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Relationship Between Fractal Dimension and Morphological Features of *Cephalosporium acremonium* M25 in a 30-l Bioreactor Culture

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Abstract In a 30-l bioreactor culture, whole differentiation occurred from 48 h, and then proceeded rapidly. As swollen hyphal fragments and arthrospores increased, cephalosporin C (CPC) production increased exponentially to 1.85 g/l^{-1} at 72 h. To explain the morphological changes of *Cephalosporium acremonium* M25 more quantitatively, specific differentiation rates and fractal analysis were employed. Specific differentiation rates of morphological factors varied greatly during the period of culture time from 48 h to 72 h, when CPC production increased significantly. Changes of fractal dimensions showed a pattern similar to that of the specific rate of arthrospores. Furthermore, it was inversely related to the specific rate of tips. Overall, it was suggested that the fractal dimension had potential for a new morphological parameter of fungal morphology, showing complex differentiation patterns.

Key words: Cephalosporin C, *Cephalosporium acremonium* M25, fractal dimension, morphology, specific differentiation rate

Filamentous fungi have been used to produce a range of important metabolites such as antibiotics, enzymes, and other chemicals. Filamentous fungi in submerged culture may grow in the form of either free mycelium or pellets [1, 2, 13]. The morphology of mycelial growth is influenced by many factors such as genetic factors [7], size and nature of inoculum [9], medium composition [8, 10], physical culture conditions [5, 18, 19], and so on. Many studies have been described on the morphology of industrially important filamentous microorganisms [5, 13, 15]. In order to describe fungal growth, microscopic morphological parameters such as total length of mycelium, diameter of mycelium, number of tips, and mycelial growth units have been used; however, it is highly inadequate to distinguish morphological differences, thus necessitating a new morphological parameter.

Fractals, self-similarities, have been applied to describe natural phenomena: deposition of inorganic material, shape of seashore, and diffusion of liquid in porous material [3]. Many quantitative results on fractals in biology and related life sciences have been reported. Moreover, the concepts derived from fractals are fundamental to the description and modeling of phenomena in biology, spanning from molecular to ecosystemic levels of organization. Fractal geometry offers a new approach to describe the morphology of organisms. In microbiological systems, fractals were introduced to describe growth patterns and morphology [16, 17]; however, most studies have been focused on the correlation between fractal dimensions and branching complexity. These studies are limited to general fungi that have a simple growth pattern. To be a useful tool in morphological studies of microorganism, fractal analysis should be able to appropriately predict the correlation between morphological change and physiological function of microorganisms that have a more complex differentiation pattern.

In this study, the complex mycelial morphology of an industrially important microorganism, *Cephalosporium acremonium*, was analyzed by an image analysis system. The relationship between fractal dimension and morphological changes that are closely related to cephalosporin C production was investigated in a 30-l bioreactor culture.

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MATERIALS AND METHODS

Strain

In our previous work [9], *C. acremonium* ATCC 20339 was mutagenized by irradiation under UV and a mutant of *C. acremonium*, strain M25, was finally selected by an agar-diffusion method. This strain was used in the current study. The stock cultures were maintained by monthly transferring the organism onto potato dextrose agar slants.

Media and Culture Conditions

The basal seed medium consisted of 2.5% (w/v) sucrose, 1.0% (w/v) glucose, 2.5% (v/v) corn steep liquor, and 0.4% (w/v) $(NH_4)_2SO_4$. To improve morphological differentiation, 3.0% (w/v) soybean meal, 1.0% (w/v) cotton seed flour, and 0.5% (w/v) $CaCO_3$ were added to the basal seed medium [10]. The main medium consisted of 1.95% (w/v) glucose, 5% (v/v) corn steep liquor, 0.8% (w/v) $(NH_4)_2SO_4$, 0.3% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, 0.5% (w/v) DL-methionine, and 0.4% (v/v) trace element solution [11]. Sugars and $(NH_4)_2SO_4$ were sterilized separately from the other components. pH was adjusted to 7.0 with 1 N NaOH prior to sterilization. $CaCO_3$ was added after pH adjustment. The seed and main cultures were carried out in a 30-l bioreactor (Kobiotech Co., Ltd., Korea) at 27°C. The operating volume was 20 l, air flow rate was 0.5 vvm, and agitation speed was 150 rpm. In addition, 5% (v/v) of seed broth at 60 h was inoculated to the main culture. To improve CPC production, 5% rice oil was fed at 24 h.

