Production of Cyclodepsipeptides Destruxin A and B from *Metarhizium anisopliae*

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Maltose and peptone were the best carbon and nitrogen sources for the production of destruxins from *Metarhizium anisopliae*. With the addition of 0.1% (w/v) β -alanine to the basal medium, the yields of cyclodepsipeptides DA and DB were 7.2 and 279 mg/ L, respectively, which was 2-fold higher than that of control experiment. Response surface methodology (RSM) was applied to optimize the compositions of maltose, peptone, β -alanine, and glucose used in a shaker-flask cultivation of *M. anisopliae* for the production of DA and DB. Estimated optimal compositions for the DA production were maltose 2.58%, peptone 0.72%, β -alanine 0.02%, and glucose 0.55%. The predicted DA yield was 18.5 mg/L. On the other hand, the optimal compositions for DB production were maltose 2.51%, peptone 0.75%, β -alanine 0.02%, and glucose 0.43%. A maximum DB yield of 232 mg/L was predicted. These were confirmed by cultivation experiments conducted at the optimized conditions for maximum destruxins production in a shaker-flask. Furthermore, a modest high level of DA (49 mg/L) and DB (268 mg/L) yields were obtained by employing the response surface methodology optimized DB production medium in a no-baffle, stirred-tank fermentor.

Introduction

Pathogenic fungi use different strategies to penetrate the outer surface of their targets and kill them. One important aspect of the mode of action of these pathogens is the production and release of mycotoxins from these pathogens (1). Among the metabolites produced by entomogeneous fungi, the destruxins are of a particular interest because these components are the only mycotoxins detected in the insect body when advance stages of infection cause death (2). The destruxins, insecticidal cyclodepsipeptides, were first isolated from Metarhizium anisopliae. Other pathogenic fungi such as Alternaria brassicae (3), Trichothecium roseum (4), or Ophisophaerella herpotricha (5) are also sources of cyclohexadesipeptide destruxins. Currently, over 25 different structurally related destruxins have been isolated, and 15 of these were isolated from *M. anisopliae* (6). The most abounding components are destruxin A (DA), B (DB), and E (DE) (7). The typical structure of destruxins is depicted in Figure 1. Some destruxins have been shown to possess immunodepressant activity in insect model systems and cytotoxic and cytostatic effects on mouse leukemia cells (8). Furthermore, several destruxins also showed a strong suppressive effect on the production of the hepatitis B surface antigen in human hepatoma cells (9).

For the mass production of these useful components, optimization of an appropriate submerged fermentation process is the best choice (10). For widespread field use, large quantities of destruxin preparations with high insecticidal potency are required. Apart from the genetic approach, another way to successfully produce these compounds is the development of an optimal fermenta-



Figure 1. Chemical structures of DA and DB.

tion medium. For this connection, statistical methodologies can be applied in bioprocesses to define the main effects and interactions of the factors that play fundamental roles in microorganism cultivation. One worthwhile technique to identify the explanatory variable in the system is response surface methodology (RSM) (11). RSM can be used to evaluate the relative significance of several factors (12), optimization of microbiological media (13–15), culture conditions (16–17), and synthesis of metabolite (18), and so on.

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Table 1. Coding and Assigned Concentrations (in %, w/v) of Variables of Different Levels of the Central-Composite Design ($\alpha = 2$)

			coded level						
factor	symbol	$-\alpha$	-1	0	1	α			
maltose peptone β -alanine Glucose	$\begin{array}{c}X_1\\X_2\\X_3\\X_4\end{array}$	2.1 0.3 0 0.04	2.3 0.5 0.01 0.20	2.5 0.7 0.02 0.36	2.7 0.9 0.03 0.52	2.9 1.1 0.04 0.6			

Accordingly, in the present study, the fermentation condition and culture medium were tested to increase the production of DA and DB. Furthermore, RSM was employed to optimize the chosen medium compositions. The optimal condition was further illustrated in a 5-L, stirred-tank fermentor.

