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

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Abstract

Chaetominine (CHA), an alkaloid with biological activity produced by *Aspergillus fumigatus* CY018, has strong anticancer activity against the human leukemia cell. However, its further research for pharmacokinetics is limited by CHA production in submerged fermentation, which is a problem urgently awaiting solutions by effective biological strategies. In this work, Ca^{2+} and Al^{3+} exhibited a strong promoting effect on CHA production after multiple metal ions screening. Then, the concentration of Ca^{2+} and Al^{3+} and the addition time was optimized. The suitable conditions were 0.5 mM Ca^{2+} and 0.5 mM Al^{3+} at 120 h, in which the CHA production reached 58.5 and 49.45 mg/L with optimized condition of Ca^{2+} and Al^{3+} respectively. The intermediate metabolites, such as DAHP, chorismate and tryptophan, were increased obviously with addition of Ca^{2+} and Al^{3+} . The activities of key enzymes of DAHPs, AroAs and TrpCs in CHA biosynthesis pathway were improved by 3.58-, 3.60- and 3.34-folds respectively. Meanwhile, the transcription levels of *laeA*, *dahp*, *cs*, and *trpC* were upregulated by 3.22-, 12.65-, 5.58- and 6.99-fold respectively by addition of Ca^{2+} and Al^{3+} . Additionally, the fermentation strategy was successfully scaled up to 5 L reactor, in which CHA production could attain 75.6 mg/L at 336 h. This work demonstrated that Ca^{2+} and Al^{3+} co-addition was an effective strategy for increasing CHA production, and the information obtained might be useful to the further improvement of CHA fermentation on large scale.

Introduction

Aspergillus sp. as an endophytic fungus was excavated, and had received widespread attention by researchers in recent years [1]. *Aspergillus fumigatus*, an endophyte existing in plants, portunus and others, possessed an excellent synthesis ability of bioactive metabolites. The secondary metabolites biosynthesized by *A. fumigatus* had multiple biological activities. Fumigaclavine C, produced by *A. fumigatus* CY018 through the two-stage culture process, was an ergot alkaloid with anti-inflammatory activity [2]. The yield of antitumor Epothilone B, arresting cancer cell at G2-M phase, was improved with response surface methodology by *A. fumigatus* [3]. Chaetominine (CHA) (Fig. S1) as a quinazolinone alkaloid from the endophytic fungi *Aspergillus fumigatus* CY018 was found to have greater cytotoxic activity than clinical drug, which implied CHA could be developed as an anti-cancer candidate [4–6]. Moreover, it's reported that CHA could inhibit the germination and growth of wheat and radish at low concentrations [7]. However, the in-depth functional research and application of CHA had been seriously limited by the low production in the liquid fermentation. Therefore, further improvement of CHA production is urgently required considering its potential application in the field of medicine.

There were many biological control strategies to enhance the product of secondary metabolites by microbe, such as optimization of nutritional components, regulation of temperature, control of oxygen concentration and others' regulation [8–10]. The effects of metal ions on growth of microbe and biosynthesis of secondary metabolites were evident in the literature. Luo et al. proposed an effective strategy of the metal ions (Zn^{2+} , Cu^{2+} and Mg^{2+}) addition and their combination could enhance the production of botrallin and TMC-264 by *Hyalodendriella* sp. Ponipode12 [11]. 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* [12]. 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., indicated demonstrated that Fe^{3+} could promote the microalgal lipid production and sedimentation in batch culture and Al^{3+} addition could enhance the sedimentation efficiency, while it had inhibitory effect on the biomass and lipid production [13]. It was evidently that the effect of metal ions would improve the biosynthesis of active metabolites in microbe.

We also found the addition of metal ions could change the ability of metabolite synthesis in the preliminary experiment. Therefore, the selection and optimization of metal ions on CHA production were investigated. Subsequently, the transcription level and enzyme activity of specific genes and production of intermediate metabolites were analyzed to demonstrate the regulatory mechanism of metal ions for CHA biosynthesis. Moreover, the addition strategy of metal ions was reproduced in the 5-L bioreactor, which was successfully performed in a lab scale-up scale. This work would be useful to enhance the production of target compounds in submerged fermentation.

Materials And Methods

Microorganism

A. fumigatus CY018 as a filamentous fungus was kindly provided by East China University of Science and Technology. The conidia were stored by 40% glycerin in $-80\text{ }^{\circ}\text{C}$ refrigerator. The mycelia were activated on potato dextrose agar (PDA) medium and stored at $4\text{ }^{\circ}\text{C}$ refrigerator BCD-256KT.

Fermentation conditions

The mycelia of *A. fumigatus* CY018 at $4\text{ }^{\circ}\text{C}$ was first transferred and cultured on PDA at $28\text{ }^{\circ}\text{C}$ for 7 days in biochemical incubator SHP-150. An agar plug (size of 1 cm^2) was cut from the PDA and cultured in 500 mL shake flask with 200 mL potato dextrose broth (PDB) at 180 rpm, $28\text{ }^{\circ}\text{C}$ for 72 h in shaking incubator MQT-60, which was cultured as seed medium. Subsequently, 7 mL of seed medium was transferred into a 250 mL Erlenmeyer flask with 50 mL fermentation medium, which was cultured in the constant temperature shaking incubator at 180 rpm, $28\text{ }^{\circ}\text{C}$ for 384 h in shaking incubator MQT-60. The composition of fermentation medium was following as: 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_2PO_4 , 0.84 g/L $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.016 g/L $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$.

