

*Effects of the Cultivation Method on the Characteristics or Gene Expression Profiles of *Aspergillus oryzae* Using mCD or DPY Media*

Hiroyuki IMANAKA, Soukichi TANAKA, Bin FENG[#], Koreyoshi IMAMURA, and Kazuhiro NAKANISHI*
Division of Chemistry and Biochemistry,
Graduate School of Natural Science and Technology,
Okayama University, 3-1-1 Tsushima-Naka, Kita-Ku,
Okayama 700-8530, Japan

(Received December 22, 2009)

We used modified Czapek-Dox (mCD) or dextrin-peptone-yeast extract (DPY) media to cultivate 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 identify the differences in cultivation behaviors and gene transcriptional profiles. The fungi cultivated by APC or MSLC secreted a greater number of different proteins/enzymes in larger quantities compared with fungi cultivated by SFC, particularly when DPY medium was used. In particular, the amounts of protease secreted by fungi cultivated via MSLC or APC were much greater compared with SFC. When mCD medium was used, α -amylase activity was barely detectable in all cultures while the activity was detected in MSLC and APC in a quantity that was several times higher than that in SFC using DPY medium. SDS-PAGE analysis and N-terminal amino acid sequences confirmed 6 proteins in the culture supernatants when DPY medium was used. Among these proteins oryzin (an alkaline protease) and α -amylase were detected at much higher levels in APC and MSLC compared with SFC, which was consistent with the measured activity of the secreted enzymes. However, when mCD medium was used, only oryzin was detected in significant amounts in MSLC and APC. Microarray analyses of the fungi cultivated by SFC, APC or MSLC using either mCD or DPY media indicated that the gene transcriptional profile of the MSLC sample was similar to that of the APC sample but different from that of the SFC sample. When mCD medium was used, most of the genes that were up-regulated 10-folds or greater in the MSLC sample relative to the SFC sample were unknown or predicted proteins. Transcription of the oryzin gene was only slightly up-regulated in the MSLC sample while transcription of the α -amylase gene was slightly down-regulated. On the other hand, when DPY medium was used, many known genes including the oryzin gene were up-regulated in the MSLC sample versus the SFC sample.

Running title: ENZYME SECRETION AND GENE EXPRESSION OF *A. ORYZAE*

*E-mail address: kazuhiro@cc.okayama-u.ac.jp

[#]Present Address; Dept. of Biotechnology, College of Basic Medical Science, Dalien Medical University, No. 9 West Section, Lyshun South, Dalien. 116044, China

1 INTRODUCTION

Filamentous fungi are widely utilized in the production of enzymes/heterologous proteins, organic

acids, and antibiotics, and they are also used in the preparation of traditional fermented foods and beverages [1, 2]. Most filamentous fungi are absolute aerobe and, therefore, require oxygen for growth. In nature,

filamentous fungi grow on solid substrates and are able to thrive under conditions of relatively low humidity by 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), which uses water-soluble nutrients. Advantages of SLC include ease of medium preparation, control of pH/temperature, and recovery of products. However, the amount of enzymes/proteins secreted by filamentous fungi, including heterologous proteins and low-molecular weight metabolites of filamentous fungi, is much larger when fungi are cultivated by solid-substrate culture (SSC), which uses water-insoluble agricultural products, such as steamed rice, ground soybeans, and wheat bran, compared with SLC using a liquid medium [1-3]. In addition, enzymes that are barely detected, when SLC is used, can sometimes be produced by SSC [1, 4-14]. Thus, based on these results, a number of researchers are currently investigating how differences in the cultivation methods might alter the transcription of various genes that control important phenotypic characteristics, such as growth, development, and mycotoxin/enzyme production [12]. Moreover, many investigators have recently attempted to identify the mechanism responsible for the increased enzyme production in SSC by investigating gene transcription [9,10,14-21] and protein expression [22]. Genes that are uniquely expressed by SSC or SLC have been screened under different cultivation conditions and discussed in the context of their function in the fungi [19,20]. However, gene expression studies have focused mainly on several particular genes, such as those encoding glucoamylase B, acid protease, oryza, and neutral protease [15,17,18,20,23]. Recently, the sequence of the entire *A. oryzae* genome [24] has been revealed, which might help researchers understand the factors that affect the higher enzyme productivity of SSC.

We previously reported that filamentous hyphae cultivated by membrane-surface liquid culture (MSLC) [25,26] shows cultivation characteristics, such as growth and enzyme productivity that are similar to those of fungi cultivated by agar-plate culture (APC). Thus, MSLC-cultivated fungi can serve as a model for SSC [26-30]. In MSLC, filamentous fungi are grown statically on the surface of a microporous membrane. The spore-forming side of the fungus faces the air with forming spores, similar to SSC while the other side is in contact with the liquid medium, as shown schematically in Figs. 1(a) and (b) [25,26,31]. We showed that neutral

protease, α -glucosidase, and kojic acid are both produced at much higher levels by MSLC-cultivated fungi than fungi grown in shaking-flask culture (SFC). In addition, we identified MSLC-specific genes [32]. Shimokawa *et al.* [33] recently showed that protein secretion, dye decoloring peroxidase activity, and aryl alcohol oxidase activity of a basidiomycete, *Thanatephorus cucumeris*, are markedly enhanced by cultivation in an air-membrane surface bioreactor, which is similar to MSLC in principle. Hence, it is expected that MSLC might be a useful tool not only for an efficient production system [26,31] and also for elucidating the mechanism by which SSC results in increased enzyme production.

In this study, we cultivated *A. oryzae* IAM 2706 by SFC, APC or MSLC, using identical cultivation conditions for each method. We used two different media, *i.e.*, mCD or DPY, to systematically investigate growth and secretion of enzyme particularly proteases and α -amylase, and we used gene transcriptional profiles by DNA microarray analysis to identify the effects of different cultivation methods on *A. oryzae*.