Materials and Methods

Microorganism and Culture Conditions. M. anisopliae var. anisopliae (Metschnikoff) was kindly supplied by Dr. S. S. Kao, Taiwan Agricultural Chemicals and Toxic Substances Research Institute (Wufeng, Taiwan). β -Alanine, L-alanine, L-valine, L-isoleucine, and L-proline were purchased from Acros (Geel, Belgium). DA and DB were purified as described previously (19). The seed culture was obtained from 5-day-old submerged cultures grown on 3.5% (w/v) Czapek-Dox broth (CD broth, Difco) supplemented with 0.5% (w/v) bactopeptone (Difco), at 200 rpm and 28 °C. For shaker-flask studies, a 5% (v/v) inocula level was used. The cultivation was performed in a set of 500-mL Erlenmeyer flasks each containing 100 mL of different composition of liquid medium, and the culture was allowed to grow at ambient temperature (28 °C) for 14 days on a rotary incubator (200 rpm).

Experimental Designs. The productions of DA and DB from *M. anisopliae* were assumed to be under the influence of the medium composition named: maltose, peptone, β -alanine, and glucose. A central-composite design (CCD) was used to find the accuracy of the results of statistical experimentation. For a 2⁴ factorial fractional design with four factors at two levels, 16 experimental runs are required. Both DA and DB in each Erlenmeyer flask run was analyzed after a growth period of 14 days, and the experimental data was subjected to the segregation of each factor and the interactions between these factors. The actual concentration of each medium component was coded to facilitate multiple regression analysis. To approach the vicinity of the optimum, a first-order model was fitted to the data obtained from the factorial fractional design experiment. The analysis of a simple polynomial model, therefore, is represented by eq 1:

$$Y = \sum a_i x_i + c_0 + \epsilon \tag{1}$$

where *Y* is the predicted response (DA or DB concentration), the parameters a_i are coefficients of x_i , c_0 is the intercept term, and the remaining term, ϵ , represents random error in the yield values.

To describe the nature of the response surface in the optimum region, a 2^4 factorial central-composite design with eight star points ($\alpha = 2$) and two replications of the central points, leading to a total 26 sets, was used at five levels of inocula. The levels of each factor are given in Table 1. The model fitted to the centered data on the response of destruxin production were of second-order polynomial function:

$$Y = c_0 + \sum_{i=1}^n a_i x_i + \sum_{j \le i}^n b_{ij} x_i x_j$$
(2)

where *Y* is the predicted response, subscripts *i* and *j* take value from 1 to the number of variables (*n*), the c_0 is the intercept term, the a_i values are the linear coefficient, the b_{ij} values are the quadratic coefficient, and x_i and x_j are the level of the independent variables.

Statistical Analysis. Data from factorial design were subjected to first- and second-order multiple regression analysis using least-squares regression methodology to obtain the parameters of the mathematical models. These analyses were performed using Statistica software (Tulsa, OK). This software also generated response surface plots.

Fermentation Studies. The inoculum (10% of the working volume) was transferred from the flask of the 4-day-old preculture to the reactor, which contained 3 L of the desired medium. Cultivations were conducted in a 5-L, stirred-tank reactor (BTF-600T, Bio-Top Inc., Taichung, Taiwan) at 28 °C, with fixed aeration rate of 0.3 vvm. The pH value during the period of fermentation was uncontrolled.

Analytical Methods. The analytical HPLC system consisted of a Hitachi (Tokyo, Japan) L-7100 pump, a 20- μ L fixed loop, and a model L-7400 variable-wavelength UV–vis detector. For destruxins analysis, a Micra (Northbrook, IL) NPS RP–C18 analytical column (33 × 4.6 mm) was employed. A gradient combination of acetonitrile/ water system was as follows: 0 min (0% acetonitrile) \rightarrow 20 min (27% acetonitrile) \rightarrow 25 min (90% acetonitrile) \rightarrow 30 min (90% acetonitrile). The eluting solvent with a flow rate of 1 mL/min was fixed throughout the studies. Injection volume was 5 μ L, and the detector monitored absorption was at 215 nm for a period of 30 min. The details for the qualitative and quantitative analysis of DA and DB by HPLC was described previously (*19*).

