Mycobiology

Research Note

Optimization of Fungal Enzyme Production by *Trichoderma harzianum* KUC1716 through Surfactant-Induced Morphological Changes

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Abstract The morphological optimization of *Trichoderma harzianum* was carried out using several surfactants to achieve increased cellulase production. Addition of the surfactants to the culture medium successfully modified the fungal morphology from an aggregated form to a dispersed form. Optimization of the fungal morphology increased cellulase activity up to 177%. The morphologically optimized conditions enhanced the accessibility of the fungus to substrates and thus promoted cellulase production.

Keywords Cellulase, Fungal morphology, Surfactant, *Trichoderma harzianum*

With growing concern about the depletion of fossil fuels, the importance of bioethanol as an alternative energy source has become increasingly evident. However, the cost of enzyme production has thus far hindered large-scale bioethanol production, and therefore many studies have been conducted in an attempt to optimize culture conditions to reduce the cost of this process. For example, Lynd *et al.* [1] and Lee *et al.* [2] valuated the effect of variations in the medium components such as the carbon and nitrogen source, and Deswal *et al.* [3] used lignocellulosic materials as carbon sources.

Filamentous fungi show the distinct but exceedingly related feature of a complex growth morphology. They can grow as freely dispersed mycelia or as densely compacted biomass granules, depending on the strain characteristics as well as the environmental conditions [4, 5]. Posch *et al.* [4] classified the morphology of filamentous fungi into two major discriminative classes: disperse growth and pellet growth. In submerged cultures, the morphology of filamentous microorganisms usually varies between the pelleted and dispersed forms, which depends on the cultivation conditions [5-7]. However, it is difficult to investigate the relationship between fungal morphology and enzyme production because many interrelated factors affect both the morphology and protein production. Nevertheless, Bhargava *et al.* [8] revealed that it is possible to improve protein production simply by controlling the fungal morphology. In addition, Reese and Maguire [9] and Domingues *et al.* [6] found that the addition of surfactant, Tween 80 (TW), to the growth medium improved the cellulase yield of *Trichoderma*. The mechanism of this yield enhancement by the surfactant is speculated to be related to the morphological features of the fungus. Moreover, Lucatero *et al.* [10] reported that the addition of Tween 40 resulted in changes to the fungal morphology from a dispersed to pellet shape. However, there remains limited knowledge on the effect of surfactants to fungal morphology and the possible relationship between morphology, growth, and enzyme production exists. Accordingly, the aim of the present study was to optimize fungal morphology for enhancement of enzyme production by adding surfactants. To our knowledge, this represents the first report to establish a relationship between enzyme production and fungal morphology induced by a surfactant.

Polyacrylic acid (PA; Sigma-Aldrich, St. Louis, MO, USA), polyethylene glycol 600 (PG; Samchun, Pyeongtaek, Korea),

Mycobiology 2017 March, **45**(1): 48-51
https://doi.org/10.5941/MYCO.2017.45.1.48
pISSN 1229-8093 • eISSN 2092-9323

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Received July 29, 2016
Revised November 30, 2016
Accepted January 4, 2017

polyvinylpyrrolidone (PV; Junsei, Tokyo, Japan), sodium dodecylbenzene sulfonate (SDS; Amresco, Solon, OH, USA), sodium hexametaphosphate (Samchun), and TW (Samchun) were used as test surfactants. *Trichoderma harzianum* KUC1716 was obtained from the Korea University Culture Collection (KUC; Seoul, Korea). For submerged cultivation, 1×10^6 spores were used to inoculate a flask containing 50 mL Mandels' medium and each of the different surfactants for 7 days. The surfactants were tested at final concentrations of 0.1%, 0.5%, 1.0%, and 1.5%. After 7 days of fermentation, the samples were centrifuged, and the supernatants were collected to determine the activities of extracellular enzymes and the protein concentration. In contrast to the supernatant, the precipitated fungal cell wall was re-suspended, washed, and then lysed by sonication to extract the intracellular protein. The mixture was then centrifuged, and the supernatant was used to determine the intracellular enzyme activity and protein concentration. The filter paper unit assay was conducted to determine the total cellulase content according to the protocol of Ghose [11], and the extracellular protein concentration was determined using the Bradford method [12]. Intracellular protein concentration was converted to the equivalent mycelium dry weight based on a previously established correlation [13]. The cell concentration and enzyme activity data were analyzed using two-way analysis of variance (ANOVA) with the type and concentration of surfactants as independent variables. The macro- and micro-morphological features of the fungus were observed after submerged cultivation. For macroscopic observation, the in-flask cultures were transferred to a Petri dish and observed visually. Microscopic observation of the fungal mycelium was carried out using an Olympus BX51 light microscope (Olympus, Tokyo, Japan).

