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Cultivation characteristics and gene expression profiles of *Aspergillus oryzae* by membrane-surface liquid culture, shaking-flask culture, and agar-plate culture

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We cultivated a filamentous fungus, *Aspergillus oryzae* IAM 2706 by three different cultivation methods, i.e., shaking-flask culture (SFC), agar-plate culture (APC), and membrane-surface liquid culture (MSLC), to elucidate the differences of its behaviors by different cultivation methods under the same media, by measuring the growth, secretion of proteases and α-amylase, secreted protein level, and gene transcriptional profile by the DNA microarray analysis. The protease activities detected by MSLC and APC were much higher than that by SFC, using both modified Czapek–Dox (mCD) and dextrin–peptone–yeast extract (DPY) media. The α-amylase activity was detected in MSLC and APC in a much larger extent than that in SFC when DPY medium was used. On the basis of SDS–PAGE analyses and N-terminal amino acid sequences, 6 proteins were identified in the supernatants of the culture broths using DPY medium, among which oryzin (alkaline protease) and α-amylase were detected at a much higher extent for APC and MSLC than those for SFC while only oryzin was detected in mCD medium, in accordance with the activity measurements. A microarray analysis for the fungi cultivated by SFC, APC, and MSLC using mCD medium was carried out to elucidate the differences in the gene transcriptional profile by the cultivation methods. The gene transcriptional profile obtained for the MSLC sample showed a similar tendency to the APC sample while it was quite different from that for the SFC sample. Most of the genes specifically transcribed in the MSLC sample versus those in the SFC sample with a 10-fold up-regulation or higher were unknown or predicted proteins. However, transcription of oryzin gene was only slightly up-regulated in the MSLC sample and that of α-amylase gene, slightly down-regulated.

[**Key words:** Filamentous fungi; *Aspergillus oryzae*; DNA microarray; Enzyme secretion; Membrane-surface liquid culture]

Filamentous fungi are widely utilized in the production of enzymes/heterologous proteins, organic acids, and antibiotics in addition to the production of traditional fermented foods and beverages (1, 2). Most filamentous fungi belong to an absolute aerobe having a strong oxygen requirement for growth. In nature, filamentous fungi grow on solid substrates and are able to thrive in relatively low humidity, extending long, thin, branched threads of mycelium, which form spores when nutrients are scarce. Filamentous fungi that are used in industrial processes are usually cultivated by submerged liquid culture (SLC) using water-soluble nutrients due to their ease of medium preparation, control of pH/temperature, and recovery of products.

One of the most important features of filamentous fungi when cultivated is that their cultivation behaviors, in particular secretion of enzymes/proteins are usually quite different by the cultivation methods. Namely, filamentous fungi cultivated by solid–substrate culture (SSC) using water-insoluble agricultural products, such as steamed rice, ground soybeans, and wheat bran, tend to secrete various metabolites and enzymes in larger amounts than those by SLC (1–3). Some enzymes, including heterologous proteins, can be produced at much higher levels by SSC as compared to SLC. Enzymes that are barely detected by SLC could be sometimes produced by SSC (1, 4–14). These facts have recently attracted the attention of a number of researchers from the standpoint that differences in cultivation conditions between SSC and SLC might alter the expression levels of various genes that affect various phenotypes, such as growth, development, mycotoxin, and enzyme production (12). Hence, recent attempts have been devoted to reveal a mechanism of higher enzyme production in SSC on the basis of gene expression (9, 10, 14–22) and proteomic analyses (23).

Genes that are expressed specifically by SSC or SLC have been screened under different cultivation conditions and are discussed in connection with functions of the fungi (19–21). However, gene expression studies have focused mainly on the transcriptional regulation of several particular genes, such as those encoding glucoamylase B, acid protease, alkaline protease, and neutral protease (15, 17, 18, 20, 24). The entire genome sequence for *Aspergillus oryzae* (25) has recently been revealed, which should help researchers understand the factors affecting the higher enzyme productivity of SSC. On the basis of the genome sequences, a DNA microarray that covers almost all protein-coding genes for *A. oryzae* is now commercially available (Fermlab Inc., Tokyo, Japan).

We previously reported that filamentous fungi cultivated by membrane-surface liquid culture (MSLC) (26–31) shows cultivation

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behaviors that are similar to those cultivated by agar-plate culture (APC). Namely, in MSLC, filamentous fungi are grown statically on the surface of a microporous membrane, one side of which faces the air with forming spores, similar to SSC, while the other side is in contact with the liquid medium. We showed that neutral protease, α-glucosidase, and kojic acid are produced at much higher levels by MSLC than those produced by shaking-flask culture (SFC) in a manner similar to SSC. In addition, we screened some MSLC-specific genes (32). Hence, MSLC would be a useful tool not only for an efficient production system but also for elucidating the mechanism of higher enzyme productivity in SSC. Shimokawa et al. (33) have recently reported that protein secretion, dye decoloring peroxidase activity, and aryl alcohol oxidase activity of a basidiomycete, *Thanatephorus cucumeris*, are markedly enhanced by cultivating in an air–membrane surface bioreactor, of which principle is similar to MSLC.

In this study, we cultivated *A. oryzae* IAM 2706 by SFC, APC, and MSLC under the same cultivation conditions and measured growth, enzyme production, particularly those of proteases and α-amylase, secreted proteins, and gene transcriptional profiles using the DNA microarray that carries the entire genome of *A. oryzae*, to elucidate the differences of behaviors of *A. oryzae* by different cultivation methods.

