Biotechnology and Bioprocess Engineering **2004**, 9: 184-190

Impeller Types and Feeding Modes Influence the Morphology and Protein Expression in the Submerged Culture of *Aspergillus oryzae*

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Abstract The influences of impeller types on morphology and protein expression were investigated in a submerged culture of *Aspergillus oryzae*. The impeller types strongly affected mycelial morphology and protein production in batch and fed-batch fermentations. Cells that were cultured by propeller agitation grew in the form of a pellet, whereas cells that were cultured by turbine agitation grew in a freely dispersed-hyphal manner and in a clumped form. Pellet-grown cells showed high levels of protein production for both the intracellularly heterologous protein (β-glucuronidase) and the extracellularly homologous protein (α-amylase). The feeding mode of the carbon source also influenced the morphological distribution and protein expression in fed-batch fermentation of *A. oryzae*. Pulsed-feeding mainly showed high protein expression and homogeneous distribution of pellet whereas continuous feeding resulted in less protein expression and heterogeneous distribution with pellet and dispersed-hyphae. The pellet growth with propeller agitation paralleling with the pulsed-feeding of carbon source showed a high level of protein production in the submerged fed-batch fermentation of recombinant *A. oryzae*.

Keywords: *Aspergillus oryzae*, filamentous fungi, morphology, propeller agitation, submerged culture, fed-batch fermentation

INTRODUCTION

Filamentous fungi have been widely used as hosts for the production of homologous and heterologous proteins because of their great productive capacity for the secretion of proteins and high adaptation of glycosylation/post-translational modification for higher eucaryote proteins [1]. Among the filamentous fungi that are suitable for industrial uses, *Aspergillus oryzae* presents a wide range of applications as a producer of highly active proteins. It secretes large amounts of industrial enzymes, such as hydrolases including amylase, xylanase, and protease [2]. *A. oryzae*, which has GRAS (generally regarded as safe) status, is suited well in industrial application due to its ability to produce numerous asexual conidiospores, to maintain a high genetic stability, to be resistant to shear stress, and to grow at a high cell density [3].

During the submerged cultivation of filamentous fungi, the type of growth varies from the spherical form, which is defined as pellet, to the filamentous form, which is defined as dispersed hyphae, depending on the genotype of the strain and on the culture conditions [4]. The morphological features of filamentous fungi have a large influence on the rheological properties of the bioreactor and protein productivity [5,6]. A dispersed filamentous form is common in industrial fermentations. However, because of the increase in impeller power for lowering the viscosity in the medium, hyper-fragmentation typically leads to damage cells and reduced productivity by releasing a lot of proteases [7]. In contrast, a pelleted form behaves in a Newtonian manner which is makes it easy to establish the mathematical model for process development [8]. It also reduces the secretion of extracellular protease, which is beneficial for heterologous protein secretion and prevents hyphal fragmentation. However, when pellets exceed a critical radius, entangled growth may result in reduced cell mass and protein productivity due to the substrate and oxygen limitation in the dense core of the pellet [9]. The characterization of the pellet formation is important in industrial processes using filamentous fungi in order to optimize the culture conditions and protein production [10]. There are conflicting reports on the relationships between morphology and protein productivity although many studies have been conducted in order to ascertain the relations. Also, many results obtained from investigating an influence of mor-

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phologies on cellular productions have been considered mostly as the impeller type using turbine agitation. As a consequence, the ability to obtain and control certain morphologies of filamentous fungi in submerged fermentation is critical. Parameters influencing morphological variation include the inoculum state, inoculum level, initial pH, medium composition, and agitation. Among them, agitation is regarded as the most important in determining morphology.

The *Escherichia coli* β-glucuronidase has been widely used as a reporter protein [11,12]. This enzyme has good stability in a wide pH range and has high sensitivity for various activity assays using fluorimetric, spectrophotometric, and histochemical methods. It also has been used in the gene fusion system in protein targeting studies [13].

