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# Effect of pH and Aeration Rate on the Production of Destruxins A and B from *Metarhizium anisopliae*

Bing-Lan Liu[1], Tzyy-Min Rou[1], Yerra Koteswara Rao[2], Yew-Min Tzeng[2]*

[1]*Department of Applied Chemistry,* [2]*Institute of Biotechnology, Chaoyang University of Technology, Wufong, Taichung County 41349, Taiwan, R.O.C.*

**Abstract:** The effects of culture medium initial pH ranging from pH 2 to 9 on the production of destruxins A (DA) and B (DB) from the fungus of *Metarhizium anisopliae* F061 were examined by shaker flask culture, in 5 L stirred tank bioreactor (STR) and 20 L airlift bioreactor (ALR). In shaker flasks, when no control was applied, whatever initial pH used in the medium, the final pH of the broth was always dropped between 3 and 4 at the end of cultivation. During this study, when initial pH was held at below 5, low concentration of DB was obtained. However, the initial pH at 9 is more favorable for the maximal DB production; in contrast, less distinction of DA concentration was observed within the pH range studied. On the other hand, the pH values showed different effects on DA and DB production in 5 L STR cultivation. Maximum yield of DA (71.0 mg/L) and DB (310.6 mg/L) were achieved at pH 9 after 12 and 14 day cultivations, respectively, and these values were three fold higher than those obtained from pH of 2 cultivation. Furthermore, culture medium final pH at 3.7 is more favorable for the high yield of DB production in the above studies. In 20 L ALR with uncontrolled pH, the maximum levels 350 and 550 mg/L of DB were obtained, after 12 and 9 day cultivations by using 6 mesh and without (bubble column mode), net-draft-tube, respectively. On the other hand, the concentration of DB was greatly increased by increasing the aeration rate from 0.5 to 1.5 vvm (volume per volume per minute). A maximum yield, nearly 700.0 mg/L of DB was obtained with an aeration rate of 1.5 vvm and controlled pH at 6 in bubble column mode cultivation.

**Keywords:** *Metarhizium anisopliae*; Destruxins; Airlift bioreactor

## 1. Introduction

The entomopathogenic fungus *Metarhizium anisopliae* is one of the most studied and applied species amongst fungal biocontrol agents and several commercial products have been developed and registered for the control of different insect pests. Among them, the destruxins (Dtxs) are a particularly interesting example as these cyclic depsipeptides are composed of one α-hydroxy acid and five amino acid residues [1-4]. Up to now, over 35 different structurally related Dtxs have been isolated from cultures of *M. anisopliae* [5]. By virtue of its structure, Dtxs exhibit a wide spectrum of biological activities including cytotoxic, anti-hepatitis B, insecticidal, V-ATPase inhibitors, phytotoxins, mitochondrial ATPase inhibitors, and calcium channel blockers [6-14]. Destruxin A (DA), and destruxin B (DB) are having the same amino acid sequence but differ in their hydroxyacid

* Corresponding author; e-mail: ymtzeng@cyut.edu.tw
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residue are reported as antiviral and immunodepressant agents in insect cells among the destruxins [15,16].

Mass production of Dtxs preparation will be required for supplying public demand and to employ for widespread usages. The secondary metabolite production from microorganisms is not only influenced by biological factors, the physicochemical environment in which the organism is grown also plays an important role in high yield production. It has been reported that pH, agitation, dissolved oxygen tension, polymer additives, surface active agents, growth rate and inoculum are the parameters that affect the secondary metabolite production by filamentous fungi [17-21]. In particular, the pH and aeration rate are the most critical parameters and plays a significant role in productivity of the process. Despite the likely importance of culture pH, most studies on Dtxs formation by *M. anisopliae* have adopted a fixed initial pH (*i.e.* pH was uncontrolled) for the cultivation process in shaker flask studies [22, 23]. In our previous papers, we have reported some of the factors affecting on the production, purification, and effect of fungal pellet size of the fungus *M. anisoplie* for production of Dtxs [24-26].

