Arch. Biol. Sci., Belgrade, 63 (3), 907-914, 2011 DOI:10.2298/ABS1103907D

OPTIMIZATION OF CYCLOSPORIN A PRODUCTION BY BEAUVERIA NIVEA IN CONTINUOUS FED-BATCH FERMENTATION

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Abstract - To develop the effective control method for fed-batch culture of cyclosporin A production, we chose fructose, L-valine and $(NH_4)_2HPO_4$ as feeding nutrients and compared their productivities in relation to different concentrations. The feeding rate of three kinds of feeding materials was controlled to maintain the suitable residual concentration. The fed-batch fermentation results indicated that the optimal concentrations of fructose, L-valine and $(NH_4)_2HPO_4$ were about 20 g/L, 0.5 g/L and 0.6 g/L for cyclosporin A production, respectively. The cultivation of *Beauveria nivea* could produce cyclosporin A up to 6.2 g/L for 240 hrs through a continuous feeding-rate-controlled-batch process under the optimal feeding conditions.

Key words: cyclosporin A, fed-batch fermentation, *Beauveria nivea*

UDC 615.01:616

INTRODUCTION

Cyclosporin A is a cyclic 11-membered fungal peptide metabolite which is widely used as a powerful immunosuppressant in transplantation surgery (Zhao et al., 1991). The organisms that are known to produce cyclosporin A include *Tolypocladium inflatum*, *Fusarium solani* and *Neocosmospora uarinfecta* (Aarino and Agathos 1989; Sawai et al., 1981; Nakajima et al., 1988). In particular, the strain *Tolypocladium inflatum* was also formally defined as *Beauveria nivea*. The biosynthesis of cyclosporin A and its analogues appears to proceed via a non-ribosomal mechanism involving the sequential activation of all 11 amino acids, their N-methylation and eventual peptide formation, by a single multifunctional enzyme, cyclosporin synthetase. Although cyclosporin A could be produced by the fungal microorganism *Beauveria nivea* in the matter of submerged cultivation of solid-state fermentation, the productivity of cyclosporin A was lower than that in the submerged fermentation. The previous studies have also proved that the exogenous supplementation of amino acids such as L-valine or L-leucine and the carbon source during fermentation could significantly enhance the production of cyclosporin A in submerged fermentation(Agathos and Parekh 1990; Agathos et al., 1987). The production of cyclosporin A generally is carried out by submerged fermentation with batch fermentation or internal feeding batch fermentation. Although the internal feeding batch fermentation has a high yield of cyclosporin A in contrast to batch fermentation, this pattern almost resulted in the unstable substrate concentration disrupting the biosynthesis of cyclosporin A. However, so far there is no perfect defined continuous fed-batch process for the

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production of cyclosporin A. In the present study, the fungal strain *Beauveria nivea* was performed to produce cyclosporin A as the matter of continuous fed-batch fermentation.

MATERIALS AND METHODS

Microorganism and culture conditions

The microorganism *Tolypocladium inflatum* (lnc1), indicated as *Beauveria nivea*, was stored in the Agricultural Culture Collection of China (ACCC) as number of 31701.The spores were obtained from a PDA agar (tomato 200 g/L, glucose 20 g/L, pH nature), and spore suspensions were prepared after 10 days of cultivation and maintained in 50% glycerol at -80°C. The viability of the deep-frozen cultures was periodically controlled by plating them onto an ISP2 agar (malt extract 10 g/L, yeast extract 4 g/L, glucose 4g/L, agar 20 g/L, pH7.2), and then counting the colonies formed to confirm the spore viability.

Fed-batch cultivation conditions

The inoculum for the shake flask was prepared by transferring the spore suspension in a 500-mL Erlenmeyer flask containing 100 mL of seed medium (containing fructose 5 g/L, glucose 12 g/L, soybean powder 30 g/L, corn steep powder 10 g/L, pH 6.0), incubating on a rotary shaker at 27°C and 240 rpm for 40 h, and inoculating the seed medium into a seed tank at a 2% v/v ratio and cultivating under the same conditions as the shake flask culture. The cyclosporin A biosynthesis was then performed using a fermentation medium (containing fructose 100 g/L, $(NH_4)_2HPO_4$ 20 g/L, $NaNO_3$ 5 g/L, KH_2PO_4 3 g/L, KCl 0.2 g/L, $MgSO_4{\cdot}5H_20$ 0.7 g/L, pH 6.5) in a 30 L stirred tank bioreactor (Biotech-2002, Shanghai Baoxing Bio-engineering Equipment Co., Ltd, China) with a working volume of 20 L. The agitation and aeration were adjusted according to the dissolved oxygen concentration. The pH was adjusted by addition of liquid ammonia. Fermentation foam was suppressed by the addition of the antifoam reagent aminoalkyl polysiloxane.