MATERIALS AND METHODS

Strain, media, and cultivation methods *A. oryzae* IAM2706 (hereafter abbreviated as *A. oryzae*) was obtained from the Institute of Molecular and Cellular Biosciences, The University of Tokyo (Tokyo). All the cultivations, including slant and main cultures, were performed at 30 °C throughout this study. The vegetative cells were cultivated for about 6 days on a slant medium (1.5% glucose, 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and 2% agar, pH 6.0) to obtain spores. Then, the main cultures (SFC, APC, and MSLC) were started. As a medium for the main culture, we usually used modified Czapek–Dox medium (mCD medium; 1.5% glucose, 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, and 0.001% $FeSO_4 \cdot 7H_2O$, pH 7.0) and dextrin–peptone–yeast extract (DPY) medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7.0) with or without agar. SFC was usually started by inoculating 200 μl of spore suspension (1×10^7 spores/ml) in 100-ml Sakaguchi flasks containing 25 ml of mCD or DPY medium on a reciprocal shaker (120 strokes/min) for 5 days. The cultivation was also done in 2-l shaking-flasks containing 150 ml of DPY medium by inoculating the spore suspension at the same concentration as used for the cultures using 100-ml flasks, to examine the effect of the scale of shaking-flasks on the cultivation characteristics. Similarly, APC was started by inoculating 200 μl of the spore suspension containing the same number of spores as used for SFC, uniformly on 25-ml agar-plate medium (2% agar), containing mCD or DPY medium in plastic dishes (94 mm × 21 mm) followed by static incubation in an incubation box (ESPEC incubator CHS-110; Tabai ESPEC, Osaka). The temperature and relative humidity inside the box were controlled at 30 °C and 98%, or higher, respectively. MSLC was conducted using the same method as reported previously (31, 32) using plastic dishes (94 mm × 21 mm) with Biodyne A (PALL Co., NY) as a porous membrane. The cultivation was started by uniformly inoculating 200 μl of the spore suspension containing the same number of spores as used for SFC and APC, onto the membrane surface. The dishes were then incubated under the same conditions as those used for APC. The hyphae grew uniformly on the membrane surface with no leakage into the liquid medium contained in the bottom dish.

For each SFC, APC, and MSLC, we usually cultivated several cultures simultaneously, and one of the cultures was withdrawn for analysis at appropriate times during the course of cultivation. In the case of APC, we crushed the agar into small pieces in a mortar, followed by suspension in a large amount of 50 mM potassium phosphate buffer, pH 7.0 at 4 °C for 12 h to extract the enzyme and glucose, by the same method as reported in our previous paper (31).

Analytical methods The glucose concentration was measured by the mutarotase–glucose oxidase/peroxidase method using a Glucose CII-test Wako (Wako Pure Chemical Industries, Ltd., Osaka). The protease activity was measured using the final 0.6% casein dissolved in potassium phosphate buffer, pH7.0 as the substrate. The reaction was stopped by the addition of trichloroacetic acid after a 60-min incubation at 30 °C followed by quantification of soluble peptides in the reaction mixture as described previously (29). One unit of the protease activity was defined as the amount of enzyme that catalyzes the solubilization of 1 μg of protein at pH 7.0 and 30 °C in 1 min, as measured using the Lowry method (34) with bovine serum albumin as the standard. The activities for α-amylase and α-glucosidase/glucoamylase were measured using an α-amylase measurement kit and an α-glucosidase/glucoamylase assay kit (Kikkoman Co., Noda), respectively. One unit of the α-amylase activity was defined as the amount of enzyme that releases 1 μmol of 2-chloro-4-nitrophenol from 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaoside at 37 °C in 1 min. On the other hand, one unit of α-glucosidase and glucoamylase was defined as the amount of enzyme that releases 1 μmol of 4-nitrophenol, in the form of 4-nitrophenyl α-D-glucopyranoside or 4-nitrophenyl *O*-α-D-glucopyranosyl (1→4)-β-D-glucopyranoside, at 37 °C in 1 min, respectively. The dry cell weight was measured using the same method as described elsewhere (26).

SDS–PAGE and N-terminal amino acid sequences SDS–PAGE was performed using a 12.5% polyacrylamide gel according to the protocol described elsewhere (35), in which Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA) were used as the molecular weight marker. The protein bands were stained with CBB Stain One (Nacalai Tesque, Inc., Kyoto). For analysis of the N-terminal amino acid sequence of proteins, the supernatant was first separated on a 12.5% SDS–PAGE gel (Mini-Protean III Ready Gels J, Bio-Rad Laboratories, Hercules, CA) and electroblotted onto a PVDF membrane (Atto Corp., Clear Blot Membrane-P, Tokyo). After washing with distilled water, the membrane was subjected to a protein sequencer (Applied Biosystems, Model 491, Foster, CA) for N-terminal sequence analysis.

Gene sequence analyses Gene sequences and annotations of *A. oryzae* were obtained from the Database of the Genome Analyzed at NITE (DOGAN: http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao) and function predictions were complementarily carried out according to the results of either the BLASTP or the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST). Homology searches of the N-terminal amino acid sequences were performed using BLAST and FASTA algorithms from DOGAN, MEROPS, and the DNA Data Bank of Japan (DDBJ) (http://www.bio.nite.go.jp/dogan/Top, http://merops.sanger.ac.uk/, http://www.ddbj.nig.ac.jp/Welcome-j.html). The signal peptide sequence was predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

Purification of mRNA and cDNA microarray analysis A DNA microarray (Fermlab Array *Aspergillus oryzae* 12k) that carries nearly the entire genome of *A. oryzae* (12,168 probes) was purchased from Fermlab (Tokyo). The total RNA was extracted from *A. oryzae* mycelia, which had been collected after 1.5 days of cultivation of 10 batches of SFC using a 100-ml flask, APC, and MSLC containing 25 ml of mCD medium, using Trizol reagent (Invitrogen, Carlsbad, CA). For APC, we recovered the whole mycelia using a spatula and pincet from the agar plate to extract the total RNA, in which we removed agar fragments, and then quickly frozen followed by grinding using a pestle, by the same method as described in our previous paper (31). Poly(A) mRNAs were purified using a FastTrack 2.0 kit (Invitrogen) according to the manufacturer's protocol, as reported elsewhere (32). We confirmed the quality of the RNA by agarose gel electrophoresis. Then, Cy3- and Cy5-labeled cDNAs were prepared using 1 μg of purified mRNA and a Cyscribe cDNA Post Labelling Kit (GE Healthcare, Piscataway, NJ). Then, approximately 60 pmol of the purified cDNA probe was dissolved in a hybridization solution (1% DNA sodium salt from salmon testes (Sigma-Aldrich, St. Louis, MO), 35% formamide, 0.3% SDS, and 3.4 × SSC) and denatured at 95 °C for 5 min, followed by overnight hybridization with a DNA microarray plate in a hybridization chamber at 42 °C. The microarray plate was then washed at room temperature with 2 × SSC and 0.03% SDS for 15 min, 0.2 × SSC for 5 min, 0.05 × SSC for 5 min, rinsed with