In this study, a recombinant *A. oryzae* strain that produces the intracellularly heterologous protein, β-glucuronidase, and the extracellularly homologous protein, α-amylase, was employed as the model system in investigating the relationship between the impeller type and the morphology relating to the protein productivity. The turbine and propeller were chosen as a model impeller mixing type in order to investigate an influence of different rheological behaviors on cellular morphology. The feeding mode was also examined to control the conditions of morphological distribution and to enhance the production of protein.

MATERIALS AND METHODS

Organism and Culture Conditions

The recombinant *A. oryzae* strain, TF-d4, which carries the β-glucuronidase gene (*uidA*), was kindly provided by Dr. Masayuki Machida of the National Institute of Bioscience and Human-Technology, Japan. The β-glucuronidase gene was integrated into the genome of *A. oryzae* and was induced by the enolase promoter [12].

The spores for inoculation were harvested by washing 5-day-grown plates with 10 mL of 0.1% Tween-80 solution.

The fermentations were carried out in a 7-L fermentor (Kobiotech Co., Korea). The growth medium contained the following compositions per liter: 4.0 g of $NaNO_3$, 5 g of KCl, 1.0 g of K_2HPO_4, 6.0 g of KH_2PO_4, 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 20 g of glucose, and 5 g of yeast extract. The fermentor had an aspect ratio H/D of 1.4 and was equipped with three baffled plates and two, Rushton style, six-bladed turbine (D = 0.09 m) or marine propellers (D = 0.086 m) depending on the experimental purposes. The aeration rate was maintained at approximately 1.0 vvm. The main culture, with a 3 L starting volume, was inoculated with 100 mL of sterilized water that contained 10 mL of inoculum.

During fermentation, the impeller speed was varied from 700 to 1,000 rpm, depending on the cell growth, and the temperature was maintained at 28°C. Foam was automatically depressed by the controlled supply of siliconic antifoamer (Shin-etsu Silicone KM72, Japan).

Fed-batch fermentation was performed by two different modes with respect to the feeding method of the carbon source, which was classified into either the pulsed-feeding mode or the continuous feeding mode. The nutrient solution was prepared with 25% glucose solution and was supplemented with 5% yeast extract. For the pulsed-feeding mode, the nutrient solution was fed intermittently by pH-stat control at the end of the batch phase. The controlling algorithm was programmed using the AFS-biocommand Bioprocessing Software (New Brunswick Scientific Co., USA). The set point for controlling pH was adjusted at 6.0, and following the feeding equation of predetermined growth rate ($< 0.1\ h^{-1}$), a pulse was activated exponentially below this value after the consumption of the initial glucose in the medium. For the continuous feeding mode, nutrient solution was supplied continuously at the end of the batch phase, following the same algorithm of the pulsed-feeding mode with the exception of the pH-stat pulse control.

The culture was sampled periodically and was stored as a frozen state in a deep freezer until analyses. To extract soluble proteins, the samples were freeze-dried and were disrupted by grinding with a mortar and pestle. The disrupted cells were suspended in extraction solution, which was prepared in the following compositions: 20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 5 mM EDTA, and 10% glycerol.

Analytical Methods

The amount of glucose in the culture medium was analyzed using the DNS method [14].

β-Glucuronidase was assayed in a buffer that consisted of 50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 0.1% Triton X-100, and 1 mM *p*-nitrophenyl β-D-glucuronide. Reactions occurred in volumes of 1 mL at 37°C and were terminated by the addition of 0.4 mL of 2.5 M 2-amino-2-methylpropanediol. The absorbance of *p*-nitrophenol was measured at 415 nm. One unit was defined as the amount of enzyme producing one nanomole of *p*-nitrophenol/min at 37°C. The molar extinction coefficient of *p*-nitrophenol is 18,053 L mol^{-1} cm^{-1}.

Concentrations of the protein from the cell extract and the supernatant were estimated by the Bradford method, using a kit supplied by Bio-Rad Laboratories (USA). SDS-12% polyacrylamide gel electrophoresis was performed following the Laemmli method [15].

The biomass concentration was determined as the dry cell weight. Samples were filtered through a preweighed Miracloth filter (Calbiochem, USA) and were dried at 85~95°C for 2 days.

