Analysis of Enzyme Production by Submerged Culture of Aspergillus oryzae Using Whole Barley

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We have reported on high enzyme production by submerged culture of Aspergillus kawachii using barley with the husk (whole barley). To elucidate the mechanism underlying this high enzyme production, we performed a detailed analysis. Aspergillus oryzae RIB40 was submerged-cultured using whole barley and milled whole barley. Enzyme production was analyzed in terms of changes in medium components and gene expression levels. When whole barley was used, high production of glucoamylase and α-amylase and high gene expression levels of these enzymes were observed. Low ammonium concentrations were maintained with nitrate ion uptake continuing into the late stage using whole barley. These findings suggest that the sustainability of nitrogen metabolism is related to high enzyme production, and that a mechanism other than that associated with the conventional amylase expression system is involved in this relationship.

Key words: Aspergillus oryzae; submerged culture; barley; enzyme production; nitrogen metabolism

The koji mold Aspergillus oryzae is traditionally used in the Japanese brewing industry, and it has also been widely accepted as being safe to ingest in the USA and Europe. A. oryzae genome sequencing was completed by a Japanese research group17 in 2005, and high-level applications of this fungus are expected in industries other than brewing.

Methods of culturing koji mold include solid-state culture, inoculating fungal conidia in a raw material with pretreatment, such as steaming, and submerged culture, inoculating fungal conidia or precultured fungal hyphae in a liquid medium containing a raw material and other nutrition sources in water. Solid-state culture for the production of many types of enzyme in the large quantities needed for brewing is very useful. On the other hand, submerged culture generally yields lower enzyme production than solid-state culture, although it has high production efficiency due to easy culture control. However, we found that submerged culture with modification of raw material processing conditions using whole barley as the raw material resulted in higher enzyme production,2,3 although the mechanism underlying this higher enzyme production has not yet been clarified in detail.

With these findings as background, this study was designed to elucidate the mechanism underlying higher enzyme production by submerged culture of A. oryzae using whole barley as the raw material. Changes in the concentrations of sugars, organic acids, and inorganic nitrogen ions in culture and gene expression levels determined using DNA microarrays were examined in two culture types, submerged culture with unmilled whole barley (UWB) and that with milled whole barley (MWB), using the production of glucoamylase and α-amylase as indexes. On the basis of this examination, the mechanism underlying high enzyme production by submerged culture with UMB was analyzed.

Materials and Methods

Microorganism and culture conditions. The A. oryzae RIB40 strain was used. For preculture, 100 ml of preculture medium containing 8% w/ v pearl white barley (65% polished white barley: crude barley was polished to a residual weight of 65% w/ v, Australia-grown Schooner species) was placed in a 500-ml Ehrlenmeyer flask with a baffle and then sterilized by autoclaving at 121°C for 15 min. After cooling to room temperature, the preculture medium was inoculated with spores using a platinum loop and incubated with rotary shaking at 100 rpm at 30°C for 24 h. For the main culture, a modified Czapek-Dox medium was used. Its composition was as follows: 2% w/ v carbon source, 0.3% w/ v NaNO3, 0.1% w/ v KH2PO4, 0.05% w/ v MgSO4·7H2O, 0.05% w/ v KCl, and 0.001% w/ v FeSO4·7H2O (all the reagents were from Wako Pure Chemical Industries, Osaka, Japan). As carbon sources, UWB (98% polished white barley, Australia-grown Sterling species) and MWB were used. The medium (100 ml, without pH adjustment) was placed in a 500-ml Ehrlenmeyer flask with a baffle and then sterilized by autoclaving at 121°C for 15 min. After cooling to room temperature, the medium was inoculated with 2 ml of preculture medium and then incubated with rotary shaking at 100 rpm at 30°C for 102 h.

Enzyme activity test. Glucoamylase (GA) activity in the culture supernatant obtained by centrifugation (1,670 × g, 10 min) was measured in accordance with the official method of the National Tax Agency of Japan. α-Amylase (AA) activity in the culture supernatant obtained by centrifugation (1,670 × g, 10 min) was measured using an α-amylase measurement kit (Kikkoman, Chiba, Japan) following the manufacturer’s instructions.