2 MATERIALS AND METHODS

2.1 Cultivation Methods and Conditions

A. oryzae IAM2706 (hereafter abbreviated to *A. oryzae*) was obtained from the Institute of Molecular and Cellular Biosciences, The University of Tokyo (Tokyo). In the present study, 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% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄•7H₂O, 0.002% FeSO₄•7H₂O, and 2% agar, pH 6.0) to obtain spores. Then the main cultures (SFC, APC, and MSLC) were started, using either modified Czapek-Dox medium (mCD medium; 1.5% glucose, 0.4% yeast extract, 0.1% K₂HPO₄, 0.05% MgSO₄•7H₂O, 0.05% NaCl, and 0.001% FeSO₄•7H₂O, pH 7.0) or DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, and 0.05% MgSO₄•7H₂O, pH 7.0) with or without agar.

Typically, SFC was started by inoculating 25 ml of mCD or DPY medium in a 100-ml Sakaguchi flask with 200 μ l of spore suspension (1×10^7 spores/ml). The flasks were placed on a reciprocal shaker (120 strokes/min), and the cultures were grown for 5 days. To investigate the effects of flask volume on the cultivation characteristics, the cultivation was also performed in 2-l

shaking flasks by inoculating 150 or 500 ml of DPY medium with the spore suspension at the same concentration as was used for the cultures in 100-ml flasks. APC was started by spreading 200 μ l of the spore suspension, which contained the same number of spores as used for SFC, uniformly on 25 ml of mCD or DPY agar plate medium (2% agar) in a plastic dish (94 mm x 21 mm) followed by static incubation in an incubation box (ESPEC incubator CHS-110; Tabai ESPEC, Osaka, Japan). The incubator temperature and relative humidity were maintained at 30°C and \geq 98%, respectively.

MSLC was conducted by the same method as reported previously [30-32] using plastic dishes (94 mm x 21 mm) with Biodyne A (PALL Co., NY) as a porous membrane as shown in Figs.1(a) and (b). Namely, the cultivation was started by uniformly spreading 200 μ l of the spore suspension, which contained the same number of spores as used for SFC and APC, onto the membrane surface. The dishes were then incubated under conditions identical to those used for APC. Hyphae growth on the membrane surface was uniform with no leakage into the liquid medium contained in the bottom dish.

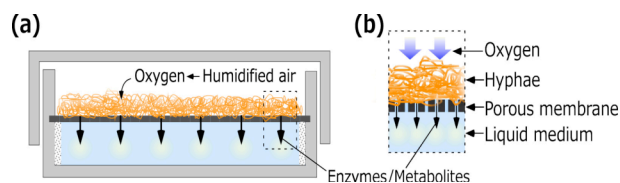


Fig. 1 Conceptual diagram for an MSLC apparatus (a) and an enlarged view (b). In actual practice, a porous membrane is set on the surface of a liquid medium and held in place by appropriate supports. MSLC using a plastic dish could be carried out just in a manner similar to an agar-plate culture.

For each of the cultivation method examined, *i.e.*, SFC, APC, and MSLC, we usually cultivated several cultures simultaneously and one of the cultures was withdrawn for analyses at the appropriate times during the course of cultivation. The glucose concentration was measured by the mutarotase-glucose oxidase/oxidase method using the Glucose CII-test Wako (Wako Pure Chemical Industries, Ltd., Osaka). Protease activity was measured using the same method as described previously [26]. One unit of the protease activity was defined as the amount of enzyme that catalyzes the solubilization of 1-mg protein at pH 7.0 and 30°C in one minute. Protein was 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 kit and an

α -glucosidase/glucoamylase assay kit (Kikkoman Co., Chiba, Japan), respectively. One unit of the α -amylase activity was defined as the amount of enzyme that releases 1 μ mol of 2-chloro-4-nitrophenol from 2-chloro-4-nitrophenyl 6⁵-azido-6⁵-deoxy- β -maltopentaoside at 37°C in one minute. One unit of α -glucosidase or glucoamylase activity was defined as the amount of enzyme that releases 1 μ mol of 4-nitrophenol, in the form of 4-nitrophenyl α -D-glucopyranoside or 4-nitrophenyl O- α -D-glucopyranosyl (1 \rightarrow 4)- β -D-glucopyranoside at 37°C in one minute, respectively. Dry cell weight was measured by the same method as described elsewhere [25].

The $k_L a$, an indicator of oxygen-uptake efficiency of the culture was measured indirectly using the sodium sulfite method. The reaction was performed in a 100-ml shaking flask containing 25-ml 0.2 N Na₂SO₃ or in a 2-l shaking flasks containing 150-ml 0.2 N Na₂SO₃, as described elsewhere [35]. The flasks were shaken at 30°C on a reciprocal shaker (120 strokes/min).

2.2 SDS-PAGE and N-Terminal Amino Acid Sequences

SDS-PAGE was performed using a 12.5% polyacrylamide gel and Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA) as the molecular weight marker, as described elsewhere [36]. Protein bands were stained with CBB Stain One (Nacalai Tesque, Inc., Kyoto, Japan). For determination 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, Japan). After sufficient washing with distilled water, the membrane was subjected to a protein sequencer (Applied Biosystems, Model 491, Foster, CA).

2.3 Purification of mRNA and cDNA-Microarray Analysis

A DNA microarray (Fermlab Array *Aspergillus oryzae* 12k) that includes nearly the entire genome of *A. oryzae* (12,168 probes) was purchased from Fermlab Inc. (Tokyo, <http://www.fermlab.com/motechinfo.html>). Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) from *A. oryzae* mycelia, which had been collected from 10 batches of SFC, APC, and MSLC, in either mCD or DPY media after 1.5 days of cultivation. Poly(A) mRNAs were purified using a FastTrack 2.0 kit (Invitrogen) as reported elsewhere [32,35,37]. The

quality of the RNA was confirmed by agarose gel electrophoresis. Then, Cy3- and Cy5-labeled cDNAs were prepared using 1 μ g of purified mRNA and a Cyscribe cDNA Post Labeling Kit (GE Healthcare, Piscataway, NJ). Approximately 60 pmol of the purified cDNA probes were dissolved in a hybridization solution (1% salmon testes (Sigma-Aldrich, St. Louis, MO)), 35% formamide, 0.3% SDS, and 3.4 x 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 washed at room temperature with 2 x SSC and 0.03% SDS for 15 min, 0.2 x SSC for 5 min, rinsed with MilliQ water, and finally dried by centrifugation. Then, the spots were detected using an Affymetrix 428TM Array Scanner (Affymetrix, Santa Clara, CA), followed by analysis 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 obtained using mCD medium and the average value for the mean intensity ratio obtained were used for the analyses while only one batch experiment was carried out for the sample using DPY medium. During the data processing, the data were filtered using the Microsoft Excel 2007.