Selection and optimization of metal ions

The biomass and CHA production were detected by 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 were sterilized in autoclave at $121\text{ }^{\circ}\text{C}$ for

25 min. After the effective metal ions were determined as CaCl_2 and AlCl_3 , the addition concentration and time of which were further optimized.

Metabolite analysis

After fermentation, growth of *A. fumigatus* CY018 was measured by dry cell weight (DCW) with total mycelium in the 250 mL Erlenmeyer flask. The determination of residual sugar in fermentation broth was utilized the anthrone-sulfuric acid method. The concentration of CHA was analyzed by HPLC, and the detailed method as Liu et al. described [14]. The concentration of CHA was measured with comparison to 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 Agilent 1260, in which the metals were added at the optimized addition time. The HPLC condition for organic acids was followed: mobile phase was 0.01% trifluoroacetate; flow rate was 1 mL/min; column temperature was 25 °C; sample injection volume was 20 μL . The concentration of organic acids was measured with comparison to the standard.

qRT-PCR for key genes

1 mL of broth from the shake flask was taken for analysis of bioactivity and transcription level of key gene at taken points. The TriZol solution was employed to extract total RNA of *A. fumigatus* CY018 for all experiments. The extracted solution was detected for RNA concentration using a spectrophotometer at A260/280. The method of Li et al. [15] was utilized to detect transcription level of *laeA*, *dahp*, *cs*, *trpC* and *actin* with feeding strategy. The total RNA as initial template and the PremixScript™ Reagent Kit (Takara) were used for reverse transcription. The Premix Ex Taq™ II (Takara) was utilized to determine transcriptional levels of some key genes according to manufacturer's procedure. The sequences of primer pairs for PCR amplification and qRT-PCR assay were displayed in Table. 1 and 2. The process of qRT-PCR followed as firstly pre-denature at 95 °C for 5 min, amplification occurred in two steps: 5 s at 95 °C for denature, 30 s at 55 °C for annealing and 60 s at 72 °C for extension for 40 cycles.

Biochemical analysis of key enzymes

1 mL of broth from the shake flask was taken for analysis of bioactivity and transcription level of key enzyme in the shikimate pathway. The bioactivity of DAHP synthase (DAHPs) was analyzed as Liu et al. described [16]. A total solution and 100 μL of treated crude enzyme solution and incubated at 37 °C for 10 min. Subsequently, the mix was added with 0.2 mL 10% trichloroacetic acid and centrifuged with 10000 rpm for 5 min. 0.25 mL mixed solution was added with HIO_4 , NaAsO_4 , thiobarbital for reaction, and the final solution was centrifuged fast and determined by measuring the decrease of PEP with spectrophotometer at 549 nm.

The bioactivity assessment of AroA (AroAs) was measured at 28 °C in 50- μL reaction volume mixtures containing 50 mM Hepes buffer (pH 7.0), 1 mM shikimate-3-phosphate, 1 mM PEP, and 0.4 μg of the

purified cell disruption, which was referred to method in Liu and Gao [17]. TrpC synthase (TrpCs) was analyzed by the reaction of indole and L-serine with purified cell disruption, in which the modified method was described as Zhao et al. [18]

Scale-up experiments

The experiments of lab-scale fermentation process were performed in 5-L stirred bioreactor as Liu et al. [19], in which was added with metal ions with optimized concentration at 120 h. Samples were taken at an interval of 48 h.

Statistical analysis

All data obtained in shake flask culture process were the mean of triplicate experiments. The statistical significances of differences in addition of metal ions, organic acid, enzyme activity and transcription level were 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.

Results

Effects of multiple metal ions for biomass and CHA production

The effects of metal ions were 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 categories metal ions (Al^{3+} , Ca^{2+} , Cu^{2+} , Mn^{2+} and Zn^{2+}) were individually researched for selection the dominant metal ion on CHA production and biomass in the Fig. 1. The effect of 0.5 mM Al^{3+} could effectively promote the CHA production, which reached 52.3 mg/L as 1.5 times that of the control. The higher concentration of Al^{3+} played little role in the biosynthesis of CHA production. The production of CHA was achieved respectively 55.31 and 40.18 mg/L by addition of 0.5 and 1 mM Ca^{2+} . Ca^{2+} and Al^{3+} could enhance CHA production, but had no effect on biomass of *A. fumigatus*. The effect of Cu^{2+} on CHA production was decreased by the increased content, meanwhile, the effect of Cu^{2+} on biomass was increased. The similar phenomenon was showed by the effect of Mn^{2+} , in which the inhibitory effect of Mn^{2+} was stronger than Cu^{2+} . The Zn^{2+} completely inhibited CHA biosynthesis and reduced biomass of *A. fumigatus*. Comparison to the effects of five metal ions, the low concentration of Ca^{2+} and Al^{3+} could significantly improve the CHA production, which indicated Ca^{2+} and Al^{3+} were dominant metal ions.