Analytical Methods

The dry cell weight of mycelium was measured as follows; 10 ml of culture broth was centrifuged at 12,000 ×*g* for 10 min and filtered through a preweighted Whatman glass-microfiber filter GF/C. After washing twice with distilled water, the cells were dried at 95°C to a constant weight prior to measuring the dry weight. The amount of glucose was measured by using a modified DNS method [12]. CPC was measured by HPLC using a reverse-phase column of μ Bondapak C-18 and 254 nm UV detector. The mobile phase was an acetonitrile-phosphate buffer. The elution mixture was 98% phosphate buffer and 2% acetonitrile at a flow rate of 0.9 ml/min.

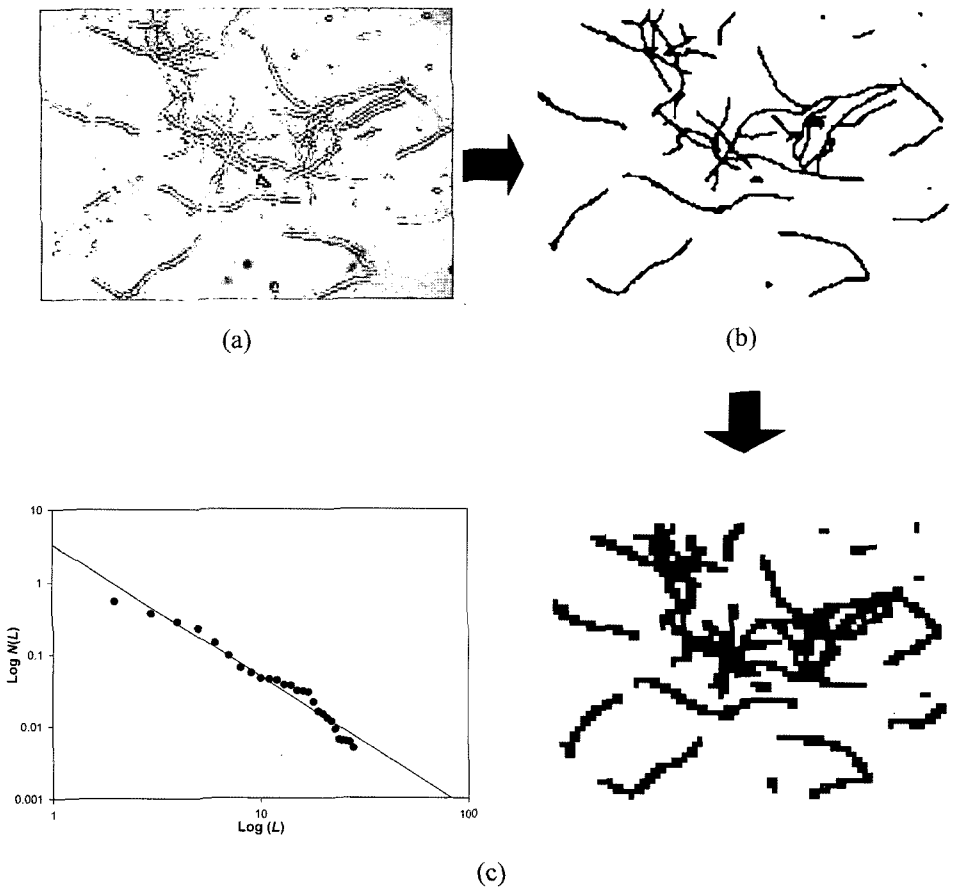

Fig. 1. Schematic diagram of determination of fractal dimension using the box counting method.
(a) Image from optical microscopy, (b) image after processing, and (c) fractal dimension determination by calculator.

Image Analysis for Cell Morphology

The cell morphology was studied on photomicrographs through optical microscopy (Samwonscientific Ind. Co. Ltd., Korea) connected with Image Pro 3.0 software (Media Cybernetics, Silver Spring, MD, U.S.A.). Morphological factors such as hyphal length, number of tips and number of arthrospores were measured manually or automatically after sorting and classifying by the image analysis process. Each sample was diluted 5-fold, and average values for hyphal length, and number of tips and arthrospores were calculated from approximately 100 observations.