2.4 Gene Sequence Analysis

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). Gene function predictions were carried out simultaneously based on 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 the DNA Data Bank of Japan (DDBJ), MEROPS, and DOGAN (<http://www.ddbj.nig.ac.jp/Welcome-j.html>, <http://merops.sanger.ac.uk/>, <http://www.bio.nite.go.jp/dogan/Top>). The signal peptide sequence was predicted using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>).

3 RESULTS

3.1 Cultivation Characteristics

Figs. 2(a)–(c) show the growth, courses of protease production, and α -amylase production, respectively, for SFC, APC, and MSLC using mCD medium. Fig. 2(a) shows that there was only a slight difference in growth among the three cultivation methods using mCD medium and the glucose consumption rates were comparable (data not shown). For both APC and MSLC, the

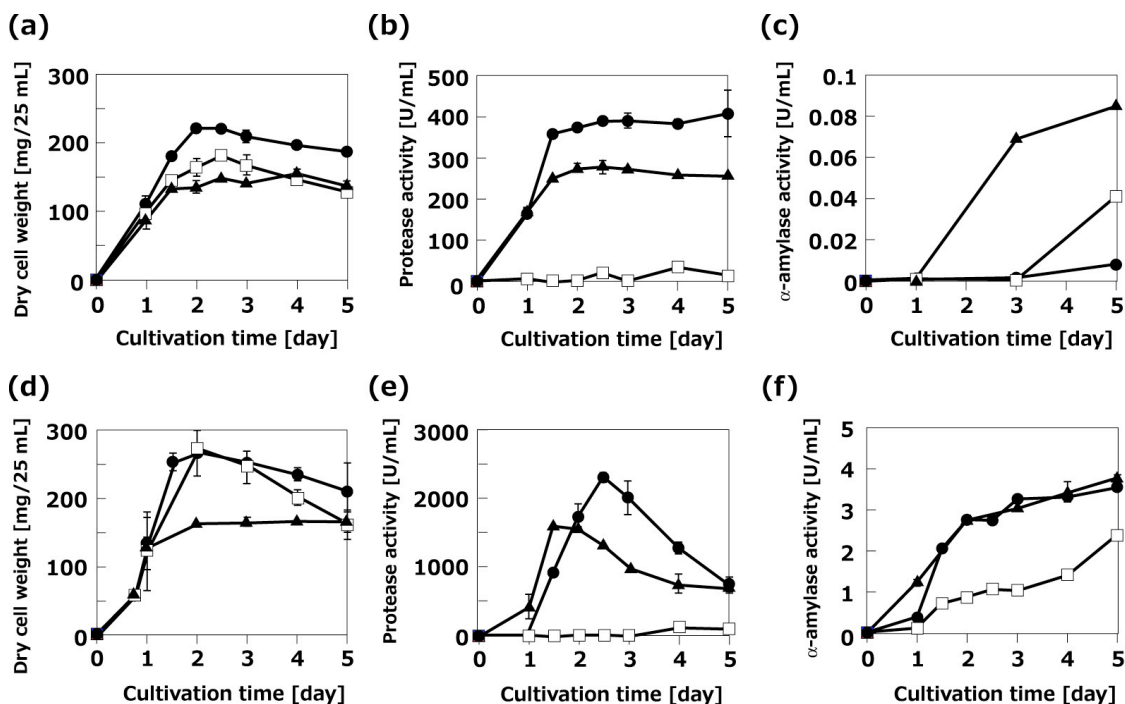


Fig. 2 Courses of cultivation by SFC (open squares), APC (closed triangles), and MSLC (closed circles) using mCD (a – c) or DPY medium (d – f). Growth (a, d), protease activity (b, e), and α -amylase activity (c, g).

concentration of protease that had accumulated in the medium was maximal after 2 days of cultivation at approximately 270 and 400 U/ml, respectively as shown in Fig. 2(b). By contrast for SFC the onset of protease production occurred after 2-3 days of cultivation, and the maximal concentration was only 20 U/ml, which was less than 1/10 the concentration produced using either APC or MSLC. α -Amylase production was very low for all three cultivation methods (Fig. 2(c)). The pH changes during SFC and MSLC cultivation were similar in tendency for both SFC and MSLC (data not shown). Namely, for SFC and MSLC, pH was initially decreased to 6.8 and 6.5 after 1 and 1.5 days of cultivation, respectively, followed by cell growth with a concomitant gradual increase to pH 8-8.5 after 4 days.

Figs. 2(d) – (f) show the growth and courses of protease and α -amylase production, respectively, for SFC, APC, and MSLC using DPY medium. The growth in APC and MSLC using DPY medium was slightly higher than growth in mCD medium, as shown in Figs. 2(a) and (d). Glucose was exhausted during the first day after the start of cultivation for each of the three cultivation methods (data not shown). As shown in Fig. 2(e), the level of protease production for SFC was very low using DPY medium, which is similar to the result obtained using mCD medium described above. The amount of protease produced by APC and MSLC reached their maxima of 1,600 U/ml and 2,500 U/ml after 1.5 and 2 days of cultivation, respectively. Subsequently, the amount of protease declined gradually probably due to autolysis or to proteolysis by other kinds of proteases. Thus, the maximal protease concentrations obtained with APC or MSLC in DPY was more than 6-fold greater than those obtained by the corresponding cultivation methods using mCD medium (Fig. 2(b)). Use of DPY medium markedly increased α -amylase production compared with mCD medium, as shown in Figs. 2(c) and (g). The amounts obtained by use of APC or MSLC after 1.5 days of cultivation were approximately 3-fold greater than the amount obtained by SFC.