Optimization of addition time and concentration by dominant metal ions

For enhancement of CHA production, the addition concentration and time of Ca^{2+} and Al^{3+} were investigated, which was based on the researched content of selection of metal ions. The different concentration (0, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7 mM) of Ca^{2+} was researched for CHA production and biomass of *A. fumigatus* in the Fig. 2A. The changing tendency of CHA production was gradually

rising and then slowly falling by the increasing concentration of Ca^{2+} . When the content of Ca^{2+} was 0.5 mM, the CHA production reached the maximum value, which was 58.8 mg/L as the optimized concentration. However, the effect of Ca^{2+} was not obvious in the biomass of *A. fumigatus*, in which the biomass was relatively lower with 0.5 and 0.55 mM Ca^{2+} than others. After the addition concentration of Ca^{2+} was determined, the addition time (0, 72, 120, 168, 216, 264, 312 h) of Ca^{2+} was subsequently studied, which results were showed in the Fig. 2B. The CHA production was fast increased and reached the peak (62.74 mg/L, at 120 h) following the time from 0-120 h, then tended towards balance. The biomass of *A. fumigatus* had an analogous tendency as optimization of concentration, which was that the tendency of biomass was not correspond to that of CHA production. Now, the optimized addition concentration and time of Ca^{2+} were respectively 0.5 mM and 120 h.

The analogous experimental methods were utilized to research the effect of Al^{3+} on CHA production and biomass. Similarly, the test concentration of Al^{3+} was designed as that of Ca^{2+} , which results were displayed in Fig. 3A. The tendency of different concentration of Al^{3+} was increasing slowly from 0 mM to 0.45 mM, then maintaining stability from 0.45 mM to 0.6 mM and decreasing tardily from 0.6 mM to 0.7 mM. When the content of Al^{3+} was 0.5 and 0.55 mM, the CHA production was getting stabilized, which was respectively 49.45 and 49.15 mg/L. the effect of addition concentration of Al^{3+} on biomass was minor fluctuations, the trend of which was generally stable. The appropriate addition concentration of Al^{3+} was defined as 0.5 mM, after that the corresponding addition time of Al^{3+} was experimented. The addition time of Al^{3+} was set as 0, 72, 120, 168, 216, 264, 312 h, which results were showed in the Fig. 3B. the effect of addition time of Al^{3+} was increasing quickly and then decreasing slowly, in which the maximum value was 50.43 mg/L at 120 h same to that of Ca^{2+} . Therefore, the optimization conditions of Ca^{2+} and Al^{3+} addition were determined, which was supplied with 0.5 mM Ca^{2+} and Al^{3+} at 120 h.

Intermediate metabolites response to metal ions addition

After the addition condition of Ca^{2+} and Al^{3+} were optimized (0.5 mM, 120 h), the change trend of intermediate metabolites was researched for exploring the mechanism of metal ions addition. The synthesis trend of pyruvate was rapidly formed in the early stage of fermentation and slowly consumed in the late stage of fermentation, in which the synthesis of pyruvate after addition of Ca^{2+} and Al^{3+} was lower than control in Fig. 4A. Before 120 h of fermentation, the change of DAHP were similar both in experimental group and control group. The biosynthesis production of DAHP was higher with addition of metal ions than control from 168 h to 312 h (Fig. 4B). The tendency of chorismate biosynthesis resembled to that of DAHP, in which the biosynthetic capacity of chorismate was also higher than control in Fig. 4C. The production of tryptophan with addition Ca^{2+} and Al^{3+} was fast increased from 0 h to 216 h and then gradually decreased from 216 h to 360 h, in which the synthesis amount of tryptophan was far more than control. Visibly, the addition of Ca^{2+} and Al^{3+} was advanced in biosynthesis of intermediate metabolites in CHA biosynthetic pathway.

Enzyme expression response to metal ions addition

After the intermediate metabolites were investigated with addition of metal ions, the activities of enzymes (DAHPs, AroA and TrpC) in CHA biosynthetic pathway were studied. The key enzymes, which played an important role in CHA production with addition of metal ions, were determined as the action sites of metal ions. DAHPs was a key enzyme in shikimate pathway, which was also an important enzyme in CHA biosynthetic pathway. After the metal ions added, the activity of DAHPs was increased first and then decreased, which were all higher than control at every time. The maximum of DAHPs activity was 3.58 folds than control at 216 h (Fig. 5A). AroAs was inducing the synthesis of 3-dehydroquinic acid, which was a key node of CHA biosynthetic pathway. The trend of AroAs activity was analogous to DAHPs in Fig. 5B, in which the AroAs activity was improved by 2.01, 3.60 and 2.10 folds than control at 168, 216 and 264 h. TrpCs induced indoleglycerol phosphate to synthesize tryptophan, which was a rate-limiting enzyme in CHA biosynthetic pathway. The addition effect of metal ions was not stronger than the first two enzymes, but in which the TrpCs activity was increased by 3.34 and 1.81 folds than control at 168 and 216 h in Fig. 5C. the effect of Ca^{2+} and Al^{3+} addition was useful in enhancing the activities of key enzymes in CHA biosynthetic pathway.

