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Differentiation of *Acremonium chrysogenum* M35 in Submerged Culture with Glass Beads or Silicone Rubbers

Hwan Hyo Lee, Hyun Yong Shin, Eun Ji Kim, and Seung Wook Kim*

Department of Chemical and Biological Engineering, Korea University, Seoul 136-701, Republic of Korea

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In this study, we investigated the effects of glass beads and silicone rubbers on the differentiation and morphological changes of *A. chrysogenum* M35 in submerged culture. Differentiation in the center of the cell pellets was improved by the addition of glass beads or silicone rubbers to the primary medium. The fragmentation rate constant (k_{frag}), which is used to estimate the tensile strength of fungal hyphae, was increased by more than 40% in baffled flasks containing glass beads. The maximum fragmentation rate was also increased by 48% when silicone rubbers were added to a 5 L bioreactor containing the culture. During the cultivation in the main medium with 6 glass beads, the value of the fractal dimension increased by about 8% when it was compared with baffled flasks without glass beads. Additionally, the number of arthrospores and the dry cell weight were increased by more than 10% in baffled flasks containing beads. The degree of roundness, which is the ratio of the object area to the longest Feret diameter, was decreased from 0.85 at day 1 to 0.77 at day 5. The differentiation of *A. chrysogenum* M35 was also supposedly closely related with the enlargement of the cell surfaces. Taken together, these results indicate that complex changes in morphology resulted in increased cell growth and differentiation in the culture broth containing glass beads and silicone rubbers.

Keywords: *A. chrysogenum*, tensile strength, fractal dimension, glass bead

Morphological changes of *Acremonium chrysogenum* are closely related to the production of β-lactam antibiotics (Bartoshevich *et al*., 1990; Suarez and Gudiol, 2009; Zahar *et al*., 2009). In addition, various morphological cell types, long slender smooth hyphae, moderately and highly swollen hyphal fragments, unicellular arthrospores and small elongated conidia are known to be produced by *A. chrysogenum* during differentiation in culture broth (Makagiansar *et al*., 1993; Matsumura *et al*., 1994). Moreover, the increased production of cephalosporin C (CPC) has been observed during the differentiation of filamentous hyphae into wide, highly swollen, metabolically-active hyphal fragments (Queener and Ellis, 1975; Sandor *et al*., 2001; Grimm *et al*., 2005). Finally, it has been shown that complex morphological differentiation can be caused by mechanical forces (Lee *et al*., 2001a, 2001b; Kim *et al*., 2005a).

Fungal hyphae formed during filamentous fungal fermentation are generally assumed to break in response to factors that produce excessive shearing forces (Li *et al*., 2002a, 2002b). Moreover, both morphological changes and hyphal fragmentation are determined by agitation and hyphal tensile strength (Li *et al*., 2002b; Kim *et al*., 2007). The fractal dimension was first introduced to describe the natural phenomena that occurs during the culture of fungi (Lim *et al*., 2005); for example, it has been used to describe the growth patterns and morphology of microbiological systems (Ryoo, 1999; Kim *et al*., 2005b); however, this concept has primarily been used to evaluate the branching complexity, and not other important physical properties of the culture broth which influence the morphological differentiation of fungi (Cruz *et al*., 1999; Kim *et al*., 2003). Accordingly, fractal analysis may be useful in the evaluation of cell differentiation in submerged culture, particularly for characterization of the correlation between physiological properties and the complex morphology of fungi (Golinski *et al*., 2008; Arelli *et al*., 2009).

It has been reported that the differentiation of *A. chrysogenum* increases the production of CPC (Lee *et al*., 2001; Kim *et al*., 2006). In addition, the tensile strength and fractal dimension have been investigated to determine if they can be used to predict the differentiation and morphology of filamentous fungi (Kim *et al*., 2005b; Lim *et al*., 2005). In this study, the fragmentation rate constant (k_{frag}), which can be used to estimate the tensile strength of fungal hyphae, was measured during the differentiation of *A. chrysogenum* and analyzed using the fractal dimension to determine if these methods could be used to predict changes in morphology and differentiation in *A. chrysogenum.*

Materials and Methods

Strain

In this study, *A. chrysogenum* M35 was developed by mutation of *A. chrysogenum* (ATCC 20339) via exposure to UV light to increase its cephalosporin C production.