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Sugar determination. For sample preparation, 0.3 ml of the culture supernatant obtained by centrifugation (1,670 \times g, 10 min) was subjected to centrifugal ultrafiltration (Ultra-free MC centrifugal filter unit with a molecular weight cutoff of 10,000, Millipore, Billerica, MA) at 13,200 \times g for 20 min. The supernatant obtained (0.1 ml) was diluted 10-fold with a solution containing 0.22% w/v \(\beta\)-d-fucose (Across Organics, Geel, Belgium) as the internal standard, and then filtered (DISMIC-13c filter with a pore size of 0.45 \(\mu\)m, Advantec, Toyo Roshi, Chiba, Japan) for use as a test sample solution.

The sample solution was analyzed using a high-performance liquid chromatography device equipped with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). A column Aminex HPX-87H (300 mm \times 7.8 mm I.D., Bio-Rad Laboratories, Hercules, CA) was used. The mobile phase was 6% H\(_2\)SO\(_4\), the flow rate was 0.4 ml/min, the oven temperature was 65 °C, and the injection volume was 10 \(\mu\)l.

Determination of organic acids. For sample preparation, 2 or 0.5 ml of the culture supernatant obtained by centrifugation (1,670 \times g, 10 min) was transferred to a test tube. A 0.5 ml solution containing piperazine-1,4-bis-(2-ethane sulfonic acid) as the internal standard was added, and this was diluted to a final volume of 10 ml with water from an ultrapure water filtration system (Milli Q). This solution was then subjected to centrifugal ultrafiltration (Ultra-free MC centrifugal filter unit with a molecular weight cutoff of 5,000, Millipore, Billerica, MA) for use as a sample solution.

The sample solution was analyzed using a capillary electrophoresis (CE) system (Agilent, Palo Alto, CA) equipped with a mass spectrometer (Agilent 1100 Series Single Quadrapole Mass Spectrometer, Agilent, Palo Alto, CA).

A column, a fused silica capillary (50 \(\mu\)m I.D., length, 100 cm; Polymicro Technologies, Phoenix, AZ), was used, and 20nm ammonium formate (pH 10.0) was used as a run electrolyte solution.

The CE voltage was +30 kV and the capillary temperature was 25 °C.

The MS conditions were as follows: ionization mode, ESI-negative; MS capillary voltage, 3,500 V; and fragmentor voltage, 120 V. The nebulizer gas pressure, dry gas rate, and temperature were 10 psi, 101/min, and 300 °C. The sheath solution was 5 nm ammonium in 50% \(\nu/\nu\) methanol-water supplied at a flow rate of 6 \(\mu\)l/min.

Determination of inorganic nitrogen ions. For sample preparation, the culture supernatant obtained by centrifugation (1,670 \times g, 10 min) was diluted 20-fold for ammonium analysis and 100-fold for nitrate ion analysis with Milli Q water, and then filtered (DISMIC-13c filter with a pore size of 0.45 \(\mu\)m, Advantec) for use as a sample solution. An ion chromatography device (Dionex, Sunnyvale, CA) equipped with a conductometric detection system (AD25, Dionex) was used.

For ammonium analysis, a separation column Ion Pac CS12A (Dionex), a guard column Ion Pac CG12A (Dionex), and a suppressor CSRS-ULTRA II (Dionex) were used. The eluent was 20nm methanesulfonic acid solution. The flow rate was 1.0 ml/min and the injection volume was 25 \(\mu\)l.

For nitrate ion analysis, a separation column Ion Pac AS14 (Dionex), a guard column Ion Pac AG14 (Dionex), and a suppressor ASRS-ULTRA II (Dionex) were used. The eluent was 3.5 nm sodium carbonate solution/1 nm sodium hydrogen carbonate solution. The flow rate was 1.2 ml/min and the injection volume was 25 \(\mu\)l.

