Solid–liquid Interface Screening System — Application to the Screening of Antibiotic and Cytotoxic Substance-producing Fungi

SHINOBU ODA¹², SEIYA NOMURA¹, MANAMI NAKAGAWA¹, KAZUO SHIN-YA³, NORITACA KAGAYA³, AND TEPPEI KAWAHARA³⁴

¹Genome Biotechnology Laboratory, Kanazawa Institute of Technology, 3-1 Yatsukaho, Hakusan, Ishikawa 924-0838, Japan
²Integrated Technology Research Center of Medicinal Science and Engineering, Kanazawa Institute of Technology, 3-1 Yatsukaho, Hakusan, Ishikawa 924-0838, Japan
³Department of Life Science and Biotechnology, National Institute of Advanced Industrial Science and Technology (AIST), 2-4-8 Aomi Koto-ku, Tokyo 135-0064, Japan
⁴Faculty of Life Science, Kumamoto University, 5-1 Oehonmachi, Kumamoto Chuo-ku, Kumamoto 860-8556, Japan

Received 17 April, 2018/Accepted 27 July, 2018

A useful tool for the screening of fungi producing biologically active secondary metabolites such as antibiotics and cytotoxic substances has been developed. An agar plate-organic solvent interface cultivation (A/S-IFC) system, which comprised a hydrophobic organic solvent (upper phase), a fungal mat (middle phase) and an agar plate (lower phase), was constructed. The metabolite profiles were compared among the A/S-IFC, a traditional submerged cultivation (SmC) and an extractive liquid-surface immobilization (Ext-LSI) system consisted of a hydrophobic solvent (upper phase), a fungal cells–ballooned microspheres (middle phase) and a liquid medium (lower phase), with high-performance liquid chromatography-photodiode array detector (HPLC-PDA). In the A/S-IFC, many hydrophobic metabolites vastly different from those in the SmC were accumulated in the organic phase as with the Ext-LSI. For example, a valuable azaphilone, sclerotiorin, was remarkably produced into the organic phase in the A/S-IFC. The A/S-IFC was applied to the screening of antibiotic-producing fungi. As a result of paper disk method, it was found that 321 isolated among 811 strains produced antifungal metabolites (hit rate, 39.6%). Furthermore, 8, 23, and 30 strains also produced cytotoxic metabolites against SKOV-3 (human ovary adenocarcinoma), MESO-1 (human malignant pleural mesothelioma), and Jurkat cells (immortalized human T lymphocyte).

Key words : Antibiotic screening / Cytotoxic metabolite / Interface cultivation / Extractive fermentation / Secondary metabolite.

INTRODUCTION

Recently, it has been anxious to accelerate development of new antibiotics effective in multi-drug-resistant pathogens such as Mycobacterium tuberculosis, Staphylococcus aureus and Pseudomonas aeruginosa. However, research and development into new antibiotics by pharmaceutical industry has declined all over the world. Nowadays, although the microbial metabolites are expected as resources of new drugs that can drive away various disease, the discovery of the metabolites having novel carbon skeletons gradually become more difficult (Bérdy, 2014; Demain, 2014).

On the other hand, filamentous fungi can produce many kinds of secondary metabolite having important pharmaceutical activities such as antibiotic, anti-inflammatory, and anticancer ones in company with actino-
fungi can actively grow on an interface between a hydrophilic gel such as a nutrient agar plate and a harmless hydrophobic organic solvent such as \(n\)-decane (Oda and Ohta, 1992; Oda et al., 2014). The microbial cells growing on the interface can efficiently catalyze various microbial transformations, such as hydrolysis (Oda et al., 1998), oxidation (Oda et al., 1996a), reduction (Oda et al., 2001), biodegradation (Oda and Ohta, 2002) and transacetylation (Oda et al., 1996b).