The cell concentration and total cellulase activity are shown in Fig. 1A and 1B, respectively. The addition of 1.0% PG resulted in the highest fungal growth. Little growth of *T. harzianum* KUC1716 was observed in cultures with SDS. It is possible that SDS which acts as a toxin to the fungus, was more effective in causing membrane leakage than the other surfactants tested [14]. Accordingly, the observation of fungal morphology in the cultures with SDS was not shown. The top two conditions promoting cellulase production were in the presence of 0.5% PG and 1.0% TW, and the enzyme activities were significantly increased by 142% and 177% under these conditions, respectively, relative to the control cultivated without a surfactant. Two-way ANOVA showed that the cell concentration depended on the type of surfactant used ($p < 0.05$), but not on the concentration of the surfactant or the interaction between the type and concentration of surfactant. In addition, the enzyme activity was significantly affected by the interaction between the type and concentration of surfactant ($p < 0.05$), although the influence of each variable was not statistically significant on its own.

Several researchers have proposed the possibility of improving enzyme production in microorganisms with the

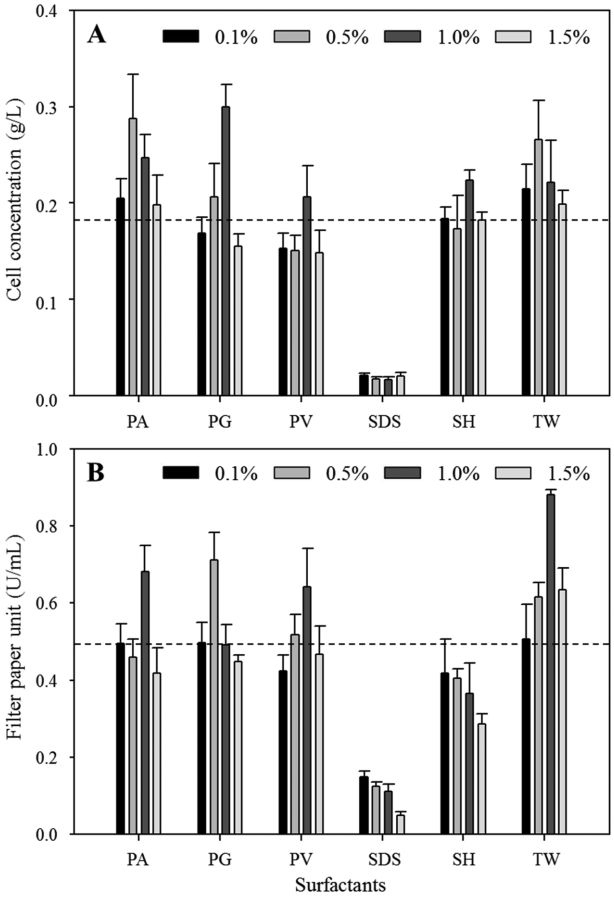

Fig. 1. Fungal growth (A) and extracellular cellulase activities (B) that were obtained from cultures containing different concentrations of six surfactants compared to the control. The dotted line indicate the cell concentration and enzyme activity of the control that was cultivated without the surfactant. PA, polyacrylic acid; PG, polyethylene glycol; PV, polyvinylpyrrolidone; SDS, sodium dodecylbenzene sulfonate; SH, sodium hexametaphosphate; TW, Tween 80.

use of surfactants. Qing *et al.* [15] reported that surfactants could affect the results of enzyme assays by reducing the irreversible binding of the enzymes to the assay substrates, which consequently results in higher enzyme activity. In addition, Ahamed and Vermette [7] reported that the increased cell permeability caused by surfactants could enhance enzyme activities by helping to export the enzyme outside of the cell. However, in the present study, the surfactants had no significant effect on the results of the cellulase assay (data not shown). In addition, intracellular enzyme activities were too low to influence the total activities (data not shown). Therefore, the observed increase in enzyme activity was likely due to increased extracellular enzyme production and not to the effects of the surfactant on the results of the cellulase assay or cell permeability. Fig. 2 shows the macro- and micro-morphological features of the cultures that were grown with TW. Fungal morphology was altered from an aggregated to a dispersed shape, and

