**Improved production of phospholipase A₁ by recombinant
Aspergillus oryzae through immobilization to control the fungal
morphology under nutrient-limited conditions**

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Abstract

Given the complex fungal morphology, we provide an alternative to enhancing secretory protein production by *Aspergillus oryzae*. Immobilized *A. oryzae*, constructed to overexpress phospholipase A1 (PLA1) and cultivated in the presence of reticulated polyurethane foams, attained the extracellular PLA1 activities of 51.2–62.0 U/ml after 96–120 h, higher than those of suspension cells (36.9–53.5 U/ml). Moreover, the extracellular PLA1 activity of the immobilized cells at 0.5% polypeptone concentration was 34.8 U/ml, which is more than one-half of the maximum activity attained using 2% polypeptone concentration. Further investigations suggested the contribution of high growth rates of immobilized cells toward the enhanced PLA1 production. The macroscopic morphology, which affects the supply of oxygen and nutrients to the interior of cell pellets, is likely the reason for the high growth rates. This is based on the findings that, at 0.5% polypeptone concentration, the suspension cells formed mycelial clumps growing to a diameter of 10 mm, whereas the immobilized cells maintained a dense film with a thickness of 0.4 mm at the surface of the reticulated matrix. Together with the potential utility of repeated-batch cultivation, immobilized cell culture can be a powerful tool for targeted control of fungal morphology in bioprocesses using *A. oryzae*.

1. Introduction

Aspergillus oryzae, one of the important koji molds used in the traditional Japanese fermentation industry, has received considerable attention as a host for the production of both homologous and heterologous proteins. The industrial importance of *A. oryzae* has prompted extensive research studies of its molecular biology including the development of transformation systems [1, 2] and strong promoters [3-5], gene transcriptional analysis [6], and observation of organelles [7]. In *A. oryzae* genomes, many hydrolytic enzyme-encoding genes (e.g., amylase, cellulase, and lipase genes) that play a crucial role in biorefining have been found [8]. An efficient enzyme production system using *A. oryzae* has, therefore, become more necessary to expand its potential in current biotechnology.

To obtain a high yield of recombinant proteins, processes in *A. oryzae* cultivation have been optimized. In a previous study, phospholipase A1 (PLA1), an enzyme that hydrolyzes the ester bond at the *sn*-1 position of lecithin to produce lysolecithin, was cloned and expressed homologously in *A. oryzae* [9, 10]. In a PLA1-producing culture, fungal morphology affected the PLA1 productivity; huge pellets (3–5 cm in diameter) under nutrient-limited conditions were not suitable for PLA1 production, whereas the increase in the concentrations of nitrogen and carbon sources changed the mycelial morphology into freely dispersed mycelia, resulting in a significant increase in PLA1 productivity [9].

Fungal metabolism is highly associated with mycelial morphology, which varies

according to various factors including the type and concentration of nutrients, pH, temperature, and other physical conditions during submerged culture [11, 12]. Variations of tip growth and branching in hyphae result in different macroscopic appearances: filamentous growth, where the hyphae are freely dispersed in the culture medium, and pellet growth, showing spherical aggregates of various diameters consisting of highly entangled hyphal networks. Because the relationship between fungal morphology and process productivity has received considerable interest in both academia and industry, extensive studies have been carried out to manipulate the morphology to attain maximal performance of filamentous fungi [9, 13, 14]. As aforementioned, increasing the concentrations of nitrogen and carbon sources is one of the useful methods of yielding the desired morphology. However, the requirement for high nutrient concentrations directly leads to the high production cost of recombinant proteins.

Recently, a unique approach to manipulate the morphology to attain considerable performance of filamentous fungi (i.e., targeted control of fungal morphology) has been reported [21, 26, 27], where the addition of microparticles (e.g., aluminum oxide, magnesium silicate, and titanium silicate oxide) to the culture medium resulted in the formation of highly productive mycelia via physical interaction that involves the collision-induced disruption of conidia aggregates and hindrance of new spore–spore interactions in the early stage of cultivation [26]. In contrast, cell immobilization is a well-established technique to retain

microorganisms in a certain space in a matrix, thus facilitating the repeated-batch cultivation based on a "draw and fill" operation [15]. In particular, passive immobilization [i.e., adsorption and colonization on porous biomass support particles (BSPs)] [16] provides a natural method of immobilizing cells without chemical addition, aseptic handling of BSPs, and significant reduction in the mass transfer rate within BSPs. Previously, a micrographic analysis using *Rhizopus* species immobilized within polyurethane foam BSPs showed the formation of a dense film near the surface of BSPs [17, 18], which can be regarded as pellet formation induced by immobilization. Although cell immobilization itself is not a new methodology, a major issue that remains unclear is an advantage of the physically driven control of fungal morphology in secretory protein production.

