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Improvement of cephalosporin C production by *Acremonium chrysogenum* M35 in submerged culture with glass beads or silicone rubber

Hwan Hyo Lee, Yoon Seok Song, and Seung Wook Kim†

Department of Chemical and Biological Engineering, Korea University, Seoul 136-701, Korea
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Abstract−Physical stimulation using a baffle or an impeller has been previously reported to improve cephalosporin C (CPC) production and cell growth. In this study, the effect of glass beads on CPC production in *Acremonium chrysogenum* M35 was investigated in baffled flasks along with the morphological properties of the culture. Addition of glass beads into the culture broth was found to significantly influence CPC production and cell growth of *A. chrysogenum* M35 in baffled shake flasks. CPC concentration increased about 30% when compared with baffled flasks without glass beads. Morphological changes such as the total perimeter and number of units, total number of differentiated hyphae or arthrospores, corresponded to varied CPC concentrations. Specifically, total perimeter and number of units increased by more than 10%. However, changes in pH had no relationship to CPC production or the number of glass beads. Pieces of silicone rubber were mixed into a 5 L bioreactor culture to assess any improvement of CPC production. Once added into the main culture, the production of CPC increased about 30% while values of dissolved oxygen (DO), which can be used to estimate oxygen transfer rate (OTR), were lower than main medium without silicone rubber. And dry cell weight was also increased about 10% when silicone rubber was added into a 5 L bioreactor.

Key words: *Acremonium chrysogenum* M35, Cephalosporin C, Glass Bead, Silicone Rubber, Oxygen Transfer Rate

INTRODUCTION

Cephalosporins are a class of β-lactam antibiotics derived from the compound CPC, which is obtained by fermentation of the mold *A. chrysogenum* [1,2]. Cephalosporins inhibit various bacterial functions and incite cell lysis by binding to penicillin-binding proteins [3,4]. Bactericidal activity is dependent upon its ability to penetrate the outer cell wall of the bacteria, bind to the penicillin-binding proteins and remain stable against β-lactamase [5]. The importance of *A. chrysogenum* is obvious, as CPC is the raw material from which antibiotics are widely obtained for treatment of bacteria disease [6].

Several investigators found through studies on CPC production and on the influence medium components and dissolved oxygen have on product formation in *A. chrysogenum* that CPC production can be increased through optimized media and culture conditions [7-11].

For optimal conditions on cultivation of *A. chrysogenum*, physical stimulation has been investigated by changes of shape, size or intensity of a baffle, an impeller or a sparger in a bioreactor [12,13]. And additives like fatty acid or rice oil were used for study on chemical stimulation in culture of *A. chrysogenum* [14,15].

Morphological or rheological changes were also derived by the physical or chemical stimulation in submerged culture of *A. chrysogenum* [16-18]. To increase CPC production, morphological characteristics and stimulation factors in the fermentation process are very important because they affect the rheological properties in a submerged culture of *A. chrysogenum* [19,20].

Currently, many industrially important metabolites such as organic acids, antibiotics and enzymes are produced by filamentous fungi.

The morphological changes of these filamentous fungi in submerged culture vary between the pellet and the free filamentous form, depending on the strain and the culture conditions [21-23].

Especially, the differentiation of hyphae into highly swollen hyphal fragments obviously occurred prior to the CPC production in *A. chrysogenum*. Gradually, highly swollen hyphal fragments were also differentiated into arthrospores during the production of CPC [24,25].

Indeed, regarding filamentous fungi, the structure of the mycelium is affected by the media conditions. Among other factors, agitation has been studied extensively and is proven to account for important morphological changes and CPC productivity.

Various studies on filamentous fungi have shown that intensive agitation leads to reduction of pellet size and enhanced microorganism differentiation [26]. However, the opposite effect has also been reported, as low metabolite production and specific growth rates result from increased impeller speed [27].

During the production of CPC, it is reported that pellet form has the considerable disadvantage of oxygen, mass or heat transfer in culture of fungi [28-30]. Therefore, experiments were performed to improve oxygen transfer rate (OTR) by using the cell dispersion of *A. chrysogenum*.

Specifically, for enhancement of CPC production, glass beads and silicone rubbers were selected for the improved movement in baffled flasks or bioreactors through the empirical difference of density between culture broth and appendages. To perform accurate experiments, glass beads were only used in baffled flasks and silicone rubbers were also used in a 5 L bioreactor, respectively.