Although Dtxs have potential application in industry and research, there are only a few reports available on the large-scale production of Dtxs and there is almost no report that deals with influence of pH and aeration rate on the DA and DB production from a native (Taiwan) strain of *M. anisopliae* F061. Thus, the aim of this study was to determine the effect of pH on DA and DB production by culturing *M. anisopliae* F061 in shaker flask and in 5 L STR. Furthermore, the influence of pH and aeration rate on the production of DB was also investigated in 20 L ALR, because each organism has its own cultivation conditions for maximum productivity. Therefore, a series of cultivations were carried out to assess the effect of pH on the DA and DB production under constant aeration rate. Similarly, the experiment was designed to evaluate the influence of aeration rate on the production of DB without changing the pH value.

## 2. Materials and Methods

### 2.1 Microorganism

A culture of *M. anisopliae* F061 var. *anisopliae* (Metschnikoff) was kindly provided by Dr. S. S. Kao, Taiwan Agricultural Chemicals and Toxic Substances Research Institute (Wufong, Taiwan) was used in this study. The spore suspension was obtained from 4-day-old submerged culture grown on 3% Czapek-Dox (CD) broth (Difco, Detroit, MI) supplemented with 0.75% bacto-peptone (Difco), at 200 rpm and 28 °C used as an inoculum. All chemicals used were of reagent-grade quality.

### 2.2 Shaker flask study

For shaker flask study, a 5% (v/v) inoculum level was used. The experiments were conducted with 500 mL Erlenmeyer flasks containing 200 mL medium (2.51% maltose, 0.75% bacto-peptone, 0.02% β-alanine, and 0.43% glucose). The initial pH was adjusted by addition of solutions of either with 1 N $H_2SO_4$ or NaOH between 2 to 9. Cultivation was then started by inoculation of 5% seed culture in each flask. The culture was allowed to grow at ambient temperature (28 °C) for 14 days on a rotary shaker (150 rpm). Samples were passed through a 0.22 μm membrane prior to HPLC analysis.

### 2.3 Stirred tank cultivation

For the stirred tank cultivation, 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, and the aeration

rate was controlled at 0.3 vvm. The culture medium pH values were varied from 2 to 9; it was kept constant by automatic addition of either 1 N NaOH or $H_2SO_4$. If not indicated otherwise, the cultivation agitation rate was 150 rpm.

### 2.4 Airlift bioreactor cultivation

The cultivations were conducted in a net-draft-tube modified 20 L airlift bioreactor (ALF-20, Bio-Top Inc., Taichung, Taiwan). Pre-culture was cultivated as stated above, then 1.5 L of the vegetative seed culture was aseptically transferred to the bioreactor previously filled with 13.5 L of sterile medium. Unless otherwise stated, the fermentation medium employed in the batch culture contained (%): 2.51 maltose, 0.75 bacto-peptone, 0.02 β-alanine, and 0.43 glucose. The temperature of the bioreactor was controlled at 28 °C and the aeration rate was varied from 0.5 to 1.5 vvm.

### 2.5 Analytical methods

The residual sugars were estimated by the phenol-sulphuric acid method [27]. Qualitative and quantitative analysis of DA and DB by HPLC was done as described previously [25]. For the dry mass content analysis, the fermentation broth (10 mL) was centrifuged at 10,000 rpm for 20 min. The pellet was washed with distilled water (30 mL) for three times. Finally, the pellet was resuspended in 10 mL RO water and the drymass was determined by means of moisture balance (HR73, Metller-Toledo, Switzerland).

## 3. Results and Discussion

### 3.1 Effect of pH on DA and DB production

Based on our previous studies on DA and DB production by *M. anisopliae* F061 [24-26], we have selected the cultivation temperature at 28 ºC, agitation speed 150 rpm for all the cultivations, in addition 5 and 10% (v/v) inoculum for shaker flask and bioreactor cultivations, respectively. In preliminary experiments, the shaken submerged cultures of *M. anisopliae* F061 were grown in medium containing maltose with other ingredients. The experiments were carried out using shaker flasks, in which rigorous control of many culture parameters was not possible. The experimental medium was adjusted to pH values from 2 to 9 by addition of either 1 N NaOH or $H_2SO_4$, and inoculated with 5% (v/v) of seed culture suspension. The culture medium pH values were monitored throughout the 14-day period. The changes in pH during the cultivations were shown in Figure. 1. In general, it can be seen that these values remain practically constant when the initial pH values were lower than 4. When the initial pH was higher than 5, however, there was a tendency for the pH to drop clearly during the cultivation. During this study, whatever initial pH used in the medium, the most final pH values dropped between 3 and 4 at the end of fermentation *i.e.* $14^{th}$ day (Figure 1).