Mycelial weight determination. Yatalase (Takara Bio, Shiga, Japan) was applied to mycelia of the koji mold by the method of Fujii et al.,* and the amount of \(N\)-acetylglucosamine (GlCNac) released from the mycelia was determined by the method of Renissi et al.** Specifically, 1 ml of the culture solution was transferred to the test tube for centrifugation (1,770 \times g, 10 min). The precipitate obtained was washed with 10 ml of 50 mm phosphate buffer (pH 7.0). The resulting solution was then subjected to centrifugation (1,770 \times g, 10 min), and 10 ml of 50 mm phosphate buffer (pH 7.0) containing 20 mg of Yatalase was added to the precipitate obtained. The precipitate obtained was subjected to enzyme reaction at 37 °C for 4 h. At the end of the enzyme reaction, the precipitate was centrifuged (1,770 \times g, 10 min), and 0.5 ml of the supernatant obtained was transferred to a test tube to which 0.1 ml of 0.8M potassium tetraborate solution (pH is adjusted to 9.1 with KOH) was added. The supernatant in the test tube was heated for 3 min in boiling water. After cooling in tap water, 3 ml of p-dimethylaminobenzaldehyde (pDMAB) reagent was added to the supernatant, and the mixture was allowed to stand at 37 °C for 20 min for color development. The mixture was cooled in tap water. The absorbance was then determined at 585 nm. pDMAB reagent stock solution was prepared by dissolving 10 g of pDMAB in 100 ml of acetic acid containing 12.5% \(\nu/\nu\) 10 N HCl and stored at 2 °C. It was diluted 10-fold with acetic acid immediately before use. Calculation was carried out on the basis of 139 \(\mu\)g of GlCNac in 1 mg of dry mycelia.*}

Gene expression analysis. mRNA preparation. For mycelia recovery, the culture solution was first filtered through a stainless mesh (2.8 mm in diameter) to remove the solids from the whole barley. The mycelia recovered were washed with cold deionized water, and excess water was removed from the mycelia with Miraculous (Merck, Whitehouse Station, NJ), and this was frozen quickly in liquid nitrogen. Frozen samples were sent to Fermlab (http://www.fermlab.com), which was commissioned to conduct the subsequent experiment. Total RNA was isolated using Isogen (Wako, Osaka, Japan) following the manufacturer’s instructions. The mycelia were crushed in liquid nitrogen and mixed with 5 ml of Isogen. Chloroform (1 ml) was added to the mixture, which was mixed well. The suspension obtained was centrifuged (10,000 \times g, 10 min), and the supernatant was transferred to a test tube to which 2.5 ml of isopropanol was added. The mixture was allowed to stand for 10 min at room temperature and then centrifuged (10,000 \times g, 10 min). The precipitate was washed with 100% ethanol (10,000 \times g, 3 min). The precipitate obtained was dissolved in 300 \(\mu\)l of RNase-free water and frozen at −80 °C for storage until use. mRNA was prepared from approximately 200 \(\mu\)g of total RNA using an Oligotex TM-dT (super) mRNA purification kit (Takara Bio) following the manufacturer’s instructions.

DNA microarray experiment. Approximately 1 \(\mu\)g of mRNA calculated on the basis of the absorbance at 260 nm was labeled with Cy3-dUTP and Cy5-dUTP using a CyScribe cDNA postlabeling kit (GE Healthcare, Buckinghamshire, UK) following the manufacturer’s instructions. The labeled complementary DNA (cDNA) was purified using a CyScribe GFX purification kit (GE Healthcare) following the manufacturer’s instructions. After purification, cDNA was mixed with an equal amount of cDNA probes labeled with Cy3- and Cy5-dUTP, and the mixture was placed in an Eppendorf tube and dried with a vacuum pump.

The labeled probe mixture was resuspended in a hybridization solution consisting of 10.2 \(\mu\)l of 20 \% SSC, 2 \% of formamide, 0.6 \% of salmon testes DNA, 1.8 \% of 10 \% SDS, and 26.4 \% of distilled water. After heat denaturation (95 °C, 5 min), the mixed solution was incubated at room temperature for 30 min, and then placed on 12k Aspergillus oryzae oligonucleotide microarrays (Fermlab, Tokyo). After hybridization (42 °C, 15 h), the microarrays were washed with buffer solution containing 2 \% SSC and 0.03 \% SDS at room temperature for 15 min. These were then washed with buffer solution containing 0.2 \% SSC for 5 min, and finally with buffer solution containing 0.05 \% SSC for 5 min. After removal of the remaining solution, DNA microarray slides were scanned with a GenePix 4000B scanner (Axon Instruments at Molecular Devices, Sunnyvale, CA).

The submerged culture with UW and that with MWB (n = 4) were compared by performing a Cy5-Cy3 dye swap using mycelia at 22 and 54 h of culture.