Generally, both growth and protein secretion levels of filamentous fungi depend on culture volume, which affects the oxygen uptake efficiency. The k_{La} values were 71 and 230 h^{-1} , for the 100-ml flask containing 25 ml of solution and the 2-l flask containing 150 ml, respectively. These results indicate that the 2-l flask containing 150 ml of medium had a much higher oxygen uptake efficiency than the 100-ml flask containing 25 ml of medium. However, during cultivation, cell growth

delayed 1 day using the 2-l flask containing 150 ml medium ($k_{La} = 230 \text{ h}^{-1}$), compared with the onset of cell growth in the 100-ml flask containing 25 ml of medium ($k_{La} = 71 \text{ h}^{-1}$) (data not shown). During cultivation in the 100-ml flask, the pH of the medium initially decreased to approximately 6.6 but gradually increased to 8 with concomitant cell growth. Cell growth was maximal after 3 days of cultivation, followed by rapid increase in the protease secretion. On the other hand, in the 2-l flask, the pH was still less than pH 8 after 5-days of cultivation. Moreover, the amount of protease secreted in the 2-l flask was approximately 1/6 that obtained in the 100-ml flask and the α -amylase production in the 2-l flask was $\leq 40\%$ that of the 100-ml flask.

3.2 SDS-PAGE Analysis

Figs. 3(a) and (b) show the results of the SDS-PAGE analysis of the culture supernatants of the broths obtained from MSLC, SFC, and APC after 1.5 days of cultivation using mCD or DPY medium, respectively. Both the number and the intensity of the protein bands were highly dependent on both the type of medium (*i.e.*, mCD or DPY medium) and the cultivation methods. More numerous bands and bands with much higher intensities were detected in the samples that were grown in DPY medium compared with corresponding samples cultivated in mCD medium. Regarding the

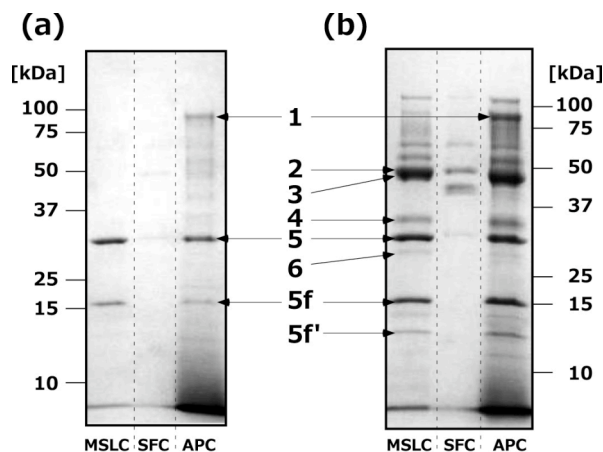


Fig.3 Results of SDS-PAGE analyses of the supernatants obtained from 1.5-day cultures of MSLC, SFC, and APC using mCD medium (a) or DPY medium (b). The number (1-6 and 5f/5f') shown by the arrow corresponds to the band number shown in Table 1. It should be noted that the supernatants of the mCD and DPY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

effects of the cultivation methods, the protein profiles and band intensities for APC and MSLC were similar, while fewer proteins with lower band intensities were detected for SFC regardless of which medium was used. Similar tendencies were observed throughout the entire cultivation period of 5 days (data not shown).

Table 1 N-Terminal amino acid sequence of proteins/polypeptides isolated by SDS-PAGE.

Band No.	N-terminal sequence	<i>A. oryzae</i> 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 ⁸	AO090011000052	Transferrin receptor and related proteins containing 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 -)
6	A ²⁹ PYQNRILET ³⁸	AO090003000935	FG-GAP repeat protein (putative) (M ¹ -A ²⁸)

Based on the N-terminal sequence analysis of the proteins extracted from the SDS-PAGE gels for the MSLC sample obtained using mCD or DPY medium and on the BLAST search, six proteins (see Fig. 3 (bands 1–6 and 5f/5f')) were identified. The six proteins are listed in Table 1 with their Gene IDs. For dipeptidyl peptidase V or alanyl dipeptidyl peptidase (band 1), α -amylase (band 2) [37,38], transferring receptor and related proteins that contain a protease-associated domain (band 4), and a putative FG-GAP repeat protein (band 6), the signal peptides were confirmed to be cleaved by analysis using Signal P 3.0 and the complete amino acid sequence confirmed cleavage of the signal peptides. 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 oryzin, respectively. These results are consistent with previously reported results [37-42]. The signal peptide and propeptide regions of the precursors of neutral protease and oryzin are deleted during secretion into the medium. Bands 5f and 5f' were found to be truncated oryzins with deletion of the C-terminal peptide region as the their N-terminal peptide sequences were identical to that of the full-length oryzin. Some other bands besides the 6 proteins mentioned above were a mixture of plural proteins (data not shown). The N-terminus of the band intermediate to the 50-kDa and 37-kDa marker proteins of the SFC lane in Fig. 3 (b) was found to be blocked and therefore could not be sequenced. Oryzin was a major protein secreted in both APC and MSLC using mCD medium was oryzin (band 5 in Fig. 3(a)), while those obtained using DPY medium contained both α -amylase (band 2 in Fig. 3(b)) and oryzin (band 5 in Fig. 3(b)). On the other hand, a neutral protease was definitively detected in the

supernatants from MSLC and APC using DPY medium, particularly after 2 days of cultivation (data not shown). Dipeptidyl-peptidase V was detected only in APC after 1.5 days of cultivation, using either mCD or DPY media, although the band intensity was much stronger when DPY medium was used, as shown in Figs. 3(a) and (b).

3.3 Gene Transcriptional Analysis

We investigated differences in the gene transcriptional profile of a filamentous fungus cultivated by SFC, APC or MSLC, using either mCD or DPY medium. Figs. 4 (a) and (b) show scatter plots of the mean intensity ratios (\log_2 transformed) for the MSLC sample (cells cultivated by MSLC) versus those for the SFC sample (cells cultivated by SFC) and APC sample (cells cultivated by APC) cultivated in mCD medium, respectively while Figs. 4 (c) and (d) show scatter plots of the mean intensity ratios for the MSLC sample versus those for the SFC and APC samples, respectively when cultivated in DPY medium.