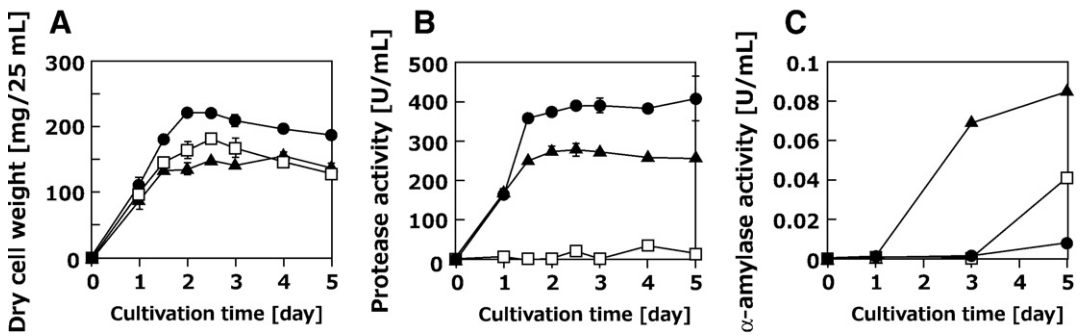

FIG. 1. Courses of growth (A), protease secretion (B), and α-amylase secretion (C), using mCD medium by SFC (open squares), APC (closed triangles), and MSLC (closed circles).

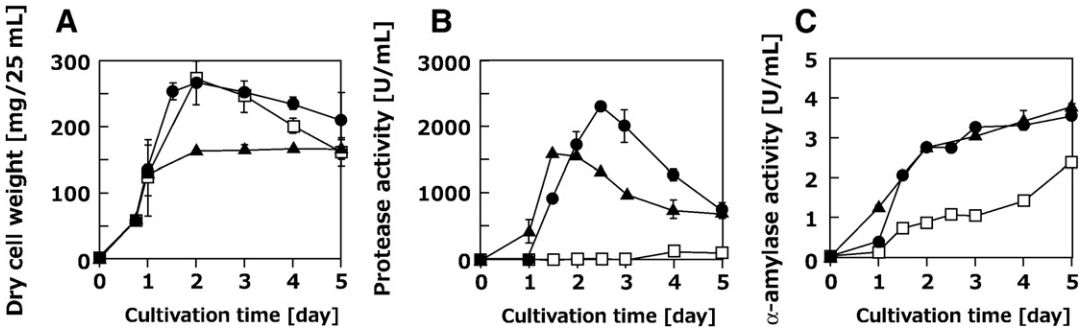

FIG. 2. Courses of growth (A), protease secretion (B), and α-amylase secretion (C), using DPY medium by SFC (open squares), APC (closed triangles), and MSLC (closed circles).

MilliQ water and dried by centrifugation. Then, the spots were detected using an Affymetrix 428™ Array Scanner (Affymetrix, Santa Clara, CA) and analyzed using ImaGene ver. 5.5 software (BioDiscovery, El Segundo, CA). The hybridization experiment using the microarray was duplicated for both MSLC–SFC and MSLC–APC samples and the average values for the mean intensity ratio obtained were used for the analyses. In the data processing, we filtered the data using Microsoft Excel 2007.

Measurement of k_La The k_La value was measured by the sodium sulfite method using a 100-ml shaking-flask containing 25-ml 0.2 N Na_2SO_3 and 2-l shaking-flask containing 150-ml 0.2 N Na_2SO_3, as described elsewhere (36), in which the flasks were shaken at 30 °C on a reciprocal shaker (120 strokes/min).

RESULTS

Cultivation characteristics of *A. oryzae* IAM 2706 Figs. 1A–C show the growth, courses of protease production, and those of α-amylase production, respectively, for SFC, APC, and MSLC, using mCD medium. There was only a slight difference with respect to growth for the three cultivation methods, as shown in Fig. 1A. In SFC, the decrease in the dry cell weight after 2 days of cultivation was slightly more appreciable, as compared to APC and MSLC. Glucose was nearly exhausted after 2 days of cultivation for the three cultivation methods (data not shown). The pH change during cultivation was also similar in tendency for both SFC and MSLC, in which pH was initially decreased to 6.8 and 6.5 from 7.0 after 1 and 1.5 days of cultivation, respectively, followed by a gradual increase with concomitant cell growth and reached to pH 8–8.5 after 4 days (data not shown). In contrast to the growth and glucose consumption, behaviors of the enzyme production were substantially different by the cultivation methods. Namely, in both APC and MSLC, the amount of protease produced in the medium expressed in terms of the activity reached its highest level after 2 days of cultivation, with approximately 270 and 400 U/ml, respectively. While, in SFC, protease seems to be produced in a very small quantity after 2–3 days of cultivation, and the maximum production level was in the order of 20 U/ml, which was around 1/20–1/10 those obtained for APC and MSLC. By the three cultivation methods, α-amylase was produced at a very low level in the mCD medium (Fig. 1C).

Figs. 2A–C show the growth, courses of protease production, and those of α-amylase production, respectively, for SFC, APC, and MSLC, using DPY medium. As shown in Fig. 2A, the growth in APC and MSLC using DPY medium was slightly higher than those using mCD medium. In the three cultivation methods, glucose was exhausted within 1 day from the start of the cultivation (data not shown). While the amount of protease produced by APC and MSLC reached their maxima of 1600 and 2500 U/ml after 1.5 and 2 days of cultivation, respectively, followed by a gradual decrease probably due to autolysis or proteolytic action by other kinds of proteases. The maximum protease concentrations for APC and MSLC were six-fold or higher than those obtained by the corresponding cultivation method using mCD medium (Fig. 1B). Using DPY medium, the α-amylase production was markedly increased compared to that using mCD medium as shown in Fig. 2C, and the enzyme activities observed after 1.5 days of cultivation in APC and MSLC were approximately 3-fold that of SFC.

The growth and protein secretion levels are usually varied with the culture size since it affects the oxygen uptake efficiency for the culture. The values of k_La, which indicates oxygen uptake efficiency, were determined to be 71 h^{-1} and 230 h^{-1}, for 100-ml and 2-l shaking-flasks with 25-ml and 150-ml Na_2SO_3 solutions, respectively. Although the cell growth was delayed 1 day in the culture using a 2-l flask as compared to that using a 100-ml flask, the maximum cell growth was similar for the both cultures. However, the pH was not increased to pH 8 until 5 days of cultivation using a 2-l shaking flask in contrast to that using a 100-ml shaking flask, as mentioned above, and the protease secretion level was still approximately 1/6 that obtained in the 100-ml shaking-flask. The α-amylase production level was approximately 40% or lower in the culture using a 2-l shaking flask as compared to that using a 100-ml shaking flask.