Our research interest is, therefore, focused on how passive cell immobilization affects morphology and secretory protein production in recombinant *A. oryzae*. To clearly investigate the effect of morphological changes on protein secretion, polypeptone concentration in a culture medium was changed, leading to a significant difference in morphological appearance in suspension culture. We herein report on the advantages of an immobilized cell culture of *A. oryzae* over suspension culture under nutrient-limited conditions.

2. Materials and methods

2.1. Strains and media

The host strain *A. oryzae* niaD300 was a *niaD* mutant derived from the wild-type strain RIB40 [19] and routinely maintained on Czapek-Dox (CD) medium [2% glucose, 0.2% NaNO₂, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.2% KCl (w/v), 0.8 M NaCl, 0.001% (v/v) trace element solution (2% CuSO₄·5H₂O, 1% FeSO₄·7H₂O, 0.1% ZnSO₄·7H₂O, 0.1% MnSO₄·7H₂O, 0.1% AlCl₃, w/v), adjusted to pH 5.5] containing 1.5% (w/v) agar. Fungal transformants were selected on 1.5% (w/v) agar-containing CD-NO₃ medium, in which NaNO₃ was used instead of NaNO₂.

2.2. Fungal expression vector and transformation

The PLA1 gene (E16314) [10] was amplified from *A. oryzae* niaD300 chromosomal DNA by polymerase chain reaction (PCR) using two primers: PLA1-fw-*SalI* (5'-ATCAGTCGACATGTTTAGTCTCGCGCGATTG-3') and PLA1-rv-*SphI* (5'-ATAGGCATGCCTATGAACATTCGCTAATATAAATAAAGTACC-3'). The amplified fragment was digested with *SalI* and *SphI*, and inserted into pNAN8142 [4, 20]. The resulting plasmid was designated pNAN8142PLA1. The successful construction of the desired plasmid was confirmed by nucleotide sequencing (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Tokyo, Japan).

The transformation of *A. oryzae* was carried out in accordance with the method described by Gomi et al. [1]. *A. oryzae* protoplasts were prepared from mycelia grown at 30°C for 48 h using Yatalase (Takara Bio Inc., Shiga, Japan). The constructed plasmids were digested with *Bam*HI prior to transformation. The single-copy plasmid integration at the *niaD* locus in the genome was confirmed by real-time PCR (Thermal Cycler Dice Real Time System II, Takara Bio Inc.) using nuclease S1 (XM_001818584) as a reference.

2.3. Growth conditions

The solution containing spores was aseptically inoculated into a Sakaguchi flask (500 ml) containing 100 ml of DP medium (2% glucose, 1% polypeptone, 0.5% KH₂PO₄, 0.2% NaNO₃, 0.05% MgSO₄·7H₂O). Polypeptone used in this study was an enzymatic digest of milk casein, manufactured by Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan), and purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). In the investigation of the effect of polypeptone concentration (0.2–2.0%), pH was initially adjusted to 6.0 using sodium hydroxide. The flasks were incubated at 30°C on a reciprocal shaker (150 oscillations/min; amplitude, 50 mm). After cultivation, the culture broth was collected by filtration, whereas the cultivated mycelia were washed thoroughly with distilled water and lyophilized.

For immobilized cell culture, Sakaguchi flasks containing 100 ml of DP medium, fungal spores, and 150 BSPs were incubated at 30°C on a reciprocal shaker (150

oscillations/min; amplitude, 50 mm). The BSPs used for cell immobilization were 6-mm cubes of reticulated polyurethane foam (Bridgestone Co., Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch. The *A. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask cultivation. Biomass in suspension culture was determined by measuring dry cell weight, whereas immobilized biomass concentration within a BSP was measured using a sodium hypochlorite solution as described previously [17]. In the repeated-batch cultivation, the culture medium was removed by filtering after 96 h and exchanged with fresh DP medium, whereas the BSP-immobilized cells were retained in the flask.