Thus, it is greatly expected that the agar plate-organic solvent interface cultivation (A/S-IFC) system above-mentioned also gives many characteristic hydrophobic metabolites, which are not produced in the SmC, similarly to the Ext-LSI system. The expectation must be very attractive because slow-growing fungi and actinomycetes cannot be applied to the Ext-LSI system. While a piece of fungal mat often sinks into a liquid medium and the slow-growing microorganism-microsphere layer easily collapses by overlaying hydrophobic organic solvent in the Ext-LSI, the phenomena make little difference in the A/S-IFC.

In this study, metabolite profiles in an aqueous phase of the SmC and Ext-LSI systems and in an organic phase of the Ext-LSI and A/S-IFC ones were exactly compared regarding 7 fungal strains (Fig. 1). The detailed determination of fungal metabolites was carried out by using a reversed phase high-performance liquid chromatography–photodiode array detector (HPLC-PDA). Furthermore, the efficacy of the A/S-IFC to the screening of antifungal and cytotoxic metabolites was confirmed.
MATERIALS AND METHODS

Fungi, seed broth and chemicals

Seven fungal strains, Aspergillus brasiliensis NBRC 9455, Monodictyon nordii NBRC 30548, Penicillium multicolor IAM 7153, Trichoderma atroviride AG2755-5NM398, Phoma exigua ATCC 14728, Rhizopus oryzae NBRC 31005, and Lecanicillium muscarium CBS 383.35 were used. All strains were maintained on potato-dextrose agar (PDA) plates. F-1 medium consisted of potato starch 20.0 g, glucose 10.0 g, soy protein (Soypro®; Inui Co., Ltd., Osaka) 20.0 g, KH₂PO₄ 1.0 g, MgSO₄·7H₂O 0.5 g in 1.0 L of reverse osmosis water (pH 6.0) for 10 days. The modified Sabouraud medium consisted of glucose 40.0 g, Bacto peptone 10.0 g, FeSO₄·7H₂O 5 mg, MnSO₄·H₂O 20 mg, CaCl₂ 10 mg in 1 L of reverse osmosis water (pH 6.0). After incubation, the broth was treated by excess NaCl and extracted with ethyl acetate (3 × 20 ml). The combined ethyl acetate layer was dehydrated with anhydrous Na₂SO₄ and evaporated in vacuo, giving a crude mixture of metabolites, which was dissolved in 300 μl of acetonitrile. The sample was named SmC:Aq.

Construction, operation and product recovery for SmC

The 3-days seed broth (300 μl) of each strain was inoculated into modified Sabouraud medium (20 ml). Incubation was done at 25°C with shaking (200 rpm) for 10 days. The modified Sabouraud medium consisted of glucose 40.0 g, Bacto peptone 10.0 g, FeSO₄·7H₂O 5 mg, MnSO₄·H₂O 20 mg, CaCl₂ 10 mg in 1 L of reverse osmosis water (pH 6.0). After incubation, the broth was treated by excess NaCl and extracted with ethyl acetate (3 × 20 ml). The combined ethyl acetate layer was dehydrated with anhydrous Na₂SO₄ and evaporated in vacuo, giving a crude mixture of metabolites, which was dissolved in 300 μl of acetonitrile. The sample was named SmC:Aq.

Construction, operation and product recovery of Ext-LSI

The 3-days seed broth (300 μl) of each strain, modified Sabouraud medium (20 ml) and the microspheres (1.3 g; Advancel HB-2051, Sekisui Chemical Co., Ltd., Tokyo) were mixed and poured into a polypropylene beaker (55 mm i.d. × 70 mm). After preincubation at 25°C for 3 days, lowly viscous dimethylsilicone oil was removed by column chromatography with silica gel 60N (2.0 g; Kanto Chemical Co., Ltd., Tokyo). After washing of the column with n-hexane, metabolites were eluted by ethyl acetate (9 ml). The ethyl acetate layer was concentrated in vacuo and dissolved in 300 μl of acetonitrile. The sample was named Ext-LSI:Org. The metabolites in an aqueous phase of the Ext-LSI (Ext-LSI:Aq) were recovered by the same manner as the SmC:Aq.