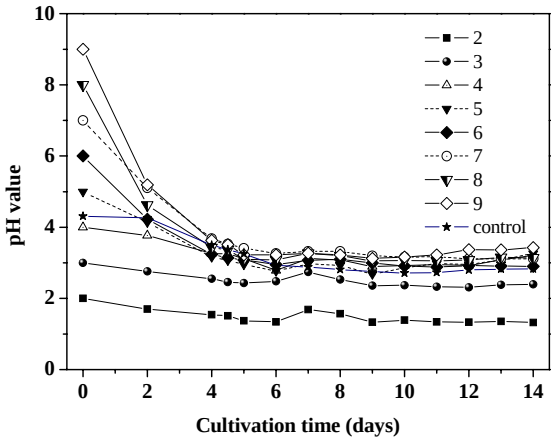


Figure 1. The pH profiles of the cultivation of *M. anisopliae* at different initial pH values in shaker flask study.

In each of the cultures, the formation of DA

and DB were followed. The fall in pH was concomitant with appreciable changes in the concentration of DA and DB (Table 1). It was found that the low pH of 5 greatly inhibited the synthesis of DB, and in the case of starting pH 2, the concentration of DB was 2-3 fold lower than that of higher pH. However, the initial pH values for the maximal synthesis of DB were established at pH 6, 7, and 9 (Table 1). The highest level of DB (240.6 mg/L) was obtained at pH 9, and this level about nearly the same yield as in 5 L STR (268 mg/L), with controlled pH experiment as reported previously [25]. On the other hand, during this study within the pH range applied, notable DA concentration variations were not observed (Table 1). This indicates that DB production is more sensitive than DA to the initial pH of the shaker flask culture medium.

Table 1. Effect of initial pH on the production of DA and DB (mg/L) in shaker flask and 5 L stirred tank cultivations

| Initial pH | Shaker flask[a] | | Stirred tank[b] | |
|---|---|---|---|---|
| | DA | DB | DA | DB |
| 2 | 8.3 | 72.7 | nd[c] | 101.6 |
| 3 | 9.9 | 155.0 | 8.9 | 108.6 |
| 4 | 8.7 | 194.0 | 9.1 | 181.4 |
| 5 | 9.8 | 106.7 | 8.4 | 185.1 |
| 6 | 8.8 | 230.4 | nd[c] | nd[c] |
| 7 | 9.5 | 203.1 | 9.2 | 239.6 |
| 8 | 9.6 | 133.2 | 9.6 | 289.8 |
| 9 | 9.9 | 240.6 | 11.9 | 310.6 |

a. Fermentations were carried out with a 5% (v/v) inocula level in 500 mL Erlenmeyer flask containing 200 mL medium (2.51% maltose, 0.75% bacto-peptone, 0.02% β-alanine, and 0.43% glucose). The cultivations were maintained at 28 °C, 200 rpm for 14 days.
b. Fermentations were carried out with a 10% (v/v) inocula level in 5 L stirred tank reactor containing 3 L medium (2.51% maltose, 0.75% bacto-peptone, 0.02% β-alanine, and 0.43% glucose). The cultivations were maintained at 28 °C, 150 rpm, and 0.3 vvm aeration rate for 14 days.
c. not detected.