Results and Discussion

Influence of Medium Composition. Table 2 shows the effects of medium compositions on the DA and DB production. Usually, peptone and the CD broth were the most popular combination found in the literature. However, this medium was not the best composition for the production of destruxins (Table 2). The highest DB level of 77 mg/L was obtained by using peptone and maltose medium. Almost no DA and DB were produced when yeast extract and the CD broth was used as carbon and nitrogen sources, respectively. Peptone and glucose, on the other hand, gave the highest DA yield (3.8 mg/L) and a relatively high concentration of DB (57 mg/L). Campbell and co-workers also reported that glucose and maltose induced good spore production for *M. anisopliae* (20). However, the relationship between sporulation and destruxin production should be studied further. Careful comparison of the carbon sources used in this study shows that maltose is the best, followed by the glucose and then the CD broth. Furthermore, whatever nitrogen source was used parallelly, maltose had the highest DB production. In contrast, less distinction of the DA concentrations was found between all other media used. This suggests that the carbon source has a significant effect on the production of destruxins. The results also revealed that the peptone is the best nitrogen source as compared with yeast and beef extracts under the same cultivation conditions.

The final pH of fermentation broth was varied between 2.7 and 8.5. No direct correlation was found between destruxins yields and final pH value or dry weight. The

 Table 2. Results of the Medium Screening Tests on the

 Various Medium Compositions^a

run	DA (mg/L)	DB (mg/L)	pH (final)	dry wt (%)
peptone + CD broth	ND^b	12	3.4	1.5
peptone + maltose	2.8	77	3.4	0.6
peptone + glucose	3.8	57	2.7	1.5
yeast extract + CD broth	ND	1.4	8.3	1.0
yeast extract + maltose	3.0	48	8.2	1.3
yeast extract $+$ glucose	2.1	24	8.5	2.4
beef extract $+$ CD broth	ND	8.9	4.8	1.1
beef extract + maltose	2.6	28	3.7	1.4
beef extract + glucose	1.3	14	3.0	1.4

 a The experiments were conducted in a 500-mL shaker flask supplement with 3% (w/v) carbon sources and 0.5% (w/v) nitrogen sources for all medium used. b ND: not detected.

final pH mostly depends on the nitrogen source used. Both peptone and beef extract media have a final acidic broth, whereas yeast extract gave an alkaline effect (Table 2). Figure 2 depicted the time course of pH and DB concentration changes in which maltose and peptone were utilized for the culture. Accordingly, the pH dropped from 5.5 at the beginning of the cultivation to about 3.5 in a span of 6 days. Subsequently, the pH value remained at a plateau for a week. DB concentration was dramatically increased during this period. The highest DB appeared on the 13th day (87 mg/L) after inoculum. The pH value was slightly increased from 3.5 to 3.8 at harvest (14th day). Meanwhile, the further production of DB was also diminished. This indicated that the acidic condition is facilitated with the production of DB. However, the reality of the effects of fermentation pH needs to be elucidated further since the biosynthesis of each destruxins seems to facilitate with a different pH or cultivation environment (21). Preliminary results demonstrated that peptone and maltose have a remarkable capability to enhance the production of destruxins in the shake flask studies.

Effect of Amino Acid. Destruxins are cyclic depsipeptides consisting of five basal amino acids and an α -hydroxy acid (contains one R-group), which constitute the basal structure of destruxins (Figure 1). Addition of amino acid is a pivotal factor in many biosynthesis studies (22–24). Accordingly, five amino acids, β -alanine, L-alanine, L-valine, L-isoleucine, and L-proline, were tested on destruxin biosynthesis in M. anisopliae cultivation. To study the influence of these amino acids, the experimental medium was composed of 3% maltose and 0.5% peptone (basal medium) supplement with 0.1% (w/ v) of each amino acid. The effects of selected amino acids on the production of destruxin are summarized in Table 3. It can be noted that L-alanine and L-valine did not provoke destruxin synthesis and had a negative effect compared to the control experiment. The introduction of L-proline and L-isoleusine in the culture medium, however, did not increase the destruxin biosynthesis. Only β -alanine introduced into the medium has allowed production of DA and DB with more activity than that of the basal medium (without amino acid, control). The yields of DA and DB were 279 and 7.2 mg/L, respectively, which are 2-fold higher than those of control experiments. This suggested that β -alanine is more efficient in provoking DA and DB synthesis in *M. anisopliae*.