Determination of Fractal Dimension

The fractal dimension of cell morphology was determined by a box counting method, derived from the method used by Obert *et al.* [14]. The binary image of mycelium was edited to remove foreign particles and correct optical errors. When the mycelium is covered by a grid of equal side length (L), the number of boxes (N) overlapped by a mycelium can be counted. The number of boxes overlapped by a mycelium image grows as the side length (L) of the box is increased. For well-defined fractal subjects, the following equation should be satisfied.

$$N(L)=\alpha L^{D} \quad (1)$$

where α and D are the proportionality constant and fractal value of the subject, respectively. Equation (1) is expressed in logarithmic form as

$$\log N(L)=D \log L+\log\alpha \quad (2)$$

For a well-defined fractal subject, the logarithmic number of the overlapped boxes is linear to the logarithmic value of the side length of the box with a slope of fractal D. Fractal Dimension calculator (ver. 1.1, Bar-Ilan University, Israel) automatically counts the number of overlapped boxes and calculates fractal dimension through linear regression. More than 30 images were processed, and the averages of fractal dimensions were derived from various culture time. A schematic diagram of determination of fractal dimension is shown in Fig. 1.

Results and Discussion

CPC Production and Morphological Changes of *C. acremonium* M25 in a 30-l Bioreactor Culture

Figure 2 shows the time course of CPC production by *C. acremonium* M25 in a 30-l bioreactor. As glucose and rice oil were consumed rapidly in the early stage of culture time, cell mass increased exponentially to 84.0 g/l^{-1} during 36 h without a lag phase, which was then maintained to the end of cultivation. However, residual glucose was not completely consumed during the stationary phase. This was thought to be due to the fact that rice oil, which was fed at 24 h, was

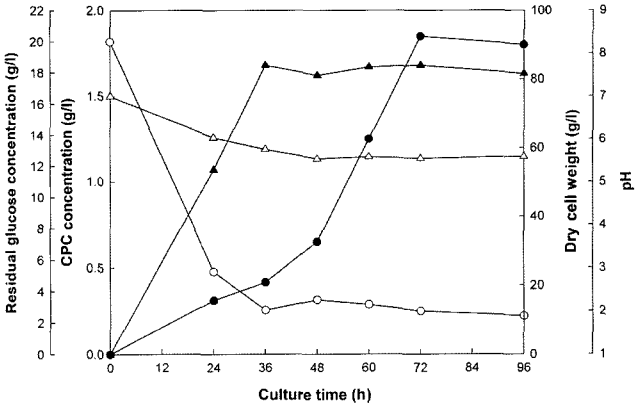

Fig. 2. Time courses of CPC production by *C. acremonium* M25 in a 30-l bioreactor.
Culture was carried out at 27°C and 150 rpm with air flow rate of 0.5 vvm. 5% rice oil was fed at 24 h to improve CPC production. Changes of CPC production (●), glucose concentration (○), cell mass (▲), and pH (△) were observed.

consumed during the CPC production phase. CPC production increased gradually to 0.65 g/l^{-1} during the initial 48 h and then increased exponentially to 1.85 g/l^{-1} by 72 h. pH decreased to 5.55 during the exponential phase and increased gradually to 5.70 during the stationary phase. Maximum CPC production and cell mass were 1.85 g/l^{-1} and 84.0 g/l^{-1}, respectively. Figure 3 shows the morphological changes of *C. acremonium* M25 in a 30-l bioreactor culture. The growth of *C. acremonium* M25 stopped very early, and differentiation proceeded rapidly in a 30-l bioreactor. During 48 h of culture, the mycelia of *C. acremonium* M25 showed long slender smooth hyphae, and these hyphae thereafter differentiated into swollen hyphal fragments and arthrospores. Overall, in a 30-l bioreactor culture, whole differentiation occurred from 48 h and then proceeded rapidly. As swollen hyphal fragments and arthrospores increased, CPC production increased exponentially. Generally, it has been reported that CPC was produced after the hyphae differentiated into swollen hyphal fragments and arthrospores [6]. However, the biochemical mechanism underlying the morphological changes of *C. acremonium* is not fully understood. Several studies demonstrated that the vacuolation and fragmentation of filamentous microorganism are closely related to fermentation conditions such as agitation intensity, aeration, and medium composition [4, 18–20]. In our previous work [11], it was also proved that the morphology of *C. acremonium* M25 changed significantly at different bioreactor scales, in agreement with the study of Jüsten *et al.* [5].