Gene expression response to metal ions addition

In the previous work, the author explored the biosynthetic way of CHA and found the predicted genes significantly affecting the biosynthesis of CHA (Liu et al. 2018, 2019). To explore further the possible mechanism of addition of Ca^{2+} and Al^{3+} on CHA production, transcription levels of four genes *laeA* (a global regulator), *dahp*, *cs* and *trpC* of CHA biosynthetic pathway were discovered by qRT-PCR. The expression levels of these genes in collaborative supplement of Ca^{2+} and Al^{3+} with samples taken at 120, 168, 216, 264, 312 and 360 h were investigated in Fig. 6.

As shown in Fig. 6A, the transcription level of *laeA* was obviously elevated at 168, 216 and 264 h, which increased multiple were respectively 2.89, 3.22 and 2.46 folds than the group without addition of metal ions. The expression level of *dahp* was increased about 12.65 folds at 168 h and 5.51 folds at 216 h (Fig. 6B), which were affected stronger than others with supplement of Ca^{2+} and Al^{3+} . The transcription level of *cs* gene was up-regulated at 216, 264 and 312 h, and the expressed folds of this gene increased 5.58, 3.32 and 2.67 with feeding Ca^{2+} and Al^{3+} in Fig. 6C. The change trend of *trpC* gene was similar to *dahp*, but the enhanced effect of *trpC* gene was worse than *dahp*. The expression level of *trpC* was increased about 6.99 folds at 168 h and 2.12 folds at 216 h (Fig. 6D). These results indicated the key genes of CHA biosynthetic pathway were up-regulated with co-addition of Ca^{2+} and Al^{3+} .

Scale-up CHA production to lab-scale bioreactor

For the supplement effect of Ca^{2+} and Al^{3+} was really worked, the scale-up experiment was performed in 5-L stirred bioreactor. As shown in Fig. 7A and B, the time profiles of 5-L bioreactor and shake flask with addition of Ca^{2+} and Al^{3+} were investigated. The trend of DCW was similar both in first 5-L bioreactor and shake flask, which was increased slowly finally declined slightly. However, DCW in 5-L bioreactor was litter lower than that in shake flask at the end of fermentation. The rate of sugar consumption was also

analogy in 5-L bioreactor and shake flask, and the descent rate of sugar consumption was faster in 5-L bioreactor after feeding metal ions than that in shake flask. The change trend of pH was consistent, which was increased fast then declined and finally around 4 in 5-L bioreactor and shake flask. When Ca^{2+} and Al^{3+} were co-added in 5-L bioreactor, the value of pH was rising and then falling quickly at 120 h. The DO continued to decline during 0-120 h in Fig. 6B, then maintained stability at 20% from 120 h to 336 h. The trend of CHA production in 5-L bioreactor and shake flask were both increased then decreased. The maximum of CHA production in 5-L bioreactor was 75.6 mg/L at 336 h, which was higher than that (37.6 mg/L, at 360 h) in shake flask. Therefore, the scale-up experiment in laboratory was successful, in which the sugar consumption, pH and CHA production in 5-L bioreactor were all better than that in shake flask.

Discussion

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

The effective factors contributing to production of metabolites were studied by many experts and scholars on the mechanism research and application development. The temperature, pH, agitation speed, surfactants and so on were optimized to improve the yield of target compound [20-22]. The metal ions had an important role in growth and metabolite synthesis of microbe [23-24]. The results of present study indicated the Ca^{2+} and Al^{3+} enhanced the production of CHA and other ions had different degrees of reduction on the synthesis of CHA in Fig. 1. Visibly, the enhancement effect of Ca^{2+} and Al^{3+} were better than Cu^{2+} , Mn^{2+} and Zn^{2+} . Lima et al. [25] screened the effects of many metal ions ($2.5 \text{ mmol L}^{-1} \text{ AgNO}_3$, 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 activities. Chauhan and Jha [26] found the metal ions Na^+ , K^+ , Pb^{2+} , Ca^{2+} , Cu^{2+} and Co^{2+} enhanced enzyme activity of laccase from *Pseudomonas* sp. S2. Therefore, the promoted effect of metal ions on biosynthesis of active metabolites was verified by numerous researchers.

The addition concentration and time of Ca^{2+} and Al^{3+} were optimized, in which the different addition condition of optimized metal ions had different influences on the biosynthesis of CHA. The optimized concentration and time of Ca^{2+} and Al^{3+} supplement were respectively 0.5 mM and 120 h, in which the maximum of CHA was respectively 62.74 and 50.43 mg/L. Wei et al. [27] explored the induced effect of Ca^{2+} on curvulamine synthesis by marine-derived fungus *Curvularia* sp. IFB-Z10, in which researchers firstly investigated the selection of metal ions and optimization of addition concentration and time of Ca^{2+} . The addition concentration and time of CaCl_2 were first investigated for exploring the mechanism of Ca^{2+} on dalesconols A and B biosynthesis in the culture of *Daldinia eschscholzii* via calcium/calmodulin signaling [28]. Therefore, the optimization of addition concentration and time of Ca^{2+} and Al^{3+} was necessary for clarifying the effect of Ca^{2+} and Al^{3+} on enhancement of CHA production.