Data analysis. Image spots on scanned microarray slides were identified using GenePix Pro 6.0 software (Axon Instruments at Molecular Devices). Read data were analyzed using R (statistic processing free software) (http://www.r-project.org/) and BioConductor (statistic processing free software) (http://www.bioconductor.org/). Locally weighted scatter plot smoothing normalization was carried out, and the expression level ratio was analyzed for microarrays, on which spots were identified.

Results and Discussion

Changes in medium components

The time courses of GA activity, AA activity, and mycelial content (mycelial weight per milliliter of medium) during culture are shown in Fig. 1. In the
submerged culture with MWB, GA and AA activities stopped increasing at and after 50 h of culture respectively. In the submerged culture using UWB, both GA and AA activities continued to increase for a longer time. The final GA and AA activities were 1.9 and 1.4 times higher respectively in the culture with UWB than in that with MWB.

Throughout the culture period, the mycelial content of the culture with MWB was higher than that of the culture with UWB, finally reaching 1.6 times. There was no significant difference between GA and AA activities in terms of mycelial content until halfway through the culture, but the final GA activity was 3.0 times and the final AA activity 2.3 times higher in the culture with UWB.

First, regarding sugar production, the glucose and disaccharide concentrations in the medium were examined (Fig. 2). The concentrations of both sugars remained low, showing suppressed sugar production, in the culture with UWB as compared with those in the culture with MWB. In the culture with MWB, the concentrations peaked at 22 h of culture, which was the first half of the culture period, rapidly decreasing until 30 h of culture. The glucose and disaccharide concentrations apparently remained at nearly 0 at and after 50 h of culture respectively.

In each culture type, the glucose concentration was higher than 10 mg/l during the first half of the culture period and lower than 10 mg/l during the second half. Carbon repression is induced at a glucose concentration of approximately 10 mg/l in A. oryzae culture.7) It was thought that enzyme production might have been affected by carbon repression during the first half of the culture period but was less likely to be affected during the second half due to the low glucose concentrations in the culture with UWB and that with MWB (Fig. 2).

To analyze further the differences in carbohydrate metabolism, organic acids in the glycolytic pathway and the TCA cycle were examined. The time courses of the concentrations of pyruvic acid, succinic acid, and malic acid are shown in Fig. 3. Some differences in the carbohydrate metabolism of all these organic acids were observed during the first half of the culture period, with a marked difference in the metabolism of pyruvic acid in particular. Shoji et al.3) reported that when MWB was mixed with an enzyme liquid in a model experiment, the release rate of glucose was 3.3-fold that when using UWB. We assumed that the glucose concentration in the medium did not increase markedly in the case of MWB during the first half of the culture period, because the glucose released was taken up rapidly by mycelia despite the high rate of glucose release. However, when glycolysis became active, pyruvic acid was produced and was secreted into the medium, leading to an increase in pyruvic acid concentration. The succinic acid and
Malic acid concentrations were also thought to have increased in the culture with MWB, because when glycolysis became active, succinic acid and malic acid were produced in the TCA cycle, following the glycolytic pathway, and were secreted into the medium. The concentrations of succinic acid and malic acid apparently remained at 0 at and after 50 h of culture with UWB and MWB respectively.

After examination of carbohydrates, nitrate ions, which were in the medium as a nitrogen source, and ammonium, a nitrogen metabolite, were examined next (Fig. 4). Differences in the time courses of the concentrations of nitrate ions and ammonium were observed between the culture with UWB and that with MWB. Specifically, the nitrate ion concentration tended to decrease throughout the period of culture in the case of UWB, whereas in the case of culture using MWB, the nitrate ion concentration decreased rapidly up to 30 h of culture and then remained stable. The ammonium concentration remained close to 0 throughout the period of culture in the case of UWB, whereas in the case of culture using MWB, the ammonium concentration began to increase at 40 h of culture and subsequently continued to increase until the end of the culture period. It appears likely that in the case of MWB, glucose became insufficient during culture due to the rapid disappearance of the carbohydrate from the raw material and that alterations in metabolism such as the use of nitrogenous compounds as the carbon source occurred, leading to an increased ammonium concentration. It was thought that for the culture using MWB, the marked alterations in the nitrogen metabolism occurred in the second half of the culture period, whereas for the culture using UWB, nitrogen metabolism was maintained without marked alterations from the first half of the culture period (Fig. 4).