Media and culture conditions

The basal seed medium was composed of 2.5% sucrose, 1.0% glucose, 2.5% corn steep liquor (CSL), 0.4% ammonium sulfate, 3.0% soy

* For correspondence. E-mail: kimsw@korea.ac.kr; Tel: +82-2-3290-3300; Fax: +82-2-926-6102

bean meal, 1.0% cotton seed flour and 0.5% $CaCO_3$. The primary medium was composed of 1.95% glucose, 5% corn steep liquor, 0.8% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.5% K_2HPO_4, 0.5% DL-methionine, and 0.4% trace element solution (Lee *et al*., 2001).

Glucose, CSL and ammonium sulfate were sterilized separately from the other components in the medium. The pH was adjusted to 7.0 prior to sterilization, and $CaCO_3$ was added after adjusting the pH. Linoleic acid was added to the main medium to a percentage of 4% (v/v). The seed culture was conducted in 2,000 ml Erlenmeyer flasks containing 200 ml of medium at 27°C and 280 rpm for 72 h. The main culture was conducted in 250 ml baffled shake-flasks containing 10 ml of medium and glass beads (glass bead 3, Glastechnique Mfg., Germany) at 130 rpm and 27°C. The 5 L bioreactor culture was conducted at 27°C, 340 rpm and 1.2 vvm. The height, upper diameter and lower diameter of a silicone rubber made from a silicone stopper (silicone stopper 1, Korea Ace Scientific Co., Ltd., Korea) in the fermenter were adjusted to 9 mm, 8 mm, and 7 mm, respectively.

Image analysis of cell morphology

Cell morphology was measured on photomicrographs (Nicon eclipse 80i, Nicon Co., Japan) connected to Image Pro 3.0 software (Media Cybernetics Inc., USA). Morphological factors were manually or automatically measured using the i-solution program for image analysis.

Analytical methods

The dry cell weight of the mycelium was measured as follows: 10 ml of culture broth was centrifuged at 12,000×g for 10 min, after which the supernatant was removed. The sediment was then added to 10 ml of distilled water and vortexed for 2 sec, followed by centrifugation under the same conditions. This was repeated twice, after which the sediment separated from the supernatant was dried at 80°C for 60 h.

Measurement of hyphal fragmentation rate constants

Ten milliliters of culture broth were centrifuged at 3,000 rpm for 10 min, after which the supernatant were removed and washed twice with deionized water. The cell pellet was then diluted with deionized water to 1 g dry wt/L, followed by vortexing (KMC-1300V Vortex Mixer, Vision Scientific Co. LTD, Korea) for 10 sec. As shown in Eq. 3, Li *et al*. (2002a, 2002b) proposed that tensile strength is inversely proportional to the fragmentation rate constant (k_{frag}), whcih can be calculated using Eq. 4.

$$\text{Hyphal tensile strength} \propto (1/k_{frag}) \quad (3)$$

$$(1/A - 1/A_0) = k_{frag}t \quad (4)$$

where A is the average projected area of all fungal elements (μm^2), t is the time spent vortexing (sec), k_{frag} is the second order fragmentation rate constant ($\mu m^2\ sec^{-1}$) and A_0 is the average projected area of all fungal elements (μm^2) at the beginning of the fragmentation test.

Fractal dimension

The fractal dimension of cell morphology was determined by the box counting method. Briefly, the number of boxes (N) overlapping microorganisms was measured when the microorganisms were covered by a grid of equal side length (L). For well-defined fractal subjects, the following equation was satisfied.