As shown in Figs. 4(a) – (d), most of the points are scattered around a line that passes through the origin with a slope of 1. This result indicates that in general, the SFC, MSLC, and APC samples had similar transcriptional levels. However, some points in the areas shaded gray in Figs. 4(a) – (d) clearly deviate from the line with a slope of 1, which satisfies the conditions, *i. e.*, $y-x > 2$, $y > 1$, $x < 0.5$) and ($y-x < -2$, $y < 0.5$, $x > 1$), where y represents the mean intensity ratios of the MSLC sample, and x those of the SFC or APC sample. The points existing in the upper left (second quadrant) gray areas in Figs. 4 (a) and (b) correspond to the genes specifically transcribed in the MSLC sample, which were up-regulated at least 4-fold compared with the SFC and APC samples, respectively. The points in the lower right gray area (fourth quadrant) represent the genes specifically transcribed in the SFC sample or APC sample, which were up-regulated at least 4-fold compared with the MSLC sample. Figs. 4(c) and (d) show scatter plots for the results obtained with DPY medium; these plots correspond to Figs. 4(a) and (b), respectively. As shown in Figs. 4(a) – (d), the number of genes specific for MSLC relative to SFC and *vice versa* was greater than the number of genes unique to MSLC versus APC and *vice versa* for both mCD and DPY media. Namely, the gene transcriptional profile obtained for MSLC was similar to that of APC for both mCD and DPY media. Table 2 lists the 8 genes that were uniquely transcribed in the MSLC sample versus the SFC sample (upper column) and the 14 genes that were uniquely

transcribed in the SFC sample versus the MSLC sample (lower column) with differences in transcription level of at least 10-fold in mCD medium.

oryzin gene (AO090003001036) in the MSLC sample was increased only 2.3-fold compared with the SFC sample, which was much less than the increase observed

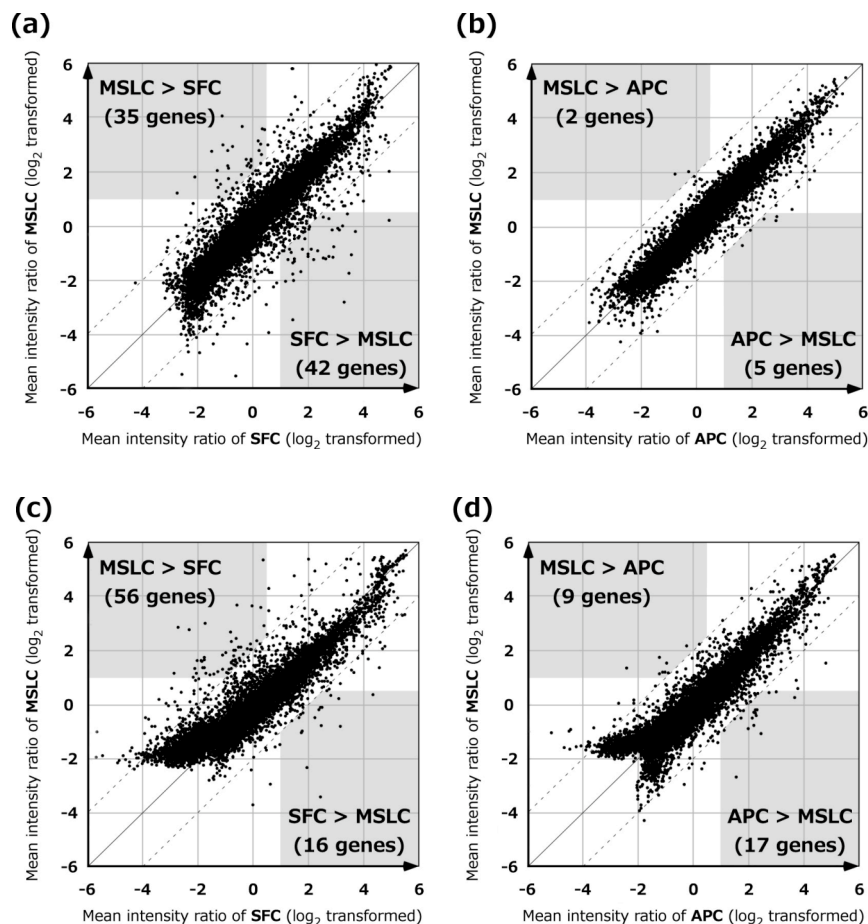


Fig. 4 Scatter plots of mean intensity ratios (log₂transformed) of MSLC versus SFC ((a) for mCD medium and (c) for DPY medium, respectively) and MSLC versus APC ((b) for mCD medium and (d) for DPY medium, respectively). The fluorescence intensities of samples were normalized by division by the average fluorescence intensity and subsequent log₂ transformation. The areas shaded gray indicate regions bound by the conditions, $x-y > 2$, $x > 1$, $y < 0.5$ and $x-y < -2$, $x < 0.5$, $y > 1$, respectively.

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, but not in the MSLC sample, included genes encoding membrane-associated proteins such as MFS monosaccharide transporter, GPI-anchored protein, and the cytochrome P450 CYP2 subfamily. There were no genes for which the difference in level of transcription between the MSLC and APC samples were 10-fold or greater. The transcription of the

for the genes listed in Table 2 despite high enzyme production during cultivation (Fig. 3(a)).

Genes that were transcribed only in the MSLC sample, but not in the SFC sample (first column) or APC samples (third column), and genes transcribed in the SFC sample (second column) or APC sample (fourth column), but not the MSLC sample, for which the transcription was increased at least 10-fold or greater in DPY medium are listed in Table 3. Most of the genes listed in Table 3 are predicted to function in cell growth, electron transfer, hydrolysis reaction, gene transcription, and protein folding. There were also three genes that encode proteins with no known function (each described as predicted

Table 2 Lists of genes specifically transcribed with more than 10-fold up-regulation in mCD medium.

<i>A. oryzae</i> Gene ID ^a	Gene product ^b	Fold change in fluorescence intensity (log ₂ -transformed mean intensity ratio ± SD)
MSLC specific genes to SFC		
AO090005000538	Alpha-1,3-glucanase	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		
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 original ID termed only in the array used in this study

^b Other candidate with E-value of lower than e^3 obtained by BLASTp search is shown in parenthesis

protein). In addition, there were two MSLC-specific genes that were either down-regulated or up-regulated relative to expression in both SFC and APC. For example, the C6 zinc finger domain protein was down-regulated and O-methyltransferase was up-regulated compared with SFC and APC. The oryzin, FG-GAP repeat protein (AO090003000935), and transferring receptor and related proteins containing the protease-associated (PA) domain (AO090011000052) were up-regulated 32.4, 13.3, and 13.2-fold, respectively. These results were coincident with enzyme production during cultivation, as evaluated by SDS-PAGE (Fig. 3), which was quite different from the results obtained using mCD medium (Table 2).