Biomass determination

A 10 mL sample of culture broth was centrifuged at 5,000 rpm for 5 min and the mycelial biomass was washed twice with distilled water after the supernatant was decanted. The centrifuged cell mass was transferred to a pre-weighed metal dish and dried at 90°C to constant weight (dry cell weight: DCW).

Total carbohydrates determination

The total carbohydrates were determined spectrophotometrically according to the method of Dubios et al. (1956).

Ammonia nitrogen determination

The ammonia nitrogen of the fermentation broth was determined spectrophotometrically according to the method described by Xie et al. (2005).

Recovery and analysis of cyclosporin A

Cyclosporin A was extracted from the culture broth according to the following process (Ly et al. 2007): 10 ml of culture was treated with 30 ml acetone at 35°C, 200 rpm for 3 h. Thereafter, the samples were centrifuged at 5,000 rpm for 10 min to delete the precipitation. The suspension was filtered using a micropore filter. The concentration of cyclosporin A was determined with the reverse phase C_{18} column by HPLC method (Ly and Margaritis 2007). 5 ml of fermentation broth was treated to collect the biomass by centrifugation at the speed of 3000 rpm. The biomass was extracted in 5 x volume of methanol for 2 h at 30°C. The supernatant of the extracted sampler was performed to detect cyclosporin A. The HPLC conditions were 70/30 (V/V) of acetonitrile and water as the mobile phase, 205 nm of ultraviolet detective wavelength, 1.5 mL/min of flow rate, 70°C of column temperature and 20 μL of sample size. The cyclosporin A was identified and quantified based on the similarity of retention time with that of standard cyclosporin A which was used for the calibration of the system.

Table 1. The comparison of $Y_{p/x}$ and $Y_{p/s}$ at the different fructose concentrations.

Initial fructose concentration (g/l)	Maximum DCW (g/l)	Final pH	cyclosporin A (g/l)	Total fructose consumption (g)	$Y_{p/x}$ (mg/g)	$Y_{p/s}$ (mg/g)
20	18.6	5.6	1.6	1360	86.02	1.17
40	25.7	4.2	2.8	2620	108.95	1.07
60	30.8	3.6	4.8	3980	155.84	1.21
80	33.6	2.8	4.5	4340	133.93	1.04
100	38.3	2.5	3.9	4900	101.83	0.79
120	42.1	2.2	3.5	5260	83.14	0.67

L-valine determination

An HPLC method was chosen to analyze the residue L-valine in the medium (Lange et al. 2003). The medium, free of cells, obtained by centrifugation for 5 min at 14,000 rpm was derivatized with dansyl chloride solution before being injected into a C_{18}-ODS reversed phase column, 4.6×250 mm (Waters, USA). The mobile phase consisted of 60/40 (v/v) of methanol/water, 0.6% (v/v) of glacial acetic acid and 0.008% (v/v) of triethylamine. The constant flow rate was 2 ml/min and derivatized L-valine was detected with a UV detector at 254 nm.