Al^{3+} and Ca^{2+} promoted metabolic parameters

The purpose of research about metabolic parameters was utilized to analyze the mechanism of Ca^{2+} and Al^{3+} on enhancement of CHA production based on the research of previous work. The accumulation of DAHP, chorismate and tryptophan after addition of Ca^{2+} and Al^{3+} was higher than non-treat group in Fig. 3B-D. And, the tryptophan production was far more than control, which was an important intermediate metabolite of CHA biosynthesis pathway and resulted to the increasing CHA production. However, the amount of pyruvate after addition of optimized metal ions was lower than control (Fig. 3A). The carbon source might flow to the CHA synthesis pathway instead of the EMP pathway, which led to the lower pyruvate production. Zhou et al. [29] researched the effect of calcium carbonate addition for improving L-methionine biosynthesis by metabolically engineered *Escherichia coli* W3110-BL, in which the biosynthesis of organic acids (α-KG, succinic acid, lactic acid, formic acid, and acetic acid) had varying degrees of promotion after addition of calcium carbonate. Meanwhile, the time course of DCW, residual sugar, pH and CHA production were compared in Fig. S2. The trend of DCW and CHA production was improved after addition of Ca^{2+} and Al^{3+} . The residual sugar was consumed rapidly than control. The pH was ephemerally increased after addition of optimized metal ions, then emerged the same trend of control. Chakraborty et al. [30] explored the effect of aluminum on chlorophyll and carotenoids, photosynthetic efficiency, starch, and sucrose for expounding physiological mechanisms of aluminum toxicity tolerance in *Azolla microphylla* Kaulf, in which the target parameters were decreased by the increasing aluminum concentration. Based on the above discussion, the addition of Ca^{2+} and Al^{3+} could promote biosynthesis of intermediate metabolite (DAHP, chorismate and tryptophan) and residual sugar consumption to increase DCW and CHA production.

The induced effect of Ca^{2+} and Al^{3+} on biosynthesis of CHA

To clarify the induced effect of Ca^{2+} and Al^{3+} on biosynthesis of CHA, the activities of key enzymes and transcriptional level of key genes were investigated. The changing trend of key enzymes (DAHPs, AroAs and TrpCs) were firstly increased and then reduced after Ca^{2+} and Al^{3+} addition following fermentation in Fig. 5, in which the point of maximum effect appeared at 216 h. And, the enzyme activity by addition of Ca^{2+} and Al^{3+} was dropped gradually along the CHA biosynthetic pathway. The ligninolytic enzyme complex and the antioxidant enzyme activity in the white-rot fungus *Trametes trogii* 4₆ were improved by lignocellulose degradation after addition of copper ions [31], in which the changing tendency of laccase, SOD and CAT activities were a close analogy to the present work. The phosphoglucosmutase (PGM) activity from *Cordyceps militaris* was investigated by addition of metal ions [32]. The results were found Ca^{2+} , Zn^{2+} , Mg^{2+} , Fe^{2+} and Fe^{3+} could enhance the activity of PGM and mRNA transcription level of *p_{pgm}* gene. These results demonstrated the activities of key enzymes were induced to promote accumulation of target compounds by addition of optimized metal ions.

The inner function of Ca^{2+} and Al^{3+} addition on CHA biosynthesis was investigated on the transcription level of key genes (*laeA*, *dahp*, *cs*, and *trpC*) in Fig. 6. The expression of *laeA*, as a global regulatory gene, was increased by Al^{3+} and Ca^{2+} addition, which meant the effect of metal ions addition could improve the production of primary and secondary metabolites. The impact of the global secondary metabolite

regulators *laeA* on echinocandin B production was enhanced in the industrial production strain *Aspergillus pachycristatus* NRRL 11440 [33]. Meanwhile, the transcription levels of *dahp*, *cs*, and *trpC* were also improved at different time and degrees. Comfortingly, the effect time of transcription level of *dahp* and *trpC* were worked in advance of DAHP and TrpCs, which indicated the effect of Ca^{2+} and Al^{3+} addition was kicked in the sequence of the central law. The similar phenomenon was observed in aluminum chloride elicitation (amendment) improved callus biomass growth and reserpine yield in *Rauvolfia serpentina* leaf callus, in which SOD, APX and CAT activities were improved by Al^{3+} addition [34]. The enhancement of omega-3 produced by *Chlorella sorokiniana* was achieved by Ca^{2+} -induced homeoviscous adaptation, in which the relative expression profiles of *accD* and *rbcL* were increased by addition of Ca^{2+} [35]. Xu et al. [36] found the induction of calcium ion could improve production of ganoderic acid and intermediate metabolites (squalene and lanosterol), which was resulted of the up-regulated the transcription levels of key genes (*hmgr*, *fps*, *sqs* and *cyp*) with addition of Ca^{2+} . Therefore, the inducing effect of Ca^{2+} and Al^{3+} played role in upregulating the transcription levels of key genes, and increasing the activities of key enzymes for enhancement of CHA production.