Construction, operation and product recovery for A/S-IFC

The 3-days broth (300 μl) was inoculated onto the surface of the modified Sabouraud agar plate of which surface area and volume were 23.7 cm² and 20 ml, respectively, in a polystyrene beaker (55 mm i.d. × 70 mm). After preincubation at 25°C for 3 days, 10 ml of KF-96L-1CS was added onto a fungal mat formed on the surface of an agar plate. Incubation was continued at 25°C without shaking for 7 days. After the incubation, the organic phase was collected and dried over anhydrous Na₂SO₄. The metabolites in the organic phase were recovered by the same manner as the Ext-LSI:Org and named A/S-IFC:Org.

Analyses of secondary metabolites

The profiles of secondary metabolite in SmC:Aq, Ext-LSI:Aq, Ext-LSI:Org, and A/S-IFC:Org were analyzed by using HPLC-PDA (LS-2010AHT-SPD-M20A, Shimadzu Co., Ltd., Kyoto). The column was Poroshell 120 (4.6 mm i.d. × 150 mm; Agilent Technologies, Santa Clara, CA), the column temperature was held at 40°C. Each sample (10 μl) was analyzed by using a 70:30 to 10:90 of 0.1% phosphoric acid–acetonitrile linear gradient for 40 min and held to the final solvent mixture for 30 min. The flow rate of the eluent was 0.4 ml/min and the spectra were monitored at 210 and 254 nm. Spectrophotometric data were collected at 200-500 nm.

A major hydrophobic metabolite (t_m, 40.55 min; Figure 3) of P. multicolor IAM 7153 was fully analyzed by Waters UPLC-DAD-ELS-HRMS system. Chromatography was performed by using a Water Acquity UPLC BEH C18 (2.1 mm i.d. × 50 mm) column at 55°C eluted with 50% acetonitrile containing 0.1% formic acid at 0.6 ml/min for 7 min (injection, 0.2 μl; ca. 2 mg/200 μl-methanol). Molecular formula was determined by the high-resolution MS data obtained by a LCT-Premier XE mass spectrometer (capirally voltage, 3.0 kV (ESI positive) or 2.6 kV (ESI negative); sample cone, 80 V; desolvation temperature, 350°C; source temperature, 120°C).

Application of A/S-IFC to the screening of antibiotic-producing fungi

Five ml of modified Sabouraud agar plate (agar, 1.5%) were prepared in a glass vial (volume, 20 ml; i.d., 24 mm). A piece (approximately 2 × 2 mm) of each fungal mat (811 strains) was inoculated onto the center of the agar surface with a toothpick. After the precultivation at 25°C for 3 days, 1.5-2 ml of volatile dimethylsil-
FIG. 2. Experimental procedure of a novel screening (A/S-IFC) system of antibiotic-producing fungi. After formation of a fungal mat on an agar plate, low viscous dimethyl-silicone oil, KF-96L-0.65CS, was added onto the fungal mat. All glass vials were packed into a container and a gas phase of the container was exchanged every other day by introducing air. After the still cultivation, each organic phase was collected and applied to paper disk assay.

FIG. 3. Comparison of profiles of secondary metabolites produced by *Penicillium multicolor* IAM 7153 among SmC, A/S-IFC and Ext-LSI systems. Aq, metabolites in an aqueous phase; Org, metabolites in an organic phase.
icone oil (KF-96L-0.65CS; boiling point, 100°C; viscosity, 0.65 cP; Shin-Etsu Chemical Co., Ltd., Tokyo) was added onto the fungal mat, and the incubation was hermetically continued for 7 days. A gas phase of the vessel was aseptically exchanged by air on the 3rd days. After the incubation, the organic phase was collected, combined with 500 µl of ethyl acetate layer used to washing a surface of fungal mat. After the removal of the organic layer by conventional air blow, metabolites were dissolved into 200 µl of ethyl acetate.