Kinetic analysis

Another method of analyzing the fed-batch fermentation was by examining the variation of specific productivity, q_p, and of specific growth rate, μ. The specific productivity, $q_p = dP/dt\ 1/X$ (P=cyclosporin A concentration, X=biomass concentration, t=time), and μ were derived from time-course data of cell mass and cyclosporin A concentration using a graphic package (Origin 7.5, origin lab company, USA, 2008)

RESULTS AND DISCUSSION

Optimization of the initial fructose concentration

In order to switch the primary metabolism of *Beauveria nivea* to the phase of cyclosporin A production rapidly and smoothly in fed-batch fermentation, it was necessary to optimize the initial carbon source concentration. It was evident from our studies that carbon sources such as glucose and sucrose, which are highly favored for biomass production by *Beauveria nivea,* do not generally provide the physiological state necessary for optimal secondary metabolite formation (data not shown). It was identified that fructose as carbon source had the advantages of improving the production of cyclosporin A (Isaac et al., 1990). Thus, fructose was selected as both the initial and feeding carbon source. The effects of the different initial fructose concentration on the cyclosporin A production in batch fermentation were investigated in a 30 L tank bioreactor. The results shown in Table 1 indicated that low or high initial fructose concentration had a negative effect on the cyclosporin A production. It was concluded that the lower dissolved oxygen concentration triggered by the large biomass in the conditions of higher initial fructose concentration was the most important factor for the weak production of cyclosporin A. These data further demonstrated that the initial fructose concentration of 60 g/L gave rise to the highest specific production rate of 1.21 mg cyclosporin A per g cell and specific consumption rate of 155.84 mg cyclosporin A per g fructose.

Fed-batch fermentation processes of cyclosporin A at different residual fructose concentrations

The biosynthesis of cyclosporin A by the fungus *Beauveria nivea* took a longer period of about 10 days in the submerged fermentation, so it was necessary to carry out the fed-batch fermentation for high production of cyclosporin A. Only a few studies about cyclosporin A production by continuous fed-batch fermentation have been reported. In this study, the continuous fed-batch fermentation processes with different residual fructose concentrations of 10 g/L, 15 g/L, 20 g/L and 25 g/L were examined. The

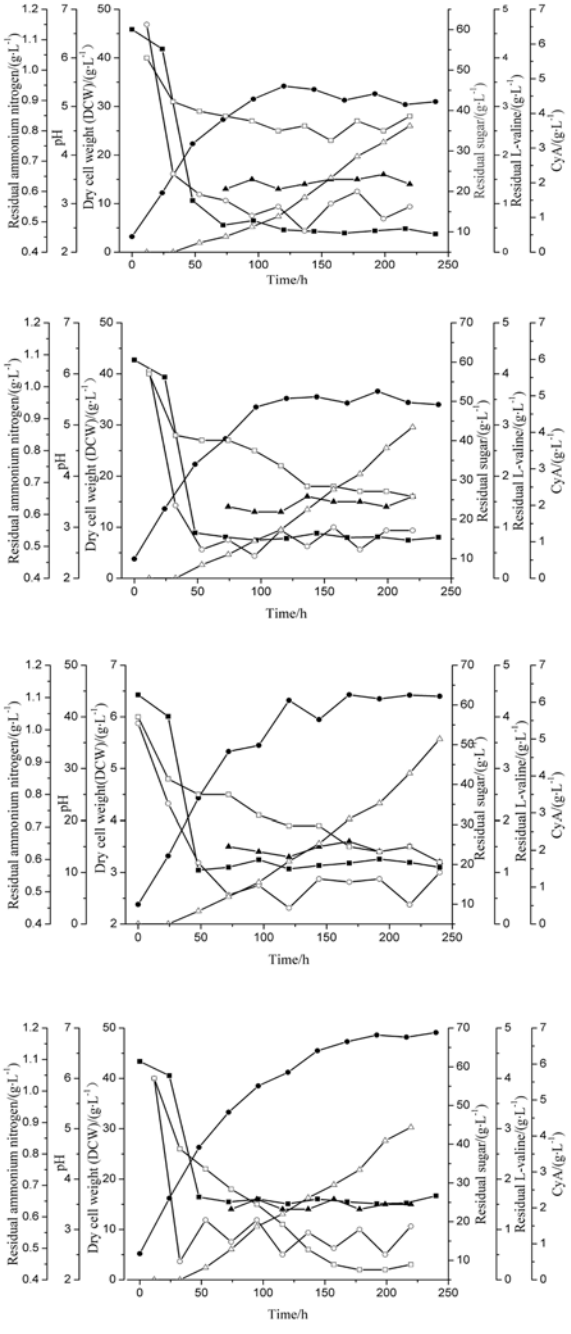

Fig. 1. Kinetics of cyclosporin A fermentation in a 30 l-stirred bioreactor. Residual sugar/▪; DCW/●; Residual L-valine/▲; pH/□; Residual ammonium nitrogen/○; cyclosporin A/△. **A**, fructose controlled about 10 g/L; **B**, fructose controlled about 15 g/L; **C**, fructose controlled about 20 g/L; **D**, fructose controlled about 25 g/L. The concentration of L-valine and $(NH_4)_2PO_4$ were controlled about 1.5 g/L and 0.6 g/L in A-D fermentations, respectively.

