Integrated strategy of temperature shift and mannitol feeding for enhanced production of echinocandin B by *Aspergillus nidulans* CCTCC M2012300

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Abstract

The production of echinocandin B (ECB) by *Aspergillus nidulans* CCTCC M2012300 was improved by integrating the temperature-shift and fed-batch control strategies. The kinetic characteristics of batch cultures were analyzed at different culture temperatures, and then a two-stage temperature control strategy was established. In the first 6 days, the temperature was maintained at 30 °C to obtain the maximal cell growth rate; subsequently, 25 °C was used to gain a high ECB formation rate. On the basis of temperature control, the ECB productivity was increased to 143.3 mg/(L day), which was a 1.3-fold improvement compared with the optimal constant-temperature cultivations. The influences of fed-batch cultures were further investigated. A maximal ECB productivity of 170.8 mg/(L day) was obtained through a three-stage mannitol pulse-feeding strategy, which was another 1.2-fold improvement than that of the batch fermentation. This is the first report of the use of a two-stage temperature control fed-batch strategy in ECB fermentation. This strategy was simple and economical to operate and may provide new guidance for the industrial-scale production of ECB.

Keywords *Aspergillus nidulans* · Echinocandin B · Two-stage temperature control · Fed-batch culture

Introduction

Echinocandins, a family of cyclic hexlipopeptides, are a new class of antifungal agents with broad-spectrum activity against *Pneumocystis carinii*, *Aspergillus species*, and *Candida* (Emri et al. 2013). These compounds can disturb the biosynthesis of β -1,3-glucan in the fungal cell walls by inhibiting β -1,3-glucan synthase activity (Bauer and Bronstrup 2014; Mattay et al. 2018). Thus, echinocandins have very few toxic side effects in humans because glucan polymers are not present in mammalian cells. Echinocandin B (ECB), an echinocandin-type compound produced by *Aspergillus nidulans*, is a key precursor for the chemical

synthesis of antifungal drug—anidulafungin (Huttel et al. 2016; Cacho et al. 2012). Owing to the low toxicity, broad antifungal activity, and excellent pharmacokinetic properties of the echinocandin-type drug, their production by microbial fermentation has been a focus of much attention (Balkovec et al. 2014; Zou et al. 2015a). Many reports are available on echinocandin fermentation (Cockshott and Hartman 2001; Kanda et al. 2009, 2010; Bills et al. 2008; Pollard et al. 2007; Hu et al. 2016). However, the ECB production by microbial fermentation continues to suffer challenges, such as high viscosity of fermentation, long fermentation periods and low ECB productivity. Therefore, developing an efficient process for ECB fermentation is necessary.

Temperature is one of the major fermentation parameters that affect the microorganism growth and biosynthesis of products in numerous microorganisms (Tubia et al. 2018; Cordova and Alper 2018; Zhang et al. 2016; Kumar et al. 2014; Peng et al. 2010). In general, temperature can affect the activity of enzymes and the direction of the synthesis of metabolites (Ullah et al. 2014). Temperature plays a significant role in the production of ECB (Emri et al. 2013). The temperature optimum for ECB production is generally between 24 and 28 °C, similar to those of other

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echinocandin-type cyclic hexlipopeptides, such as pneumocandin B₀ and FR901379, which are produced by *Glarea lozoyensis* and *Coleophoma empetri*, respectively (Hu et al. 2016). However, the ECB-producing strain grows poorly below 30 °C (Tóth et al. 2011). ECB was a type of intracellular metabolite, and its concentration was associated with cell growth. Thus, simultaneous high cell growth rate and high product formation rate are necessary for gaining high ECB accumulation. However, obtaining a maximal production by controlling a constant temperature in the whole fermentation is difficult. An efficient method for solving this problem is the use of a two-stage control strategy for simultaneously acquiring optimal temperatures for cell growth and product formation (Liu et al. 2018; Feng et al. 2016; Zhang et al. 2016; Lee et al. 2015; Zhen et al. 2013; Wei et al. 2012; Peng et al. 2010). Recently, two-stage control strategies have been widely applied in many fermentations of desired metabolites (Si et al. 2017; Wang et al. 2016; Dias et al. 2015; Ying et al. 2014; Zheng et al. 2013). Previous studies on ECB production addressed the optimization of the medium composition (Tóth et al. 2012; Cockshott and Sullivan 2001; Cockshott and Hartman 2001) or focused on strain screening (Zou et al. 2015b; Hu et al. 2016). However, no reports have been made on the effects of a temperature-shift strategy on ECB fermentation.