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

where α is a proportionality constant and D is the fractal value of the subject. In logarithmic form, Eq. 1 is expressed as follows:

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

The fractal dimension was automatically calculated by linear regression using a fractal dimension calculator (Ver. 1.1, Bar-Ilan University, Israel) (Ryoo, 1999). More than 20 images were processed, and the averages of fractal dimensions were derived at various culture times.

Results and Discussion

Typical morphological changes in the 5 L bioreactor containing silicone rubbers were evaluated over 6 days using a phase microscope equipped with a camera (Fig. 1). Some filamentous hyphae and swollen hyphal fragments were presented in the early stage of the main culture in all cases. Differentiation of *A. chrysogenum* M35 was observed on day 4, at which time many arthrospores were also produced. Due to the limited availability of oxygen, the centers of cell pellets generally undergo limited cell differentiation (Grimm *et al*., 2005). However, cell differentiation in the center of the cells pellets was increased when silicone rubbers were added to the main culture. As shown in our previous study, glass beads or silicone rubbers stimulate the swollen hyphal fragments of cell pellets by increasing oxygen availability and mass transfer during fermentation (Lee *et al*., 2010). Therefore, it was assumed that the additives used in this study enhanced the

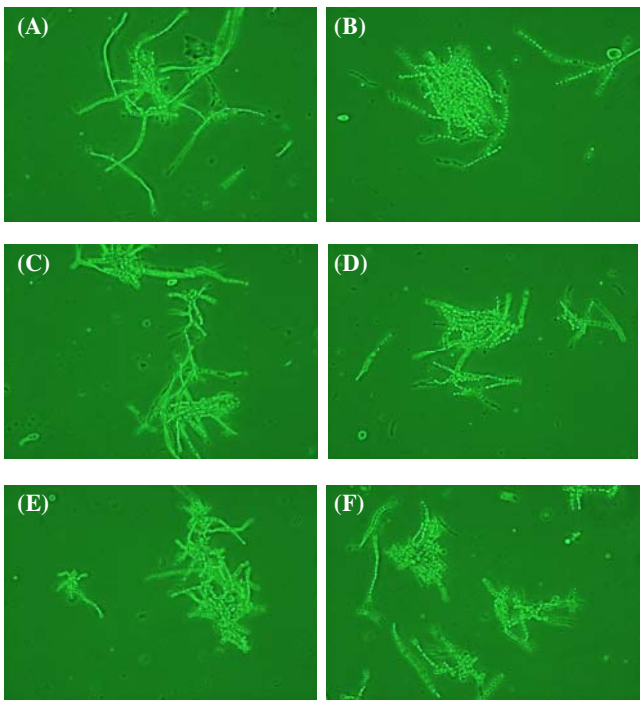

Fig. 1. Typical changes in morphology during the cultivation of *A. chrysogenum* M35 in a 5 L bioreactor with silicone rubbers. (A) no silicone rubber at day 2, (B) no silicone rubber at day 4, (C) 6 silicone rubbers at day 2, (D) 6 silicone rubbers at day 4, (E) 12 silicone rubbers at day 2, (F) 12 silicone rubbers at day 4.