Further study on the influence of pH on the production of DA and DB was investigated in a 5 L STR, and results are summarized in Table 1. The pH controlled fermentations were carried out by addition of solutions either1 N NaOH or $H_2SO_4$. From the time course of the

formation of DA, it appeared in day 4 after inoculation with pH at 9, and reached to its highest yield of 71.0 mg/L after 12 days of cultivation (data not shown). In contrast, when the pH value was controlled at 2, the maximum level of DA (30.0 mg/L) appeared at day 9 after inoculation, which was one-third of the yield as compared with pH 9 cultivation. In addition, the first detectable DA (37.0 mg/L) was found at day 10 with control experiment (no pH control). On the other hand, however during this study the best yield of 310.6 (mg/L) of DB was obtained with pH 9 experiment, and was nearly three fold and 30 % higher than that from pH 2, and shaker flask cultivations, respectively (Table 1). Nevertheless, when the pH was not controlled, the maximum concentration of DB was only 190.0 mg/L at day 14. A common observation in all these runs was that the yields of DA and DB were affected to different extent by application of pH control. This might be explained by that the culture pH of the uncontrolled cultivation decreasing towards an acidic environment during the course of cultivation, which would not favor the formation of DA and DB. Moreover, the pH values showed different effects on DA and DB production in shaker flask and in 5 L STR cultivations. This might be due to the difference of environmental conditions between a flask and bioreactor; for instance, auto-shifting of culture pH, oxygen transfer rate, and shear rate. However, further experiments are required to substantiate the arguments. Furthermore, the concentration of residual sugars decreased during the cultivation, coinciding with an increase in dry mass and DB yield (Figure 2). The minimum residual sugar and maximum dry mass content was observed at day 9, this may be due to the concomitant increase in the cell autolysis.

The effects of final pH on the production of DB were also evaluated in shaker flask and STR cultivations. The results indicate that the optimal final pH is in the range from 2.8 to 3.9 (Figure 3). Direct correlation was observed between DB yield and final pH value. The maximum DB yield of 330.0 mg/L was achieved at a final pH of 3.7 in 5 L STR cultivation. This was also found in the shaker flasks studied.

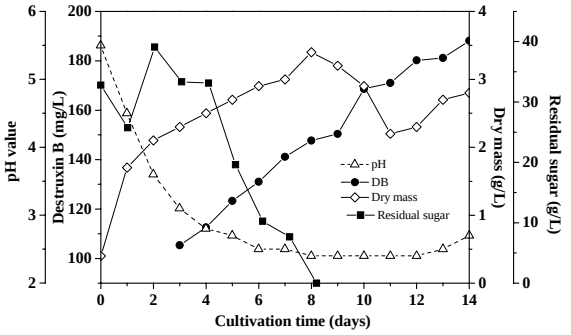


Figure 2. Time course of destruxin B production by *M. anisopliae* at 28 °C, 150 rpm with 5 L stirred tank bioreactor under pH uncontrolled condition: destruxin B (DB, ●), residual sugar (■), dry mass (◇), and pH value (△) as a function of the cultivation time.

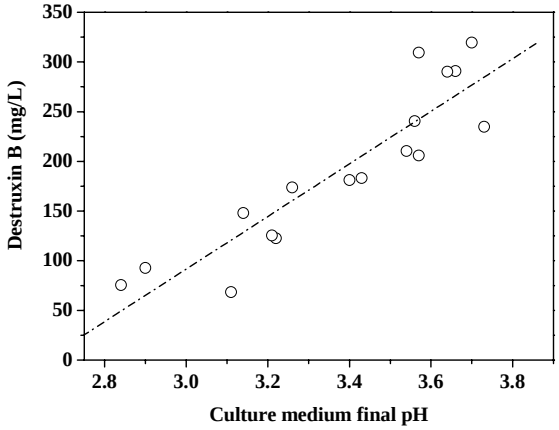


Figure 3. Influence of final pH on DB production. The data points were collected from the shaker flask and 5 L stirred tank cultivations. The straight line was the least square fitting result of each data set.