Photographs of mycelia were obtained using an EOS Kiss X5 digital camera (Canon Inc., Tokyo, Japan). A stereoscopic microscope (model YS05Z; Microscope Network Co., Ltd., Saitama, Japan) was used for cross-sectional imaging of the immobilized cells.

2.4. Enzyme assays

For the measurement of PLA1 activity, a 2.5- μ l portion of the culture broth was added to a reaction mixture [0.25 ml of substrate solution containing 4% Triton X-100 and 1% phosphatidylcholine from egg yolk (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.25 ml of 0.1 M sodium acetate buffer (pH 5.6), and 0.05 ml of 0.05 M CaCl_2]. The reaction mixture was incubated at 37°C for 10 min followed by the addition of 0.05 ml of 1 N HCl to

terminate the reaction. The concentrations of released fatty acids were measured photometrically using Detaminar NEFA755 (Kyowa Medex Co., Ltd., Tokyo, Japan) in accordance with the protocol specified by the supplier. One unit of PLA1 activity was defined as the amount of the enzyme catalyzing the formation of 1 μ mol of free fatty acids per minute. Unless otherwise noted, data show averages and standard deviations of triplicates.

Analytical polyacrylamide gel electrophoresis of culture broth (15 μ l) in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed using a 12.5% gel. The separated proteins were stained with Coomassie brilliant blue R-250.

3. Results

3.1. Comparison between suspension and immobilized cell cultures

Figure 1 shows the time courses of PLA1 production using DP medium (1% polypeptone) and *A. oryzae* transformants. The extracellular PLA1 activity of the suspension cells carrying pNAN8142PLA1 increased with cultivation time, whereas no significant increase in the extracellular PLA1 activity of *A. oryzae* niaD300 was observed, suggesting the successful expression of the PLA1 gene under the control of the strong promoter P-No8142 [4, 20]. Additionally, the immobilized cells carrying pNAN8142PLA1 showed an approximately two fold higher PLA1 activity than the suspension cells (24–72 h, Fig. 1). Although the

extracellular PLA1 activities of the suspension cells increased significantly to reach 36.9–53.5 U/ml after 96–120 h, they fluctuated across the experiments, whereas, in case of the immobilized cells, high activities of 51.2–62.0 U/ml with a good standard deviation of 3.5 were obtained.

Table 1 shows a comparison of cell growth between the suspension and immobilized cell cultures. In the case of the immobilized cell culture, the immobilized cell weight (mg/BSP) was converted into the cell concentration in the culture medium (g-dry cell/L). At 72–120 h of the immobilized cell culture, the cell concentrations were comparable to those of the suspension culture. At the early stage of cultivation (24–48 h), however, the immobilized cell culture attained higher cell concentrations than the suspension culture, suggesting the contribution of cell growth to the increased PLA1 activity (Fig. 1).

3.2. PLA1 production using different concentrations of polypeptone

To investigate the effect of nutrients on PLA1 production, the polypeptone concentration in the culture medium was changed from 0.2 to 2.0 (% w/v). Figure 2 shows the extracellular PLA1 activities of *A. oryzae* carrying pNAN8142PLA1 during suspension and immobilized cell cultures (96 h) using different concentrations of polypeptone. Generally, the higher the polypeptone concentration, the higher the PLA1 activity, which reached the maximum values of 67.2 U/ml (suspension) and 66.4 U/ml (immobilization). The

extracellular PLA1 activity of the suspension cells at 2% polypeptone concentration was comparable to that of the immobilized cells; however, decreasing the polypeptone concentration led to the significantly low PLA1 activities of the suspension cells. The immobilized cells at 0.5% polypeptone concentration attained a PLA1 activity of 34.8 U/ml, which is more than one-half of the maximum activity attained using 2% polypeptone concentration. On the other hand, the PLA1 activity of the suspension cells at 0.5% polypeptone concentration was 10.6 U/ml, which is only 15% of the maximum activity attained using 2% polypeptone concentration. As can be seen in [Fig. 3](#), SDS-PAGE analysis of the culture medium at 2% polypeptone concentration clearly showed the presence of PLA1 with a molecular mass of 36 kDa (lanes 2 and 4). However, the suspension culture at 0.5% polypeptone concentration showed a small band (lane 1), whereas a considerable amount of PLA1 was observed in the immobilized cell culture at 0.5% polypeptone concentration (lane 3). Thus, these results show that the immobilized cells attain higher extracellular PLA1 activities than the suspension cells cultured at relatively low concentrations of polypeptone and suggest that the increased PLA1 activity is relevant to the increased amount of PLA1 secreted extracellularly.