Specific Differentiation Rate of Morphological Factors and Fractal Analysis of *C. acremonium* M25 in a 30-l Bioreactor Culture

To explain more quantitatively morphological changes of *C. acremonium* M25, a specific differentiation rate and fractal

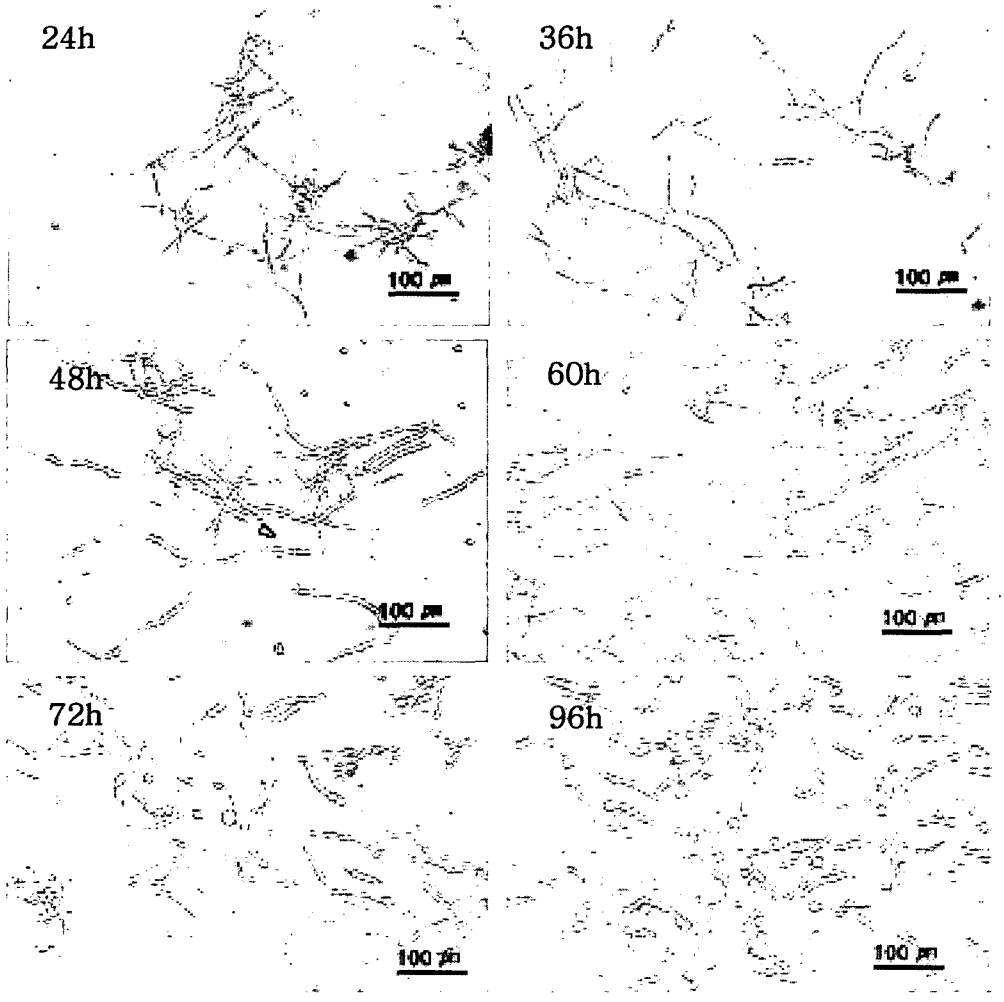

Fig. 3. Typical morphological changes of *C. acremonium* M25 in a 30-l bioreactor.

