2+}$ and Al$^{3+}$ did not have very obvious effect compared with the other two enzymes, but the TrpCs activity was increased 3.34- and 1.81-fold than control at 168 and 216 h, respectively (Figure 5C). The effect of Ca$^{2+}$ and Al$^{3+}$ coaddition was useful in enhancing the activities of key enzymes in the CHA biosynthetic pathway.

### 3.5 Gene expression response to metal ions addition

In the previous work, the author explored the biosynthetic pathway of CHA and found that the key genes significantly
The effect of Ca\(^{2+}\) and Al\(^{3+}\) addition on the accumulation of intermediate metabolites in the CHA biosynthesis pathway. (A) Pyruvate; (B) DAHP; (C) chorismate; (D) tryptophan. Data points are the average of \(n = 3\); error bars represent standard error about the mean. The addition condition of Ca\(^{2+}\) and Al\(^{3+}\) were both 0.5 mM and at 120 h of fermentation time.

affected the biosynthesis of CHA).\(^{16,19}\) To further explore the underlying mechanism of coaddition of Ca\(^{2+}\) and Al\(^{3+}\) in CHA production, transcription levels of four genes \(\text{laeA}\) (a global regulator), and \(\text{dahp}\), \(\text{cs}\), and \(\text{trpC}\) (CHA biosynthetic pathway) were analyzed by qRT-PCR. The expression levels of these genes with coaddition of Ca\(^{2+}\) and Al\(^{3+}\) at 120, 168, 216, 264, 312, and 360 h were sampled and investigated (Figure 6).

As shown in Figure 6A, the transcription level of \(\text{laeA}\) was obviously upregulated at 168, 216, and 264 h, which were, respectively, 2.89-, 3.22-, and 2.46-fold than the control. The expression level of \(\text{dahp}\) was increased by about 12.65-fold at 168 h and 5.51-fold at 216 h (Figure 6B), which were affected stronger than other genes with a supplement of Ca\(^{2+}\) and Al\(^{3+}\). The transcription level of the \(\text{cs}\) gene was upregulated at 216, 264, and 312 h and expressed with 5.58-, 3.32-, and 2.67-fold increase with the addition of Ca\(^{2+}\) and Al\(^{3+}\) (Figure 6C). The change in the trend of \(\text{trpC}\) gene was similar to \(\text{dahp}\), but the effect of the \(\text{trpC}\) gene was less than \(\text{dahp}\). The expression of \(\text{trpC}\) was increased by about 6.99-fold at 168 h and 2.12-fold at 216 h (Figure 6D). These results indicated that the key genes of the CHA biosynthetic pathway were upregulated by coaddition of Ca\(^{2+}\) and Al\(^{3+}\).

3.6 Scale-up CHA production to a lab-scale bioreactor

The supplement effect of Ca\(^{2+}\) and Al\(^{3+}\) was observed, and the scale-up experiment was carried out in the 5-L stirred bioreactor. As shown in Figure 7A, B, the time profiles of the 5-L bioreactor and shake flask with coaddition of Ca\(^{2+}\) and Al\(^{3+}\) were investigated. The trend of DCW was similar to both the 5-L bioreactor and shake flask, and it was increased slowly then declined slightly. However, the value of DCW in the 5-L bioreactor was little lower than that in the shake flask at the end of fermentation. The change in the trend of residual sugar was also analogous in the 5-L bioreactor and shake flask, and the consumption rate of sugar in the 5-L bioreactor was faster than that in the shake flask. The tendency of pH was consistent, which was increased fast then declined and finally stabilized at around 4 in the 5-L bioreactor and shake flask. When Ca\(^{2+}\)
and Al$^{3+}$ were coadded in the 5-L bioreactor, the value of pH rose and then declined quickly at 120 h. The DO continued to decrease during 0–120 h as shown in Figure 6B, then it became constant at 20% from 120 to 336 h. The trend of CHA production in the 5-L bioreactor and shake flask was both first increased then slowly decreased. The maximum CHA production in the 5-L bioreactor was 75.6 mg/L at 336 h, which was higher than that (37.6 mg/L, at 360 h) in the shake flask. Therefore, the scale-up experiment was successfully performed in the laboratory, in which the residual sugar, pH, and CHA production in the 5-L bioreactor were all better than the shake flask.