SDS–PAGE analysis of secreted proteins Figs. 3A and B show the results of the SDS–PAGE analysis for the supernatants of the broths obtained from MSLC, SFC, and APC after 1.5 days of cultivation using mCD and DPY media, respectively. The numbers of the protein bands and their intensities differed with differing cultivation methods and types of media. The more bands with much higher intensities were detected from the samples using DPY medium than those from

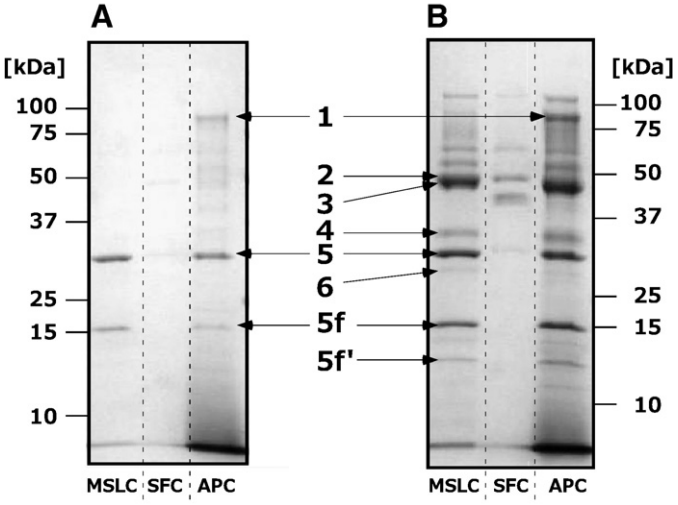

FIG. 3. Results of the SDS-PAGE analyses for the supernatants obtained from 1.5-day cultures of MSLC, SFC, and APC using mCD medium (A) and DPY medium (B). The number (1–6 and 5f/5f') shown by the arrow corresponds to the band number shown in Table 1. It should be noted that the supernatants of the broths obtained using mCD and DPY media were concentrated 5 times, using an Ultrafree-0.5 PBCC Centrifugal Filter Unit (Biomax-5 Membrane, Millipore, USA).

TABLE 1. Identification of proteins/polypeptides isolated from SDS–polyacrylamide gels.

Band no.	*N*-terminal sequence	*A. oryzae* gene ID	Protein
1	L^{19}NPEGLISAP^{28}	AO070340000111	Dipeptidyl-peptidase V (M^1-A^{18})
2	A^{22}TPADWRSQS^{31}	AO090120000196	α-Amylase (M^1-A^{21})
3	A^{246}DYQVYAWGI^{255}	AO090011000036	Neutral protease I (M^1-A^{18})
4	V^{78}TYPDSVQH^{86}	AO090011000052	Transferrin receptor and related proteins containing the protease-associated (PA) domain (M^1-A^{18})
5	G^{122}LTTQKSAPW^{131}	AO090003001036	Oryzin (alkaline protease) (M^1-A^{21})
5f, 5f'	GLTTQKSAPW	AO090003001036	Truncated oryzin deleting C-terminal peptides (M^1-A^{21})
6	A^{29}PYQNRILET^{38}	AO090003000935	FG-GAP repeat protein (putative) (M^1-A^{28})

the corresponding samples using mCD medium. However, for both mCD and DPY media, the protein profiles and their band intensities for APC and MSLC seemed to be similar, while proteins detected for SFC were few and had much lower intensities. Similar tendencies were observed throughout the entire cultivation period of 5 days (data not shown).

On the basis of the N-terminal sequence analysis of the proteins extracted from the SDS–PAGE gels for the MSLC samples obtained using both mCD and DPY media and the BLAST search, 6 proteins (see Fig. 3 (1–6 and 5f/5f′)) were identified and listed in Table 1 with their gene IDs. The protein bands obtained for the APC and SFC samples except for band 1 were predicted on the basis of the results obtained for the MSLC samples. In dipeptidyl peptidase V or alanyl dipeptidyl peptidase (band 1), α-amylase (band 2) (37), transferring receptor and related proteins containing the protease-associated domain (transferring receptor) (band 4), and putative FG-GAP repeat protein (band 6), the signal peptides were confirmed to be cleaved by analysis using Signal P 3.0 on the basis of their entire amino acid sequences. On the other hand, 245 and 121 amino acid residues were found to be deleted from the N-terminal peptide sequences of the precursors of neutral protease I and alkaline protease (oryzin, EC 3.4.21.63), respectively. These findings are in agreement with previously reported results (38–41) that the signal peptide and propeptide regions for the precursors of neutral protease and oryzin are deleted during secretion into the medium. The bands 5f and 5f′ were found to be truncated oryzins deleting the C-terminal peptide region since their N-terminal peptide sequences were identical to that of the full-length oryzin. Some other bands in addition to these 6 proteins were found to be a mixture of plural proteins. The N-terminus of the band existing between the 37-kDa and 50-kDa marker proteins of the SFC lane in Fig. 3B was found to be blocked and therefore could not be sequenced in this study. A major protein secreted in both APC and MSLC using mCD medium was oryzin (band 5 in Fig. 3A), while those obtained using DPY medium contained α-amylase (band 2 in Fig. 3B) and neutral protease (band 3) in addition to oryzin (band 5). Neutral protease was distinctly detected in the supernatants from MSLC and APC, particularly after 2 days of cultivation using DPY medium (data not shown). Dipeptidyl-peptidase V was detected only in APC with 1.5 days of cultivation, using both mCD and DPY media, as shown in Figs. 3A and B, although the band intensity was much stronger when DPY medium was used.