†To whom correspondence should be addressed.
E-mail: kimsw@korea.ac.kr
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MATERIALS AND METHODS

1. Strain

A. chrysogenum M35, a mutant strain developed from *A. chrysogenum* ATCC 20339, was used for CPC production [14].

2. Media and Culture Conditions

Stock culture was maintained by transferring the organism bi-weekly onto potato dextrose agar (PDA) slants. The basal seed medium was composed of 2.5% sucrose, 1.0% glucose, 2.5% corn steep liquor and 0.4% $(NH_4)_2SO_4$. To improve morphological differentiation, 3.0% soy bean meal, 1.0% cotton seed flour and 0.5% $CaCO_3$ were added to the basal seed medium [15]. The main 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 [11,14].

Sugars, corn steep liquor (CSL) and $(NH_4)_2SO_4$ were sterilized separately from the other components in the basal seed medium, as were sugars, $(NH_4)_2SO_4$ and DL-methionine in the main medium. The pH was adjusted to 7.0 with 1 N NaOH prior to sterilization followed by addition of $CaCO_3$ [14]. Linoleic acid, 4% (v/v), was added to the main medium [31,32].

A. chrysogenum M35 was previously cultured in a 2 L Erlenmeyer flask containing the basal seed medium of 200 mL at 27 °C and shaken at 280 rpm for 72 h. And then the *A. chrysogenum* M35 was cultured in 250 mL baffled shake-flasks containing the main medium of 10 mL on a rotary shaking incubator [27]. Flask culture was shaken with 3 mm diameter glass beads (glass bead 3, Glastechnique Mfg., Germany) at 27 °C and 130 rpm. Fermentation was performed in a 5 L stirred-tank bioreactor (Kobiotech Co., Ltd., Korea) at 27 °C and 340 rpm. The operating volume was 3.0 L and the air flow rate was 1.2 vvm.

The height, upper diameter, and lower diameter of the silicone rubber made from a silicone stopper (silicone stopper 1, Korea Ace Scientific Co., Ltd., Korea) were adjusted to 9 mm, 8 mm, and 7 mm in the bioreactor, respectively (Fig. 1).

3. Analytical Methods

The dry cell weight of mycelium was measured as follows: 60 mL of culture broth was centrifuged at 12,000 g for 10 min, and supernatant was removed from culture broth. This culture broth was added to 30 mL of distilled water and shaken by a vortex mixer for 10 s and then centrifuged at the same conditions. After being repeated twice, the sediment separated from supernatant was dried at 80 °C for 60 h.

CPC concentration was measured by high-performance liquid chromatography (HPLC, Young Lin M930, Young Lin Instrument Co., Korea) using a reverse phase column of μBondapak C-18 and a UV detector set at 254 nm. The mobile phase used was acetonitrile-phosphate buffer. The elution mixture was 98% (v/v) phosphate buffer and 2% (v/v) acetonitrile with a flow rate of 0.9 mL/min and CPC zinc salt (Sigma, USA) was used as a standard [33].

Cell morphology was studied on photomicrographs using an optical microscope (Nicon eclipse 80i, Nicon Co., Japan) connected with Image Pro 3.0 software (Media cybernetics, Inc. USA).

RESULTS AND DISCUSSION

1. Effect of Glass Beads on CPC Production and Cell Growth

Typical morphological changes of the 6 day culture in baffled flasks were photographed by a camera mounted on a phase microscope (Fig. 2). All of the broth culture in early stages contained many filamentous hyphae and several swollen hyphal fragments. Differentiation of cells was clearly observed from day 3 in which many swollen hyphal fragments and arthrospores were produced. However, several morphological factors displayed completely different tendencies during the cultivation of *A. chrysogenum* M35 in baffled flasks with glass beads.

The production of CPC was clearly affected by the number of glass beads. The concentration of CPC during cultivation of *A. chrysogenum* M35 without glass beads in baffled flasks increased from 0.5 g/L at day 1 to 4.4 g/L after 5 days (Fig. 3(a)). When the main medium was cultured with 6 glass beads, the concentration of CPC increased from 0.7 g/L at day 1 to 5.8 g/L after 5 days, followed by a slight decrease to 5.6 g/L at day 6. From these results, it can be assumed that a close relationship exists between production of CPC and differentiation of *A. chrysogenum* M35 (Figs. 2 and 3(a)). Indeed, when the number of glass beads was increased to 8, 10, 12, 14 and 16 glass beads, CPC production at day 5 was likewise increased to 5.9 g/L, 6.0 g/L, 6.1 g/L, 6.1 g/L and 6.1 g/L, respectively.