4 | DISCUSSION

4.1 Optimization of Ca$^{2+}$ and Al$^{3+}$ concentration and addition time

The effective factors improving the production of metabolites were studied by many researchers. The temperature, pH, agitation speed, surfactants, metal ions, and so on were optimized to enhance the yield of the target compound.\textsuperscript{20–22} The metal ions play an important role in the growth and metabolite synthesis in the microbe.\textsuperscript{23,24} The results of the present study indicated that Ca$^{2+}$ and Al$^{3+}$ increased the production of CHA, and other ions had different degrees of reduction of the production of CHA in Figure 1. Clearly, the effect of Ca$^{2+}$ and Al$^{3+}$ on CHA production was better than that of Cu$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$. Lima et al.\textsuperscript{25} reported the effects of different metal ions (2.5 mmol L$^{-1}$ AgNO$_3$, CuSO$_4$, CaCl$_2$, CoCl$_2$, NaCl, FeSO$_4$, MgSO$_4$, MnSO$_4$, and ZnSO$_4$) on β-mannanase activities and found different metal ions had different auxo-action on enzyme activity. Chauhan and Jha\textsuperscript{26} found the metal ions (Na$^+$, K$^+$, Pb$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, and Co$^{2+}$) enhanced enzyme activity of laccase from \textit{Pseudomonas} sp. S2. Therefore, the promoted effect of metal ions on the biosynthesis of active metabolites was verified by researchers.

The addition concentration and time of Ca$^{2+}$ and Al$^{3+}$ were optimized, in which the effect of different addition of optimized Ca$^{2+}$ and Al$^{3+}$ had different influence on the biosynthesis of CHA. The optimized concentration and time of Ca$^{2+}$ and Al$^{3+}$ were both 0.5 mM and 120 h, in which the production of CHA was, respectively, 62.74 and 50.43 mg/L. A similar phenomenon has been reported in other studies. Wei et al.\textsuperscript{27} studied the induced effect of Ca$^{2+}$ on curvulamine synthesis by a marine-derived fungus \textit{Curvularia} sp. IFB-Z10, in which authors first investigated the selection of metal ions and optimization of addition concentration and time of Ca$^{2+}$. Homoplastically, the addition concentration and time of CaCl$_2$ were first investigated by exploring the mechanism of Ca$^{2+}$ on
The effect of Ca\(^{2+}\) and Al\(^{3+}\) addition on the transcriptional level of key genes in the CHA biosynthesis pathway. (A) laeA; (B) dahp; (C) cs; (D) trpC. Data points are the average of \(n = 3\); error bars represent standard error about the mean. The addition condition of Ca\(^{2+}\) and Al\(^{3+}\) were both 0.5 mM and at 120 h of fermentation time. * Indicates statistical significance (\(p < 0.05\)) compared to the control without inhibitors.

Therefore, the optimization of addition concentration and time of Ca\(^{2+}\) and Al\(^{3+}\) was necessary for further clarifying the mechanism of Ca\(^{2+}\) and Al\(^{3+}\) and enhancement of CHA production.

4.2 Effect of Al\(^{3+}\) and Ca\(^{2+}\) on metabolic parameters

The purpose of metabolic parameters was to analyze the mechanism of Ca\(^{2+}\) and Al\(^{3+}\) on the improvement of CHA production based on previous work. The accumulation of DAHP, chorismate and tryptophan with coaddition of Ca\(^{2+}\) and Al\(^{3+}\) was higher than the control group (Figure 3B–D). Meanwhile, the production of tryptophan was far higher than the control, which was an important intermediate metabolite in the CHA biosynthesis pathway and resulted in increasing CHA production. However, the amount of pyruvate with the addition of optimized metal ions was lower than the control (Figure 3A). The carbon source might flow to the EMP pathway instead of the CHA synthesis pathway, which led to lower pyruvate production. Analogously, Zhou et al.\(^{29}\) investigated the addition effect of calcium carbonate for promoting biosynthesis of L-methionine by metabolically engineered *Escherichia coli* W3110-BL, in which the concentration of organic acids (a-KG, succinic acid, lactic acid, formic acid, and acetic acid) had varying degrees of change with the addition of calcium carbonate.