Fifty µl of each ethyl acetate fraction was charged to a paper disk (diameter, 8 mm; thickness, 1.5 mm). The paper disks were stored at room temperature overnight and put on the surface of yeast extract-peptone-dextrose (YPD) agar plates (agar content, 1% w/v) inoculated with a cell suspension of Wickerhamomyces anomalala NBRC 10213 (approximately 20 µl of cell paste in 10 ml of physiological saline solution). After preservation at 4°C for 12 h, the agar plates were incubated at 30°C for 48 h. The diameter of a halo formed was measured with a digital caliper. The schematic diagram for the application of the A/F-IFC to interfacial screening system is shown in Fig. 2.

Cytotoxic assay
SKOV-3 (human ovary adenocarcinoma) cells were cultured in DMEM medium (Wako Pure Chemicals Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific K.K., Yokohama), penicillin (50 U/ml) and streptomycin (50 mg/ml). MESO-1 (human malignant pleural mesothelioma) cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 mg/ml). Jurkat (immortalized human T lymphocyte) cells were cultured in RPMI 140 medium supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 mg/ml) and glutamam (Gibco). As a cell lines were seeded in 384-well plate at a density of 1000 cells/well in 20 ml media and incubated at 37°C in a humidified incubator with 5% CO2. After 4 h, samples were added to cell culture at the concentration of 0.5% and incubated for 72 h. Cell viabilities were measured by using CellTiter-Glo Luminescend Cell Viability Assay (Promega Corporation, Madison, WI) and EnVision Multilabel Plate Reader (PerkinElmer, Inc., MA).

RESULTS AND DISCUSSION
Comparison of secondary metabolites of P. multicolor
As shown in Fig. 3, the drastic differences of metabolism profile in the aqueous and organic phases of three cultivation systems, the SmC, Ext-LSI and A/S-IFC of P. multicolor IAM 7153, were observed. This strain can produce some azaphilone-related compounds containing sclerotiorin in the Ext-LSI system (Oda et al., 2015). Although an aqueous phase of SmC (SmC:Aq) contained relatively low yield of two metabolites (peaks 7 and 8) abundantly present in bothe organic phases of Ext-LSI (Ext-LSI:Org) and A/S-IFC (A/S-IFC:Org), the both metabolites were not detected in an aqueous phase of Ext-LSI (Ext-LSI:Aq).

Four kinds of hydrophobic predominant metabolite (peaks 7-10) of which UV-Vis spectra were similar to azaphilones (Fujimoto et al., 1995) were produced in both the A/S-IFC:Org and the Ext-LSI:Org. A metabolite of peak 8 (tR, 40.55 min; λmax, 218.9, 287.4 and 364.5 nm) was identified as sclerotiorin by LC-MS. Sclerotiorin has various biological activities, such as inhibitory action toward many enzymes (Chindananda et al., 2007), anti-HIV activity (Arunpanichlert et al., 2010), antibacterial and antifungal activities (Lucas et al., 2007), and anti-cancer activity (Giridharan et al., 2012). The accumulation of azaphilones, especially sclerotiorin (peak B) in the Ext-LSI:Org was higher than that in the A/S-IFC:Org (Fig. 3).

Comparison of secondary metabolites of T. atroviride
It is known that some species of Trichoderma produce a fungicidal aroma metabolite, 6-pentyl-α-pyrene (6PP), in SmC and liquid-surface fermentation (Kalyani et al., 2000), solid-state fermentation (Ladeira et al., 2010), and the Ext-LSI system (Oda et al., 2009; Oda et al., 2012). Thus, for T. atroviride AG2755-5NM398, the secondary metabolite profiles of SmC:Aq, Ext-LSI:Aq, Ext-LSI:Org, and A/S-IFC:Org were minutely compared to each other.