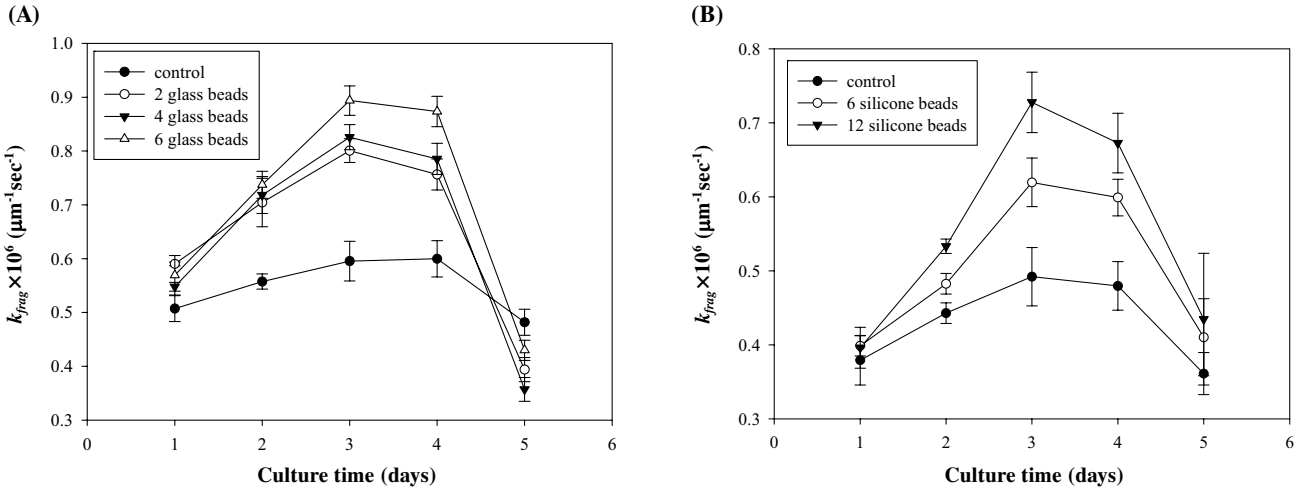

Fig. 2. Variation in the fragmentation rate constant during cultivation of *A. chrysogenum* M35 in a 250 ml baffled flask with glass beads (A) or silicone rubbers (B).

differentiation of *A. chrysogenum* M35 in the center of cell pellets by improving oxygen transfer.

Several studies have been conducted to determine if decreased tensile strength is correlated with enhanced differentiation of microbes (Li *et al*., 2002b; Kim *et al*., 2007). In the present study, the fragmentation rate constant (k_{frag}), which can be used to estimate the tensile strength of fungal hyphae, was measured to determine if there was a relationship between the morphological changes and the glass beads in the *A. chrysogenum* M 35 cultures. To determine the hyphal k_{frag}, baffled shake-flask cultures were conducted using glass beads (Fig. 2A). The k_{frag} values were increased until day 3 or 4, but the k_{frag} of the control was lower than the values of the treatments, except for day 5.

The maximum k_{frag} value was increased by about 48% compared to the fermenter without a silicone rubber (Fig. 2B). In other words, the tensile strengths of the fungal hyphae were weaker in the 5 L bioreactor containing 6 silicone rubbers and 12 silicone rubbers than in the reactor lacking silicone rubbers.

Figure 3A shows the variance of the fractal dimension of the main *A. chrysogenum* M35 culture in baffled shake-flasks containing glass beads. During culture of the main medium without glass beads, the fractal dimension increased from 1.40 on day 1 to 1.65 on day 5, after which it decreased to 1.57 on day 6. When 6 glass beads were added to the main medium, the fractal dimension showed a steep curve from 1.46 on day 1 to 1.70 on day 4, followed by a decrease to 1.68 on day 5.

The fractal dimension inside the fermenter was improved remarkably by the presence of silicone rubbers (Fig. 3B). Specifically, the fractal dimension of the culture broths in the 5 L bioreactor not containing silicone rubbers increased from 1.39 on day 1 to 1.62 on day 5, followed by a decrease to 1.56 on day 6. Conversely, the maximum value of the fractal dimension was 1.64 in the bioreactor containing 12 silicone additives on day 4. Evaluation of the fractal dimension of the 5 L bioreactor containing silicone rubbers revealed similar results to those obtained when the fractal dimension of baffled flasks with glass beads was measured. The increased