Analysis using DNA microarray We investigated differences in the gene transcriptional profile for the filamentous fungi cultivated by SFC, APC, and MSLC, using mCD medium that is a much simpler medium than DPY medium, to make the analysis easier. Figs. 4A and B show the scattered plots of the microarray analyses obtained for the cells cultivated for 1.5 days using mCD medium, in which the mean intensity ratios ($\log_2$ transformed) for the MSLC sample is plotted versus those for the SFC and APC samples, respectively. As shown in Figs. 4A and B, most of the points scatter around a linear line passing through an origin with a slope of 1, which indicates that the MSLC, SFC, and APC samples show a similar transcriptional level, in general. While some points in the areas painted gray in Figs. 4A and B clearly deviate from the line with a slope of 1, which tentatively satisfies the conditions, ($y-x>2$, $y>1$, $x<0.5$) and ($y-x<-2$, $y<0.5$, $x>1$), where y represents the mean intensity ratios for the MSLC sample, and x those for the SFC or APC sample. Namely, the points existing in the upper left (second quadrant) gray area in Figs. 4A and B, respectively, correspond to the genes specifically transcribed in the MSLC sample with at least 4-fold up-regulation versus the SFC or APC sample, while

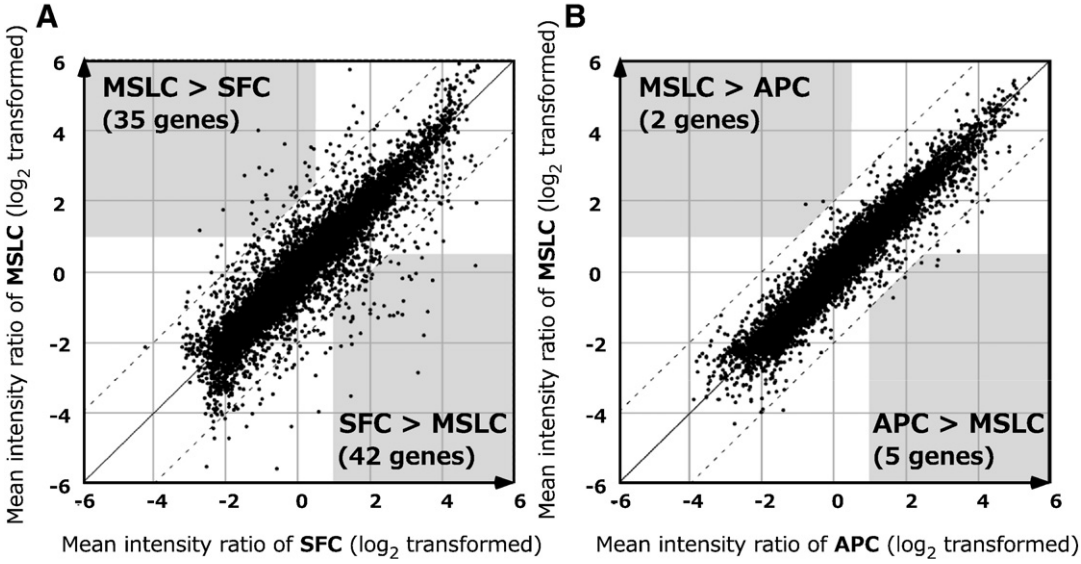

FIG. 4. Scatter plot of the mean intensity ratios ($\log_2$-transformed) for the MSLC sample versus SFC sample (A) and that of the MSLC sample versus APC sample (B). All samples used for the cDNA microarray analyses were prepared from MSLC, SFC, and APC using mCD medium. The fluorescence intensities of the samples were normalized by dividing by the average fluorescence intensities and transformed into $\log_2$. The areas painted gray indicate the regions that are encircled by the conditions, ($y-x>2$, $y>1$, $x<0.5$) and ($y-x<-2$, $y<0.5$, $x>1$), respectively.

TABLE 2. Lists of genes specifically transcribed (more than 10-fold up-regulation) in MSLC or SFC.

A. oryzae gene ID[a]	Gene product[b]	Fold change in fluorescence intensity (log_2-transformed mean intensity ratio ± SD)
MSLC-specific genes to SFC		MSLC/SFC
AO090005000538	Glycosyl hydrolase, family 71	35.8 (5.16 ± 0.21)
AO090701000865	Predicted protein	19.2 (4.26 ± 0.34)
AO090026000409	Predicted protein	16.8 (4.07 ± 0.16)
AO090701000862	Predicted protein	16.6 (4.05 ± 0.68)
AO090011000391	Predicted protein	15.4 (3.95 ± 0.35)
AO090001000235	GTP cyclohydrolase I	15.0 (3.91 ± 0.41)
AOX00000000398	Predicted protein	12.9 (3.69 ± 0.20)
AO090701000545	Predicted protein	12.6 (3.65 ± 0.46)
SFC-specific genes to MSLC		SFC/MSLC
AO090005000583	Predicted protein	70.2 (6.13 ± 1.61)
AO090003000984	Predicted protein	37.9 (5.24 ± 0.39)
AO090103000472	Predicted protein	30.3 (4.92 ± 0.39)
AO090003000661	Pyruvate decarboxylase	26.7 (4.74 ± 0.08)
AO090026000494	MFS monosaccharide transporter	25.5 (4.67 ± 0.44)
AO090003001282	Predicted protein (GPI-anchored protein)	17.0 (4.08 ± 0.47)
AO090009000634	Alcohol dehydrogenase	15.2 (3.93 ± 0.12)
AO090038000578	Indoleamine 2,3-dioxygenase family protein	14.8 (3.88 ± 0.58)
AO090023000525	RNA polymerase Rbp1 C-term repeat domain (endoglucanase)	14.5 (3.86 ± 0.47)
AO090003000519	Predicted protein	13.4 (3.75 ± 0.09)
AO090020000514	Predicted protein	13.1 (3.71 ± 0.47)
AO090003001361	Cytochrome P450 CYP2 subfamily	11.4 (3.51 ± 0.63)
AO090038000281	Predicted protein	11.1 (3.47 ± 0.36)
AO090026000168	Predicted protein	10.8 (3.44 ± 0.48)

[a] AOX00000000398 is an ID termed only in the array used in this study.
[b] Other candidate with *E*-value of lower than e^{-5} obtained by BLASTP search is shown in parenthesis.

the points in the lower right (fourth quadrant) gray area indicate the genes specifically transcribed in the SFC or APC sample versus the MSLC sample, respectively. As seen from Fig. 4, there are many more specific genes for the MSLC or SFC sample versus the SFC or MSLC sample (in the second or fourth quadrant in Fig. 4A) than those for the MSLC or APC sample versus the APC or MSLC sample (in the second or fourth quadrant in Fig. 4B). These findings indicate that the gene transcriptional patterns for the MSLC and APC samples are similar but substantially different from the SFC sample.