Pellet diameters were measured using a graduated microscope slide. The average diameter of 30-50 pellets per sample was calculated.

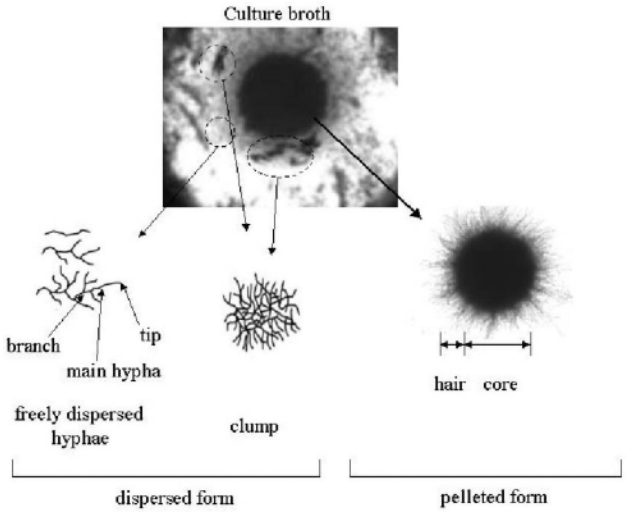

Fig. 1. Schematic diagrams of the typical morphology of filamentous fungi in submerged culture.

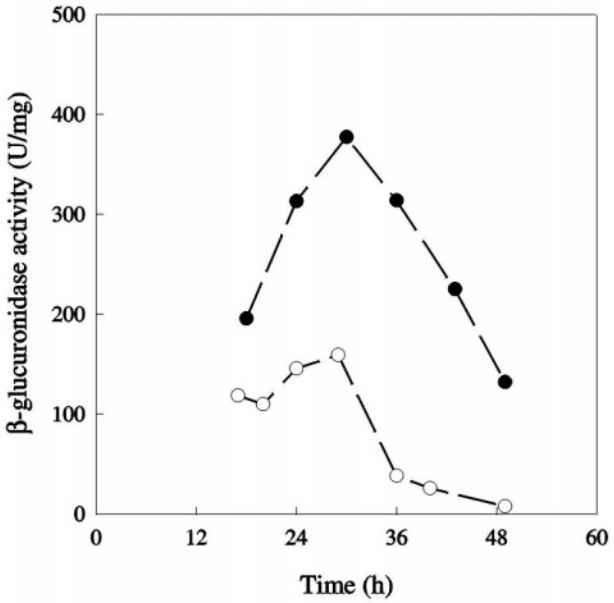

Fig. 2. Effect of the impeller type on β-glucuronidase production in batch fermentation of the recombinant *A. oryzae.* ●, propeller agitation; ❍, turbine agitation.

RESULTS AND DISCUSSION

Effect of Impeller Type on the Morphology

The rheological properties of many fungal fermentation broths appear to be linked to the mycelial morphology [5]. In the case of submerged fermentation of filamentous fungi, two extreme types of morphology, pellets and dispersed hyphae, were formed as shown in Fig. 1. In this study, the agitation caused by a turbine produced dispersed hyphae, such as free hyphae and clumps. In contrast, the agitation caused by a propeller formed pellets even at high agitation speeds of over 1,000 rpm. This suggests that the mechanical forces caused by impeller type would have a profound effect on the morphology of mycelia.

Effect of Impeller Type on Protein Production

In order to investigate the effect of impeller type on the production of different proteins, both intracellularly heterologous protein (β-glucuronidase from *E. coli*) and extracellularly homologous protein (α-amylase) were used as reporter proteins in batch and fed-batch fermentation.