Further studies about the effect of pH on the production of DB were investigated in 20 L ALR. This reactor has several advantages compared with other bioreactors: the fermentation process can be precisely controlled, energy saving from the required bulk mixing and mass transfer, resulting in lower cost. The cultivations were carried out with an aeration rate of 1 vvm, and no pH control. Under the STR without net-draft-tube (bubble column) mode, the production of DB appeared from the day 3 and its concentration increased greatly up to the 10$^{th}$ day, and then decreased noticeably on the following days. Nearly 550.0 mg/L of DB was obtained after 9 day cultivation (Figure 4), while the pH increased constantly up to the end of cultivation (12 days). On the other hand, dry mass content increased till 7$^{th}$ day and then dramatically decreased. Figure 5 shows the DB concentration, residual sugar, dissolved oxygen (DO), and pH time course for the cultivation of *M. anisopliae* F061 in 20 L ALR using 6-mesh net-draft-tube. As can be seen from this figure, the production and the appearance of DB was started after 3 days, and was steadily rose up to the harvest (12$^{th}$ day). The DB reached its maximum concentration of 350.0 mg/L after 12 days cultivation, while pH increased from 4.2 to 6.4. In contrast, the residual sugar decreased rapidly was accompanied by dramatic fall in DO from 100 to 20 %.

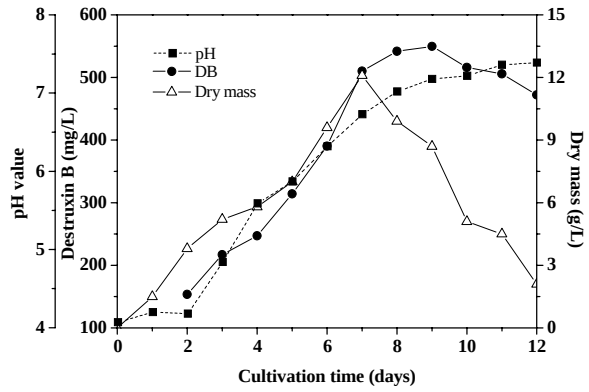


Figure 4. Time course of destruxin B production by *M. anisopliae* at 28 °C, 1 vvm, without net-draft tube (bubble column mode) in 20 L airlift bioreactor under pH uncontrolled condition: destruxin B (DB, ●), dry mass (△), and pH value (■) as a function of the cultivation time.

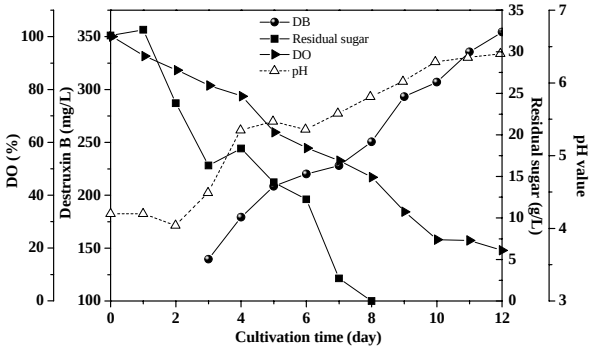


Figure 5. Time course of destruxin B production by *M. anisopliae* at 28 °C, 1 vvm, with 6 mesh net-draft tube in 20 L airlift under pH uncontrolled condition: destruxin B (DB, ●), residual sugar (■), dissolved oxygen (DO, ▶), and pH value (△) as a function of the cultivation time.

The concentration of DB in 20 L ALR was more profound than that in the shaker flask and in 5 L STR cultivation. This was probably due to the better-controlled environment for microbial growth in the ALR. Taking all the results into account, in 20 L ALR the alkaline conditions with bubble column mode operation were more favorable for maximal production of DB. However, the reality of the effects of culture medium pH needs to be elucidated further since the biosynthesis of each destruxin seems to require a different pH or cultivation environment [23]. It has become challenging to systematically develop an optimal expression strategy for the production of Dtxs from *M. anisopliae*. In addition, secondary metabolite production in filamentous cultivations varies with physiology and morphology. This metabolic event not only reflects nutrient conditions, such as nitrogen

limitation and amino acid starvations, but also important for initiating both physiological and morphological differentiation. Practically, any enzyme mediated reaction in the external medium, whether truly external or in the periplasm, will be influenced by the culture pH. Structures such as membranes in contact with the external environment were also subject to chemical changes in response to pH. Microorganism may need to adapt their function in order to cope with a change in hydrogen ion concentration. The pH may also determine the solubility of some components of the medium. Thus a modification in the pH might also cause some micronutrients to precipitate and become impossible to be assimilated [28].