Interestingly, L-alanine and β -alanine have entirely different effects on the production of destruxins. One possible interpretation is that the induction of destruxin is a stereospecific mechanism. The least destruxin yield was observed when the additive amino acid is far away from α -hydroxy acid group (cf. Figure 1). This was found



Figure 2. pH and DB profiles during the cultivation of *M. anisopliae* in shaker-flask studies. A basal medium containing maltose (3%) and peptone (0.5%) were used as sole carbon and nitrogen sources, respectively. Each point represents the mean of three determinations.

 Table 3. Influence of Selected Amino Acid on the

 Production of Destruxins by *M. anisopliae*^a

	yie	eld
additive	DA (mg/L)	DB (mg/L)
control	ND	106
L-isoleusine	3.0	116
L-alanine	ND	61
L-valine	2.1	82
L-proline	0.6	114
β -alanine	7.2	279

 a M. anisopliae was grown in the basal medium (3% maltose + 0.5% peptone) supplement with 0.1% (w/v) amino acid. b ND: not detected.

in the case of L-alanine and L-valine (Table 3). Similarly, less effect was generated in the experimental results for L-proline and L-isoleusine. However, β -alanine is next to the α -hydroxy acid in the basal ring structure of destruxin. As a result, a significant increase in the DA and DB productivity was obtained. This implied that the existence of β -alanine in the culture medium could produce a sensitive effect on the production of destruxins. Thus, the key step in the formation of the destruxin ring could be attributed to the conjunction of α -hydroxy acid and β -alanine during the biosynthesis. However, the distribution of the destruxin derivatives may depend on the cultivation conditions employed and the species used.

Optimization of Culture Medium. 2^4 fractional factorial designs were performed in order to approach the optimum response region. The range of the variables tested was as follows: maltose (X_1), 1–3.8%; peptone (X_2), 0.1–0.7%; β -alanine (X_3), 0.05–0.35%; glucose (X_4), 0.2–1%. After 14 days cultivation, concentrations of DA and DB were found to be in the ranges of 2.5–9.0 and 2–256 mg/mL, respectively, for the different media corresponding to the statistical design. The experimental results were fitted with multiple regression analysis as given by eq 1. Corresponding equation related to the DA (\hat{Y}_A) and DB (\hat{Y}_B) yields are given below:

\hat{Y}_{Λ}	= 2.48	+1.05	$5X_{1} +$	$0.84X_{2}$	$-2.08X_3$ -	$-0.6X_{4}$	(3)
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$$\hat{Y}_B = 86.24 + 3.71X_1 + 59.85X_2 - 85.37X_3 - 40.1X_4$$
(4)

Effect estimates from eqs 3 and 4 point out that the X_1 and X_2 have positive effects; X_3 and X_4 exhibited

Table 4. Experimental Design and Results of theCentral-Composite Experiments

	coded level		yield (mg/L)			coded level			yield (mg/L)				
run	$\overline{X_1}$	\mathbf{X}_2	X_3	X_4	DA	DB	run	X_1	\mathbf{X}_2	X_3	X_4	DA	DB
1	-1	-1	-1	-1	5.7	92	14	1	-1	-1	1	8.2	165
2	-1	-1	-1	1	6.2	148	15	1	-1	1	-1	5.1	141
3	-1	-1	1	-1	10.9	145	16	1	-1	1	1	16.4	150
4	-1	-1	1	1	8.6	125	17	-2	0	0	0	1.9	56
5	-1	1	-1	-1	5.0	85	18	2	0	0	0	11.2	144
6	-1	1	-1	1	10.5	184	19	0	-2	0	0	5.4	75
7	-1	1	1	-1	3.6	185	20	0	2	0	0	6.4	125
8	-1	1	1	1	12.5	218	21	0	0	-2	0	8.7	121
9	1	-1	-1	-1	6.9	99	22	0	0	2	0	13.4	148
10	1	-1	-1	1	14.6	203	23	0	0	0	-2	11.6	141
11	1	-1	1	-1	6.0	146	24	0	0	0	2	14.2	163
12	1	$^{-1}$	1	1	12.3	94	25	0	0	0	0	17.0	223
13	1	-1	-1	-1	12.9	155	26	0	0	0	0	17.0	233