As shown in Fig. 2, the impeller type greatly influenced protein expression. The activity of β-glucuronidase, which was cultured with the agitation driven by a propeller, was 2.6 times higher than the activity caused by the agitation of a turbine in batch fermentation. In a stirred bioreactor, shear stress significantly affects the microbial growth, metabolic responses, morphology, and protein productivity [16-18]. In general, conventional six-bladed turbine impeller can disperse gas to a superficial velocity of around 400 ft/h, compared with 70 ft/h for a propeller [19]. In addition, turbine agitation shows a power number of 5.75, compared with 0.32 for a propeller in a turbulent flow regime [20]. The power number is proportional to the ratio of the drag force acting on a unit area of the impeller and inertial stress. These relations means that turbine agitation shows higher shear stress and impact to the cell than that of propeller agitation. Toma *et al.* reported that excess turbulence caused by high-intensity stirring inhibited microbial growth and metabolism causing decreased adenosine triphosphate generation, lower oxygen uptake, and decreased lysine biosynthesis in *Brevibacterium flavum* [15]. Sahoo *et al.* [17] obtained the result that the specific intracellular catalase and protease levels increased and an intracellular transcriptional level decreased at a high shear rate in *Bacillus subtilis*. In addition, the specific intracellular reactive oxygen species level in *Bacillus subtilis* was increased 9.3-fold at the highest shear rate. They suggested the main reason for the inhibition was shear stress effects.

From the results of the dry cell weight and glucose consumption as shown in Fig. 3, the two extreme types of impellers showed no apparent difference in nutrition uptake and cell growth in batch fermentation. However, the activity of β-glucuronidase, intracellularly recombinant protein, was lower in turbine agitation than that of propeller agitation. A high shear stress seemed to give crucial effect on the protein production in the submerged culture of *A. oryzae*.

Fig. 4 shows the effect of impeller type on β-glucuronidase production in fed-batch fermentation of recombinant *A. oryzae*. In fed-batch fermentation, β-glucuronidase activity from the expression of the pellet formed by propeller agitation was higher than that of the dispersed hyphae by 3.2 times. In contrast, the dry cell weight was higher in the case of dispersed hyphal formation. The SDS-polyacrylamide gel electrophoresis analysis of the α-amylase that was secreted from pellet-grown

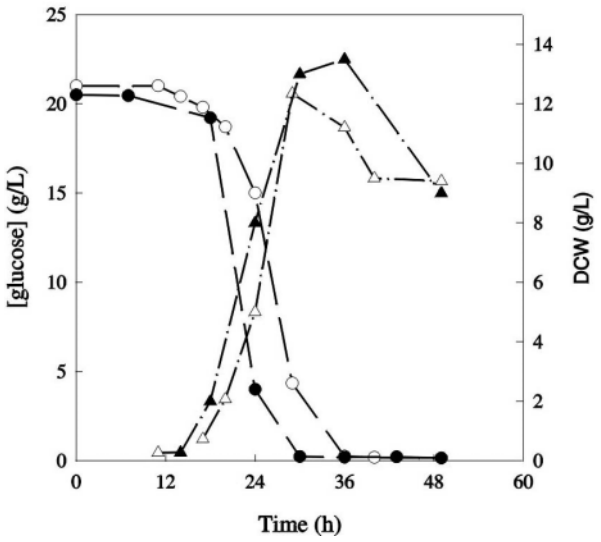

Fig. 3. Effect of the impeller type on glucose consumption and cell growth in batch fermentation of the recombinant *A. oryzae*. ●, ○, glucose concentration; ▲, △, dry cell weight (DCW); ●, ▲, propeller agitation; ○, △, turbine agitation.