In addition, the time course of DCW, residual sugar, pH, and CHA production are compared in Figure S2 in the Supporting Information. The trend of DCW and CHA production was improved after the addition of Ca\(^{2+}\) and Al\(^{3+}\). The residual sugar was consumed more quickly than the control. The pH was briefly increased after coaddition of optimized Ca\(^{2+}\) and Al\(^{3+}\), then showed the same trend of control. Chakraborty et al.\(^{30}\) explored the effect of aluminum on multiple parameters (chlorophyll and carotenoids, photosynthetic efficiency, starch, and sucrose) for expounding physiological mechanisms of aluminum toxicity tolerance in *Azolla microphylla* Kaulf.
The strategy of Ca and Al on the biosynthesis of CHA

And the change tendency of laccase, SOD, and CAT activities with the addition of Cu2+ were closely analogous to the present work. The phosphoglucomutase (PGM) activity from Cordyceps militaris was investigated with the addition of metal ions. And, results showed Ca2+, Zn2+, Mg2+, Fe2+, and Fe3+ could improve the activity of PGM and mRNA transcription level of the pgm gene. These results demonstrated that the activities of key enzymes helped to accumulate the target compounds with the addition of optimized metal ions.

The inner function of Ca2+ and Al3+ coaddition in the CHA biosynthesis was investigated at the transcription level of key genes (laeA, dahp, cs, and trpC) (Figure 6). The expression of laeA, as a global regulatory gene, was increased by coaddition of Al3+ and Ca2+, which meant the effect of metal ions addition might promote the cell development and production of CHA/intermediate metabolites in the CHA biosynthesis pathway. The impact of the global secondary metabolite regulators LaeA on echinocandin B production was researched, and enhanced with the use of industrial production strain Aspergillus pachycristatus NRRL 11440. Meanwhile, the transcription levels of dahp, cs, and trpC were also upregulated at different times and degrees. Clearly, the effect of time on the transcription level of dahp and trpC was reacted in advance of enzyme activities of DAHPs and TrpCs, which indicated that the effect of Ca2+ and Al3+ coaddition was in accordance with the the central law. A similar phenomenon was observed when elicitation (amendment) of aluminum chloride improved callus biomass growth and reserpine yield in Rauwolfia serpentina leaf callus, in which SOD, APX, and CAT activities were promoted by Al3+ addition. The enhancement of omega-3 fatty acids by Chlorella sorokiniana was obtained via Ca2+-induced homeoviscous adaptation, in which the relative expression profiles of accD and rbcL were upregulated by the addition of Ca2+. Xu et al. found that the induction of calcium ions could improve the production of ganoderic acid and intermediate metabolites (squalene and lanosterol), which resulted in upregulation of the transcription levels of key genes (hmgr, fps, sqs, and cyp) with the addition of Ca2+. Therefore, the inducing effect of Ca2+ and Al3+ lead to upregulate the transcription levels of key genes (laeA, dahp, cs, and trpC) and improving the activities of key enzymes (DAHPs, AroAs, and TrpCs) for enhancement of CHA production.

In conclusion, CHA could be taken as a drug candidate worthy of study owing to its attractive anticancer activity. Herein, the selected Ca2+ and Al3+ were optimized with
the addition concentration and time as 50 mM and 120 h, in which CHA production reached 62.74 and 50.43 mg/L with the suitable condition of Ca\(^{2+}\) and Al\(^{3+}\), respectively. The effect of Ca\(^{2+}\) and Al\(^{3+}\) coaddition improved the concentration of intermediate metabolites (DAHP, chorismate, and tryptophan) and activities of key enzymes (DAHPs, AroAs, and TrpCs). Simultaneously, the transcription levels of laeA, daph, cs, and trpC were upregulated by 3.22-, 12.65-, 5.58-, and 6.99-fold, respectively. Furthermore, the fermentation process was successfully scaled up to the 5-L bioreactor, in which CHA production could achieve 75.6 mg/L at 336 h and higher than that in the shake flask. This work will be helpful in further research and development of CHA and active secondary metabolites produced by other filamentous fungi.