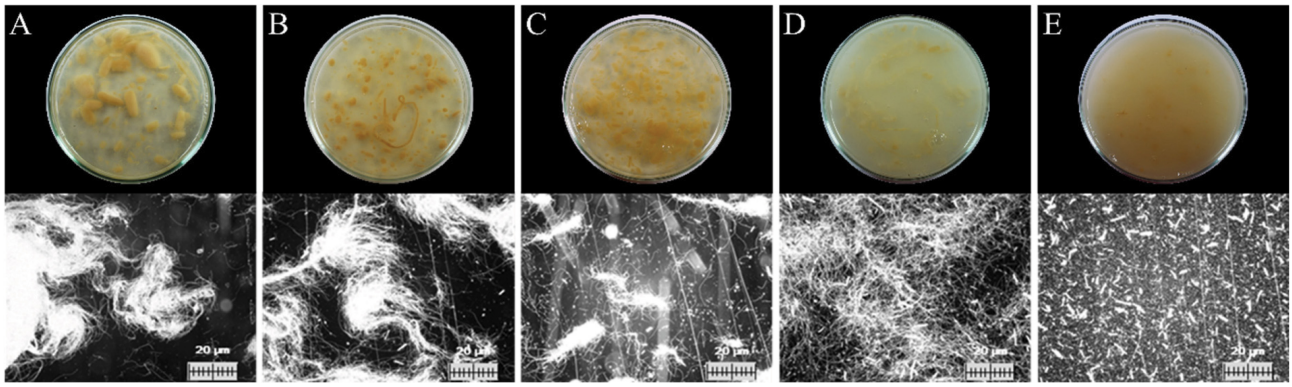

Fig. 2. Macro (upper)- and micro (bottom)-morphology comparison of *Trichoderma harzianum* KUC1716 samples that were collected after 7 days with the addition of no surfactant (A) or 0.1% (B), 0.5% (C), 1.0% (D), or 1.5% (E) Tween 80.

the mycelial pellets gradually loosened and dispersed with an increase in surfactant concentration. At the highest surfactant concentration tested (Fig. 2E), the fungal mycelia were so fine that the cellulose powder could be easily observed. The effects of other surfactants, except SDS, showed a similar pattern to those of TW. Although fungal growth was interrupted at a high surfactant level, *T. harzianum* KUC1716 was morphologically modified by all of the surfactants. Interestingly, at the highest enzyme activities achieved in the presence of each surfactant, the cultured colonies showed similar shapes to those shown in Fig. 2D. Specifically, the fungi cultivated with 1.0% PA, 0.5% PG, 1.0% PV, and 1.0% TW all showed dispersed mycelia and a high level of cellulase activity. This result indicates that the fungus was morphologically optimized to increase enzyme production after adding the surfactants. It is possible that the surfactants increase the surface area of *T. harzianum* KUC1716 by dispersing its mycelia, thus facilitating accessibility to nutrients, thereby inducing the production of more enzymes.

In this study, we added various types of surfactants at a range of concentrations to the culture medium of the filamentous fungus *Trichoderma harzianum* KUC1716 to determine the optimized culture conditions for altering the fungal morphology to promote cellulase production. SDS was highly toxic to the cells, whereas 1.0% TW 80 and 0.5% PG resulted in the highest cellulase production and activity levels. Increased surfactant concentrations resulted in a morphological shift from an aggregated to a more dispersed form, which likely improves accessibility of the fungus to nutrients to improve the enzyme production level and activity overall. We here provide the first report that establishes a clear relationship between enzyme production and fungal morphology induced by a surfactant. These results demonstrate that simply optimizing the culture conditions can have a large effect on the fungal enzyme production process, and therefore further exploration of these effects and the underlying mechanisms can help to simply reduce the cost of bioethanol production toward large-scale industrial applications.

ACKNOWLEDGEMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (NRF-2013R1A1A2A10011390)

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