In our previous work, a wild-type *Aspergillus nidulans* was treated with random mutagenesis and several high ECB-producing mutants were obtained (Zou et al. 2015b). This work aims to establish an efficient fermentation process to improve the productivity and efficiency of ECB production by *Aspergillus nidulans* CCTCC M2012300. First, the influence of temperature on ECB production was investigated, and a two-stage temperature control strategy was proposed. Then the influence of mannitol feeding strategies on ECB production was studied based on the two-stage temperature control strategy, and an efficient fermentation process with integrated temperature control and mannitol pulse feeding was developed to enhance ECB productivity. This integrated fermentation process was simple and economical to operate, and it may be suitable for industrial-scale production of ECB.

Materials and methods

Materials

Echinocandin B (ECB) was purchased from BioAustralis (Smithfield, NSW, Australia). Acetonitrile and methanol were HPLC grade and purchased from Honeywell Burdick & Jackson Chemical Ltd., USA. All other chemicals and reagents were of analytical grade and purchased from Mike Chemical Reagents Co., Ltd. (Hangzhou, China).

Microorganism

Aspergillus nidulans CCTCC M2012300 was preserved in our laboratory. It was maintained on potato dextrose agar (PDA) slants stored at 4 °C and subcultured periodically.

Batch culture medium

Seed medium contained (g/L) cottonseed meal 25, glucose 10, glycerol 10, pH 6.8–7.0. Fermentation medium contained (g/L) mannitol 97, glycerol 10, peanut oil 20, peptone 40, and tomato powder 15, K₂HPO₄·3H₂O 8, MgSO₄·7H₂O 0.5, MnSO₄·H₂O 0.2, FeSO₄·7H₂O 0.05, CaCl₂ 0.5, CuSO₄·5H₂O 0.6, pH 6.8–7.0. All the media were autoclaved at 115 °C for 30 min.

Fermentation kinetic studies

A loop of mycelium of *A. nidulans* CCTCC M2012300 was inoculated into a fresh PDA slant and incubated at 25 °C. After 12–14 days of incubation, a lot of green spores were generated on mycelium and eluted with sterile water. The spores were broken into single spores by 3–4-mm glass beads and filtered through three layers of sterile lens paper to obtain the spore suspension. One milliliter spore suspension (1×10^8 cells/mL) was inoculated to seed medium. Inoculation was done in 500-mL flasks containing 100 mL seed medium for 48 h at 25 °C with shaking at 200 rpm. In the shake flasks experiments, 250-mL flasks containing 50 mL fermentation medium were inoculated at 10% (v/v) and incubated on a shaker at 25 °C and 200 rpm for 12 days. In the fermenter experiments, batch fermentation was carried out in a 15-L fermenter (New Brunswick Scientific, USA) with a working volume of 10 L. The fermenter was equipped with one, six-bladed disc turbine impellers. The agitation speed was 350 rpm and the aeration rate was controlled at 1.0 vvm (volume of air per volume of culture per minute).

In the fed-batch culture of *A. nidulans* CCTCC M2012300, four feeding strategies were studied. The initial mannitol concentration was 48.5 g/L. In the one-time feeding fed-batch fermentation, mannitol of 10 g was added at day 3 (mode A); mannitol of 10 g was added at day 6 (mode B) and mannitol of 10 g was added at day 9 (mode C). In the pulse-feeding fed-batch fermentation, mannitol of 3.3 g was added at day 3, 6, 9, respectively (mode D). All the four feeding experiments gave the total mannitol equal and mannitol concentration is 97 g/L. All the experiments were carried out in triplicates.

Analytical methods

The fermentation broth was harvested by filtration and washed twice with distilled water. The dry cell weight

(DCW) was determined by drying the wet mycelium at 60 °C to obtain a constant weight. The chromatographic conditions were as follows: ODS column (Shim-pack, VP-ODS, 4.6 mm × 250 mm, Shimadzu Corporation, Kyoto, Japan); UV detector; detection wavelength, 223 nm; flow rate, 1.0 ml/min; mobile phase, methanol:acetonitrile:water in the ratio of 70:10:20 (v/v); injection volume, 10 µL. The retention times for ECB were around 13.5 min. Moreover, all samples were filtered through a 0.45-µm one-off filtration membrane prior to injection into the HPLC system to get accurate results. The ECB concentration was determined using standard plots obtained with pure ECB. Mannitol was determined by HPLC (Waters 2414, USA) equipped with a refractive index detector and a Bio-rad Aminex HPX-87H (300 × 7.8 mm) column as described in the previous publication (Li et al. 2014).