As shown in Fig. 4, three kinds of relatively hydrophobic metabolite (peaks 13-15) were remarkably accumulated in the A/S-IFC:Org and Ext-LSI:Org compared with the SmC:Aq and Ext-LSI:Aq. A metabolite detected as peak 14 (tR, 22.49 min; λmax, 217.8 and 300.5 nm; λmin, 208.7 and 240.1 nm) is 6PP and two kinds of metabolite detected as peak 13 (tR, 21.44 min; λmax, 227.2 and 335.2 nm; λmin, 213.2 and 262.3 nm) and peak 15 (tR, 30.44 min; λmax, 227.9 and 335.0; λmin, 213.5 and 263.0 nm) of which UV-Vis spectra are similar to that of peak 14 may be structurally related to 6PP. It is supposed that an organic phase plays as an extractant and reservoir of toxic 6PP in the A/S-IFC and Ext-LSI systems (Oda et al., 2009).

Comparison of secondary metabolites of M. nordinii
It is known that some strains belonging to M. nordinii produce antifungal (Ayer and Peña-Rodriguez 1987), anti-cancer (Arai et al., 2003) and hair-growth stimulant metabolites (Shinonaga et al., 2009) such as monorden
30548, were compared among the SmC:Aq, Ext-LSI:Aq, Ext-LSI:Org, and A/S-IFC:Org. and monocillins which are slightly soluble in water. Thus, metabolite profiles of the useful fungus, *M. nordinii* NBRC 30548, were compared among the SmC:Aq, Ext-LSI:Aq, Ext-LSI:Org, and A/S-IFC:Org.
As shown in Fig. 5, while some relatively polar metabolites (peaks 16-19) were detected in the SmC:Aq and the Ext-LSI:Aq, many hydrophobic metabolites (peaks 20-24) were accumulated in the Ext-LSI:Org and the A/S-IFC:Org. While the $\lambda_{\max}$ of peaks 21, 22 and 24 ranged from 222 to 232, from 261 to 279, and from 333 to 354 nm, the $\lambda_{\max}$ of peaks 23 and 25 ranged from 263 to 264 nm and from 334 to 339 nm. The $\lambda_{\max}$ of peaks 21-25 having three tops were close to those of radicicol and monocillins (Shinonaga et al., 2009).

Comparison of secondary metabolites of other fungal strains.

Similarly, the metabolites accumulated in the SmC:Aq, Ext-LSI:Aq, Ext-LSI:Org, and A/S-IFC:Org were compared to each other for the following 4 strains, A. brasiliensis NBRC 9455, P. exigua ATCC 14728, R. oryzae NBRC 31005, and L. muscarium CBS 383.35 (Fig. 6). In all strains, while many hydrophilic metabolites preferentially accumulated in an aqueous phase of the SmC (SmC:Aq) and Ext-LSI (Ext-LSI:Aq) systems, various hydrophobic metabolites were detected in an organic phase of the Ext-LSI (Ext-LSI:Org) and A/S-IFC (A/S-IFC:Org).

As shown in Fig. 6, while A. brasiliensis NBRC 9455 produced some hydrophobic metabolites such as peaks 26 ($t_{R}$, 45.09 min; $\lambda_{\max}$, 275.5 and 349.2 nm) and 27 ($t_{R}$, 49.28 min; $\lambda_{max}$, 346.4 nm), P. exigua ATCC 14728 gave some hydrophobic metabolites such as peaks 28 ($t_{R}$, 46.51 min; $\lambda_{\max}$, 242.5 nm) and 29 ($t_{R}$,
49.56 min; $\lambda_{\text{max}}$ 210.0 nm).