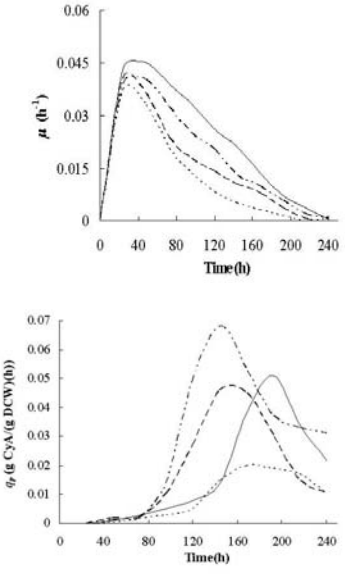

Fig. 2. Analysis of cell growth and cyclosporin A production kinetics on the control of different fructose concentration. μ, specific growth rate; q_p, specific productivity.

..... 10 g/L; – – 15 g/L; –·– 20 g/L; ——— 25 g/L;

other conditions and parameters, including nitrogen source and L-valine, were controlled in the same way. The process curves of different feeding conditions shown in Fig. 1 indicated that a high residual fructose concentration over 20 g/L resulted in high biomass and low cyclosporin A production. 10 g/L of the residual fructose also led to poor cyclosporin A production. Furthermore, the data of kinetic analysis shown in Fig. 2 revealed that the increase of the residual fructose concentration was accompanied by a rise in the specific growth rate of *Beauveria nivea*, and that the specific production rate of cyclosporin A in the condition of high residual fructose was higher than that with lower residual fructose. The 20 g/L of residual fructose concentration was optimal, and had a relatively low specific growth rate and the maximum specific production rate. It was interesting that the pH was maintained over 4.0 when the residual fructose concentration was controlled at 10 g/L and 15 g/L. However, the suitable pH environment of cyclosporin A biosynthesis ranged from 3.0 to 4.0, as reported by Agathos et al. (1986). It was

concluded that the control of feeding fructose generated the principal effects on the biomass and pH condition, and further influenced the cyclosporin A production.

Fed-batch fermentation processes of cyclosporin A at the different residual ammonium nitrogen concentrations

It has been revealed by Margaritis and Chahal (1989) that when fructose was used as a carbon source, the medium containing $(NH_4)_2HPO_4$ resulted in high cyclosporin A production compared to other nitrogen sources such as urea, $(NH_4)_2SO_4$, $NaNO_3$ and even both $(NH_4)_2SO_4$ and KH_2PO_4 in medium. Although the mechanism of $(NH_4)_2HPO_4$ promoting the cyclosporin A production was unknown, it was still favorable as a feeding nitrogen source in this study. In this test the concentration of carbon source fructose was maintained at 20 g/L, and the different residual ammonium nitrogen contents, such as 0.3 g/L and 1.0 g/L, were examined. The fed-batch fermentation processes under different ammonium conditions are presented in Fig. 3. The results demonstrated that the 1.0 g/L nitrogen concentration obviously inhibited the production of cyclosporin A (Fig. 3B). The curves of kinetic analysis shown in Fig. 4 indicated that the optimal $(NH_4)_2HPO_4$ content was 0.6 g/L, which originated from the data of Fig. 1C and presented the maximum specific production of cyclosporin A and suitable specific growth rate. The changes of ammonium nitrogen content had no significant influence on the biomass of *Beauveria nivea* and pH trace. This inhibition phenomenon might be caused by the lower activity of cyclosporin A synthetase in the environment of a high ammonium ion concentration.