Table 2 lists 8 genes specifically transcribed in the MSLC sample versus the SFC sample (upper column) and 14 genes specifically transcribed in the SFC sample versus the MSLC sample (lower column), with the up-regulation fold of 10 or higher. Most of the genes listed in Table 2 were unknown or predicted proteins except for two hydrolases for the genes specifically transcribed in the MSLC sample versus the SFC sample and six enzymes or proteins for the genes specifically transcribed in the SFC sample versus the MSLC sample. The genes specifically transcribed in the SFC sample versus the MSLC sample contained some membrane-associated proteins such as MFS monosaccharide transporter, GPI-anchored protein, and cytochrome P450 CYP2 subfamily. There were no genes that specifically transcribed in the MSLC sample versus the APC sample with a 10-fold up-regulation or higher. The up-regulation fold for the oryzin gene transcribed in the MSLC sample versus the SFC sample was approximately 2.3 and much lower than those for the genes listed in Table 2.

DISCUSSION

MSLC is a cultivation method that is similar to SSC with respect to the static and surface culture mode, although it uses liquid medium. Since a liquid medium is used in MSLC, the medium component profile is nearly uniform in the culture medium as opposed to that in SSC. Therefore, cultivation behaviors of SFC and MSLC could be compared under the substantially same conditions except for the hyphae growing in the submerged state with vigorous shaking in SFC and those growing on the surface statically, facing the atmosphere in MSLC. In this study, we showed that MSLC is similar to APC and substantially different from SFC, in terms of enzyme productions particularly those of proteases and secreted protein levels although there was only a slight difference in the growth for the three cultivation methods. The SDS–PAGE results indicated that the amounts of proteins secreted in MSLC and APC were much higher than that in SFC using both mCD and DPY media, which is very similar to the findings observed for SSC using wheat bran as the solid substrate (23), in which *A. oryzae* RIB40 secreted 4.0- to 6.4-fold more protein per milligram of mycelium by SSC than that secreted by SLC. The secretion levels of oryzin (alkaline protease) in MSLC and APC were much more than those in SFC. te Biesebeke et al. (20) showed that *alpA* (alkaline protease gene) was highly expressed in a wheat-based agar medium, which is in agreement with the results obtained in this study. The amount of the secreted enzyme was much higher using DPY medium as compared to mCD medium particularly for APC and MSLC. When 1.5% glucose, which is a carbon source for mCD medium, was used in place of 1.5% dextrin in DPY medium, the highest protease concentration was approximately 2200 U/ml in MSLC, which was similar to that using dextrin (Fig. 2B). Thus, the reason for the higher protease production using DPY medium, as compared to that using mCD medium, could be tentatively explained by the higher content of the nitrogen sources.

Since the growths for the three cultivation methods were similar, the extremely lower protease production level observed in SFC could not be explained by the differences in the growth. Furthermore, the fact that sufficient oxygen might not be available in SFC as compared to APC and MSLC would not be responsible for the lower enzyme production in SFC, because the secretion levels of proteases and α-amylase by cultivation in 2-l flasks containing 150-ml medium with k_La of 230 h^{-1} were much lower than those in 100-ml flasks with 25-ml medium with k_La of 71 h^{-1}, although the maximum amounts of cells were similar for all the cultures, consistent with the findings reported previously (31).

Although MSLC and APC showed a similar tendency in the secretion of most proteins/enzymes studied here, alanyl dipeptidyl peptidase (band 1 in Fig. 3) was not clearly detected in MSLC in contrast to the result for APC for an unknown reason. The glucoamylase activity in the culture supernatant was weak and in the order of 0.05 U/ml in the three cultivation methods, even using DPY medium. These results could probably be ascribed either to the fact that water activity was not low enough, as in the SSC using solid substrates such as wheat bran, considering the results obtained by Ishida et al. (15), or to the fact that maltose was not added at a high concentration, according to te Biesebeke et al. (20), although they measured only its transcriptional level. Furthermore, in this study, we used a different *Aspergillus* strain from those used by Ishida et al. (15) and te Biesebeke et al. (20), which might affect the glucoamylase expression.

We also elucidated differences in the gene transcriptional level obtained for the different cultivation methods using the DNA microarray that covers almost all protein-coding genes for *A. oryzae*. Although some researchers have investigated so far the gene transcriptional level of some particular enzyme secreted by filamentous fungi cultivated by different conditions (15, 20, 42, 43), there have been no reports that are focused to the changes in the transcriptional levels of the whole gene by the kinds of the cultivation methods, as far as the authors know. The microarray analyses indicated that many genes were specifically transcribed either in the MSLC or SFC sample versus the SFC or MSLC sample, while genes that were specifically transcribed either in the MSLC or APC sample

versus the APC or MSLC sample were much fewer. However, most of the genes that were specifically transcribed in the MSLC or SFC sample versus SFC or MSLC sample were those of unknown or predicted proteins except for some particular genes for hydrolases and membrane-associated proteins (Table 2). It should be noted that the gene transcriptional levels of oryzin for the MSLC and APC samples versus the SFC sample were appreciably lower than those of the genes listed in Table 2. Namely, the cultivation methods would not simply affect the transcriptional levels of secreted enzymes such as oryzin for this study but substantially change those of a number of the other different genes. There might be some particular proteins among the unknown or predicted proteins that are the clue to make clear the reason for efficient secretion of enzymes in MSLC, APC, or SSC, and detailed and comprehensive study are requisite.

In this study, we used mCD medium for cultivation of *A. oryzae* IAM2706 for the first step to carry out DNA microarray analyses, since mCD medium is a very simple medium containing glucose as a carbon source, yeast extract as a nitrogen source, and some salts. Some investigators reported that the gene transcriptional level or the production level of some particular enzymes is varied by the kinds of medium (20, 42–44), in a way similar to the enzymes found in this study such as oryzin, neutral protease, and alanyl dipeptidase. Moreover, in our previous study, we identified 22 genes that are preferentially expressed in the MSLC sample prepared from *A. oryzae* such as Ser/Thr kinase and phosphatase genes, by the suppression subtractive hybridization method (32). However, transcription of these genes identified was not up-regulated in this study probably because of different strains and culture medium used. Therefore, differences in the gene transcription profile by MSLC, SFC, and APC using different kinds of medium would provide further useful information to understand enzyme/protein secretion behaviors of filamentous fungi, which will remain as a future task.

In conclusion, the findings obtained in this study indicate that the *A. oryzae* cultivated by MSLC possess characteristics common to those of APC in terms of the protein/enzyme secretion and gene transcription, and probably to those of SSC as well. MSLC would be a very simple method for conducting proteomic, as well as genomic, analyses, with no possibility of contamination by RNA/proteins derived from water-insoluble agricultural substrates. Therefore, MSLC would be not only an efficient production system using filamentous fungi but also a powerful tool for revealing the molecular mechanism of higher enzyme productivity in SSC.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiosc.2009.09.004.