On the other hand, as shown in Fig. 6, R. oryzae NBRC 31005 preferentially gave hydrophobic metabolites such as peaks 30 ($t_{\text{R}}, 40.30 \text{ min}; \lambda_{\text{max}}$, 200 nm), 31 ($t_{\text{R}}, 44.59 \text{ min}; \lambda_{\text{max}}$, 200 nm), 32 ($t_{\text{R}}, 47.84 \text{ min}; \lambda_{\text{max}}$, 200 nm), and 33 ($t_{\text{R}}, 68.05 \text{ min}; \lambda_{\text{max}}$, 200 nm) in an organic phase of both the interface cultivation systems, Ext-LSI:Org and the A/S-IFC:Org. Among these peaks, while the peak 30 was detected only in the Ext-LSI:Org, the peak 33 was found only in the A/S-IFC:Org. Similarly, L. muscarium CBS 383.35 also preferentially produced hydrophobic metabolites such as peaks 34 ($t_{\text{R}}, 46.11 \text{ min}; \lambda_{\text{max}}$, 200 nm), 35 ($t_{\text{R}}, 48.00 \text{ min}; \lambda_{\text{max}}$, 200 nm), and 36 ($t_{\text{R}}, 49.10 \text{ min}; \lambda_{\text{max}}$, 200 nm) into the Ext-LSI:Org and A/S-IFC:Org.

As mentioned above, the Ext-LSI and the A/S-IFC systems enable much accumulation of hydrophobic metabolites into an organic phase compared with an aqueous phase of the SmC. It is assumed that serious feed-back inhibition by the hydrophobic metabolites occurs in the SmC because the release of the hydrophobic ones from cells to an aqueous phase is difficult. The authors expect that the interface cultivation systems, especially the A/S-IFC system may be applicable to actinomycetes and slow-growing fungi, give various biologically active substances having novel carbon skeletons.

**Application of A/S-IFC to the screening of antibiotic and cytotoxic metabolite-producing fungi**

Finally, in order to estimate the availability of the A/S-IFC, it was applied to the screening of antibiotic-producing fungi from 811 isolates. In our protocol (Fig. 2), antifungal activity against W. anomalaf NBRC 10213 was assayed by paper disk method. Although slow-growing fungi and actinomycetes cannot be applied to the Ext-LSI system because of a collapse of microsphere-fungal mat, the A/S-IFC enables to maintain even a small fungal colony of slow-growing fungi and actinomycetes.

As a result, 321 kinds of fungal metabolites from 811 isolates exhibited antifungal activity against W. anomalaf NBRC 10213 biofilm. Some results of the bioassay are shown in Fig. 7. The hit rate surprisingly reached to 39.6%. Thus, the A/S IFC fulfilled very high sensitivity because of the higher accumulation of metabolites in the organic phase.

Furthermore, 8, 23, and 30 strains also produced cytotoxic metabolites against SKOV-3 (human ovary adenocarcinoma), MESO-1 (human malignant pleural mesothelioma; cell viability), and Jurkat (immortalized human T lymphocyte) among fungi tested, respectively (cell viability <25%). Typical results of the cytotoxicity test are shown in Table 1.

**TABLE 1.** Cytotoxicity of typical fungal metabolites toward 3 kinds of human cancer cell lines.

| Isolated fungi | Viability (%) |
|---------------|--------------|
|               | SKOV-3 | MESO-1 | Jurkat |
| KIT-H0050     | 2.0    | 2.2    | 2.2    |
| KIT-C0335     | 24.5   | 7.5    | 2.3    |
| KIT-H0263     | 1.9    | 2.3    | 2.3    |
| KIT-D0861     | 1.2    | 1.5    | 1.4    |
| KIT-D0165     | 24.2   | 7.3    | 2.4    |
| KIT-D0250     | 35.9   | 11.1   | 3.3    |
| KIT-F1980     | 19.5   | 8.1    | 2.4    |
| KIT-CZ0176A   | 53.1   | 6.9    | 5.9    |
| KIT-AA0126A   | 22.1   | 10.8   | 31.0   |

SKOV-3, human ovary adenocarcinoma cells; MESO-1, human malignant pleural mesothelioma cells; Jurkat, immortalized human T lymphocyte cells.