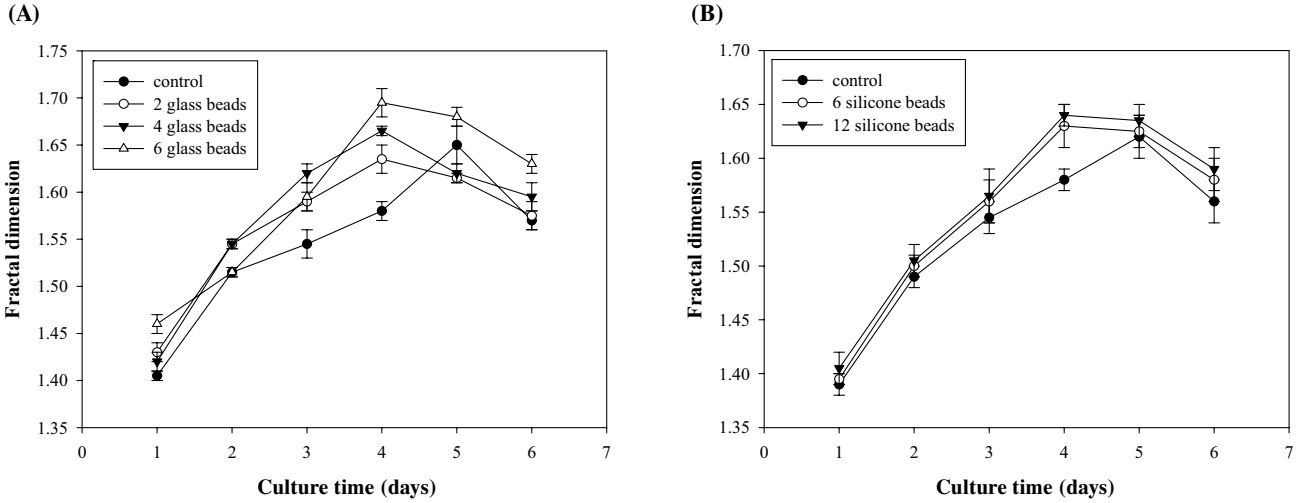

Fig. 3. Changes in the fractal dimension of *A. chrysogenum* M35 in 250 ml baffled shake-flasks with glass beads (A) or silicone rubbers (B).

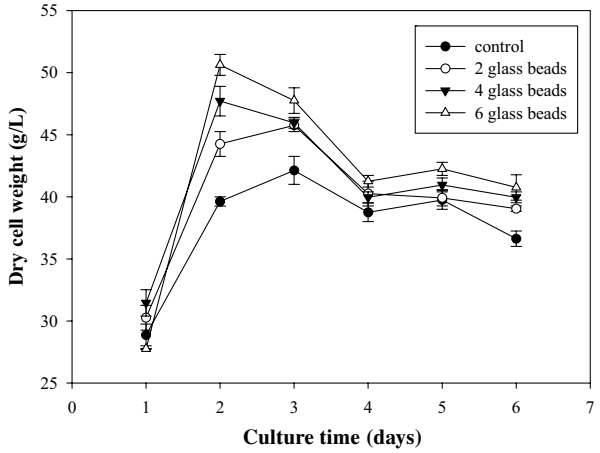

Fig. 4. Variation in dry cell weight during the cultivation of *A. chrysogenum* M35 in a 250 mL baffled flask with glass beads.

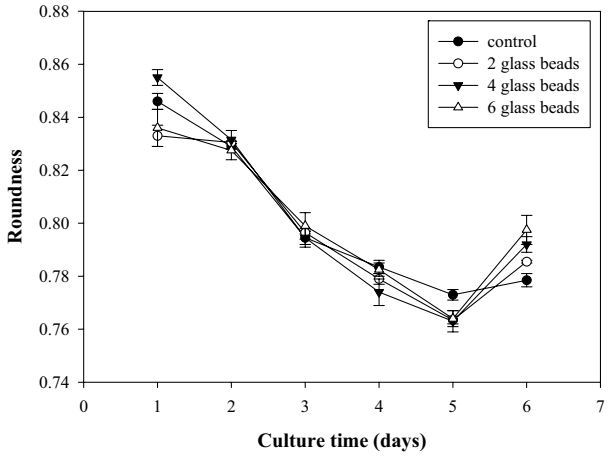

Fig. 6. Changes in the roundness spores in culture broths of *A. chrysogenum* M35 in 250 ml baffled shake-flasks with glass beads.

fractal dimension indicates that there were many complex morphological forms in the culture broth. Indeed, these findings indicate that most filamentous hyphae differentiated into complex swollen hyphal fragments during the main culture (Fig. 1, 3A, and 3B).