### 3.2 Effect of aeration rate on DB production

Amongst many parameters which affect the fungal morphology and product yield, the effect of aeration has been studied by a number of investigators [29-31]. Successful aerobic fermentations require the maintenance of an environment sufficient in dissolved oxygen to avoid limitation or impairment of the normal respiratory activities. In addition, aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, products/byproducts, and oxygen. Aeration results in better mixing of the production medium, thus helping maintain a concentration gradient between the interior and the exterior of the cells. This concentration gradient works in both directions; through better diffusion as it helps to maintain a satisfactory supply of sugars and other nutrients to the cells, while it facilitates the removal of gases and other byproducts of catabolism from the microenvironment of the cells.

Further studies have been carried out to determine the effect of aeration rate that would result in the maximum quantity of DB production. Based on the above results, experiments were conducted in 20 L ALR without net-draft-tube mode and pH at 6, between 0.5 and 1.5 vvm aeration rates. This range was chosen as our earlier studies on Dtxs production by the same organism revealed that the aeration rate at below 0.5 vvm and above 1.5 vvm, no significant changed on Dtxs production were obtained [24-26]. Hence, detailed investigation on the influence of aeration rate in the range 0.5 and 1.5 vvm would be sufficient to explain the effect of aeration on Dtxs fermentation process.

Figure 6 depicted the influence of aeration rate on destruxin B production in 20 L ALR without net-draft-tube mode. As can be seen in this figure, the aeration rate had a positive effect on the DB production. During this study we isolated nearly 220, 450, and 700 mg/L of DB for 0.5, 1.0, 1.5 vvm of aeration rate, respectively. In general, an increase in aeration rate above 2 vvm led to high shearing stresses, which could damage the growing hypha or mycelia. Maximum yield of DB with 1.5 vvm aeration rate may be due to adequate mixing with combined hyphal and pelleted morphology, and carbohydrate consumption.

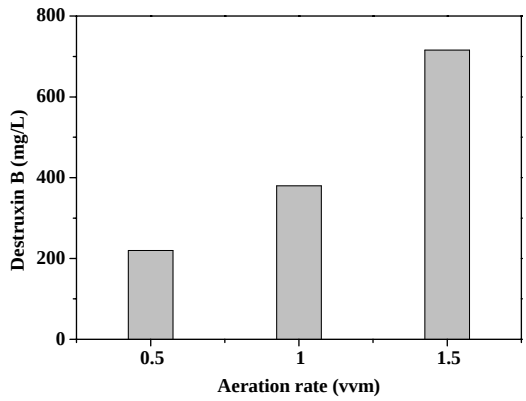


Figure 6. Influence of aeration rate on destruxin B production in 20 L ALR without net-draft-tube mode. Cultivation pH was controlled at 6. The medium used as described in the materials and methods section.

## 4. Conclusions

The present results showed that the operating conditions pH and aeration rate were important factor that should be considered in the optimization of DA and DB production. In the shaker flask, initial pH at 9 was suitable for highest level 240.6 mg/L of DB production, a more than 3-fold higher than that to pH 2. Similar results were obtained in 5 L STR (pH 9), and in 20 L ALR without net-draft-tube mode (pH uncontrolled). The corresponding maximum yields of DB were 310.6 and 550 mg/L, respectively. This led us to conclude that pH 9 in shaker flask and 5 L STR for DA and DB, and the alkaline pH (pH 6) in 20 L ALR was more favorable for highest level of DB production, respectively. In addition, it has been shown that in 20 L ALR, the aeration rate has significant effect on DB production, with a maximum nearly 700.0 mg/L at 1.5 vvm that was 3-fold higher than at 0.5 vvm and 2-fold higher than at 1 vvm aeration rate, respectively. Based on the above results, the ALR (pH 6) with bubble column mode operation was shown to be an appropriate cultivation system for the production of DB.

## Acknowledgments

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