Fed-batch fermentation processes of cyclosporin A at the different residual L-valine concentrations

The precursor role of L-valine as its concentration increased in the medium matched well with the findings obtained with L-leucine in bacitracin fermentation by *B. licheniformis* (Haavik and Vessia 1978). In short, the constituent amino acids of the cyclosporin A molecule, such as L-valine and

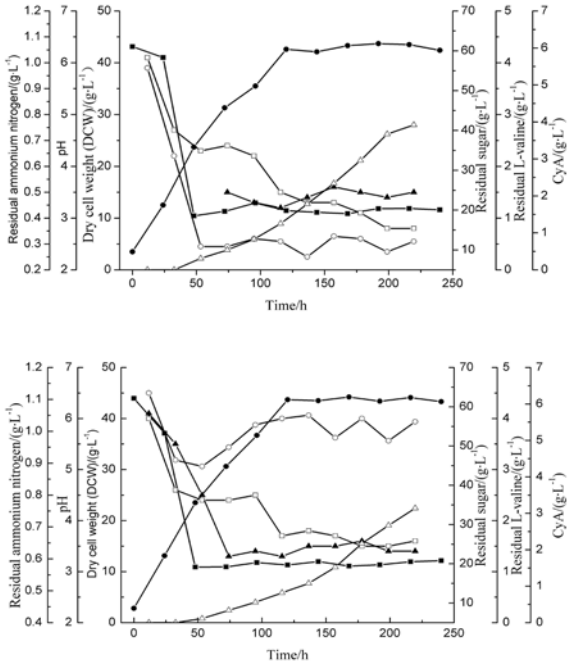

Fig. 3. Kinetics of cyclosporin A fermentation in 30 l-stirred bioreactor – control of the $(NH_4)_2HPO_4$ content. Residual sugar/▪; DCW/●; Residual L-valine/▲; pH/□; Residual ammonium nitrogen/○; cyclosporin A/△. A, $(NH_4)_2HPO_4$ controlled about 0.3 g/L; B, $(NH_4)_2HPO_4$ controlled about 1 g/L.

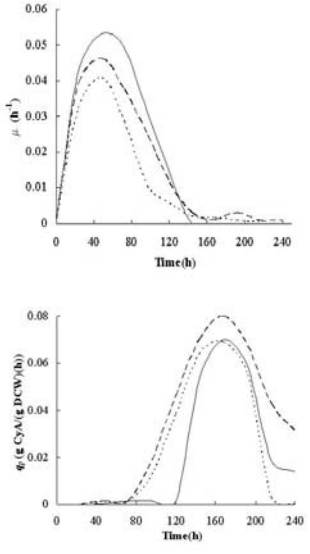

Fig. 4. Analysis of cell growth and cyclosporin A production kinetics on the control of different $(NH_4)_2HPO_4$ concentration. μ, specific growth rate; q_p, specific productivity.

⋯⋯ 0.3 g/L; – – 0.6 g/L; ‾‾ 1.0 g/L.

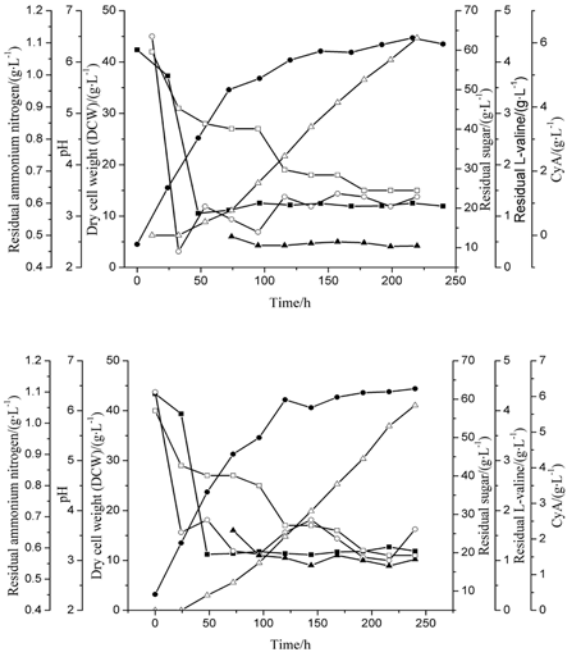

Fig. 5. Kinetics of cyclosporin A fermentation in 30 l-stirred bioreactor on the control of different L-valine concentration. Residual sugar/▪; DCW/●; Residual L-valine/▲; pH/□; Residual ammonium nitrogen/○; cyclosporin A/△. A, L-valine controlled about 0.5 g/L; B, L-valine controlled about 1.0 g/L.

