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NOTE

Change in enzyme production by gradually drying culture substrate during solid-state fermentation

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The influence of drying the culture substrate during solid-state fermentation on enzyme production was investigated using a non-airflow box. The drying caused a significant increase in enzyme production, while the mycelium content decreased slightly. This suggests that changes in the water content in the substrate during culture affect enzyme production in fungi.

[**Key words:** Solid-state fermentation; Substrate drying; Enzyme production; Non-airflow box culture; *Aspergillus oryzae*]

Solid-state fermentation (SSF) has been known to be suitable for the large-scale production of enzymes and useful materials (1–3). Free water content in the culture substrate is an important parameter for the production (4–6). For example, the glucoamylase-encoding genes *glaA* and *glaB* in *Aspergillus oryzae* were greatly expressed in the presence of at high and low water content in the substrate, respectively (6–8). In Sake brewing, a decrease in the water content in the substrate and maintenance of a high temperature in the later stages of culture (koji-making) are traditionally thought to be necessary for increasing glucoamylase production (8). In miso brewing, protease activity has been shown to increase by approximately 1.3-fold by replacing water in the substrate during culture (9). It is known that enzyme production per mycelium is traditionally higher in the presence of low water content in the substrate. However, there are no scientifically-confirmed data on the relationship between changes in the water content during culture and the production of various enzymes. Furthermore, traditional SSF results in the unguaranteed reproducibility by a complex combination of various unfavorable factors. It is impossible to uniformly change culture conditions of the entire substrate during culture because of the limited water availability (2,3). They interfere with the further elucidation of enzyme production mechanisms on changes in the water content of the substrate.

Recently, a novel SSF method using a non-airflow box (NAB) was developed to maintain a uniform state of the entire substrate during culture (10). NAB is a wooden box that opens at the top and bottom sides and the sides are fitted with a moisture-permeable expanded polytetrafluoroethylene (ePTFE) membrane. Vapor arising from fermentation heat gradually dissipates from NAB through the ePTFE membranes by establishing a difference in vapor partial pressure between the inside and outside of the box, followed by uniform drying of the substrate. This leads to a uniform state of the substrate, which thereby promotes a high degree of reproducibility. The SSF technique is close to the actual methods used by the brewing industry, with the exclusion of various unfavorable factors during culture. In addition, this technique enables the control of favorable culture conditions during culture by adjusting for ambient conditions of NAB (10). Therefore, we adopted the NAB culture method to investigate the influence of the substrate drying process on enzyme production during culture progression. The obtained data are more scientific, reliable, and conclusive in comparison with the data obtained using traditional SSF.

In this study, the fungal strain *A. oryzae* AOK 11 (Akita Konno Co., Ltd., Akita, Japan) and a dried wheat bran with a water content of approximately 13% (Odazo Seihun, Kurashiki, Japan) were combined for the fermentation process. The initial water content of the substrate was adjusted to 56.5% by the addition of 100 mL of distilled water per 100 g of substrate. Following this, the substrate was mixed with pre-incubated seed-koji of the wheat bran and then incubated at 25°C under 95% relative humidity (RH) for 24 h in NAB, which was placed in a chamber to maintain constant temperature and humidity (PL-3SP and PL-3KT, ESPEC Corp., Osaka, Japan). The detailed culture methods are described in our previous reports (10,11).

Under these experimental conditions, the production of several enzymes was significantly increased in NAB culture. In addition, the mycelial content increased by more than three-quarters of the maximum value by 24 h since the start of the culture, while the water activity (Aw) and water content in the substrate were also preserved at high levels (11). As shown in Fig. 1A, the culture conditions were gradually changed after 24 h to promote drying of the culture by decreasing the ambient RH (drying range from 60% to

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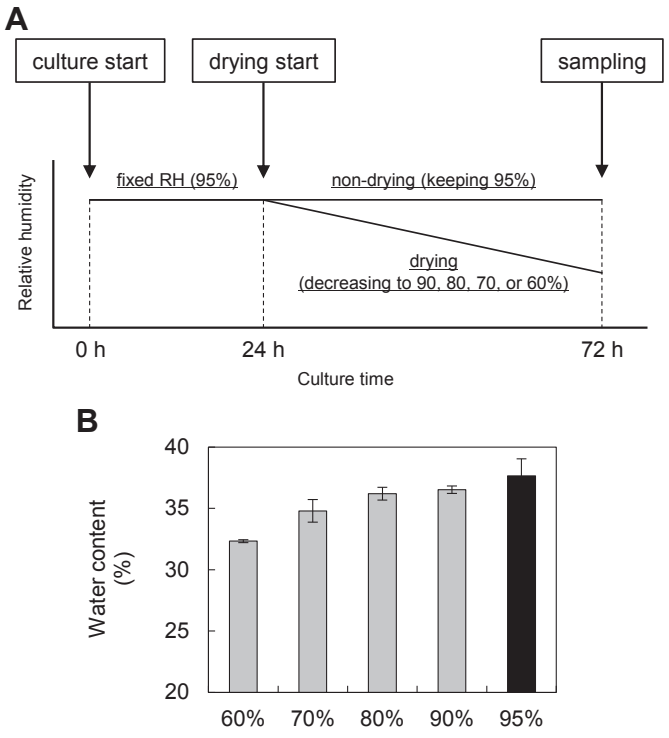

FIG. 1. Culture scheme in this study (A) and water content after culture (B). Culture conditions were controlled in a chamber with programmed temperature and humidity. After 24 h of culture, the culture conditions in the chamber were gradually altered to facilitate drying from 24 to 72 h, as shown in the scheme. Water content in the substrate was calculated by measuring the weight after drying for 48 h in the drying oven. The presented values were obtained from three independent experiments. Error bars indicate standard deviations (SDs).