analysis were employed. From the results of changes in morphological factors, the specific differentiation rate of hyphal length, number of tips, and number of arthrospores were defined as follows:

$$\mu_H = -\frac{1}{H} \cdot \frac{dH}{dt} \quad (3)$$

$$\mu_T = -\frac{1}{T} \cdot \frac{dT}{dt} \quad (4)$$

$$\mu_A = \frac{1}{A} \cdot \frac{dA}{dt} \quad (5)$$

where μ_H, μ_T, and μ_A denote specific rates of mycelial differentiation, tip differentiation, and arthrospores formation, respectively, and H, T, and A denote mean hyphal length, the number of tips, and the number of arthropsores, respectively. Figure 4 shows changes in the specific differentiation rate of morphological factors of *C. acremonium* M25: The specific rate of hyphal length increased exponentially from -9.66×10^{-3} h^{-1} at 36 h to 0.091 h^{-1} at 48 h and remained constant. Thereafter, it decreased rapidly to 0.0159 h^{-1} at 72 h. This result indicates that hyphal length decreased rapidly until 60 h of cultivation and then the hyphae differentiated into swollen hyphal fragments and arthrospores. In the

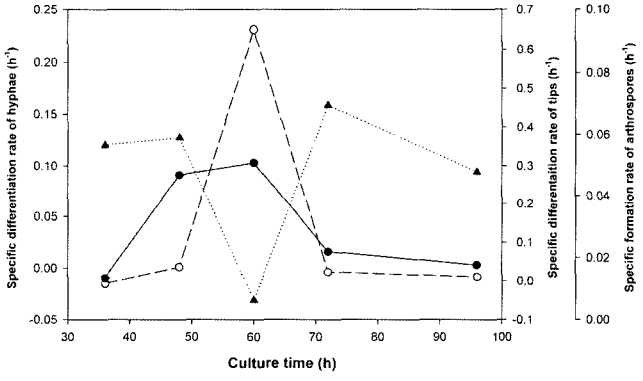

Fig. 4. Time courses of specific differentiation rates of hyphae (●), tips (○), and arthrospores (▲) in a 30-l bioreactor culture of *C. acremonium* M25.

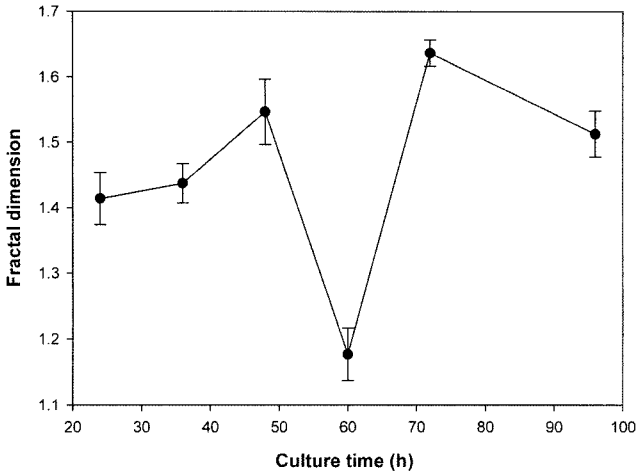

Fig. 5. Time courses of fractal dimensions in a 30-l bioreactor culture of *C. acremonium* M25.

case of tips, the specific rate increased from 0.036 h^{-1} at 48 h to 0.6495 h^{-1} at 60 h and then decreased to 0.0234 h^{-1} at 72 h, indicating that tips differentiated faster from the main hyphae than from the hyphae themselves. The specific rate of arthrospores showed patterns opposite to that of hyphae and tips: It decreased from 0.059 h^{-1} at 48 h to 6.27×10^{-3} h^{-1} at 60 h and then increased exponentially to 0.69 h^{-1} at 72 h. During this phase of cultivation, arthrospores were mainly differentiated from swollen hyphal fragments. Overall, during the cultivation from 48 h to 72 h, the specific differentiation rates of morphological factors of *C. acremonium* M25 showed significant variation. To investigate the relationship between fractal dimensions and morphological changes, fractal dimensions were determined (Fig. 5). The changes of fractal dimensions showed a pattern similar to that of the specific rate of arthrospores. It was also inversely related to the specific rate of tips. The average fractal dimension decreased from 1.546 at 48 h to 1.177 at 60 h as the hyphae differentiated into swollen hyphal fragments. It then increased rapidly to 1.637 at 72 h as swollen hyphal fragments differentiated to arthrospores. This result suggested that simple analysis using fractals would facilitate the quantification of morphological changes of filamentous microorganisms, as described by several researchers [14, 16, 17]. As shown in Fig. 2, CPC production increased significantly during the cultivation phase from 48 h to 72 h. The above results demonstrate that the change in fractal dimension is a good predictor of morphological changes of *C. acremonium* M25 that are closely related to CPC production.

Acknowledgments

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