Kinetic parameter calculation

The cell growth rate and ECB production rate were estimated from experimental or fitted data of cell growth and ECB production as previously described (Jin et al. 2015). The fitted data were obtained by interposing between experimental data of cell growth, or ECB production at definite time ($dt = 1$ h) with the approximation method of cubic spline interpolation in Origin software (OriginLab Corp., Northampton, MA, USA).

Statistics

All experiments were repeated at least three times with three batches of cells and data are expressed as the mean value $\pm$ standard deviation.

Results and discussion

Influences of temperature on ECB fermentation in batch culture

Figure 1a–c shows the influences of varying culture temperature (23, 25, 27, and 30 °C) on cell growth, mannitol consumption, and ECB production in batch ECB fermentation by *A. nidulans* CCTCC M2012300. As shown in Fig. 1a, the dry cell weight (DCW) of *A. nidulans* CCTCC M2012300 increased quickly in the initial stage of fermentation and reached the maximum at the stationary phase for all operating temperatures. Higher temperatures resulted in higher biomass. Only 56.8 g/L of DCW was achieved when the culture temperature was 23 °C, whereas a relatively higher DCW (67.6 g/L and 72.8 g/L) was obtained when the culture temperature was 27 and 30 °C. Similar to enhanced cell

Fig. 1 Effects of culture temperature on DCW (a), residual mannitol concentration (b), and ECB production (c) of *A. nidulans* CCTCC M2012300 in batch fermentation. The symbols used were 23 °C (unfilled diamond), 25 °C (filled triangle), 27 °C (unfilled circle) and 30 °C (filled inverted triangle)

growth, mannitol consumption significantly increased from 23 to 30 °C (Fig. 1b).

As shown in Fig. 1c, the maximum ECB production of 1.43 g/L was achieved after 12 days of cultivation at 25 °C; however, the maximum ECB concentration was only 0.92 g/L at 23 °C at the same fermentation time. Although the lag time of ECB biosynthesis was obviously shorter at higher temperatures (27 and 30 °C) than that at lower temperatures (23 and 25 °C), the ECB concentrations at 27 and 30 °C were only 1.22 and 0.98 g/L, respectively. This finding indicates that a relatively high fermentation temperature has a disadvantage in the production of ECB. This result was consistent with the previous reports, in which the optimum temperature for ECB production is between 24 and 28 °C (Tóth et al. 2011; Klich et al. 2001).

The above results suggested that culture temperature was vital in cell growth and product formation during ECB fermentation. The final ECB yield was a complex combination of biomass and specific ECB production rate. The results in Fig. 1 indicated that the production of ECB was not accompanied by the increase in DCW. Although the low content of DCW did not result in high ECB formation, a high DCW did not lead to high ECB production. ECB content initially increased at the beginning of the stationary phase, whereas cell growth mainly occurred at an earlier fermentation stage. When metabolite production was independent of cell growth, a novel two-stage cultivation strategy can be applied to maximize the level of target metabolite. In that case, optimal cell growth and metabolite production would be achieved simultaneously by separately optimized conditions.

Analysis of kinetic characteristics of cell growth and ECB production

During the entire ECB fermentation process, the suitable temperatures for cell growth and ECB formation were different. Thus, using a temperature-shift control process instead of a constant-temperature process was favorable. Furthermore, determining the temperature-shifted point was also important, and learning the time profile of the rate of cell growth and ECB formation would be helpful to determine this key point. To determine the temperature-shift point, the kinetic characteristics of cell growth and ECB production were investigated.

Figure 2 shows the kinetic characteristics of cell growth and ECB production at various culture temperatures. As shown in Fig. 2a, the cell growth rate increased sharply at the initial phase of fermentation, and the maximum cell growth rate was achieved more quickly at higher temperatures. Figure 2a also shows that the maximum cell growth rate increased with increasing culture temperature, and the highest growth rate of *A. nidulans* was achieved at 30 °C.

Fig. 2 Kinetic parameters of ECB fermentation by *A. nidulans* CCTCC M2012300: cell growth rate (a) and ECB production rate (b) under different culture temperature

Therefore, a relatively high temperature is obviously favorable for the growth of *A. nidulans*. Figure 2b indicates the time profile of ECB production rate at various culture temperatures. The maximum ECB production rates were achieved after 8, 7.5, 6 and 4 days of cultivation at 23, 25, 27, and 30 °C, respectively. These results indicate that ECB biosynthesis will be accelerated when the culture temperature rises and the highest ECB formation rates were achieved at 25 °C. Given that the formation of ECB was at the middle and late stages of fermentation (Kanda et al. 2009), maintaining a low culture temperature (e.g. 25 °C) in the anaphase of fermentation was beneficial for maximizing ECB formation rates. These results were in agreement with those of Peng et al. (2010), who reported that fungi *Mortierella alpina* require more high temperature (25 °C) for cell growth and low temperature (20 °C) for the formation of arachidonic acid. The various optimal temperatures for different phases of fermentation were also reported by Zhou et al.