In conclusion, the A/S-IFC has many important advantages over the SmC and the Ext-LSI systems, such as unique hydrophobic metabolite profiles, much production and easy recovery of metabolites, still cultivation, and possibility of application to slow-growing fungi and actinomycetes. It is expected that many hydrophobic secondary metabolites having unique carbon skeleton and important biological activities such
as antibiotic, antitumor and anti-inflammatory ones are discovered by using the A/S-IFC system.

ACKNOWLEDGMENTS

The authors wish to thank to members of The Circle of Natural Drug Discovery Group in Kanazawa Institute of Technology, Miss Kaho Ohsawa, Miss Chiaka Yamashita, Mr. Kazuki Shinya, Miss Haruka Kuboki, Miss Honami Murakawa, Miss Kaede Nakano, Miss Kyouko Sugimoto, Miss Riko Aoki, Miss Mei Matsumura, Mr. Yusuke Hayashi, and Mr. Yujunshi Okada, for helpful technical assistance in the experiments. This work was supported by a Grant-in-Aid for Science Research (C) (No.25450115) from the Japan Society for the Promotion of Science.

REFERENCES

Aguilar CN, Favela-Torres E, Viniegra-González G. (2001) Induction and repression patterns of fungal tannase in solid-state and submerged cultures. Process Biochem., 36, 565-570.

Arai, N., Shimo, K., Tomoda, H., Tabata, N., Yang, D. J., Masuma, R., Kawakubo, T., Ōmura, S. (1995) Isocromophones III-VI, inhibitors of acyl-CoA:cholesterol acyltransferase produced by Penicillium multicolor FO-3216. J. Antibiot., 48, 696-702.

Arai, M., Yamamoto, K., Namatsame, I., Tomoda, H., and Ōmura, S. (2003) New monordens produced by amidesine-producing Humicola sp. FO-2942. J. Antibiot., 56, 526-532.

Arupananchiert, J., Rukachaisirikul, V., Sukpondma, B., Babitha, S., Carvahlo, J. C., Soccol, C. R., and Pandey, A. (2008) Changes of Rhizopus chinenis 12 in submerged culture and its relationship with antibiotic production. Process Biochem., 38, 1643-1646.

El-Sabbagh, N., Harvey, L. M., and McNeil, B. (2008) Effects of dissolved carbon dioxide on growth, nutrient consumption, cephalosporin C synthesis and morphology of Acremonium chrysogenum in batch cultures. Enzyme Microb. Technol., 42, 315-324.

Ginicharan, P., Verekar, S. A., Khanna, A., Mishra, P. D., and Deshmukh, S. K. (2012) Antitumor activity of sclerotiorin, isolated from an endophytic fungus Cephalotheca faveolata Yaguchi, Nishim. & Udagawa. Indian J. Exp. Biol., 50, 464-468.

Hewage, R. T., Aree, T., Mahidol, C., Ruhrcriawat, S., and Kittakoop, P. (2014) One strain-many compounds (OSMAC) method for production of polyketides, azaphilones, and an isochromanone using the endophytic fungus Dothideomycete sp. Phytochemistry, 108, 87-94.

Kalyani, A., Propulla, S. G., Karanth, N. G. (2000) Study on the production of 6-pentyl-α-pyrene using two methods of fermentation. Appl. Microbiol. Biotechnol., 53, 610-612.

Kim, H. J., Kim, J. H., Oh, H. J., and Shin, C.S. (2002) Morphology control of Monascus cells and scale-up of pigment fermentation. Process Biochem., 38, 649-655.

Ladeira, N. C., Peixoto, V. J., Penha, M. P., de Paula Barros, E. B., and Leite. S. G. F. (2010) Optimization of 6-pentyl-α-pyrene production by solid state fermentation using sugar cane bagasse as residue. Bioresources, 5, 2297-2306.

Lai, L. -S. T., Tsai, T. -H., Wang, T. C., and Cheng, T. -Y. (2005) The influence of culturing environments on lovastatin production by Aspergillus terreus in submerged cultures. Enzyme. Microb. Technol., 36, 737-748.