ACKNOWLEDGMENTS
This work was supported by Funding for school-level research projects of Yancheng Institute of Technology (XJR2019066) and Jiangsu Science and Technology Special Project-Enriching Civilization and Enhancing the County (SZ-YC202040).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ORCID
Changqing Liu https://orcid.org/0000-0001-5274-8609

REFERENCES
1. Minh LTH, Hue NT, Linh NT, Ngan TB, Quyen VT, Anh NM, et al. Antimicrobial secondary metabolites from the marine-derived fungus Aspergillus sp. M28. Chem Nat Compd. 2020;56:1173-5.
2. Zhu YX, Yao LY, Jiao RH, Lu YH, Tan RX. Enhanced production of fumigaclavine C in liquid culture of Aspergillus fumigatus under a two-stage process. Bioresource Technol. 2014;152:162-8.
3. El-Sayed ASA, Shindia AA, Ali GS, Yassin MA, Hussein H, Awad SA, et al. Production and bioprocess optimization of antitumor epothilone B analogue from Aspergillus fumigatus, endophyte of Catharanthus roseus, with response surface methodology. Enzyme Microb Technol. 2021;143:109718.
4. Yao JY, Jiao RH, Liu CQ, Zhang YX, Yu WG, Lu YH, et al. Assessment of the cytotoxic and apoptotic effects of Chaetominine in human leukemia cell line. Biomol Ther. 2016;24:147-55.
5. Yao J, Wei X, Lu H. Chaetominine reduces MRPI-mediated drug resistance via inhibiting PI3K/Akt/Nrf2 signaling pathway in K562/Adr human leukemia cells. Biochem Biophys Res Commun. 2016;473:867-73.
6. Yao J, Xiao J, Wei X, Lu H. Chaetominine induces cell cycle arrest in human leukemia K562 and colon cancer SW116 cells. Oncol Lett. 2018;16:4671-8.
7. Gui RY, Xu L, Kuang Y, Chung III-M, Qin JC, Liu L, et al. Chaetominine, (+)-alantrypinone, questin, isorhodoptilometrin, and 4-hydroxybenzaldehyde produced by the endophytic fungus Aspergillus sp. YT-6 inhibit wheat (Triticum aestivum) and radish (Raphanus sativus) germination. J Plant Interact. 2015;10:87-92.
8. Ammar EM, Martin J, Brabo-Catala L, Philippidis GP. Propionic acid production by Propionibacterium freudenreichii using sweet sorghum bagasse hydrolysate. Appl Microbiol Biotechnol. 2020;104:9619-29.
9. Long R, Yang W, Huang G. Optimization of fermentation conditions for the production of epothilone B. Chem Biol Drug Des. 2020;00:1-5.
10. Liu M, Yang X, Ren Y, Xia H, Huang J, Ke C. Two-stage oxygen supply strategy for enhancing fed-batch production of pyrrolquinoline quinone in Hyphomicrobium denitrificans FJNU-6. Appl Microbiol Biotechnol. 2020;104:6615-22.
11. Luo H, Xu D, Xie R, Zhang X, Wang J, Dong X, et al. Enhancement of botrallin and TMC-264 production in liquid culture of endophytic fungus Hyalodendriella sp. Ponipodefl2 after treatments with metal ions. Electron J Biotechnol. 2016;24:12-20.
12. Dzurendova S, Zimmermann B, Tafintseva V, Kohler A, Horn SJ, Shapaval V. Metal and phosphate ions show remarkable influence on the biomass production and lipid accumulation in oleaginous Mucor circinelloides. J Fungi. 2020;6:260.
13. Kong F, Ren HY, Zhao L, Nan J, Ren NQ, Liu BF, et al. Semi-continuous lipid production and sedimentation of Scenedesmus sp. by metal ions addition in the anaerobic fermentation effluent. Energy Conver Manage. 2020;203:112216.
14. Liu CQ, Jiao RH, Yao LY, Zhang YX, Lu YH, Tan RX. Adsorption characteristics and preparative separation of chaetominine from Aspergillus fumigatus mycelia by macroporous resin. J Chromatogr B. 2016;1015:335-41.
15. Li SB, Liu LM, Chen J. Mitochondrial fusion and fission are involved in stress tolerance of Candida glabrata. Bioresour Bioprocess. 2015;2:12-20.
16. Liu CQ, Pan ZH, An FL, Lu YH. Co-addition strategy for enhancement of chaetominine from submerged fermentation of Aspergillus fumigatus CY018. Appl Biochem Biotechnol. 2018;186:384-99.
17. Liu F, Cao Y. Expression of the 5-enoylpyruvylshikimate-3-phosphate synthase domain from the Acremonium sp. aroM complex enhances resistance to glyphosate. Biotechnol Lett. 2018;40:855-64.
18. Zhao G, Liu J, Dong K, Zhang F, Zhang H, Liu Q, et al. Enzymatic synthesis of L-tryptophan from hair acid hydrolysis industries wastewater with tryptophan synthase. Bioresource Technol. 2011;102:3554-7.
19. Liu CQ, Wei XC, An FL, Lu YH. Ammonium acetate supplement strategy for enhancement of chaetominine production in liquid culture of marine-derived Aspergillus fumigatus CY018. J Microbiol Biotechnol. 2019;29:587-95.
20. Mummalani G, Sarma C, Kalakandan SK, Sivanandham V, Rawson A, Anandharaj A. Optimization and extraction of edible microbial polysaccharide from Fresh Coconut Inflorescence Sap: An alternative substrate. LWT-Food Sci Technol. 2021;138:110619.
21. Zhang H, Wang L, Wang H, Yang F, Chen L, Hao F, et al. Effects of initial temperature on microbial community succession rate and volatile flavors during Baijiu fermentation process. Food Res Int. 2021;410:109887.
22. Wang GL, Din AU, Qiu YS, Wang CL, Wang DH, Wei GY. Triton X-100 improves co-production of β-1,3-D-glucan and pul- lulan by Aureobasidium pullulans. Appl Microbiol Biotechnol. 2020;104:10685-96.