Conclusion

CHA could be taken as a drug candidate worthy of study owing to its attractive anticancer activity. Herein, the selected Ca^{2+} and Al^{3+} were refined in the addition concentration and time to 50 mM and 120 h, in which CHA production reached 49.45 and 58.5 mg/L with optimized condition of Ca^{2+} and Al^{3+} respectively. The effect of Ca^{2+} and Al^{3+} supplement improved production of intermediate metabolites (DAHP, chorismate and tryptophan) and activities of key enzymes (DAHPs, AroAs and TrpCs). Simultaneously, the transcription levels of *laeA*, *dahp*, *cs*, and *trpC* were upregulated by 3.22-, 12.65-, 5.58- and 6.99-fold respectively. Additionally, the fermentation process was scaled up to 5 L reactor, in which CHA production could achieve 75.6 mg/L at 336 h higher than control. This work would promote the further development and research of CHA, and provide valuable experience for research on yield improvement of other filamentous fungi.

Declarations

Authors' contributions CL, JC, JX, JG, HL and ZL designed the research. TC and LD supervised the project. CL, JC and JX conducted the main experiments. CL, TC, JC and JX wrote the manuscript. All authors read and approved the manuscript.

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Availability of Data and Materials The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests The authors declare no competing interests.

References

1. Minh, L. T. H., Hue, N. T., Linh, N. T., Ngan, T. B., Quyen, V. T., Anh, N. M. ... Huong, D. T. M. (2020). Antimicrobial Secondary Metabolites from the Marine-Derived Fungus *Aspergillus* sp. M28. *Chem. Nat. Compd*, 56, 1173–1175
2. Zhu, Y. X., Yao, L. Y., Jiao, R. H., Lu, Y. H., & Tan, R. X. (2014). Enhanced production of Fumigaclavine C in liquid culture of *Aspergillus fumigatus* under a two-stage process. *Bioresource Technol*, 152, 162–168
3. El-Sayed, A. S. A., Shindia, A. A., Ali, G. S., Yassin, M. A., Hussein, H., Awad, S. A., & Ammar, H. A. (2021). Production and bioprocess optimization of antitumor Epothilone B analogue from *Aspergillus fumigatus*, endophyte of *Catharanthus roseus*, with response surface methodology. *Enzyme Microb. Tech*, 143, 109718
4. Yao, J., Jiao, R., Liu, C., Zhang, Y., Yu, W., Lu, H., & Tan, R. (2016). Assessment of the Cytotoxic and Apoptotic Effects of Chaetominine in a Human Leukemia Cell Line. *Biomol. Ther*, 24, 147–155
5. Yao, J., Wei, X., & Lu, H. (2016). Chaetominine reduces MRP1-mediated drug resistance via inhibiting PI3K/Akt/Nrf2 signaling pathway in K562/Adr human leukemia cells. *Biochem. Biophys. Res. Co*, 473, 867–873
6. Yao, J., Xiao, J., Wei, X., & Lu, H. (2018). Chaetominine induces cell cycle arrest in human leukemia K562 and colon cancer SW1116 cells. *Oncol. Lett*, 16, 4671–4678
7. Gui, R. Y., Xu, L., Kuang, Y., Chung, I. I. I. M., Qin, J. C., Liu, L. ... Zhao, L. C. (2015). Chaetominine, (+)-alantrypinone, questin, isorhodoptilometrin, and 4-hydroxybenzaldehyde produced by the endophytic fungus *Aspergillus* sp. YL-6 inhibit wheat (*Triticum aestivum*) and radish (*Raphanus sativus*) germination. *J. Plant Interact*, 10, 87–92
8. Ammar, E. M., Brabo-Catala, M. J., & Philippidis, L., G. P. (2020). Propionic acid production by *Propionibacterium freudenreichii* using sweet sorghum bagasse hydrolysate. *Appl. Microbiol. Biot*, 104, 9619–9629
9. Long, R., Yang, W., & Huang, G. (2020). Optimization of fermentation conditions for the production of epothilone B. *Chem. Biol. Drug Des*, 00, 1–5
10. Liu, M., Yang, X., Ren, Y., Xia, H., Huang, J., & Ke, C. (2020). Two-stage oxygen supply strategy for enhancing fed-batch production of pyrroloquinoline quinone in *Hyphomicrobium denitrificans* FJNU-6. *Appl. Microbiol. Biot*, 104, 6615–6622