(Zhou and Kang 2012), who also suggested that a higher temperature (34 °C) was beneficial for cell growth and that a low temperature (32 °C) was beneficial for producing the yellow pigments.

The results above indicated that the growth of *A. nidulans* is mainly before day 6, whereas the ECB formation is mainly after day 6. Thus, we set the sixth day as the shift-temperature point to make both phases obtain optimum results. The optimal values of cell growth and ECB formation rates would be attained simultaneously in the temperature stage-controlled fermentation process.

Development of a two-stage temperature control strategy for ECB fermentation

Based on the above analysis of kinetic characteristics of ECB fermentation at different culture temperatures, a two-stage temperature control strategy was proposed as follows: in the first 6 days, the temperature was maintained at 30 °C to obtain the maximal cell growth rate; subsequently, 25 °C was used to obtain a high ECB formation rate to enhance the ECB. Figure 3 shows the time course of this two-stage

Fig. 3 Batch fermentation with culture temperature shifted from 30 to 25 °C in a 5-L fermenter by *A. nidulans* CCTCC M2012300

Table 1 Comparison of different fermentation parameters at different temperature control conditions

Parameters	Temperature (°C)				
	23	25	27	30	30→25
DCW (g/L)	56.8 ± 3.4	63.7 ± 4.2	67.6 ± 2.1	72.8 ± 3.3	65.5 ± 5.7
ECB (g/L)	0.92 ± 0.07	1.43 ± 0.08	1.22 ± 0.06	0.98 ± 0.05	1.72 ± 0.08
$Y_{\text{ECB/DCW}}$ (%)	1.62	2.24	1.81	1.34	2.62
Period (day)	15	13	12	12	12
ECB productivity [mg/(L day)]	61.3	110	102	81.6	143

DCW dry cell weight, ECB echinocandin B; $Y_{\text{ECB/DCW}}$ the mass ratio of ECB to DCW

^aStarting time of ECB synthesis was defined as the time when ECB was obtained and the ECB concentration was more than 0.1 g/L

temperature control strategy. As shown in Fig. 3, the DCW increased rapidly during the first 6 days of fermentation, and it reached the maximum value (65.5 g/L) at the sixth day. During the initial stage of fermentation, a few ECB were biosynthesized. Nevertheless, it improved rapidly after 4 days of fermentation, and a high ECB formation rate was maintained after the temperature-shift point. The fermentation parameters at different temperature control conditions are listed in Table 1. ECB production was significantly enhanced using the two-stage temperature control strategy. The highest ECB production reached 1.72 g/L under the two-stage temperature strategy, which was an improvement of 26.2% compared with that of the maximum obtained under the constant-temperature condition (25 °C). In addition, using the two-stage temperature strategy, the starting time of ECB biosynthesis was advanced by 1 day and the fermentation period was shortened compared with that of constant-temperature condition (25 °C). Thus, the temperature-shift strategy can considerably improve the ECB concentration and shorten the lag time of ECB biosynthesis. Considering that both increased DCW and ECB percentage in DCW ($Y_{\text{ECB/DCW}}$) contributed to a higher ECB concentration, the value of $Y_{\text{ECB/DCW}}$ from different fermentation processes has also been provided in Table 1. The value of $Y_{\text{ECB/DCW}}$ was 2.62% using the temperature-shift strategy, whereas the values of $Y_{\text{ECB/DCW}}$ under the single temperature 23, 25, 27 and 30 °C were only 1.62%, 2.24%, 1.81% and 1.34%, respectively. The ECB productivity reached 143.3 mg/(L day) using the two-stage temperature strategy, which corresponds to a 1.3-fold increase compared with that of the optimal single temperature cultivation at 25 °C.

By applying this temperature-shift strategy, cell reproduction and ECB accumulation can be optimized simultaneously with a two-stage culture, rather than one stage, in which ECB accumulation conflicts with cell proliferation. Dissolved oxygen concentration was also possibly increased by low-temperature cultures at the later stage of fermentation to mitigate cell autolysis (Kanda et al. 2009; Klich et al. 2001). It might be the reason why the value of DCW did

not significantly decrease when the culture temperature was switched to a lower value at the later stage of fermentation. It was clear that the proposed temperature-shift strategy can improve ECB fermentation level obviously.