**Gene expression analysis**

Analysis of gene expression in the mycelia was conducted using DNA microarrays. It was carried out at 22 h of culture, which was the first half of the culture period when the glucose concentration peaked for the culture using MWB (Fig. 2), and at 54 h of culture, which was the second half of the culture period when enzyme activity started to saturate for the culture using MWB (Fig. 1). The genome database of *A. oryzae* RIB40 (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao) was referred to for gene functions.

Table 1 shows the glycolytic genes that had a more than 2-fold difference in expression level between the culture with UWB and that with MWB at 22 h of culture (in the first half of the culture period) at a significance level of \( p < 0.05 \). In the culture with MWB, high gene expression levels of hexokinase, glucose-6-phosphate isomerase, and pyruvate kinase were observed. How-
ever, the gene expression level of fructose-1,6-bisphosphatase, which catalyzes the conversion from fructose-1,6-diphosphate to fructose-6-phosphate, was low. The present results are in agreement with those of northern blot analysis of the glycolytic gene group expression in the presence and the absence of glucose as reported by Nakajima et al.11 As mentioned above, in the case of the culture using MWB, glycolysis was thought to have been occurred more quickly, because the rate of glucose release from the MWB was higher than that from the UWB as raw material. With regard to AA-related gene expression, as Table 2 shows, the gene expression levels were more than 2-fold higher in the culture with UWB than in that with MWB at a significance level of \( p < 0.05 \). Because probe sequences were devised for this experiment, we could distinguish homologous genes of AA. Low AA-related gene expression levels were observed in the culture with MWB, and this was thought to be attributable to the larger effect of carbon repression due to the high rate of glucose release. At 22 h of culture, AA activity was higher in the culture with UWB due to the high rate of glucose release. At 22 h of Culture, enzyme activities continued to increase in the culture with UWB, whereas they saturated in the culture with MWB. There was also a clear difference in amylase-related gene expression levels between them. Tables 3 and 4 show GA-related and AA-related gene groups respectively. These showed a more than 2-fold difference in the levels of amylase-related gene expression at 54 h of culture at a significance level of \( p < 0.05 \). As shown in Tables 3 and 4, GA-related and AA-related genes expression levels were markedly higher in the culture with UWB, and these higher levels were in agreement with the trend of changes related to enzyme activities in the medium. Regarding the GA genes, not only \( glaA \) expression but also \( glaB \) expression, which is unique to solid-state culture, was enhanced in the submerged culture with UWB. In this experiment, the quantitative ratio of glucoamylase encoded by the \( glaA \) gene and the \( glaB \) gene was not determined.

Concerning gene expression in relation to the inorganic nitrogen components in the medium showing a time-course change in the second half of the culture period as shown in Fig. 4, Table 5 shows the genes that had a more than 2-fold difference in expression levels between the culture using UWB and that using MWB in the second half of the culture period at a significance level of \( p < 0.05 \). Higher gene expression levels of the nitrate transporter related to nitrate ion uptake and glutamine synthetase, which catalyzes the biosynthesis of glutamine from ammonium and glutamic acid, for the culture using UWB than for the culture using MWB were observed. Differences not only in the time course of inorganic nitrogen ion concentration but also in related gene expression levels were observed between the culture using UWB and that using MWB. These findings indicate the maintenance of nitrogen metabolism with little change from the first half to the second

**Table 1.** Differences in Glycolytic Gene Group Expression Intensity (at 22 h of Culture)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function description</th>
<th>Fold change</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO090011000659</td>
<td>Glucose-6-phosphate isomerase</td>
<td>2.54</td>
<td>0.010</td>
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<tr>
<td>AO090005000003</td>
<td>Hexokinase</td>
<td>2.50</td>
<td>0.014</td>
</tr>
<tr>
<td>AO090005001556</td>
<td>Pyruvate kinase</td>
<td>2.05</td>
<td>0.045</td>
</tr>
<tr>
<td>AO090003001189</td>
<td>Fructose-1,6-bisphosphatase</td>
<td>0.26</td>
<td>0.021</td>
</tr>
</tbody>
</table>

*The E-value was \( < 10^{-30} \) for each.
Expression ratio (MWB/UWB)
Corrected p-value with multiple comparison taken into consideration