23. Buracco S, Peracino B, Andreini C, Bracco E, Bozzaro S. Differential effects of iron, zinc, and copper on Dictyostelium discoideum cell growth and resistance to Legionella pneumophila. Front Cell Infect Microbiol. 2018;7:536.

24. Jiang L, Song M, Yang L, Zhang D, Sun Y, Shen Z, et al. Exploring the influence of environmental factors on bacterial communities within the rhizosphere of the Cu-tolerant plant, Elsholtzia splendens. Sci Rep-UK. 2016;6:36302.

25. Lima AC, Silva D, Silva V, Godoy M, Cammarota M, Gutarra M. β-Mannanase production by Penicillium citrinum through solid-state fermentation using açai residual biomass (Euterpe oleracea). JChemTechnolBiotechnol. 2021;96:2744-54.

26. Chauhan PS, Jha B. Pilot scale production of extracellular thermo-alkali stable laccase from Pseudomonas sp. S2 using agro waste and its application in organophosphorous pesticides degradation. J Chem Technol Biotechnol. 2018;93:1022-30.

27. Wei X, Liu C, An F, Lu Y. Induced effect of Ca²⁺ on curvulamine synthesis by marine-derived fungus Curvularia sp. IFB-Z10 under submerged fermentation. Process Biochem. 2019;83:18-26.

28. Lu Y, Pan Z, Tao J, An F. Induced effect of Ca²⁺ on dalesconols A and B biosynthesis in the culture of Daldinia eschscholzii via calcium/calmodulin signaling. J Biosci Bioeng. 2018;125:205-10.

29. Zhou HY, Wu WJ, Xu YY, Zhou B, Niu K, Liu ZQ, et al. Calcium carbonate addition improves L-methionine biosynthesis by metabolically engineered Escherichia coli W3110-BL. Front Bioeng Biotechnol. 2020;8:300.

30. Chakraborty S, Mishra A, Verma E, Tiwari B, Mishra AK, Singh SS. Physiological mechanisms of aluminum (Al) toxicity tolerance in nitrogen-fixing aquatic macrophyte Azolla microphylla Kauff: phytoremediation, metabolic rearrangements, and antioxidative enzyme responses. Environ Sci Pollut Res Int. 2019;26:9041-54.

31. Kostadinova N, Krumova E, Boteva R, Abrashev R, Miteva-Stalева J, Spassova B, et al. Effect of copper ions on the ligninolytic enzyme complex and the antioxidant enzyme activ-

### SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

**How to cite this article:** Liu C, Chen T, Chen J, Zhou Y, Deng L, Feng G, et al. Induced effect of Ca²⁺ and Al³⁺ on chaetominine synthesis by Aspergillus fumigatus CY018 under submerged fermentation. Biotechnol Appl Biochem. 2022;69:2733–2744. [https://doi.org/10.1002/bab.2318](https://doi.org/10.1002/bab.2318)