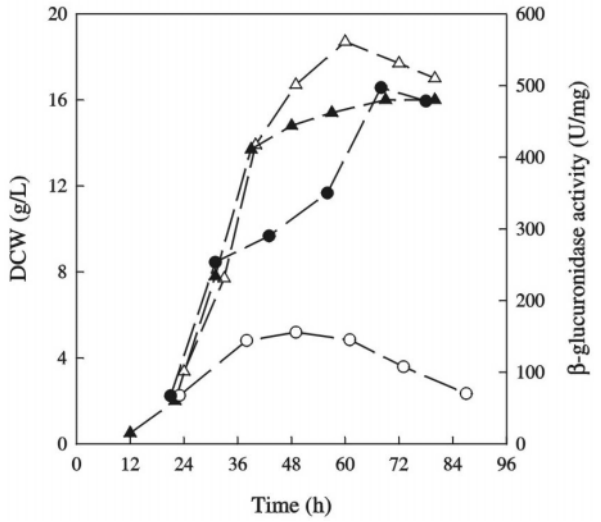

Fig. 4. Effect of morphology on β-glucuronidase production in fed-batch fermentation of the recombinant *A. oryzae*. ●, ○, β-glucuronidase activity; ▲, △, dry cell weight (DCW), ●, ▲, propeller agitation, ○, △, turbine agitation.

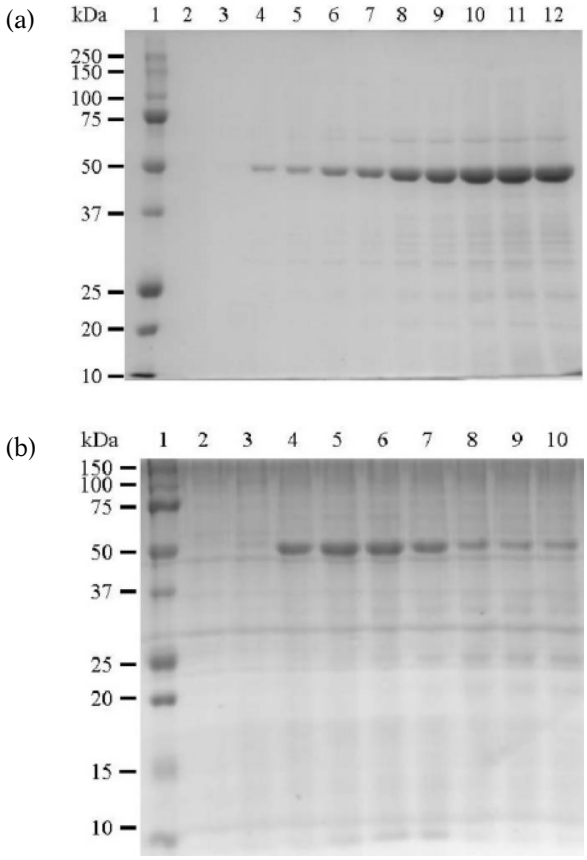

Fig. 5. SDS-12% polyacrylamide gel electrophoresis analysis of α-amylase that was either secreted from pellet-grown cells (a) or dispersed-hyphae-grown cells (b) in fed-batch fermentation of the recombinant *A. oryzae*. (a) lane 1, molecular marker; lane 2, 12 h; lane 3, 18 h; lane 4, 30 h, lane 5, 34 h; lane 6, 48 h; lane 7, 54 h; lane 8, 68 h; lane 9, 78 h; lane 10, 92 h; lane 11, 104 h; lane 12, 118 h; (b) lane 1, molecular marker; lane 2, 12 h; lane 3, 23 h; lane 4, 38 h; lane 5, 49 h; lane 6, 61 h; lane 7, 73 h; lane 8, 87 h; lane 9, 97 h; lane 10, 103 h. The culture supernatant (10 μL) of the cells grown in fed-batch fermentation was taken at the indicated times and then was analyzed by Coomassie Blue staining.

cells in Fig. 5 also shows similar profiles of protein production with respect to impeller type. The α-amylase expression from pellet-grown cells gradually increased with respect to the culture time as shown in Fig. 5(a). On the other hand, the α-amylase expression from dispersed-hyphae-grown cells illustrated a peak production at 49 h of culture and faded out with respect to culture time as shown in Fig. 5(b).