[Table 2](#) shows a comparison of cell growth rate between the suspension and immobilized cell cultures (96 h). In accordance with the results in [Fig. 2](#), the cell concentrations increased with increasing concentrations of polypeptone. In the suspension

culture, polypeptone concentrations of 0.2% and 0.5% produced significantly low cell growth rates even after 96 h. Note also that the addition of 1% polypeptone, which led to a high cell concentration during suspension culture (8.93 g/L), resulted in a PLA1 activity lower than those of the immobilized cultures (Fig. 2). These findings suggest that high cell growth rate during immobilized culture is one of the major reasons, but not the sole reason, for the high extracellular PLA1 activity.

To investigate whether the different nutrient conditions caused the morphological change during suspension and immobilized cell cultures, the fungal mycelia were observed (Fig. 4). At 2% polypeptone concentration, the suspension cells aggregated into small pellets and showed partially dispersed mycelial forms (Fig. 4b), whereas at 0.5% polypeptone concentration, they produced mycelial clumps growing to a diameter of approximately 10 mm (Fig. 4a). However, the immobilized cells formed a dense film with a thickness of approximately 0.4 mm near the surface of BSPs and, except for the center region of the matrix, showed no significant difference in morphological appearance at the surface of BSPs between the conditions examined (Figs. 4c and 4d). These findings, together with the cultivation data presented above, suggest that the use of BSPs during fungal growth can control the morphology of *A. oryzae*, thus leading to the efficient production of PLA1 even with a limited nutrient supply.

3.3. Continuous PLA1 production by immobilized cells

To investigate the possibility of producing PLA1 continuously under nutrient-limited conditions, *A. oryzae* carrying pNAN8142PLA1 was cultivated by three draw-and-fill operations using DP medium with 0.5% polypeptone concentration (Fig. 5). The extracellular PLA1 activities when using 0.5% polypeptone concentration reached 51–55 U/ml at the end of the batch cultivation (96 h for each cycle), which correspond to the values obtained by the single-batch cultivation using 1% polypeptone concentration (Figs. 1 and 2). This finding thus suggests that repeated-batch production of PLA1 can be achieved by immobilized cell culture even with a limited nutrient supply.

4. Discussion

The complex morphology of filamentous fungi is problematic because the productivity in bioprocesses often correlates with the morphological form [11, 12]. Thus far, the importance of obtaining the desired morphology has prompted researchers to manipulate the growth characteristics of filamentous fungi by controlling parameters such as inoculum size, stirring rate, and pH [13, 14]. Here, we have demonstrated the possibilities of enhancing secretory protein production via physical immobilization of fungal cells within a reticulated matrix.

The finding of interest is that decreasing the concentration of a nutrient (polypeptone) distinguished the performance of BSP-immobilized cells from that of suspension cells (Fig. 2 and Table 2). SDS-PAGE analysis also confirmed the difference in the amount of PLA1 secreted (Fig. 3). In contrast to the extracellular PLA1 production, the cell-bound PLA1 activity did not show a significant difference among the conditions examined [Supplementary data (Fig. S1)]. Although detailed data on the expression levels of PLA1 and protease are not provided, the above results of experiments using BSP-immobilized cells show a marked preference for PLA1 secretion even with a limited nutrient supply.

The growth characteristics strongly suggested a contribution of increased cell weight to the enhanced PLA1 secretion (Tables 1 and 2). The limited supply of oxygen and nutrients to the interior of cell pellets might underlie the high growth rates. It is generally regarded that there exists a critical diameter determining the efficiency of nutrient supply. In *Aspergillus* species, the critical diameter of pellets appears to be in the range of 370–410 μm , that is, the availability of oxygen and nutrients to the interior of cell pellets is significantly limited beyond the critical penetration depth of approximately 200 μm [21]. This theory explains well why cells with highly entangled mycelia (on the order of cm in diameter) in suspension culture showed low growth rates (Fig. 4a), whereas the BSP-immobilized cells forming a film with a thickness of 0.4 mm at the surface of the reticulated matrix showed relatively high growth rates even with a limited nutrient supply. Thus, the physically driven control of

morphology using a reticulated matrix is considered effective for preventing limited cell growth. However, other mechanisms underlying the enhanced PLA1 secretion might exist because the addition of 1% polypeptone, which led to the high cell concentration during suspension culture (Table 2), resulted in a PLA1 activity lower than those of immobilized cultures (Fig. 2).