However, the total volume of the glass beads falls out of the error range when the number of glass beads is over 8. Furthermore, glass

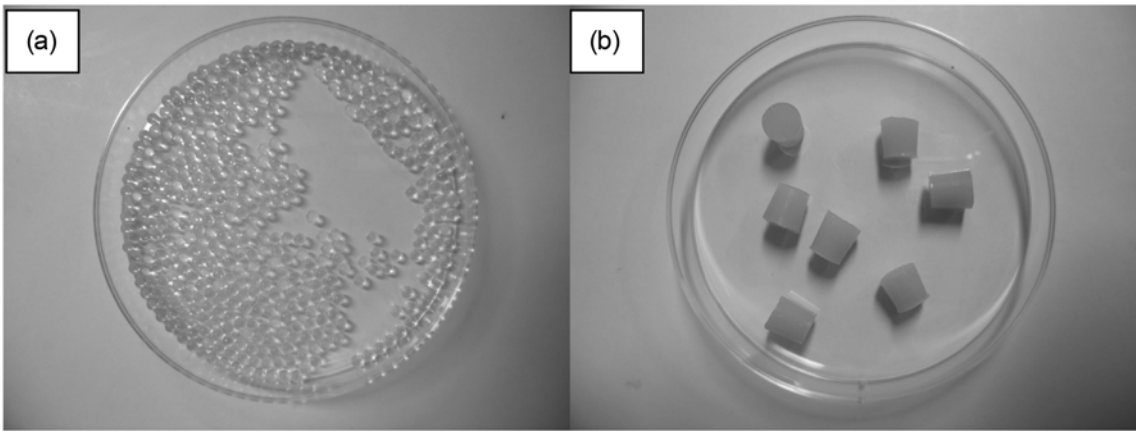

Fig. 1. Glass beads (a) and silicone rubber (b) were added into main culture.

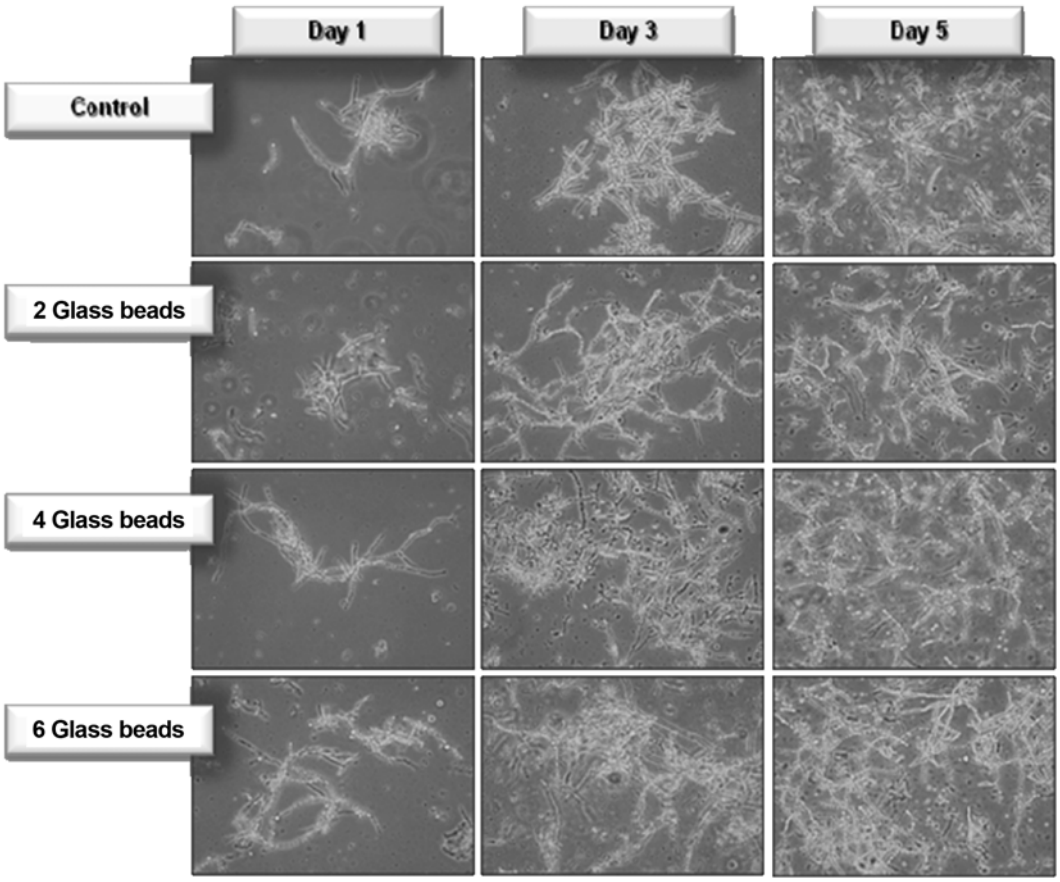

Fig. 2. Typical morphological changes during *A. chrysogenum* M35 cultivation in a 250 mL baffled flask with glass beads.