Transcription of a neutral protease gene (AO090011000036) was high in both the MSLC and APC samples when DPY medium was used. MSLC and APC samples showed similar mean intensity ratios of approximately 4, and transcription of the gene was up-regulated 5-fold in the MSLC sample compared with the SFC sample. On the other hand, the transcription level of the α -amylase gene (AO090120000196) was high in the SFC, MSLC, and APC samples with mean intensity ratios of approximately 4-5. However, the amount of α -amylase was greater in MSLC and APC compared with SFC during the 5-day cultivation (Fig. 2(f)), as discussed later.

Table 3 Lists of genes specifically transcribed with more than 10-fold up-regulation in DPY medium.

<i>A. oryzae</i> Gene ID	Gene product	Fold change in fluorescence intensity (log ₂ -transformed mean intensity ratio)
MSLC specific genes to SFC		
AO090103000450	Gibberellin 20-oxidase family protein	50.0 (5.64)
AO090012000034	Predicted flavoprotein involved in K ⁺ transport	35.4 (5.15)
AO090003001036	Oryzin (Alkaline protease)	32.4 (5.02)
AO090020000438	Predicted protein	27.8 (4.80)
AO090023000804	Hydrophobic surface binding protein A	19.9 (4.31)
AO090003000045	CFEM domain protein	16.9 (4.08)
AO090003000935	FG-GAP repeat protein	13.3 (3.73)
AO090011000052	Transferrin receptor and related proteins containing the protease-associated (PA) domain	13.2 (3.72)
AO090005000817	Predicted protein	12.0 (3.58)
AO090138000075	L-aminopeptidase D-esterase	11.9 (3.58)
AO090103000494	Cytochrome P450	11.7 (3.53)
AO090005000538	Alpha-1,3-glucanase	11.5 (3.53)
AO090701000528	O-methyltransferase	11.2 (3.49)
AO090011000041	RTA1 domain protein	11.2 (3.49)
AO090026000175	Predicted protein (Cyanovirin-N family protein)	10.3 (3.36)
SFC specific genes to MSLC		
AO090020000262	C6 zinc finger domain protein	56.5 (5.82)
AO090003001005	F-box domain protein	19.2 (4.26)
AO090701000554	Heat shock protein 30	15.5 (3.96)
AO090003000018	Heat shock Hsp30-like protein	15.3 (3.94)
MSLC specific genes to APC		
AO090701000528	O-methyltransferase	13.9 (3.79)
APC specific genes to MSLC		
AO090020000262	C6 zinc finger domain protein	18.6 (4.22)
AO090020000420	Predicted protein	11.5 (3.53)
AO090020000179	Molybdenum cofactor synthesis protein 2B	10.6 (3.40)

^b Other candidate with E-value of lower than e^3 obtained by BLASTp search is shown in parenthesis

4 DISCUSSION

MSLC and SSC are similar cultivation methods in that they both are static surface cultures, although MSLC uses liquid medium. Because MSLC uses liquid medium, the medium component of the culture is nearly uniform, as opposed to that in SSC. Therefore, the cultivation behaviors of SFC and MSLC result from very similar conditions, except that hyphae submerged and vigorous shaking. Results of the present study suggest that in general MSLC is similar to APC, but substantially different from SFC, in terms of enzyme secretion – particularly protease secretion and gene transcriptional profile. These differences in cultivation methods were observed regardless of the type of medium used, *i.e.*, mCD or DPY medium. The level of protease secretion in SFC was extremely low while MSLC and APC resulted in high protease secretion. These differences in protease secretion occurred though there was no appreciable difference in growth among the cultivation methods using either mCD or DPY medium. However, use of DPY medium increased the level of protease secretion in MSLC and APC to a greater extent than use of mCD medium. These results were supported by the SDS-PAGE results. Similar to the results of the present

study, Oda *et al.* [22] reported that *A. oryzae* RIB40 secreted 4.0 to 6.4-fold more protein per milligram of mycelium when cultured by SSC compared with SLC. The higher level of oryzin secretion in MSLC and APC compared with SFC was not consistent with the level of gene transcription when mCD medium was used, but gene transcription and protein secretion were consistent when DPY medium was used. These results clearly suggest that the relationship between the enzyme secretion and gene transcriptional levels depends on the kinds of medium composition, although the component(s) of the medium that affect the relationship are not known at present.

Because the three cultivation methods resulted in similar growth, the significant reduction in protease production 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 with APC and MSLC does not explain the lower enzyme production in SFC. The levels of protease and α -amylase secretion during cultivation in a 2-l flask containing 150-ml medium (k_{La} of 230 h⁻¹) were much lower than those in a 100-ml flask with 25-ml medium (k_{La} of 71 h⁻¹), with similar maximal cell numbers in all cultures. These results were obtained with use of DPY medium.

The glucoamylase activity in the culture supernatant was very low (0.05 U/ml) in each of three cultivation methods, even use of DPY medium. These results could probably be ascribed either to the fact that water activity was not as low as that in SSC, which uses solid substrates, as suggested by the results of Ishida *et al.* [15]. Alternatively, maltose was not added to the liquid medium at a high concentration, as suggested by te Biesebeke *et al.* [20], although they measured only the level of glucoamylase transcription. Furthermore, in the present study, we used a different *Aspergillus* strain than used by both Ishida *et al.* [15] and te Biesebeke *et al.* [20], which might affect the glucoamylase expression in addition to the differences in the cultivation conditions as mentioned above. Both the α -amylase-encoding gene and its activity were barely detectable when mCD medium was used. On the other hand, when DPY medium was used, the level of α -amylase secretion in MSLC and APC were similar and larger than that obtained with SFC, according to the SDS-PAGE analysis and activity measurement. However, the microarray analysis showed similar intensities for the three cultivation methods. These results suggest that secretion of α -amylase through the cell wall into the medium is

hindered in SFC, as reported by Oda *et al.* [22].