References

1. **H. W. Doelle, D. A. Mitchell, C. E. Rolz (Eds.),** Solid substrate cultivation, Elsevier Applied Science, London and New York, 1992, pp. 1–16 (1992).
2. **Abe, K., Gomi, K., Hasegawa, F., and Machida, M.:** Impact of *Aspergillus oryzae* genomics on industrial production of metabolites, Mycopathologia, **162**, 143–153 (2006).
3. **Raimbault, M.:** General and microbiological aspects of solid substrate fermentation, Electronic J. Biotechnol., **1**, 174–188 (1998).
4. **Sakaguchi, K., Okazaki, H., and Takeuchi, M.:** A note on the comparison of koji and submerged culture, Nippon Nougeikagaku Kaishi, **29**, 349–353 (1955).
5. **Grajek, W.:** Comparative studies on the production of cellulases by thermophilic fungi in submerged and solid-state fermentation, Appl. Microbiol. Biotechnol., **26**, 126–129 (1987).
6. **Sudo, S., Ishikawa, T., Sato, K., and Oba, T.:** Comparison of acid-stable alpha-amylase production by *Aspergillus kawachii* in solid-state and submerged cultures, J. Ferment. Bioeng., **77**, 483–489 (1994).
7. **Tsuchiya, K., Nagashima, T., Yamamoto, Y., Gomi, K., Kitamoto, K., Kumagai, C., and Tamura, G.:** High level secretion of calf chymosin using a glucoamylase–prochymosin fusion gene in *Aspergillus oryzae*, Biosci. Biotechnol. Biochem., **58**, 895–899 (1994).
8. **Acuna-Arguelles, M. E., Gutierrez-Rojas, M., Viniegra-Gonzalez, G., and Favela-Torres, E.:** Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid-state fermentation, Appl. Microbiol. Biotechnol., **43**, 808–814 (1995).
9. **Hata, Y., Ishida, H., Kojima, Y., Ichikawa, E., Kawato, A., Suginami, K., and Imayasu, S.:** Comparison of two glucoamylases produced by *Aspergillus oryzae* in solid-state culture (Koji) and in submerged culture, J. Ferment. Bioeng., **84**, 532–537 (1997).
10. **Minetoki, T., Kumagai, C., Gomi, K., Kitamoto, K., and Takahashi, K.:** Improvement of promoter activity by the introduction of multiple copies of the conserved region III sequence, involved in the efficient expression of *Aspergillus oryzae* amylase-encoding genes, Appl. Microbiol. Biotechnol., **50**, 459–467 (1998).
11. **Pandey, A., Selvakumar, P., Soccol, C. R., and Nignam, P.:** Solid state fermentation for the production of industrial enzymes, Curr. Sci., **77**, 149–162 (1999).
12. **Iwashita, K.:** Recent studies of protein secretion by filamentous fungi, J. Biosci. Bioeng., **94**, 530–535 (2002).
13. **Holker, U., Hofer, M., and Lenz, J.:** Biotechnological advantages of laboratory-scale solid-state fermentation with fungi, Appl. Microbiol. Biotechnol., **64**, 175–186 (2004).
14. **Obata, H., Ishida, H., Hata, Y., Kawato, A., Abe, Y., Akao, T., Akita, O., and Ichishima, E.:** Cloning of a novel tyrosinase-encoding gene (melB) from *Aspergillus oryzae* and its overexpression in solid-state culture (rice koji), J. Biosci. Bioeng., **97**, 400–405 (2004).
15. **Ishida, H., Hata, Y., Ichikawa, E., Kawato, A., Suginami, K., and Imayasu, S.:** Regulation of the glucoamylase-encoding gene (glaB), expressed in solid-state culture (koji) of *Aspergillus oryzae*, J. Ferment. Bioeng., **86**, 301–307 (1998).
16. **Akao, T., Gomi, K., Goto, K., Okazaki, N., and Akita, O.:** Subtractive cloning of cDNA from *Aspergillus oryzae* differentially regulated between solid-state culture and liquid (submerged) culture, Curr. Genet., **41**, 275–281 (2002).
17. **Kitano, H., Kataoka, K., Furukawa, K., and Hara, S.:** Specific expression and temperature-dependent expression of the acid protease-encoding gene (pepA) in *Aspergillus oryzae* in solid-state culture (rice-koji), J. Biosci. Bioeng., **93**, 563–567 (2002).
18. **te Biesebeke, R., Ruijter, G., Rahardjo, Y. S., Hoogschagen, M. J., Heerikhuisen, M., Levin, A., van Driel, K. G., Schutyser, M. A., Dijksterhuis, J., Zhu, Y., Weber, F. J., de Vos, W. M., van den Hondel, K. A., Rinzema, A., and Punt, P. J.:** *Aspergillus oryzae* in solid-state and submerged fermentations. Progress report on a multi-disciplinary project, FEMS Yeast Res., **2**, 245–248 (2002).
19. **te Biesebeke, R., Levin, A., Sagt, C., Bartels, J., Goosen, T., Ram, A., van den Hondel, C., and Punt, P.:** Identification of growth phenotype-related genes in *Aspergillus oryzae* by heterologous macroarray and suppression subtractive hybridization, Mol. Genet. Genomics, **273**, 33–42 (2005).
20. **te Biesebeke, R., van Biezen, N., de Vos, W. M., van den Hondel, C. A., and Punt, P. J.:** Different control mechanisms regulate glucoamylase and protease gene transcription in *Aspergillus oryzae* in solid-state and submerged fermentation, Appl. Microbiol. Biotechnol., **67**, 75–82 (2005).
21. **te Biesebeke, R., Record, E., van Biezen, N., Heerikhuisen, M., Franken, A., Punt, P. J., and van den Hondel, C. A.:** Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates, Appl. Microbiol. Biotechnol., **69**, 44–50 (2005).
22. **Kobayashi, A., Sano, M., Oda, K., Hisada, H., Hata, Y., and Ohashi, S.:** The glucoamylase-encoding gene (glaB) is expressed in solid-state culture with a low water content, Biosci. Biotechnol. Biochem., **71**, 1797–1799 (2007).
23. **Oda, K., Kakizono, D., Yamada, O., Iefuji, H., Akita, O., and Iwashita, K.:** Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions, Appl. Environ. Microbiol., **72**, 3448–3457 (2006).
24. **Hata, Y., Ishida, H., Ichikawa, E., Kawato, A., Suginami, K., and Imayasu, S.:** Nucleotide sequence of an alternative glucoamylase-encoding gene (glaB) expressed in solid-state culture of *Aspergillus oryzae*, Gene, **207**, 127–134 (1998).
25. **Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., and other 54 authors:** Genome sequencing and analysis of *Aspergillus oryzae*, Nature, **438**, 1157–1161 (2005).
26. **Yasuhara, A., Ogawa, A., Tanaka, T., Sakiyama, T., and Nakanishi, K.:** Production of neutral protease from *Aspergillus oryzae* by a novel cultivation method on a microporous membrane, Biotechnol. Tech., **8**, 249–254 (1994).
27. **Nakanishi, K.:** Membrane surface liquid culture, microorganisms, fungi, in: M. C. Flickinger, S. W. Drew (Eds.), The encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation, John Wiley and Sons, New York, 1999, pp. 1706–1713 (1999).