beads would clump together and not move until scattered by an increased power level. Therefore, the relevant results in this study were limited to those produced by 2, 4 and 6 glass beads.

The number of units is the total number of differentiated hyphae or arthrospores. Fig. 3(b) shows changes in the number of units during cultivation of *A. chrysoenum* M35 in baffled flasks with glass beads. The number of units increased about 40% in medium with 6 glass beads compared with that in without glass beads. Generally, the number of units was drastically increased after 2 days.

Specifically, when 6 glass beads were added to the medium the maximum number of units produced was 1330.0/μL at day 4. This figure actually increased to 48.9% when compared with the maximum number of units grown without glass beads. The increase in the number of units implies that differentiation of *A. chrysogenum* M35 in baffled flasks is improved with glass beads.

It has been investigated that the increase of differentiation of hyphae into highly swollen hyphal fragments improves the CPC production in a microorganism [24].

This data supports the idea that cell differentiation enhances CPC production (Figs. 3(a) and 3(b)). Moreover, it seems additional glass beads enhance CPC production and the number of units in the main culture from these results. Therefore, CPC production could somehow be related to the number of units.

The total perimeter is defined as the sum of the perimeters of every object observed by microscopic analysis. Fig. 3(c) shows variations in the total perimeter in culture grown with glass beads. The value of the total perimeter in baffled flasks without glass beads increased from 9.9 mm at day 1 to 39.1 mm at day 5, followed by a rapid decrease to 31.4 mm at day 6. However, 6 glass beads added into the medium produced a maximum total perimeter of 44.6 mm at day 4. The average value of the total perimeter increased about 13%.

This increase in total perimeter implies either the number of objects or the size of each object was increased.

The increase of the number of units or total perimeter indicated that highly swollen hyphal fragments are also differentiated into arthrospores during the CPC production in *A. chrysogenum* M35.

Therefore, these results support the idea that the increase in the total perimeter means that growth or differentiation enhancement of *A. chrysogenum* M35 and the production of CPC was derived from this cell growth or differentiation of *A. chrysogenum* M35.

Therefore, changes of the number of units or the total perimeter show similar tendency to the variation of CPC concentration.

Conversely, variation of pH had no corollary relationship with either CPC production or the number of the glass beads (Fig. 3(d)). The pH of culture broth demonstrated similar tendencies when with or without glass beads. Physical stimulation by glass beads was assumed to have no relationship with change of the acidity in these experiments.

2. Effect of Morphological Changes with Silicone Rubber

Fig. 4(a) shows the time course of CPC production by *A. chrysogenum* M35 in a 5 L bioreactor containing the main culture with silicone rubber.