11. Luo, H., Xu, D., Xie, R., Zhang, X., Wang, J., Dong, X. ... Liu, Y. (2016). Enhancement of botrallin and TMC-264 production in liquid culture of endophytic fungus *Hyalodendriella* sp. Ponipodef12 after treatments with metal ions. *Electron. J. Biotechnol*, 24, 12–20
12. Dzurendova, S., Zimmermann, B., Tafintseva, V., Kohler, A., Horn, S. J., & Shapaval, V. (2020). Metal and Phosphate Ions Show Remarkable Influence on the Biomass Production and Lipid Accumulation in Oleaginous *Mucor circinelloides*. *J. Fungi*, 6, 260
13. Kong, F., Ren, H. Y., Zhao, L., Nan, J., Ren, N. Q., Liu, B. F., & Ma, J. (2020). Semi-continuous lipid production and sedimentation of *Scenedesmus* sp. by metal ions addition in the anaerobic fermentation effluent. *Energ. Convers Manage*, 203, 112216
14. Liu, C., Jiao, R., Yao, L., Zhang, Y., Lu, Y., & Tan, R. (2016). Adsorption characteristics and preparative separation of chaetominine from *Aspergillus fumigatus* mycelia by microporous resin. *J. Chromatogr. B*, 1015, 135–141
15. Li, S. B., Liu, L. M., & Chen, J. (2015). Mitochondrial fusion and fission are involved in stress tolerance of *Candida glabrata*. *Bioresour. Bioprocess*, 2, 12–20
16. Liu, C. Q., Pan, Z. H., An, F. L., & Lu, Y. H. (2018). Co-addition Strategy for Enhancement of Chaetominine from Submerged Fermentation of *Aspergillus fumigatus* CY018. *Appl. Biochem. Biotechnol*, 186, 384–399
17. Liu, F., & Cao, Y. (2018). Expression of the 5-enoylpyruvylshikimate-3-phosphate synthase domain from the *Acremonium* sp. aroM complex enhances resistance to glyphosate. *Biotechnol. Lett*, 40, 855–864
18. Zhao, G., Liu, J., Dong, K., Zhang, F., Zhang, H., Liu, Q., & Jiao, Q. (2011). Enzymatic synthesis of L-tryptophan from hair acid hydrolysis industries wastewater with tryptophan synthase. *Bioresour. Technol*, 102, 3554–3557
19. Liu, C. Q., Wei, X. C., An, F. L., & Lu, Y. H. (2019). Ammonium Acetate Supplement Strategy for Enhancement of Chaetominine Production in Liquid Culture of Marine-Derived *Aspergillus fumigatus* CY018. *J. Microbiol. Biotechnol*, 29, 587–595
20. Mummaleti, G., Sarma, C., Kalakandan, S. K., Sivanandham, V., Rawson, A., & Anandharaj, A. (2021). Optimization and extraction of edible microbial polysaccharide from Fresh Coconut Inflorescence Sap: An alternative substrate. *LWT-Food Sci. Technol*, 138, 110619
21. Zhang, H., Wang, L., Wang, H., Yang, F., Chen, L., Hao, F. ... Xu, Y. (2021). Effects of initial temperature on microbial community succession rate and volatile flavors during Baijiu fermentation process. *Food Res. Int*, 141, 109887
22. Wang, G. L., Din, A. U., Qiu, Y. S., Wang, C. L., Wang, D. H., & Wei, G. Y. (2020). Triton X-100 improves co-production of β -1,3-D-glucan and pullulan by *Aureobasidium pullulans*. *Appl. Microbiol. Biot*, 104, 10685–10696
23. Buracco, S., Peracino, B., Andreini, C., Bracco, E., & Bozzaro, S. (2018). Differential Effects of Iron, Zinc, and Copper on *Dictyostelium discoideum* Cell Growth and Resistance to *Legionella pneumophila*. *Front. Cell Infect. Mi*, 7, 536

24. Jiang, L., Song, M., Yang, L., Zhang, D., Sun, Y., Shen, Z. ... Zhang, G. (2016). Exploring the Influence of Environmental Factors on Bacterial Communities within the Rhizosphere of the Cu-tolerant plant, *Elsholtzia splendens*. *Sci. Rep.-UK*, 6, 36302
25. Lima, A. C., Silva, D., Silva, V., Godoy, M., Cammarota, M., & Gutarra, M. (2021). β -Mannanase production by *Penicillium citrinum* through solid-state fermentation using açai residual biomass (*Euterpe oleracea*). *J. Chem. Technol. Biotechnol.* DOI: 10.1002/jctb.6818.
26. Chauhan, P. S., & Jha, B. (2018). 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*, 93, 1022–1030
27. Wei, X., Liu, C., An, F., & Lu, Y. (2019). Induced effect of Ca^{2+} on curvulamine synthesis by marine-derived fungus *Curvularia* sp. IFB-Z10 under submerged fermentation. *Process Biochem*, 83, 18–26
28. Lu, Y., Pan, Z., Tao, J., & An, F. (2018). Induced effect of Ca^{2+} on dalesconols A and B biosynthesis in the culture of *Daldinia eschscholzii* via calcium/calmodulin signaling. *J. Biosci. Bioeng*, 125, 205–210
29. Zhou, H. Y., Wu, W. J., Xu, Y. Y., Zhou, B., Niu, K., Liu, Z. Q., & Zheng, Y. G. (2020). Calcium Carbonate Addition Improves L-Methionine Biosynthesis by Metabolically Engineered *Escherichia coli* W3110-BL. *Front Bioeng, Biotech*, 8, 300
30. Chakraborty, S., Mishra, A., Verma, E., Tiwari, B., Mishra, A. K., & Singh, S. S. (2019). Physiological mechanisms of aluminum (Al) toxicity tolerance in nitrogen-fixing aquatic macrophyte *Azolla microphylla* Kaulf: phytoremediation, metabolic rearrangements, and antioxidative enzyme responses. *Environ. Sci. Pollut. R*, 26, 9041–9054
31. Kostadinova, N., Krumova, E., Boteva, R., Abrashev, R., Miteva-Staleva, J., Spassova, B., & Angelova, M. (2018). Effect of copper ions on the ligninolytic enzyme complex and the antioxidant enzyme activity in the white-rot fungus *Trametes trogii* 46. *Plant. Biosyst*, 152, 1128–1133
32. Geng, X. Q., Ji, Y. P., Liu, C. Y., & Zhu, Z. Y. (2021). Enzymatic characterization and validation of gene expression of phosphoglucomutase from *Cordyceps militaris*. *Biotechnol. Lett*, 43, 177–192
33. Lan, N., Yue, Q., An, Z., & Bills, G. F. (2019). Apc.LaeA and Apc.VeA of the velvet complex govern secondary metabolism and morphological development in the echinocandin-producing fungus *Aspergillus pachycristatus*. *J. Ind. Microbiol. Biot*, 47, 155–168
34. Zafar, N., Mujib, A., Ali, M., Tonk, D., & Gulzar, B. (2017). Aluminum chloride elicitation (amendment) improves callus biomass growth and reserpine yield in *Rauvolfia serpentina* leaf callus. *Plant Cell Tiss. Organ. Cult*, 130, 357–368
35. Shim, S. J., Hong, M. E., Chang, W. S., & Sim, S. J. (2020). Repeated-batch production of omega-3 enriched biomass of *Chlorella sorokiniana* via calcium-induced homeoviscous adaptation. *Bioresource Technol*, 303, 122944
36. Xu, J. W., Yue, T. H., Yu, X., Zhao, P., Li, T., & Li, N. (2014). Enhanced production of individual ganoderic acids by integrating *Vitreoscilla* haemoglobin expression and calcium ion induction in liquid static cultures of *Ganoderma lingzhi*. *Microb. Biotechnol*, 12, 1180–1187