**Table 2.** Differences in AA-Related Gene Group Expression Intensity (at 22 h of Culture)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function description</th>
<th>Fold change</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO090003000234</td>
<td>Alpha-amylase</td>
<td>17.89</td>
<td>0.021</td>
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<tr>
<td>AO0900103000378</td>
<td>Alpha-amylase</td>
<td>3.68</td>
<td>0.009</td>
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<tr>
<td>AO090026000034</td>
<td>Alpha-amylase</td>
<td>3.25</td>
<td>0.035</td>
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<tr>
<td>AO090003001498</td>
<td>Alpha-amylase</td>
<td>2.31</td>
<td>0.027</td>
</tr>
<tr>
<td>AO090005000884</td>
<td>Alpha-amylase</td>
<td>2.23</td>
<td>0.016</td>
</tr>
</tbody>
</table>

*The E-value was \( < 10^{-30} \) for each.
Expression ratio (UWB/MWB)
Corrected p-value with multiple comparison taken into consideration

**Table 3.** Differences in GA-Related Gene Group Expression Intensity (at 54 h of Culture)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function description</th>
<th>Fold change</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO090010000746</td>
<td>glaA</td>
<td>10.79</td>
<td>0.006</td>
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<tr>
<td>AO090003000321</td>
<td>glaB</td>
<td>4.62</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Expression ratio (UWB/MWB)*
Corrected p-value with multiple comparison taken into consideration

**Table 4.** Differences in AA-Related Gene Group Expression Intensity (at 54 h of Culture)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function description</th>
<th>Fold change</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO090003000234</td>
<td>Alpha-amylase</td>
<td>35.20</td>
<td>0.001</td>
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<tr>
<td>AO090002600034</td>
<td>Alpha-amylase</td>
<td>8.40</td>
<td>0.012</td>
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<tr>
<td>AO090012000196</td>
<td>Alpha-amylase</td>
<td>7.69</td>
<td>0.002</td>
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<tr>
<td>AO090003001498</td>
<td>Alpha-amylase</td>
<td>6.03</td>
<td>0.005</td>
</tr>
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</table>

*The E-value was \( < 10^{-30} \) for each.
Expression ratio (UWB/MWB)
Corrected p-value with multiple comparison taken into consideration

**Table 5.** Differences in Nitrogen Metabolism-Related Gene Group Expression Intensity (at 54 h of Culture)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function description</th>
<th>Fold change</th>
<th>adj.P.Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO0900012000623</td>
<td>Nitrate/nitrite transporter</td>
<td>31.48</td>
<td>0.005</td>
</tr>
<tr>
<td>AO090009000269</td>
<td>Glutamine synthetase</td>
<td>2.73</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*The E-value was \( < 10^{-30} \) for each.
Expression ratio (UWB/MWB)
Corrected p-value with multiple comparison taken into consideration
half of the period of culture using UWB. This is in contrast to the marked change in nitrogen metabolism in the second half of the period of culture using MWB. That is, the maintenance of nitrogen metabolism appeared to lead to high enzyme production. The potential involvement of nitrogen metabolism in amylase production was assessed. It is another mode of the amylase enzyme expression system, which is different from the conventional mode previously explained for AmyR and CreA, and in another aspect different from the glycolgen-related metabolism in the mycelia.

On the basis of the above-described assessment results, the following hypothesis that might explain the high enzyme production by A. oryzae in the submerged culture with UWB was considered: First, in the first half of the period of culture using MWB, rapid glucose release from the raw material owing to milling activates glycolysis. In the case of culture using UWB, carbohydrate metabolism is gradual owing to slow sugar release from the raw material.

In the second half of the period of culture using MWB, sugars are released rapidly from the raw material, and accordingly the disappearance of carbohydrate from the medium occurs early. The ammonium concentration in the medium increases owing to a marked change in the metabolism of nitrogen compounds such as amino acids, which leads to saturation of enzyme production. In the second half of the period of culture using UWB, slow carbohydrate release makes it possible to supply sugar continuously for a long time. Consequently, nitrogen metabolism is maintained, and this allows the biosynthesis of amino acids from nitrate ions, contributing to high enzyme production. This hypothesis was supported not only by the time-course changes of sugars and inorganic nitrogen ions in the medium but also by the results of microarray analysis.

In this study, the mechanism underlying high enzyme production by A. oryzae in submerged culture using UWB was analyzed. The maintenance of nitrogen metabolism, particularly up to the second half of the culture period, is thought to be related to high enzyme production. The involvement of a mechanism other than the conventional mode of amylase expression explained for AmyR and CreA is assumed.

References