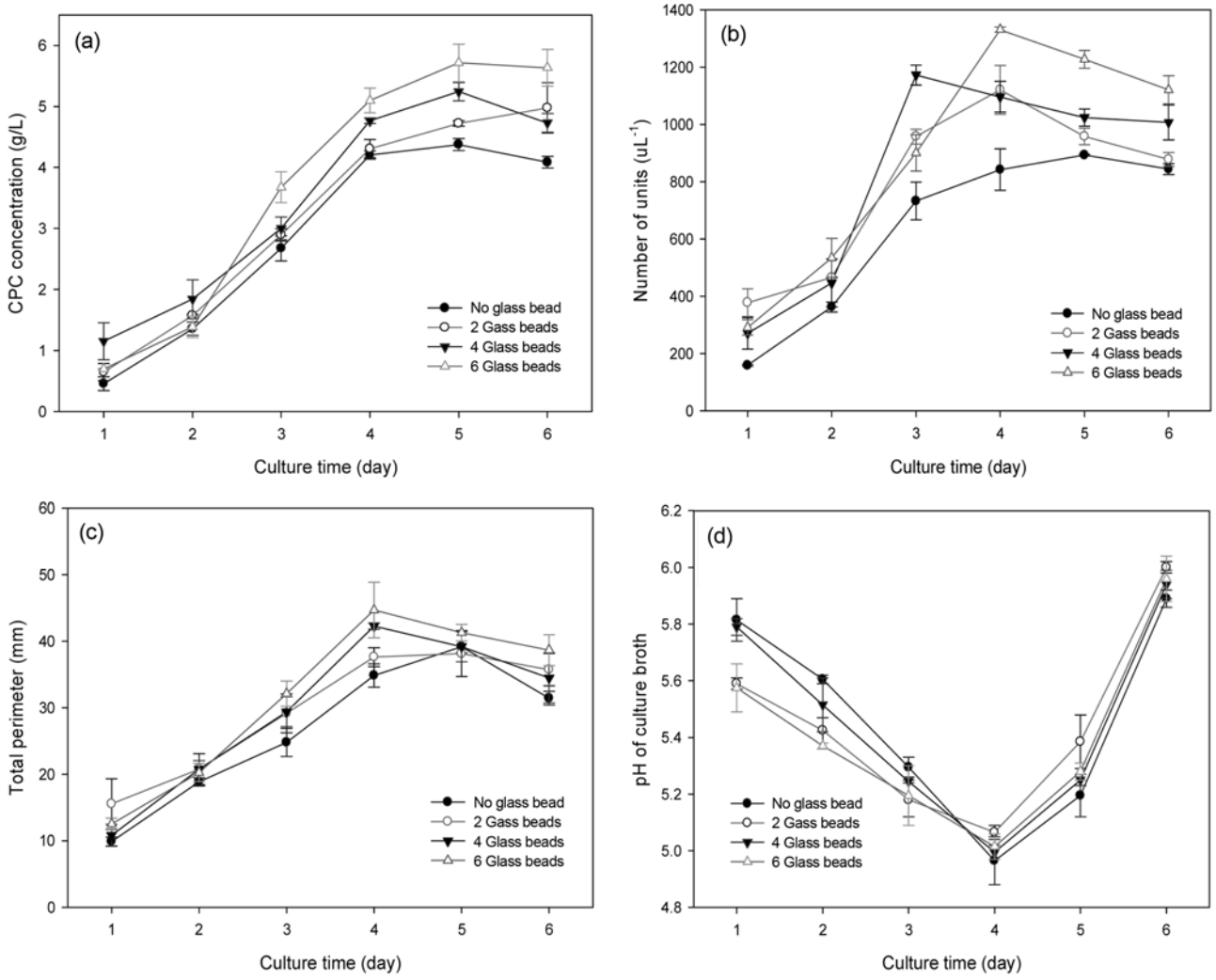

Fig. 3. Effects of glass beads on CPC production (a), number of units (b), total perimeter (c) and culture pH (d) during *A. chrysogenum* M35 cultivation in a 250 mL baffled flask.

The maximum level of CPC produced in the main medium with 12 silicone rubbers was 1.7 g/L in a 5 L bioreactor at day 5. This value was increased 30.2% when compared to the same conditions without silicone rubbers.

The dry cell weight during cultivation of *A. chrysogenum* M35 in a 5 L bioreactor was improved by the use of silicone rubber (Fig. 4(b)). The dry cell weight of the culture broth grown without silicone rubber increased from 14.2 g/L at day 1 to 25.2 g/L at day 4. The maximum dry cell weight was 27.8 g/L when grown with 12 pieces of silicone rubber in a 5 L bioreactor at day 4.

Fig. 4(c) shows the time course of DO, which can be used to estimate OTR, changed by *A. chrysogenum* M35 in a 5 L bioreactor. The level of DO in the main medium with silicone rubber was roughly lower than without.

Moreover, the values of DO decreased once the log phase of *A. chrysogenum* M35 commenced. At this time, the presence of swollen hyphal fragments sharply increased.

It was indicated that the dispersion of mycelia was considerably derived from the unpredictable movement of the silicone rubber in a 5 L bioreactor. And it is assumed that this dispersion of mycelia by silicone rubber has the advantage of the mass or heat transfer of *A. chrysogenum* M35, a kind of filamentous type fungus, in submerged culture and that leads to the improvement of cell growth or differentiation of microorganisms [28-30].

Especially, the effect of silicone rubber was assumed to improve the surface area of a mycelium for OTR in the center of cell pellets. Also, it was assumed that enhancement of cell growth was affected by oxygen transfer in a 5 L bioreactor with silicone rubber.