The maximum values of the fragmentation rate constant or fractal dimension in the main medium containing glass beads or silicone rubbers were observed on day 3 or 4. In addition, differentiation of cells primarily occurred during this period. These results indicate that the addition of glass beads or silicone rubbers impacted the differentiation of *A. chrysogenum* M35.

The dry cell weight during cultivation of *A. chrysogenum* M35 in a 250 ml baffled flask containing glass beads was remarkably higher than the weight of the control (Fig. 4). Specifically, the dry cell weight of the culture broths in baffled flasks without glass beads was increased from 28.9 g/L on day 1 to 42.1 g/L on day 3, followed by a decrease to 38.8 g/L on day 4, which is similar to the results obtained when the culture was conducted using 2 glass beads. However, the dry cell weight of other glass bead treatments was increased by at least 10 % when being compared to the control.

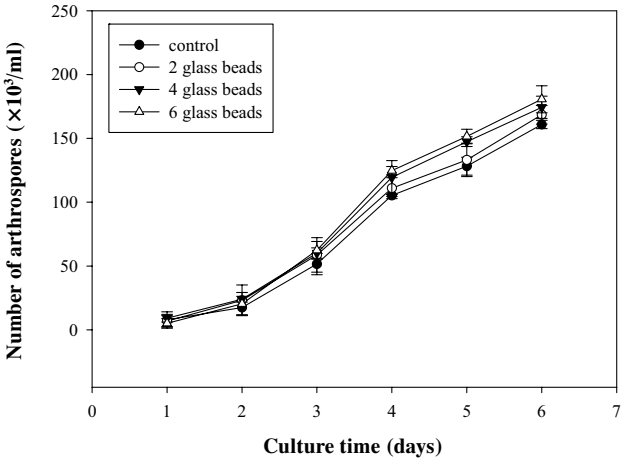

Fig. 5. Time course of the number of arthrospores.

The number of arthrospores in the baffled flask cultures without glass beads was increased from 7.9×10^3 ml^{-1} on day 1 to 160.9×10^3 ml^{-1} on day 6 (Fig. 5). When the cultivation of *A. chrysogenum* M35 was conducted in baffled flasks containing 6 glass beads, the number of arthrospores was increased from 5.1×10^3 ml^{-1} on day 1 to 180.7×10^3 ml^{-1} on day 6. The number of arthrospores increased until the end of fermentation period, and the number of arthrospores was higher in cultures that contained 6 glass beads than in those which contained no glass beads. Taken together, these results indicate that glass beads or silicone rubbers affected the cell growth and differentiation of *A. chrysogenum* M35.

The roundness is the ratio of the object area to the object with the longest Feret diameter. The value of the roundness of the arthrospores in culture broths which did not contain glass beads decreased from 0.85 at day 1 to 0.77 at day 5, after which it increased slightly to 0.78 at day 6 (Fig. 6). In this study, the minimum roundness was 0.76, which was obtained using 4 glass beads in a baffled flask on day 5. Based on these results, it is assumed that the differentiation of *A. chrysogenum* M35 led to decreased roundness. Furthermore, the differentiation of *A. chrysogenum* M35 is assumed to be closely related to enlargement of cell surfaces.

The results of this study indicate that an increased fragmentation rate constant (k_{frag}) and fractal dimension were correlated with the additives in the submerged culture of *A. chrysogenum* M35. In addition, it is assumed that the additives stimulated the swollen hyphal fragments of cell pellets via increased oxygen and mass transfer (Bai *et al*., 2003). Furthermore, it is expected that the effects of additives observed in this study will be similar in other filamentous fungi.

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