Further improvement of ECB production by a combined fed-batch strategy

The two-stage temperature control has been proved to be an efficient strategy for improving ECB production. However, the ECB productivity of whole batch fermentation remained relatively low because of the low level of mannitol that remained in ECB synthesis phase, which could have led to the insufficiency of carbon source. On the other hand, a high initial mannitol concentration had an inhibitory effect on cell growth and ECB production (Zou et al. 2015b; Tóth et al. 2012). A fed-batch fermentation process may relieve the above problem by controlling the substrate concentration below the inhibitory levels (Dolejs et al. 2014; Le Meur et al. 2014). Previous studies

showed that the fed-batch strategy was effective to increase the productivity of echinocandins (Kanda et al. 2010). In this study, to explore the potential of ECB production, the two-stage temperature control strategy was further investigated to improve ECB productivity by combining different mannitol feeding modes. Figure 4 and Table 2 show the effects of four pulse-feeding modes (modes A, B, C, and D) on the ECB production. For each mode, the initial mannitol concentration was 48.5 g/L and the total addition of mannitol was equal. As shown in Table 2, a three-stage mannitol pulse-feeding mode (mode D) led to the highest ECB productivity [170.8 mg/(L day)] among four feeding modes (modes A–C). By contrast, the obvious difference between the ECB productivities for four feeding modes suggested that both mannitol feeding frequency and feeding time were important for ECB biosynthesis. By three-stage mannitol pulse feeding, the residual of mannitol was maintained at a great concentration for young mycelia growing in the ECB synthesis period. The maximal mannitol conversion efficiency ($Y_{\text{ECB/mannitol}}$) of 2.1% and

Fig. 4 Time course of ECB fed-batch fermentation with different feeding modes based on two-stage temperature control strategy by *A. nidulans* CCTCC M2012300 in a 5-L fermenter. The initial mannitol

concentration was 48.5 g/L. 2.5 g mannitol was fed at day 3 (a); 2.5 g mannitol was fed at day 6 (b); 2.5 g mannitol was fed at day 9 (c); 0.83 g mannitol was fed at 3, 6, 9 days, respectively (d)

Table 2 Comparison of ECB production using different feeding strategies under two-stage temperature control

Feeding mode ^a	Period (d)	ECB (g/L)	$Y_{\text{ECB/mannitol}}$ (%)	$Y_{\text{ECB/DCW}}$ (%)	ECB productivity [mg/(L day)]
Mode A	12	1.68 ± 0.09	1.7	3.6	140
Mode B	12	1.87 ± 0.07	1.9	4.4	156
Mode C	12	0.9 ± 0.06	0.9	2.0	75
Mode D	12	2.05 ± 0.09	2.1	5.1	171

ECB echinocandin B, $Y_{\text{ECB/Mannitol}}$ the mass ratio of ECB to mannitol, $Y_{\text{ECB/DCW}}$ the mass ratio of ECB to DCW

^aMode A, feeding 10 g mannitol at 3 days; mode B, feeding 10 g mannitol at 6 days; mode C, feeding 10 g mannitol at 9 days; mode D, feeding 3.3 g mannitol at 3, 6, 9 days, respectively

the maximal $Y_{\text{ECB/DCW}}$ of 5.1% were obtained in fed-batch mode D (Table 2), respectively, which increased by 16.7% and 94.6% compared with the batch fermentation. This result suggested that mannitol was mainly used for synthesizing ECB instead of cell growth. These findings were consistent with Kanda et al.'s report (Kanda et al. 2010) in that control of carbon source concentration is necessary for effective production. Generally, plentiful mannitol concentrations in the fermentation prophase were beneficial for cell growth. However, during the middle- and later fermentation phases, sufficient mannitol will be necessary for the biosynthesis of ECB. By the fed-batch fermentation process, the productivity of ECB was 170.8 mg/(L day), which is a 1.2-fold improvement compared with that of batch fermentation.

Conclusion

This study described a two-stage temperature control method based on kinetic analysis for efficient ECB fermentation from *A. nidulans* CCTCC M2012300. The temperature was controlled at 30 °C for the first 6 days to speed up the growth of filamentous fungus and switched to 30 °C after 6 days to maintain high ECB production rate. Furthermore, a three-stage mannitol pulse-feeding strategy was used to enhance ECB productivity based on the proposed temperature control strategy. This strategy was simple and economical to operate and may be possible to apply this approach for the industrial-scale production of ECB.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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