The dispersed-hyphal culture broth behaved in a non-Newtonian manner and showed high viscosity resulting in the decrease of oxygen transfer [21]. This behavior of the broths necessitated the use of high agitation speeds in order to provide adequate mixing and mass transfer. However, at high agitation speeds, cellular damage by shear stress can reduce protein expression. Pelleted fermentation broth can possibly lead to Newtonian behavior and low viscosity, but problems might arise during the transport of nutrients and of oxygen into the pellet core [7]. However, it was reported that pelleted growth reduced extracellular protease secretion, which is beneficial for heterologous protein secretion [9]. These results showed that high agitation speed of the propeller seemed to prevent the culture broth from the limitation of mass transfer, and also showed that pellet growth might reduce the protease attack from the cell disruption. The dispersed hyphal growth by turbine agitation caused the

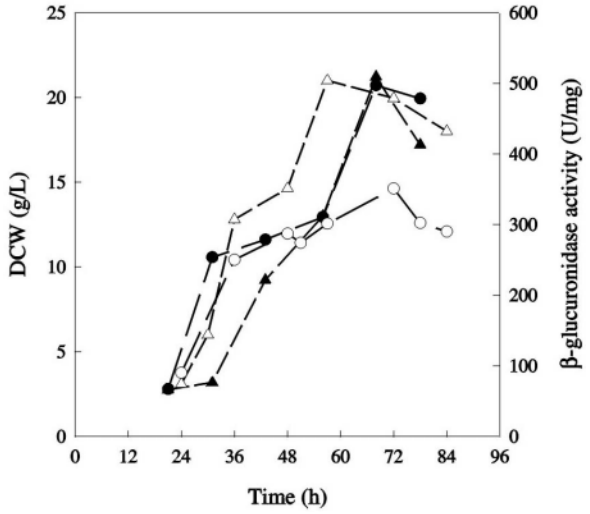

Fig. 6. Effect of the feeding modes of the carbon source on β-glucuronidase production in fed-batch fermentation of the recombinant *A. oryzae*. ●, ○, β-glucuronidase activity; ▲, △, dry cell weight (DCW); ●, ▲, propeller agitation; ○, △, turbine agitation.

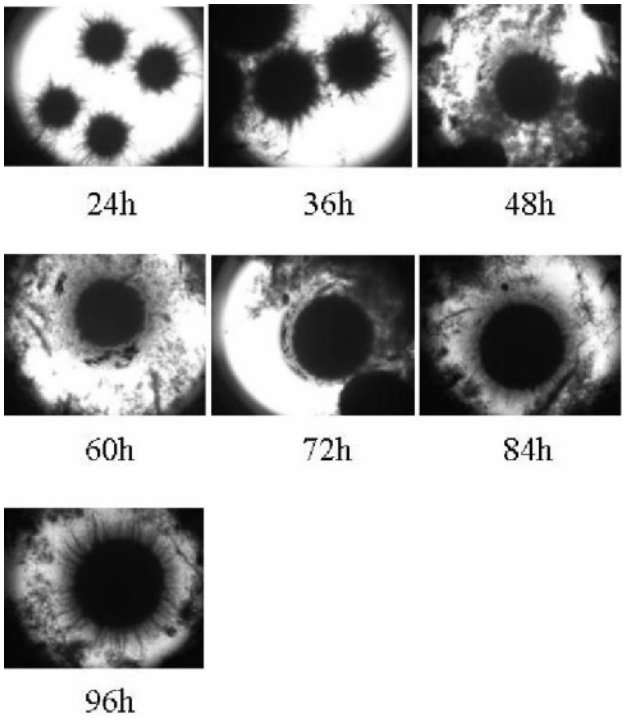

Fig. 7. Change of pellet formation and cell distribution patterns by time course under microscopic observation in continuously fed fermentation of the recombinant *A. oryzae*.

fragmentation of cells, and subsequently, this type of growth showed an increase in cell density (Fig. 4). The hyper-fragmentation, which occurred over a long period of culture time, nevertheless, seemed to occur. Cell disruption, which was caused by the continuous high level of shear stress of turbine agitation, produced a large amount of proteolytic enzymes and degraded extracellular protein. Thus, from these results, the impeller type was a great influence on the intracellular and extracellular protein production in submerged fermentation of recombinant *A. oryzae*.