Tables

Table 1. Sequences of primer pairs for PCR amplification.

Target gene	Primer name	Primer sequence (5'-3')
<i>laeA</i>	<i>laeA</i> -forward	CCCAAGCTTATGTTTCTCAACGGGCAGGGCGGA
	<i>laeA</i> -reverse	CGCGGATCCTCAATTGGCGAGAGGTTTC
<i>dahp</i>	<i>dahp</i> -forward	CCCAAGCTTATGTTGAGCCCAGACCTCCAG
	<i>dahp</i> -reverse	CGCGGATCCTCACAGACCATGGGCCTTTG
<i>cs</i>	<i>cs</i> -forward	CCCAAGCTTATGTGACGTGGGGAGAATACTT
	<i>cs</i> -reverse	CGCGGATCCCTATGAAGAGGCGGGAGCAC
<i>trpC</i>	<i>trpC</i> -forward	CCCAAGCTTATGGCGACCTCCGATCTCG
	<i>trpC</i> -reverse	CGCGGATCCTTAAGCTTCATAGGCTTTCACAGC

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

Target gene	Primer name	Primer sequence (5'-3')
<i>laeA</i>	<i>laeA</i> -forward	TTCTTTTCGAGCTGCCGTCAA
	<i>laeA</i> -reverse	TCCATGGTATGTTTCGTCCGT
<i>dahp</i>	<i>dahp</i> -forward	GACAAGGGCCCATCTTCACA
	<i>dahp</i> -reverse	TGGCAGACGTGTTATAGCGG
<i>cs</i>	<i>cs</i> -forward	CCCCCGAACAATGAATCGC
	<i>cs</i> -reverse	GGTACTTCTCCGCAATGGCT
<i>trpC</i>	<i>trpC</i> -forward	AGGTCGACGTTTTCGGTGAA
	<i>trpC</i> -reverse	AGTCACTTCAACCGCAGGAG
<i>actin</i>	<i>actin</i> -forward	TTCGAGACCTTCAACGCTCC
	<i>actin</i> -reverse	ATGGGGACAACGTGAGTGAC

Figures

Figure 1

The effect of 0.5 and 1 Mm of different metal ions on CHA production and DCW of *A. fumigatus* CY018 in shake flask. Data points are the average of $n = 3$; error bars represent standard error about the mean. The samples were harvested at 336 h of fermentation.

Figure 2

The effect of Ca^{2+} addition on CHA production and DCW of *A. fumigatus* CY018 in shake flask. A: Effect of Ca^{2+} concentration; B: Effect of Ca^{2+} addition time. Data points are the average of $n = 3$; error bars represent standard error about the mean. The samples were harvested at 336 h of fermentation.

Figure 3

The effect of Al^{3+} addition on CHA production and DCW of *A. fumigatus* CY018 in shake flask. A: Effect of Al^{3+} concentration; B: Effect of Al^{3+} addition time. Data points are the average of $n = 3$; error bars represent standard error about the mean. The samples were harvested at 336 h of fermentation.

Figure 4

The effect of Ca^{2+} and Al^{3+} addition on accumulation of intermediate metabolites in 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.

Figure 5

The effect of Ca^{2+} and Al^{3+} addition on activity of key enzymes in CHA biosynthesis pathway. A: DAHPs; B: AroAs; C: TrpCs. 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.

Figure 6

The effect of Ca^{2+} and Al^{3+} addition on transcriptional level of key genes in 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.

Figure 7

The strategy of Ca^{2+} and Al^{3+} addition scaled up in 5 L bioreactor. A: Ca^{2+} and Al^{3+} addition in shake flask; B: Ca^{2+} and Al^{3+} addition in 5 L bioreactor. The addition condition of Ca^{2+} and Al^{3+} were both 0.5 mM and at 120 h of fermentation time.

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