Lucas, E. M. F., de Castro, M. C. M., and Takahashi, J. A. (2007) Antimicrobial properties of sclerotiorin, isochromone VI and penicolide, metabolites from a Brazilian Cerrado isolate of Penicillium sclerotiorum van Beyma. Braz. J. Microbiol., 38, 785-789.

Miao, L., Kwong, T. F. N., and Qian, P. -Y. (2006) Effect of culture conditions on mycelial growth antibacterial activity, and metabolite profiles of the marine-derived fungus Arthemium c.f. sacchariaca. Appl. Microbiol. Biotechnol., 72, 1063-1073.

Oda, S., and Ohta, H. (1992) Alleviation of toxicity of poisonous organic compounds on hydrophilic carrier/ hydrophobic organic solvent interface. Biosci. Biotechnol. Biochem., 56, 1515-1517.

Oda, S., Kato, A., Matsudomi N. and Ohta, H. (1996a) Enantioselective oxidation of racemic citronellol with an interface bioreactor. Biosci. Biotechnol. Biochem., 160, 83-87.

Oda, S., Inada, Y., Kobayashi, A., Kato, A., Matsudomi, N., and Ohta, H. (1996b) Coupling of metabolism and bioconversion: microbial esterification of citronellol with acetyl coenzyme A produced via metabolism of glucose in an interface bioreactor. Appl. Environ. Microbiol., 62, 2216-2220.

Oda, S., Tanaka, J., and Ohta, H. (1998) Interface bioreactor packed with synthetic polymer pad: application to hydrolysis of neat 2-ethylhexyl acetate. J. Ferment. Bioeng., 86, 84-89.

Oda, S., Sugai, T., and Ohta, H. (2001) Preparation of methyl ursodeoxycholate via microbial reduction of methyl 7-keto-lithocholate in an anaerobic interface bioreactor. J. Biosci. Bioeng., 91, 202-207.

Oda S, and Ohta H. (2002) Biodesulfurization of dibenzothio- phene with Rhodococcus erythropolis ATCC 53968 and its mutant in an interface bioreactor. J. Biosci. Bioeng., 94, 474-477.

Oda, S., Ishikshi, K., and Ohashi, S. (2009) Production of
6-pentyl-α-pyrone with *Trichoderma atroviride* and its mutant in a novel extractive liquid-surface immobilization (Ext-LSI) system. *Process Biochem.*, **44**, 625-630.

Oda, S., Araki, H., and Ohashi, S. (2012a) Derepression of carbon catabolite repression in an extractive liquid-surface immobilization (Ext-LSI) system. *J. Biosci. Bioeng.*, **113**, 742-745.

Oda, S., Michihata, S., Sakamoto, N., Horibe, H., Kono, A., and Ohashi, S. (2012b) Enhancement of 6-pentyl-α-pyrene fermentation activity in an extractive liquid-surface immobilization (Ext-LSI) system by mixing anion-exchange resin microparticles. *J. Biosci. Bioeng.*, **114**, 596-599.

Oda, S., Sugitani, A., and Ohashi, S. (2014) Solvent-tolerance of fungi located on an interface between an agar plate and an organic solvent. *Biosci. Biotechnol. Biochem.*, **78**, 1971-1974.

Oda, S., Kameda, A., Okanan, M., Sakakibara, Y., and Ohashi, S. (2015) Discovery of secondary metabolites in an extractive liquid-surface immobilization system and its application to high-throughput interfacial screening of antibioic-producing fungi. *J. Antibiot.*, **68**, 691-697.

Shinonaga, H., Noguchi, T., Ikeda, A., Aoki, M., Fujimoto, N., and Kawashima, A. (2009) Synthesis and structure-activity relationships of radicicol derivatives and WNT-5A expression inhibitory activity. *Bioorg. Med. Chem.*, **17**, 4622-4735.