negative coefficients. The low coefficient obtained appears to indicate that the concentrations used were not large enough to detect appreciable changes in the destruxins synthesis. Accordingly, more maltose and peptone need to be added to the medium. These results could be expected, as higher maltose and peptone concentrations would provide more DA and DB production. In addition, the concentration of β -alanine and glucose should be reduced in the factorial central-composite design experiments. Equations 3 and 4 also indicate that β -alanine is the most significant factor due to its coefficient effect being heavily pronounced. In summary, preliminary studies implied that all the variables have to be rearranged to search for the direction toward the optimum region.

Table 1 shows the four independent variables and their concentrations at the different levels of the centralcomposite design chosen on the basis of the results of the previous factorial design experiments. Experimental design and results for 26 runs are given in Table 4. The fitting of the quadratic model (eq 2) along with the variables of maltose, peptone, β -alanine, and glucose from factorial central-composite experiments are given in Tables 5 for DB. The corresponding second-order response model for DA production using eq 2 that was found after analysis for the regression is

$$\hat{Y}_1 = 16.98 + 3.24X_1 - 5.2X_1^2 - 5.52X_2^2 - 2.95X_3^2 + 3.31X_4 \quad (p \ge 0.1)$$
 (5)

Similarly, the equivalent equation for the DB production is

$$\hat{Y}_2 = 227.74 - 55.85 X_1^2 - 55.68 X_2^2 - 38.68 X_3^2 - 37.49 X_3 X_4 \quad (p \ge 0.05)$$
 (6)

Statistical significance of the second-order model equation was checked by *F*-test. It was found that it is an adequate approximation to the experimental data at the 5% probability level (Table 5). The fit of the model was also expressed by the coefficient of determination R^2 , which was 0.785, indicating that 78.5% of the variability in the response can be explained by the model. This revealed that eqs 5 and 6 are the suitable models to describe the response of the experiment pertaining to DA and DB production. The calculated values for eqs 5 and 6 were the best estimates obtained from the experimental data. It would be inappropriate to conceive a hypothesis that these coefficients are equal to zero. For the analysis of the fitted surface, eqs 5 and 6 were transformed into their canonical forms:

Table 5. Coefficient Estimates in the Regression ModelSelected through Variable Selection for DB

parameter	parameter estimate	standard error	T-ratio	probability
intercept	227.74	22.52	10.11	0.0001*
X ₁	12.27	13.00	0.94	0.3654
X_2	27.40	13.00	2.10	0.0588
X_3	10.54	13.00	0.81	0.4347
X_4	23.66	13.00	1.82	0.0962*
$X_1 imes X_1$	-55.85	12.25	-3.66	0.0037*
$X_2 imes X_1$	-11.42	15.92	-0.72	0.4884
$\mathrm{X}_2 imes \mathrm{X}_2$	-55.68	15.25	-3.65	0.0038*
$X_3 imes X_1$	-31.86	15.93	-2.00	0.0707
$X_3 imes X_2$	17.38	15.93	1.09	0.2985
$X_3 \times X_3$	-38.67	15.25	-2.53	0.0276*
$X_4 imes X_1$	-12.16	15.93	-0.76	0.4612
$\mathrm{X}_4 imes \mathrm{X}_2$	7.88	15.93	0.49	0.6305
$X_4 imes X_3$	-37.48	15.93	-2.35	0.0382*
$X_4 \times X_4^{^{-}}$	-29.99	15.24	-1.96	0.0748

$$Y_1 = 18.47 - 0.68Z_1^2 - 1.57Z_2^2 - 2.74Z_3^2 - 2.86Z_4^2$$
(7)

$$Y_2 = 232.23 - 6.99Z_1^2 - 17.08Z_2^2 - 30.21Z_3^2 - 35.82Z_1^2$$
(8)