Although MSLC and APC showed a similar tendency in the secretion of most proteins studied here, alanyl dipeptidyl peptidase (band 1 in Fig. 3) was not clearly detected in MSLC for an unknown reason. One possible explanation for the different levels of alanyl dipeptidyl peptidase secretion by MSLC and APC is that, in MSLC hyphae grow over the membrane surface without entering the membrane pores. By contrast, in APC, the hyphae grow into the agar plate where they secrete enzymes to take in the nutrients in a manner similar to SSC using natural agricultural products.

In the present study, we elucidated differences in the gene transcriptional levels among the samples obtained by the different cultivation methods using a DNA microarray that included almost all protein-encoding genes for *A. oryzae*. Although much research has investigated the effects of different methods and conditions on gene transcription of specific secreted enzymes [15,20,43,44], no reports have focused on changes in the level of transcription of the entire gene, as far as the authors know. The microarray analyses indicated that the number of genes specific for MSLC relative to SFC and *vice versa* was greater than the number of genes unique to MSLC versus APC and *vice versa* for both mCD and DPY media. When mCD medium was used, most of the genes that were uniquely transcribed in the MSLC, but neither SFC nor *vice versa*, were genes encoding unknown or predicted proteins. Even oryzin, which was present in much higher quantities in the MSLC and APC samples in comparison with that for the SFC sample, was only slightly up-regulated in the MSLC and APC samples based on the microarray analyses, as mentioned previously. Thus, specific proteins might be included among the unknown or predicted proteins that are the clue to understanding the efficient secretion of enzymes in MSLC, APC or SSC. Additional detailed and comprehensive studies are requisite.

On the other hand, when DPY medium was used, many known genes were specifically transcribed only in the MSLC sample, but not the SFC sample with up-regulation of 10-fold or greater, including genes for oryzin, FG-GAP proteins, and transferring receptor and related proteins that contain the protease-associated domain. These observations are consistent with the results obtained by SDS-PAGE. *O*-methyltransferase gene was up-regulated in the MSLC sample versus both the SFC and APC samples. In addition, there were also genes that were uniquely transcribed in the SFC and/or

APC sample versus the MSLC samples compared with the MSLC sample with up-regulation of 10-fold or greater. In particular, the C6 zinc finger domain protein gene was markedly up-regulated in the SFC and APC samples versus the MSLC sample by 57- and 19-fold, respectively. However, mechanism for the up-regulation is not known at present. The results obtained above provide us novel and interesting information regarding the gene expression of *A. oryzae*. In addition, the present study also revealed similarities between MSLC and APC in terms of the enzyme secretion level and a gene transcription profiles, as mentioned above. The types of medium had a very strong impact on the transcriptional profile for each of the three cultivation methods - MSLC, APC, and SFC. Namely, function of most of the genes that were highly up-regulated or down-regulated in the MSLC when mCD medium was used, are unknown while that of many genes that were highly up-regulated with use of DPY medium are known.

In conclusion, the results of the present study indicate that the *A. oryzae* cultivated by MSLC possesses characteristics similar to fungi cultivated by APC and probably by SSC as well, but quite different from fungi cultivated by SFC. The amount of enzyme secreted and the gene transcription profiles were strongly dependent on the type of medium. 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 might be both an efficient production system for filamentous fungi and a powerful tool for revealing the molecular mechanism of higher enzyme production in fungi cultivated by SSC.

Abbreviations

APC	=	Agar-plate culture
mCD medium	=	modified Czapek-Dox medium
MSLC	=	Membrane-surface liquid culture
SFC	=	Shaking-flask culture
SLC	=	Submerged liquid culture
SSC	=	Solid-substrate culture

REFERENCES

- [1] D. A. Mitchell and B. K. Lonsane: In: H. W. Doelle, D. A. Mitchell, and B. K. Lonsane (ed.), *Solid Substrate Cultivation*, Elsevier Applied Science, London and New York (1992), 1-16,
- [2] K. Abe, K. Gomi, F. Hasegawa and M. Machida: *Mycopathologia*, **162** (2006), 143-153.
- [3] M. Raimbault: *Electronic J. Biotechnol.*, **1** (1998), 174-188.
- [4] K. Sakaguchi, H. Okazaki, and M. Takeuchi: *Nippon Nougakagaku Kaishi*, **29** (1955), 349-353.
- [5] W. Grajek: *Appl. Microbiol. Biotechnol.*, **26** (1987), 126-129.
- [6] S. Sudo, T. Ishikawa, K. Sato and T. Oba: *J. Ferment. Bioeng.*, **77** (1994), 483-489.
- [7] K. Tsuchiya, T. Nagashima, Y. Yamamoto, K. Gomi, K. Kitamoto, C. Kumagai and G. Tamura: *Biosci. Biotechnol. Biochem.*, **58** (1994), 895-899.
- [8] M. E. Acuna-Arguelles, M. Gutierrez-Rojas, G. Viniegra-Gonzalez and E. Favela-Torres: *Appl. Microbiol. Biotechnol.*, **43** (1995), 808-814.
- [9] Y. Hata, H. Ishida, Y. Kojima, E. Ichikawa, A. Kawato, K. Suginami and S. Imayasu: *J. Ferment. Bioeng.*, **84** (1997), 532-537.
- [10] T. Minetoki, C. Kumagai, K. Gomi, K. Kitamoto, and K. Takahashi: *Appl. Microbiol. Biotechnol.*, **50** (1998), 459-467.
- [11] A. Pandey, P. Selvakumar, C. R. Soccol and P. Nigam: *Curr. Sci.*, **77** (1999), 149-162.
- [12] K. Iwashita: *J. Biosci. Bioeng.*, **94** (2002), 530-535.
- [13] U. Holker, M. Hofer, and J. Lenz: *Appl. Microbiol. Biotechnol.*, **64** (2004), 175-186.
- [14] H. Obata, H. Ishida, Y. Hata, A. Kawato, Y. Abe, T. Akao, O. Akita and E. Ichishima: *J. Biosci. Bioeng.*, **97** (2004), 400-405.
- [15] H. Ishida, Y. Hata, E. Ichikawa, A. Kawato, K. Suginami and S. Imayasu: *J. Ferment. Bioeng.*, **86** (1998), 301-307.
- [16] T. Akao, K. Gomi, K. Goto, N. Okazaki and O. Akita: *Curr. Genet.*, **41** (2002), 275-281.
- [17] R. te Biesebeke, G. Ruijter, Y. S. Rahardjo, M. J. Hoogschagen, M. Heerikhuisen, A. Levin, K. G. van Driel, M. A. Schutyser, J. Dijksterhuis, Y. Zhu, F. J. Weber, W. M. de Vos, K. A. van den Hondel, A. Rinzema and P. J. Punt: *FEMS Yeast Res.*, **2** (2002), 245-248.
- [18] H. Kitano, K. Kataoka, K. Furukawa and S. Hara: *J. Biosci. Bioeng.*, **93** (2002), 563-567.
- [19] R. te Biesebeke, A. Levin, C. Sagt, J. Bartels, T. Goosen, A. Ram, C. van den Hondel and P. Punt: *Mol. Genet. Genomics*, **273** (2005), 33-42.
- [20] R. te Biesebeke, N. van Biezen, W. M. de Vos, C. A. van den Hondel, P. J. Punt: *Appl. Microbiol. Biotechnol.*, **67** (2005), 75-82.
- [21] A. Kobayashi, M. Sano, K. Oda, H. Hisada, Y. Hata and S. Ohashi: *Biosci. Biotechnol. Biochem.*, **71** (2007), 1797-1799.