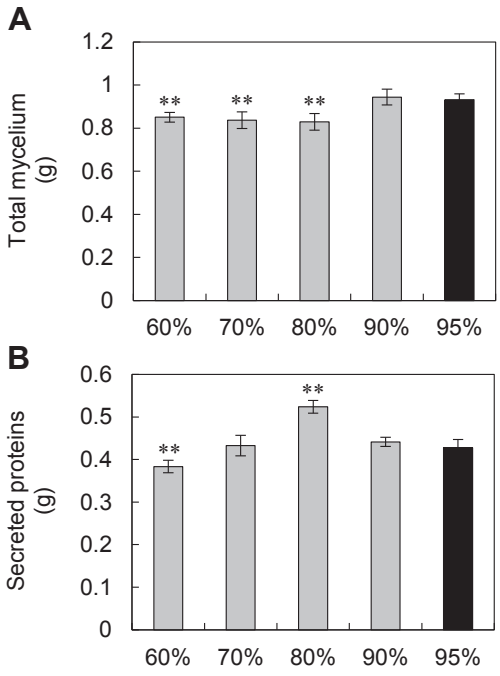

FIG. 2. Total mycelium content (A) and total secreted proteins (B) under gradual drying conditions during culture. Closed bar, non-drying condition during culture (95% RH for 72 h). The content was determined by the amount of *N*-acetylglucosamine (GlcNAc) released from fungal cell walls using the lytic enzyme Yatalase (12). The amount was measured according to the method described by Reissig et al. (13). The total mycelial content was calculated from a previous report (139 μg of GlcNAc/mg of dry mycelium) (14). The total amount of extracellular proteins was determined using the Quick Start Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The presented values were obtained from three independent experiments. Error bars indicate standard deviations (SDs). $^{**}p < 0.01$ compared with non-drying condition (closed bar) during culture.

90%) of NAB. The temperature of the substrate was virtually maintained throughout changes in all RH (data not shown). The water content of the substrate after the culture process was noticeably lower under drying conditions than under non-drying condition (60%, 70%, 80%, and 90% vs. 95% RH; Fig. 1B). Drying of the substrate was in proportion to the decrease in ambient RH of NAB.

Because the water content is closely associated with mycelial growth, the influence of substrate drying on growth was assessed by measuring the total mycelial content after culture. As shown in Fig. 2A, total mycelial content slightly decreased with increasing drying conditions (60%, 70%, and 80% RH) in comparison with non-drying condition (95% RH). The mycelial content was slightly influenced by gradually drying the substrate despite almost constant growth from 24 h in NAB culture at 25°C under 95% RH (11). In contrast, total secreted proteins were significantly increased by the drying process, particularly at 80% RH (Fig. 2B). However, the growth profile was distinctly different from that of the mycelial content. These results suggest a potential for higher productivity of various enzymes by drying the substrate during culture. The enzymatic activity was relatively low on the substrate, which contained aerial hyphae, and it was high in the substrate, which contained non-aerial hyphae (15). The substrate at 72 h contained many conidia under high RH conditions (>80% RH), while there were relatively few conidia under low RH conditions (<80% RH). This observation is consistent with those of a previous study that reported that conidia were easily formed under conditions of high water content in the substrate (16). These findings suggest that in the NAB culture, there is improved growth of aerial hyphae on the substrate under moist conditions, and growth of many non-aerial hyphae in the substrate under dry conditions.

The activity levels of various hydrolytic enzymes were measured after culture to elucidate the influence of the substrate drying process. With wheat bran as a substrate, the activity levels of cellulosic polysaccharide-degrading enzymes were also measured besides those related to brewing. The cultured substrate was suspended in 1 L of distilled water at 4°C for 3 h to extract the produced enzymes. Following this, all large particles were filtered from the suspension, which was 4-fold diluted for enzymatic analysis. One unit (U) of enzymatic activity per the initial substrate (g) was defined as 1 μmol of product/min for each enzyme. To further elucidate the influence of the substrate drying process during culture on enzyme production, the calculated enzymatic activity value reported in Fig. 3 was represented as units per total mycelial content (g). As shown in Fig. 3, compared with non-drying condition (95% RH), the activity levels of all enzymes were the highest at 80% RH, while the levels decreased at <80% RH. Even if the initial water content in the substrate was equal under all RH conditions, changes in the water content during the culture process impacted enzyme production. The profiles showed a similar trend as that of total secreted proteins. Although there were significant differences in the productivity of each enzyme in the initial water content of the substrate (10), various enzymes shown in Fig. 3 widely varied during the substrate drying process. The substrate in NAB continuously tended to dry during the culture process because the vapor continued to dissipate from the box even under extremely moist conditions (95% RH) (11). Hence, enzyme production tended to increase under comparatively high RH condition (80% RH).

Measured enzymes are classified into two types on the basis of increasing activity: those that exhibit an increase in activity in proportion to the mycelial content (e.g., α-amylase, α-glucosidase, glucoamylase, xylanase) and other enzymes (e.g., cellulase, β-glucosidase, acid peptidase, acid protease). The activity levels of