Since all coefficients showed in the eqs 7 and 8 are negative, the fitted surface has a true maximum value. On the basis of this observation, the concentration ranges of four components were toward the maximal region as seen in the surface plot (Figure 3). This plot also indicated that the yield of DA and DB near the optimum was more sensitive to changes in maltose and peptone concentration. The relationship between any two variables and destruxin concentrations can also be quantitated and displayed in the form of a contour plot (Figure 4). Estimated optimal compositions for DA production are as follows: maltose 2.58%, peptone 0.72%, β -alanine 0.02%, and glucose 0.55% (see Table 6). The predicated DA yield was 18.5 mg/L. Similarly, the optimal compositions for DB production are as follows: maltose 2.51%, peptone 0.75 \hat{k} , β -alanine 0.02%, and glucose 0.43% (RSM medium). A maximum DB yield of 232 mg/L was predicted under this condition.

To confirm the reality of empirical models, the parallel experiments were carried out on shaker flask incorporated with these two optimum points into the culture medium. The yields of 19.5 mg/mL of DA and 220 mg/L of DB were observed. Good correlation between the predicted and the measured result of this experiment verifies the validity of the response model and the ability to reach an optimum point in the system. The optimum concentration for these factors also can easily be found from the surface and contour plots of DA and DB production as given in Figures 3 and 4 obtained from the CCD experiments. The fitted models and correlation coefficients for the variables as given in eqs 7 and 8 show that the productions of DA and DB were strongly affected by β -alanine and glucose. By examination of the surface and contour plots presented in Figures 3 and 4, different effect levels were found in each case on the production of DA and DB. This suggests that the whole system is extremely difficult to assess and to give the clear interactions between the factors. Although the actual situation may be more complicated than that we have presented here, an attempt for the medium optimization has been made by RSM. In particular, the regression model is adequate to the response taken from the experimental data and can define a process that allows optimization of the culture medium.

Fermentation Studies. Further studies on the fer-



Figure 3. Surface plot of maltose versus peptone for DA (A) and DB (B) production in submerged culture.

mentor production of destruxins were investigated in a 5-L, stirred-tank reactor (STR). Influence of reactor's baffle on the production of destruxin is presented in Figure 5. As shown in this figure, the pH was dropped during the first week of cultivation and then kept constant thereafter for both with and without baffle studies. Obviously, the baffle affected the growth of *M. anisopliae* as well as the release of carboxyl acid into the medium. Once the growth of organism archived the stationary phase and the synthesis of destruxin was initiated, the pH remains unchanged.

Regardless of whether the baffle was adapted, the pH profiles were unique. However, only one pH unit of difference could be found in the harvested sample. Large differences in the DB yield were also found when compared to the DB concentration in shaker-flask cultivation (Figure 2). The highest DB concentrations were 120 and 223 mg/L for reactors with and without baffle, respectively. The STR adapted with the baffle is unfavorable to the destruxin synthesis. Theoretically, the relative oxygen transfer of the reactor with baffle is better than that without a baffle and should provide a better environment for the growth of *M. anisopliae*. This means the tank adapted with a baffle will afford better mass/gas transfer efficiency. However, the baffle also caused an aggregation of *M*. anisopliae during the cultivation. Therefore, the growth of fungus and synthesis of destruxins were hindered. Consequently, a nearly 2-fold higher DB yield was found in the STR without baffle. Furthermore, relatively low aeration (0.3 vvm) was evidence that the oxygen supplement is not a crucial factor for the



Figure 4. Contour plot of the model equation fitted to the data of the central-composition design. Estimated destruxins yield as a function of β -alanine and maltose in submerged culture. The isoresponse contours represent the DA (A) and DB (B) yields.

Table 6. Predicted and Experimental Results ofDestruxins Produced from *M. anisopliae* underCalculated Optimize Conditions^a

	optin	num comp	yield (mg/L)			
	maltose	peptone	β -alanine	glucose	predicted	experimental
DA	2.57	0.72	0.025	0.55	18.47	48.9
DB	2.51	0.75	0.020	0.43	232.23	268.4

 a The cultivated conditions selected: a eration rate, 0.3 vvm; agitation speed, 150 rpm; temperature, 28 °C.

destruxin synthesis. No significant difference was observed when comparing the yield of DA or DB between the shaker-flask (with RSM medium) and 5-L fermentor (with basal medium). Again, the aeration condition seems not to be the main effect on the production of destruxins. One possibility is that the agitation enhanced the synthesis of secondary metabolites during the cultivation. Thus, the basal medium with ambient agitation rate could obtain a similar yield of destruxins.