Consequently, the cultivation by using silicone rubber could enhance the dispersion of mycelia and it influenced oxygen or mass transfer in submerged culture of *A. chrysogenum* M35 (Figs. 4(b) and 4(c)). And these results were expected for other microorganisms of filamentous types.

CONCLUSIONS

Considering that addition of glass beads or silicone rubber was more effective in improving CPC production, these data show that differentiation of *A. chrysogenum* M35 is closely related with CPC production. Furthermore, glass beads or silicone rubber stimulates

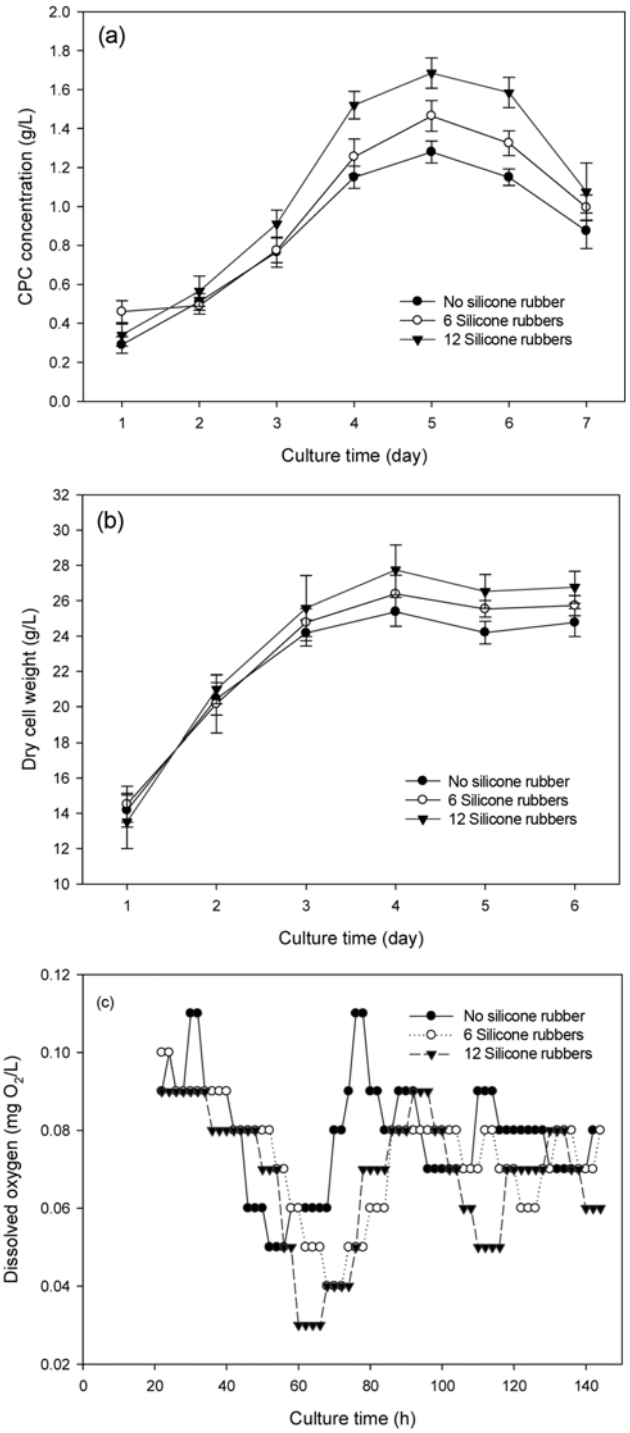

Fig. 4. Variations of CPC production (a), dry cell weight (b) and DO (c) during *A. chrysogenum* M35 cultivation with silicone rubber in a 5 L bioreactor.

morphological changes of mycelium.

Morphological data, including the number of units and total perimeter, suggest that glass beads enhance differentiation of *A. chrysogenum* M35 and improve dispersion of cells in baffled flasks. As differentiation of *A. chrysogenum* M35 and cell dispersion are related to CPC production, it can be concluded these morphological changes also influence CPC production.

Dispersion of cell pellets in culture broth is assumed to be significant since the main disadvantage of the pellet form is that its center is subject to autolysis due to oxygen limitation and therefore does not participate in product synthesis. Therefore, a culture broth containing mycelium with glass beads or silicone rubber could influence oxygen and mass transfer of the submerged culture. Through these results, cultivation with additives like beads or balls into media is expected to improve the growth of other filamentous fungi.

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