As for other enzyme-encoding genes, the expression of endo-1,4- β -glucanase (*celB*, DOGAN accession number AO090010000314) and β -glucosidase (*bgl*, DOGAN accession number AO090009000356) in *A. oryzae* [22] was also investigated using pNAN8142 as a vector. The extracellular enzyme activities of the immobilized cell culture at 0.5% polypeptone concentration were 1.58- (*celB*) and 1.96-fold (*bgl*) higher than those of the suspension culture (data not shown). In our previous studies using *A. oryzae* as a host, the secretion of only triacylglycerol hydrolase is inhibited significantly by cell immobilization within BSPs [23, 24]. The adsorption of lipase onto the cell wall might occur as was observed in the case of α -amylase [25], but this is beyond the scope of the current study.

Regarding other approaches to control fungal morphology, the addition of microparticles (e.g., aluminum oxide, magnesium silicate, and titanium silicate oxide) to the culture medium successfully led to the dispersion of fungal hyphae and enhanced enzyme production [21, 26, 27]. Compared with other alternatives that require increases in operational parameters such as stirring rate [13] and nutrient concentration [9], morphology engineering

using microparticles or BSPs can minimize the increase in cultivation cost. An additional advantage of the immobilized cell culture presented in this study is that a laborious separation of products from cells can be avoided by applying the draw-and-fill operation [28], resulting in the repeated-batch production of extracellular proteins (Fig. 5). These findings would thus provide a new insight into the process design as well as control of fungal morphology.

4. Conclusions

The immobilized cell culture of *A. oryzae* showed a marked preference for extracellular PLA1 production. One promising feature of immobilized *A. oryzae* is the relatively high growth rate of cells even with a limited nutrient supply. Despite the significantly increased diameter of pellets during suspension culture under nutrient-limited conditions, immobilized *A. oryzae* maintained the formation of a thin film at the surface of the reticulated matrix, thus facilitating the repeated-batch cultivation by the draw-and-fill operation. We herein propose that immobilized cell culture can be a powerful tool for targeted control of fungal morphology, which opens up new solutions to the design and optimization of bioprocesses using *A. oryzae*.

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References

- [1] K. Gomi, Y. Iimura, S. Hara, Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene, *Agric. Biol. Chem.* 51 (1987) 2549–2555.
- [2] O. Yamada, B.R. Lee, K. Gomi, Transformation system for *Aspergillus oryzae* with double auxotrophic mutations, *niaD* and *sC*, *Biosci. Biotechnol. Biochem.* 61 (1997) 1367–1369.
- [3] H. Ishida, Y. Hata, A. Kawato, Y. Abe, Y. Kashiwagi, Isolation of a novel promoter for efficient protein production in *Aspergillus oryzae*, *Biosci. Biotechnol. Biochem.* 68 (2004) 1849–1857.
- [4] T. Minetoki, C. Kumagai, K. Gomi, K. Kitamoto, K. Takahashi, 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 gene, Appl. Microbiol. Biotechnol. 50 (1998) 459–467.
- [5] H. Tsuboi, A. Koda, T. Toda, T. Minetoki, M. Hirotsune, M. Machida, Improvement of the *Aspergillus oryzae* enolase promoter (P-*enoA*) by the introduction of *cis*-element repeats, Biosci. Biotechnol. Biochem. 69 (2005) 206–208.
- [6] K. Abe, K. Gomi, F. Hasegawa, M. Machida, Impact of *Aspergillus oryzae* genomics on industrial production of metabolites, Mycopathologia 162 (2006) 143–153.
- [7] J. Maruyama, S. Kikuchi, K. Kitamoto, Differential distribution of the endoplasmic reticulum network as visualized by the BipA–EGFP fusion protein in hyphal compartments across the septum of the filamentous fungus, *Aspergillus oryzae*, Fungal Genet. Biol. 43 (2006) 642–654.
- [8] T. Kobayashi, K. Abe, K. Asai, K. Gomi, P.R. Juvvadi, M. Kato, K. Kitamoto, M. Takeuchi, M. Machida, Genomics of *Aspergillus oryzae*, Biosci. Biotechnol. Biochem. 71 (2007) 646–670.
- [9] Y. Shiba, C. Ono, F. Fukui, I. Watanabe, N. Serizawa, K. Gomi, H. Yoshikawa, High-level secretory production of phospholipase A₁ by *Saccharomyces cerevisiae* and *Aspergillus oryzae*, Biosci. Biotechnol. Biochem. 65 (2001) 94–101.
- [10] I. Watanabe, R. Koishi, Y. Yao, T. Tsuji, N. Serizawa, Molecular cloning and expression of the gene encoding a phospholipase A₁ from *Aspergillus oryzae*, Biosci. Biotechnol.