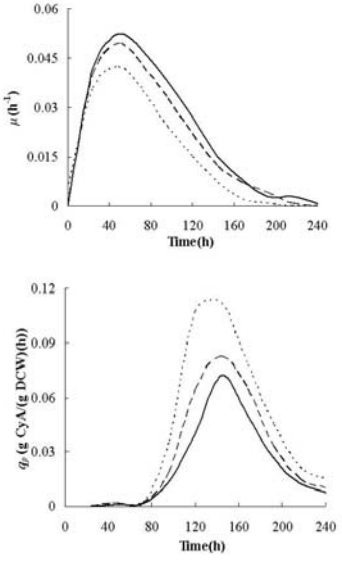

Fig. 6. Analysis of cell growth and cyclosporin A production kinetics on the control of different L-valine concentration. μ, specific growth rate; q_p, specific productivity.
..... 0.3 g/L; – – 0.6 g/L; ‾‾ 1.0 g/L.

L-leucine, may affect the production of the drug by assuming one or more roles such as precursor, inducer and/or developmental regulator. Some amino acids are believed to act as inducers that must be added in the exponential growth phase in order to enhance secondary-metabolite production by directing cell development towards the transcription of the genes concerned, a process that occurs during vegetative cell growth. Previous studies have reported that the addition of L-valine and L-leucine could obviously promote the biosynthesis of cyclosporin A (Lee and Agathos 1989). However, our studies indicated that L-leucine had no distinct positive effect on cyclosporin A production as a precursor (data not shown). In this study it was found that the cyclosporin A titer continued to increase after cell growth ceased and exogenous L-valine was depleted, which was consistant with the earlier findings (Balakrishnan and Pandey 1996). Therefore, the feed of L-valine was arranged at the last stage of logarithmic growth. It was assumed that an intermediate compound accumulated during exponential cell growth and was utilized to produce cyclosporin A after depletion of the internal substrate. Furthermore, we investigated at which level the residual L-valine concentration should be maintained. The results in Fig. 5 showed that the precursor L-valine had no positive function with its concentration increasing during the feeding process. When the residual L-valine in the fermentation broth was maintained at the lower concentration of 0.5 g/L, it obviously promoted cyclosporin A production. However, when the concentration of L-valine rose to 1.5 g/L, the biomass of *Beauveria nivea* increased rapidly and the cyclosporin A production decreased significantly. The kinetic analysis data clearly proved that the lower L-valine content had a lower specific growth rate and higher specific production rate as is shown in Fig. 6. The above results were consistent with the previous study showing that the addition of L-valine during the initial stage of

cell growth has a negative effect on cyclosporin A production, but results in somewhat increased cell growth (Agathos and Lee 1993). This suggests an incompatibility between the primary and secondary metabolic networks involved in cyclosporin A biosynthesis after addition of the amino acid.

In conclusion, many previous researches have attempted to optimize the production of cyclosporin A in submerged cultures and were mostly concerned about medium optimization and the pulsed addition of nutrients (El Enshasy et al., 2008; Abdel-Fattah et al., 2007). The present study focused on continuous fed-batch fermentation based on providing the carbon source, nitrogen source and the amino acid L-valine, which has not been done so far. In order to synthesize cyclosporin A in abundance, the lower initial level and optimal continuous feeding concentration of fructose were determined. This study verifies that the carbon source control was the important factor influencing cyclosporin A production. As for the feed of nitrogen, the regulatory scale seemed to be larger than that of fructose and L-valine. However, the higher level of $(NH_4)_2HPO_4$ over 1 g/L obviously hindered the production of cyclosporin A. The continuous addition of a lower level L-valine during the later stage of the fermentation had a stimulatory effect on cyclosporin A. That this not mediated through additional cellular growth was ruled out since the higher level L-valine could promote cell mass and inhibited the production of cyclosporin A.

Acknowledgment -This work was financially supported by the Ministry of Science and Technology, PR China (grant no. 2007BAI29B01)

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