- [22] K. Oda, D. Kakizono, O. Yamada, H. Iefuji, O. Akita and K. Iwashita: *Appl. Environ. Microbiol.*, **72** (2006), 3448-3457.
- [23] Y. Hata, H. Ishida, E. Ichikawa, A. Kawato, K. Suginami and S. Imayasu: *Gene*, **207** (1998), 127-134.
- [24] M. Machida, K. Asai, M. Sano, T. Tanaka, T. Kumagai, G. Terai, K. Kusumoto, T. Arima, O. Akita, Y. Kashiwagi and other 54 authors: *Nature*, **438** (2005), 1157-1161.
- [25] A. Yasuhara, A. Ogawa, T. Tanaka, T. Sakiyama and K. Nakanishi: *Biotechnol. Tech.*, **8** (1994), 249-254.
- [26] K. Nakanishi: Membrane Surface Liquid Culture, Microorganisms, Fungi. In: M. C. Flickinger and S. W. Drew (ed.), *The Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, John Wiley and Sons, New York, (1999), 1706-1713.
- [27] A. Ogawa, Yasuhara, T. Tanaka, T. Sakiyama and K. Nakanishi: *J. Ferment. Bioeng.*, **80** (1995), 35-40.
- [28] A. Ogawa, Y. Wakisaka, T. Tanaka, T. Sakiyama and K. Nakanishi: *J. Ferment. Bioeng.*, **80** (1995), 41-45.
- [29] Y. Wakisaka, T. Segawa, K. Imamura, T. Sakiyama and K. Nakanishi: *J. Ferment. Bioeng.*, **85** (1998), 488-494.
- [30] M. Morita, H. Shimamura, N. Ishida, K. Imamura, T. Sakiyama and K. Nakanishi: *J. Biosci. Bioeng.*, **98** (2004), 200-206.
- [31] K. Nakanishi, H. Imanaka, and S. Tanaka: Membrane Surface Liquid Culture, Microorganisms, Fungi. In: M. C. Flickinger and C. Michael (eds.), *Encyclopedia of Industrial Biotechnology, Bioprocess, Bioseparation, and Cell Technology*. John Wiley and Sons, New York, (2010), *in press*.
- [32] B. Feng, M. Morita, H. Imanaka, K. Imamura and K. Nakanishi: *J. Biosci. Bioeng.*, **102** (2006), 470-473.
- [33] T. Shimokawa, M. Hirai, M. Shoda and Y. Sugano: *J. Biosci. Bioeng.*, **193** (2008), 481-487.
- [34] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall: *J. Biol. Chem.*, **193** (1951), 265-275.
- [35] J. Nielsen, J. Villadsen, and G. Liden: *Bioreaction Engineering Principles*. Second ed, Kluwer Academic/Plenum Publishers, New York, (2003), 423-475.
- [36] B. D. Hames: One-dimensional polyacryl amide gel electrophoresis. In: B. D. Hames and D. Rickwood (ed.), *Gel Electrophoresis of Proteins*, Oxford University Press, Oxford, (1990), 1-147.
- [37] H. Imanaka, S. Tanaka, B. Feng, K. Imamura and K. Nakanishi: Cultivation characteristics and gene expression profiles of *Aspergillus oryzae* *J. Biosci. Bioeng.*, doi:10.1016/j.jbiosc.2009.09.004 (2009),.
- [38] S. Wirsal, A. Lachmund, G. Wildhardt and E. Ruttkowski: *Mol. Microbiol.*, **3** (1989), 3-14.
- [39] H. Tatsumi, O. Ogawa, S. Murakami, Y. Ishida, K. Murakami, A. Masaki, H. Kawabe, H. Arimura, E. Nakano and H. Motai: *Mol. Gen. Genet.*, **219** (1989), 33-38.
- [40] S. Cheevadhanarak, D. V. Renno, G. Saunders and G. Holt: *Gene*, **108** (1991), 151-155.
- [41] A. Dumas, R. Cramer, B. Léchenne and M. Monod: *J. Food Mycol.*, **2** (1999), 271-279.
- [42] K. Murakami, Y. Ishida, A. Masaki, H. Tatsumi, S. Murakami, E. Nakano, H. Motai, H. Kawabe and H. Arimura: *Agric. Biol. Chem.*, **55** (1991), 2807-2811.
- [43] H. Maeda, M. Sano, Y. Maruyama, T. Tanno, T. Akao, Y. Totsuka, M. Endo, R. Sakurada, Y. Yamagata, M. Machida, O. Akao, F. Hasegawa, K. Abe, N. Gomi, T. Nakajima and Y. Iguchi: *Appl. Microbiol. Biotechnol.*, **65** (2004), 74-83.
- [44] K. Tamano, M. Sano, N. Yamane, Y. Terabayashi, T. Toda, M. Sunagawa, H. Koike, O. Hatamoto, G. Umitsuki, T. Yakahashi, Y. Koyama, R. Arai, K. Abe and M. Machida: *Genet. Biol.*, **45** (2008), 135-151.