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Improved production of phospholipase A_1 by recombinant *Aspergillus oryzae* through immobilization to control the fungal morphology under nutrient-limited conditions

Shinji Hama[a], Kaoru Onodera[a,b], Ayumi Yoshida[a], Hideo Noda[a], Akihiko Kondo[b,*]

[a] *Bio-energy Corporation, Research and Development Laboratory, 2-9-7 Minaminanamatsu, Amagasaki 660-0053, Japan*
[b] *Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan*

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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 A_1 (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 A_1 (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

* Corresponding author. Tel.: +81 78 803 6196; fax: +81 78 803 6196.
E-mail address: akondo@kobe-u.ac.jp (A. Kondo).

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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% $NaNO_2$, 0.1% KH_2PO_4, 0.05% $MgSO_4\,7H_2O$, 0.2% KCl (w/v), 0.8 M NaCl, 0.001% (v/v) trace element solution (2% $CuSO_4\,5H_2O$, 1% $FeSO_4\,7H_2O$, 0.1% $ZnSO_4\,7H_2O$, 0.1% $MnSO_4\,7H_2O$, 0.1% $AlCl_3$, 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-NO_3 medium, in which $NaNO_3$ was used instead of $NaNO_2$.

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-*Sal*I (5′-ATCAGTCGACATGTTTAGTCTCGCGCGATTG-3′) and PLA1-rv-*Sph*I (5′-ATAGGCATGCCTATGAACATTCGCTAATATAAATAAAGTACC-3′). The amplified fragment was digested with *Sal*I and *Sph*I, 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% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4\,7H_2O$). 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-μl 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 μmol 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 μl) 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.