28. **Ogawa, A., Wakisaka, Y., Tanaka, T., Sakiyama, T., and Nakanishi, K.:** Production of kojic acid by membrane-surface liquid culture of *Aspergillus oryzae* NRRL 484, J. Ferment. Bioeng., **80**, 41–45 (1995).
29. **Ogawa, A., Yasuhara, A., Tanaka, T., Sakiyama, T., and Nakanishi, K.:** Production of neutral protease by membrane-surface liquid culture of *Aspergillus oryzae* IAM 2704, J. Ferment. Bioeng., **80**, 35–40 (1995).
30. **Wakisaka, Y., Segawa, T., Imamura, K., Sakiyama, T., and Nakanishi, K.:** Development of a cylindrical apparatus for membrane-surface liquid culture and production of kojic acid using *Aspergillus oryzae* NRRL484, J. Ferment. Bioeng., **85**, 488–494 (1998).
31. **Morita, M., Shimamura, H., Ishida, N., Imamura, K., Sakiyama, T., and Nakanishi, K.:** Characteristics of alpha-glucosidase production from recombinant *Aspergillus oryzae* by membrane-surface liquid culture in comparison with various cultivation methods, J. Biosci. Bioeng., **98**, 200–206 (2004).
32. **Feng, B., Morita, M., Imanaka, H., Imamura, K., and Nakanishi, K.:** Identification of genes from *Aspergillus oryzae* that are preferentially expressed in membrane-surface liquid culture, J. Biosci. Bioeng., **102**, 470–473 (2006).
33. **Shimokawa, T., Hirai, M., Shoda, M., and Sugano, Y.:** Efficient dye decolorization and production of dye decolorizing enzymes by the basidiomycete *Thanatephorus cucumeris* Dec 1 in a liquid and solid hybrid culture, J. Biosci. Bioeng., **193**, 481–487 (2008).
34. **Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.:** Protein measurement with the folin phenol reagent, J. Biol. Chem., **193**, 265–275 (1951).
35. **Hames, B. D.:** One-dimensional polyacryl amide gel electrophoresis, in: B. D. Hames, D. Rickwood (Eds.), Gel Electrophoresis of Proteins, Oxford University Press, Oxford, 1990, pp. 1–147 (1990).
36. **Nielsen, J., Villadsen, J., and Liden, G.:** Bioreaction Engineering Principles, Second edition, Kluwer Academic/Plenum Publishers, New York, 2003, pp. 423–475 (2003).
37. **Wirsel, S., Lachmund, A., Wildhardt, G., and Ruttkowski, E.:** Three alpha-amylase genes of *Aspergillus oryzae* exhibit identical intron–exon organization, Mol. Microbiol., **3**, 3–14 (1989).
38. **Tatsumi, H., Ogawa, Y., Murakami, S., Ishida, Y., Murakami, K., Masaki, A., Kawabe, H., Arimura, H., Nakano, E., and Motai, H.:** A full length cDNA clone for the alkaline protease from *Aspergillus oryzae*: structural analysis and expression in *Saccharomyces cerevisiae*, Mol. Gen. Genet., **219**, 33–38 (1989).
39. **Cheevadhanarak, S., Renno, D. V., Saunders, G., and Holt, G.:** Cloning and selective overexpression of an alkaline protease-encoding gene from *Aspergillus oryzae*, Gene, **108**, 151–155 (1991).
40. **Murakami, K., Ishida, Y., Masaki, A., Tatsumi, H., Murakami, S., Nakano, E., Motai, H., Kawabe, H., and Arimura, H.:** Isolation and characterization of the alkaline protease gene of *Aspergillus oryzae*, Agric. Biol. Chem., **55**, 2807–2811 (1991).
41. **Doumas, A., Crameri, R., Léchenne, B., and Monod, M.:** Cloning of the gene encoding neutral protease I of the koji mold *Aspergillus oryzae* and its expression in *Pichia pastoris*, J. Food Mycol., **2**, 271–279 (1999).
42. **Maeda, H., Sano, M., Maruyama, Y., Tanno, T., Akao, T., Totsuka, Y., Endo, M., Sakurada, R., Yamagata, Y., Machida, M., Akita, O., Hasegawa, F., Abe, K., Gomi, K., Nakajima, T., and Iguchi, Y.:** Transcriptional analysis of genes for energy catabolism and hydrolytic enzymes in the filamentous fungus *Aspergillus oryzae* using cDNA microarrays and expressed sequence tags, Appl. Microbiol. Biotechnol., **65**, 74–83 (2004).
43. **Tamano, K., Sano, M., Yamane, N., Terabayashi, Y., Toda, T., Sunagawa, M., Koike, H., Hatamoto, O., Umitsuki, G., Takahashi, T., Koyama, Y., Asai, R., Abe, K., and Machida, M.:** Transcriptional regulation of genes on the non-syntenic blocks of *Aspergillus oryzae* and its functional relationship to solid-state cultivation, Fungal. Genet. Biol., **45**, 139–151 (2008).
44. **Ohtaki, S., Maeda, H., Takahashi, T., Yamagata, Y., Hasegawa, F., Gomi, K., Nakajima, T., and Abe, K.:** Novel hydrophobic surface binding protein, HsbA, produced by *Aspergillus oryzae*, Appl. Environ. Microbiol., **72**, 2407–2413 (2006).