Figure 6 shows the pH, DO, and residual sugar time course for the cultivation of *M. anisopliae* with RSM medium in 5-L fermentor (without baffle). As can be seen from this figure, the pH value dropped from 5.1 (day 0) to 2.3 (day 6) and moved back slightly to 3 until harvest, which is similar to previous observations. On the other hand, the DO value also decreased rapidly during the first 5 days, and then switched back to a 60% level. The analysis of residual sugar showed the consumption of the carbon source almost exhibits a negative linear profile. Nevertheless, about 10% of the carbon nutrient was still left in the medium after harvest. This indicates that the growth of *M. anisopliae* did not stop after the 8th day of



Figure 5. Effects of baffle on the pH (top panel) and DB production (bottom panel) profile of fermentation time course. The experiments were carried out in a 5-L STR with basal medium. For operating condition, see Materials and Method for details.



Figure 6. Time course of pH $(-\nabla -)$, DO (--), and residual sugar $(\cdots \bullet \cdots)$ profiles during the cultivation of *M. anisopliae* in a 5-L STR with RSM medium.

cultivation, even though the DO was maintained at 60% during this period. Figure 7 shows the time course of the DA and DB production from the 5-L fermentation (with RSM medium). Clearly, both pH and DO values were kept as constant (Figure 6) once the destruxins synthesis was commenced. The DA and DB yields, after 14 days cultivation, were 48.9 and 268 mg/L, respectively, with the yields being 150% and 22% higher than that of shaker-flask cultivation (with RSM medium) for DA and DB, respectively.



Figure 7. Time course of DA ($\dots \oplus \dots$) and DB ($- \bigcirc -$) concentrations (mg/L) in RSM medium during cultivation in a 5-L STR. Each point is average values from two separate runs.

Conclusions

In this study, we tested several carbon and nitrogen sources to increase the production of destruxins from *M*. anisopliae. The experimental results suggest that the CD broth, which has been reported in the most literatures, could entirely be substituted by maltose. Moreover, the effect of amino acid additive has also been investigated using different kinds of amino acids. When the basal medium was supplemented with 0.1% β -alanine, a more than 2-fold increase of DA and DB was observed (Table 3) as compared to the results of the control experiment. Obviously, additive of β -alanine in the culture medium improved the production of destruxins. This led us to conclude that the key step in the formation of the destruxin ring could possibly be attributed to the conjunction of α -hydroxy acid and β -alanine during the biosynthesis.

Four factors (maltose, peptone, β -alanine, and glucose) were examined for optimization of destruxin production using response surface methodology. The present study shows that the RSM has proved satisfactory in the medium-screening assessment. Estimate optimal compositions for the DA production were as follows: maltose 2.58%, peptone 0.72%, β -alanine 0.02%, and glucose 0.55%. The predicated DA yield was 18.5 mg/L. Similarly, the optimal compositions for DB production are as follows: maltose 2.51%, peptone 0.75%, β -alanine 0.02%, and glucose 0.43%. A maximum DB yield of 232 mg/L was predicted under this condition. The experiments were examined by RSM medium in the shaker flask, in which 19.5 mg/L of DA as well as 220 mg/L of DB were observed. Fitted models provide a suitable prediction in the response (destruxins yield), indicating that the improvement of medium optimization can be made through the RSM procedure. RSM was able to describe the effects of maltose, peptone, β -alanine, and glucose on the production of destruxins by *M. anisopliae*. Further study on the effect of the baffle for destruxin production shows that the STR without baffle is more suitable for the production of destruxin under experimental conditions. Approximately twice higher DB concentration was found in the STR without baffle as compared to that resulting from the reactor with baffle. In conclusion, applying the RSM medium without baffle (0.3 vvm aeration rate) in the STR is the best condition for the production of DA and DB in the present study. The highest DA and DB concentrations at the end of cultivation were 48.9 and 268 mg/L, respectively. This is almost twice as high as that of the basal medium with baffle in STR fermentation.

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