- Biochem. 63 (1999) 820–826.
- [11] M. Papagianni, Fungal morphology and metabolite production in submerged mycelial processes, *Biotechnol. Adv.* 22 (2004) 189–259.
- [12] P. Žnidaršič and A. Pavko, The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter, *Food Technol. Biotechnol.* 39 (2001) 237–252.
- [13] B. Jin, J. van Leeuwen, B. Patel, Mycelial morphology and fungal protein production from starch processing wastewater in submerged cultures of *Aspergillus oryzae*, *Process Biochem.* 34 (1999) 335–340.
- [14] M. Papagianni and M. Moo-Young, Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects, *Process Biochem.* 37 (2002) 1271–1278.
- [15] H. Fukuda, Immobilized microorganism bioreactors, *Bioprocess Technol.* 21 (1995) 339–375.
- [16] B. Atkinson, G.M. Black, P.J.S. Lewis, A. Pinches, Biological particles of given size, shape, and density for use in biological reactors, *Biotechnol. Bioeng.* 21 (1979) 193–200.
- [17] K. Ban, M. Kaieda, T. Matsumoto, A. Kondo, H. Fukuda, Whole cell biocatalyst for biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles, *Biochem. Eng. J.* 8 (2001) 39–43.
- [18] T. Nakashima, H. Fukuda, S. Kyotani, H. Morikawa, Culture conditions for intracellular

- lipase production by *Rhizopus chinensis* and its immobilization within biomass support particles, J. Ferment. Technol. 66 (1988) 441–448.
- [19] S.E. Unkles, E.I. Campbell, Y.M.J.T. Ruiter-Jacobs, M. Broekhuijsen, J.A. Macro, D. Carrez, R. Contreas, C.A.M.J.J. Hondel, J.R. Kinghorn, The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: a convenient and general selection system for filamentous fungal transformation, Mol. Gen. Genet. 218 (1989) 99–104.
- [20] K. Ozeki, A. Kanda, M. Hamachi, Y. Nunokawa, Construction of a promoter probe vector autonomously maintained in *Aspergillus* and characterization of promoter regions derived from *A. niger* and *A. oryzae* genomes, Biosci. Biotechnol. Biochem. 60 (1996) 383–389.
- [21] H. Driouch, R. Hänsch, T. Wuchterpfennig, R. Krull, C. Wittmann, Improved enzyme production by bio-pellets of *Aspergillus niger*. Targeted morphology engineering using titanate microparticles, Biotechnol. Bioeng. 109 (2012) 462–471.
- [22] A. Kotaka, H. Bando, M. Kaya, M. Kato-Murai, K. Kuroda, H. Sahara, Y. Hata, A. Kondo, M. Ueda, Direct ethanol production from barley β -glucan by sake yeast displaying *Aspergillus oryzae* β -glucosidase and endoglucanase, J. Biosci. Bioeng. 105 (2008) 622–627.
- [23] S. Hama, S. Tamalampudi, Y. Suzuki, A. Yoshida, H. Fukuda, A. Kondo, Preparation and