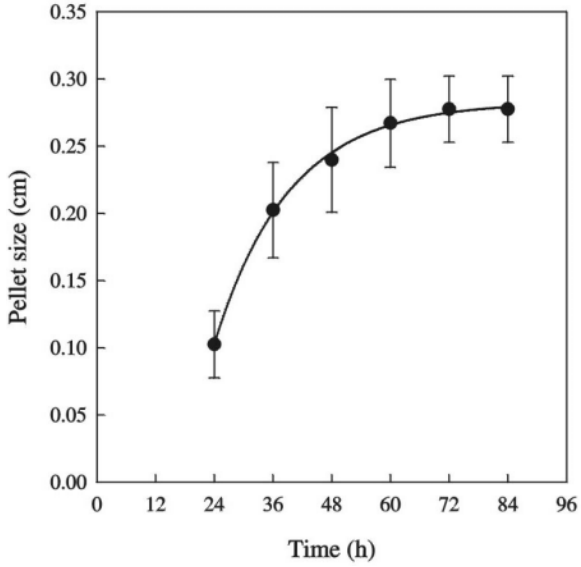

Fig. 8. The time course of pellet size distribution in pulse-fed fermentation of recombinant *A. oryzae*.

Effect of Feeding Mode on Protein Production in Fed-batch Fermentation of Recombinant *A. oryzae*

Fig. 6 shows the profiles of recombinant β-glucuronidase activity in pulse-fed fermentation and in continuously fed fermentation. Enzyme activity increased in a similar manner in both cases, until approximately the 60th hr. At this point, however, activity in pulse-fed fermentations continued to rise rapidly while that in continuously fed fermentation reached a plateau. As a result, the β-glucuronidase activity with the pulse-fed fermentation increased 1.5 times higher than the activity with the continuously fed fermentation. Data in Figs. 7 and 8 explain the difference in productivity of the recombinant protein between pulse-fed and continuously fed fermentations. Fig. 7 shows the changes of cell morphology in the continuously fed fermentation broth. At the end of batch fermentation and also at the initial stage of continuously fed fermentation, for the most part, cells grew as pellets, but dispersed hyphae and clumps accumulated in the culture broth with respect to the increase of culturing time. The pellet increased steadily in the size and swelled in the late growth phase of continuously fed fermentation, which resulted in the formation of pellets with long hair length. These phenomena led to the increase in viscosity of the broth and produced plenty of proteolytic proteins. In contrast, pulse-fed fermentation showed little formation of observable dispersed hyphae (data not

shown) with the increase of size to some extent, and pellet size reached to a plateau (Fig. 8). Thus, pulsed-feeding did not appear to have changed the morphological distribution and expression pattern.

The observations reported by Paragianni *et al*. [22] suggested that the formation of mycelial pellets was regarded as a prerequisite for successful production of microbial metabolites and proteins. Bhargava *et al.* obtained the result that fungal elements grown using the method of pulsed-feeding of a carbon source were significantly smaller. As a consequence, viscosity was up to 50% lower and the activity titer value of recombinant glucoamylase was up to 75% higher during the fed-batch fermentation of *A. oryzae* [23,24]. These observations suggest that the feeding mode considerably affects the cell morphology and rheological properties of the broth, as well as of protein production.

CONCLUSION

In *A. oryzae* cultures, mechanical stress and carbon source uptake was sensitively related to the cellular morphology and metabolism. Impeller type for agitation and feeding mode of carbon source showed a direct relationship to protein production. The propeller agitation can reduced the shear stress causing cell damage and metabolism. These two factors mainly controlled the mycelial morphology as pellet in fed-batch fermentation. Overall, the results indicate that pellet formation by propeller agitation paralleling the pulsed-feeding of carbon source sustained a high level of protein production in the submerged fed-batch fermentation of recombinant *A. oryzae*.

Acknowledgements This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Science & Technology (Grant MG02-0303-002-2-2-0), Republic of Korea. The authors deeply appreciate their financial assistance. The authors thank Dr. Masayuki Machida of the National Institute of Bioscience and Human-Technology, Japan for kindly providing us with the recombinant *A. oryzae* TF-d4 strain.

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[Received February 20, 2004; accepted May 15, 2004]