- comparative characterization of immobilized recombinant *Aspergillus oryzae* expressing *Fusarium heterosporum* lipase for enzymatic biodiesel production, Appl. Microbiol. Biotechnol. 81 (2008) 637–645.
- [24] S. Tamalampudi, M.M.R. Talukder, S. Hama, T. Tanino, Y. Suzuki, A. Kondo, H. Fukuda, Development of recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing lipase-encoding gene from *Candida antarctica*, Appl. Microbiol. Biotechnol. 75 (2007) 387–395.
- [25] H. Sato, Y. Toyoshima, T. Shintani, K. Gomi, Identification of potential cell wall component that allows Taka-amylase A adsorption in submerged cultures of *Aspergillus oryzae*, Appl. Microbiol. Biotechnol. 92 (2011) 961–969.
- [26] H. Driouch, B. Sommer, C. Wittmann, Morphology engineering of *Aspergillus niger* for improved enzyme production, Biotechnol. Bioeng. 105 (2010) 1058–1068.
- [27] B.A. Kaup, K. Ehrich, M. Pescheck, J. Schrader, Microparticle-enhanced cultivation of filamentous microorganisms: increased chloroperoxidase formation by *Caldariomyces fumago* as an example, Biotechnol. Bioeng. 99 (2008) 491–498.
- [28] C. Ogino, M. Kanemasu, M. Fukumoto, T. Kubo, T. Yoshino, A. Kondo, H. Fukuda, N. Shimizu, Continuous production of phospholipase D using immobilized recombinant *Streptomyces lividans*, Enzyme Microb. Technol. 41 (2007) 156–161.

Figure captions

Fig. 1 Time courses of PLA1 production by *A. oryzae*. *A. oryzae* carrying pNAN8142PLA1 was cultivated in (●) suspension and (▲) immobilized cell cultures. Suspension culture using *A. oryzae* niaD300 was used as (○) control.

Fig. 2 Effect of polypeptone concentration on extracellular PLA1 activity of *A. oryzae*. *A. oryzae* carrying pNAN8142PLA1 was cultivated for 96 h in suspension (white bar) and immobilized (gray bar) cell cultures. Prior to use, pH of DP medium was adjusted to 6.0.

Fig. 3 SDS-PAGE of culture supernatant. Lane 1, suspension culture at 0.5% polypeptone; lane 2, suspension culture at 2.0% polypeptone; lane 3, immobilized cell culture at 0.5% polypeptone; lane 4, immobilized cell culture at 2.0% polypeptone. *A. oryzae* carrying pNAN8142PLA1 was cultivated in DP medium for 96 h.

Fig. 4 Photographs of mycelia of *A. oryzae* cultivated by suspension and immobilized cell cultures. (a) Suspension cells at 0.5% polypeptone; (b) suspension cells at 2.0% polypeptone; (c) immobilized cells at 0.5% polypeptone; and (d) immobilized cells at 2.0% polypeptone. The cross sections of BSPs retaining *A. oryzae* are shown (c, d). The scale bars are 10 mm (white) and 1 mm (black).

Fig. 5 Repeated-batch cultivation of *A. oryzae* carrying pNAN8142PLA1. Culture medium in the first batch was DP medium (2.0% polypeptone, pH6.0), which was then exchanged with fresh DP medium (0.5% polypeptone, pH6.0). The broken lines show the time points of exchanging culture medium. The data show averages of two independent experiments.

Figure 1

Table 1

Comparison of cell growth between suspension and immobilized cell cultures.

Cultivation		Cultivation time (h)				
		24	48	72	96	120
Suspension	Cell concentration ^a (g-dry cell/L)	1.94±0.11	4.81±0.94	6.91±0.50	9.56±0.82	9.17±0.98
Immobilization	Cell concentration (g-dry cell/L)	3.80±0.69	5.21±1.31	6.82±0.55	8.87±1.31	9.61±1.09
	Immobilized cell weight ^b (mg/BSP)	2.53±0.46	3.47±0.87	4.55±0.37	5.91±0.87	6.40±0.72

^a Dry cell weight obtained in 1 liter of culture medium.

^b Dry cell weight immobilized within biomass support particle.

Figure 2

Figure 3

Table 2

Comparison of cell growth between suspension and immobilized cell cultures at various concentrations of polypeptone.

Cultivation		Polypeptone concentration (%)			
		0.2	0.5	1.0	2.0
Suspension	Cell concentration ^a (g-dry cell/L)	1.24±0.02	3.92±0.17	8.93±0.81	10.3±0.96
Immobilization	Cell concentration (g-dry cell/L)	5.20±1.08	6.26±1.54	8.90±0.39	9.96±0.83
	Immobilized cell weight ^b (mg/BSP)	3.47±0.72	4.17±1.03	5.93±0.26	6.64±0.55
Prior to use, pH of DP medium was adjusted to 6.0.					
^a Dry cell weight obtained in 1 liter of culture medium.					
^b Dry cell weight immobilized within biomass support particle.

Figure 4

Figure 5