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Inhibition of extracellular protease secretion by *Aspergillus niger* using cell immobilization

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A wild-type *A. niger* strain was employed as a model to investigate the effect of cell immobilization on extracellular protease secretion during fermentation. A metal-coated pad of polyester latex felt was used to immobilize the cells in shake flasks. Compared with free suspension culture, the maximum specific activity of the extracellular protease from immobilized cells was reduced from 129 units/g to 28 units/g.

Introduction

The ability to secrete glycosylated proteins makes filamentous fungi attractive hosts for recombinant protein production (Lin *et al*., 1993). They have been widely used for the production of heterologous proteins as well as homologous proteins. Production levels of grams per liter have been reported. However, compared with homologous fungal protein productions, the production levels of heterologous non-fungal proteins usually do not exceed a few tens of milligrams per liter (Gouka *et al*., 1997). In attempts to improve the yield of heterologous proteins in fungal fermentations, several strategies have been developed, such as using gene fusion (Gouka *et al*., 1996; Ward *et al*., 1992), protease deficient mutants (Broekhuijsen *et al*., 1993; van den Hombergh *et al*., 1995), strong fungal promoters and efficient secretion signals and the introduction of a large number of gene copies (Verdoes *et al*., 1995).

In nature, filamentous fungi are able to utilize a great variety of carbon and nitrogen sources by secreting a wide range of different enzymes in large amounts into their environment. Unfortunately, degradation of heterologous proteins by extracellular proteases secreted by fungi into culture media has been considered a major problem for the efficient production of recombinant proteins (Archer *et al*., 1992; Enfors, 1992; Yadwad *et al*., 1996). Although some protease deficient host strains have been used, reduced secretion of extracellular proteases by process engineering strategies has not been evaluated in the literature.

In this work, a wild-type *A. niger* strain was used as a model system to investigate the effect of cell immobilization on the level of extracellular protease secretion. To reduce mass transfer limitations and for ease of immobilization, immobilization by passive cell adhesion to surfaces was investigated.

Materials and methods

Strain and media

A wild-type *A. niger van Tieghem* strain (ATCC 13496) producing homologous glucoamylase was employed as a model system in this study. The fermentation medium was YM broth (Difco) having the following composition (g/L): 3 yeast extract, 3 malt extract, 5 peptone, and 10 dextrose.

Culture conditions

Shake flasks were used to study cell immobilization. All shake flasks in the same set of experiments were inoculated from the same preculture plate. A 5-day-old plate was prepared at 24°C in an incubator (Model 3956, Forma Scientific Inc.) The spores were harvested by adding 20 mL of sterilized distilled water to the plate. The 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium with and without immobilization materials were inoculated with 0.5 mL spore suspension to give a final density of at least 10^5 spores/mL. The flasks were placed in a shaker (Innova 4000, New Brunswick Scientific Co.) at 24°C and 200 rpm.

Analytical methods

Glucose concentration and glucoamylase activity measurement

Glucose concentration was determined using a glucose assay kit (Catalog No. 315–100, Sigma). Glucoamylase activity was assayed according to Zhang *et al*. (1997).

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Fungal biomass measurement

Fungal biomass was determined by measuring its dry cell weight (DCW). For free suspension cultures, 1 mL of culture broth was first centrifuged at 6000 rpm for 10 min, followed by washing with distilled water twice and then drying at 80°C for 48 h. For the immobilization cultures, the supernatant was removed by decanting, then following the same steps used for free suspension. The immobilized biomass was determined from the difference between total weight of the dried flask containing cells and that of the flask without cells. The supernatant collected was stored at −20°C for later analysis of its protease level and glucose concentration. Two replicate samples were taken for each time point.

Protease activity measurement

Extracellular proteolytic activity was determined by incubating a 450 μL sample with 50 μL 1% (w/v) BSA (fraction V, Sigma) in 0.1 M sodium acetate buffer (pH 4.0) at 37°C. The substrate solution of BSA contained 5 mM sodium azide to prevent microbial growth. At different time points, the reactions were terminated with 500 μL of 10% (w/v) trichloroacetic acid (TCA). After incubation at 0°C for 0.5 h, the precipitated proteins were removed by centrifugation at 6000 rpm for 5 min, and the optical density of the TCA-soluble fraction was measured at 280 nm. Under these conditions, good linearity was observed up to an optical density of 1.00. One unit of protease activity was defined as a change of one absorbance unit per h at 280 nm for 1 mL reaction precipitation mixture as described above (van den Hombergh *et al.*, 1995). Extracellular protease activities were expressed in units per L of culture broth. The specific activity of extracellular proteases was defined as units per g of dry cell weight.

Results and discussion

Free suspension culture in shake flask

The time course for cell growth, glucose consumption, and extracellular protease secretion by *A. niger* cultivated in shake flasks is illustrated in Figure 1.

It was observed (by microscopy) that spore germination occurred at about 10 h. After spore germination, the cells grew rapidly. The maximum specific growth rate (μ_{max}) was found to be 0.04 h^{-1}, and the corresponding biomass yield from glucose ($Y_{X/S}$) was 0.6 g cell/g glucose. In free suspension fermentation, the cells grew in pellets. There were many hyphae in the outer zone of the pellets. After 3 days, the glucose was completely consumed, and cell concentration reached a maximum of 8.5 g/L. The cells started to secrete extracellular proteases after 2 days of culture. The extracellular protease activity increased

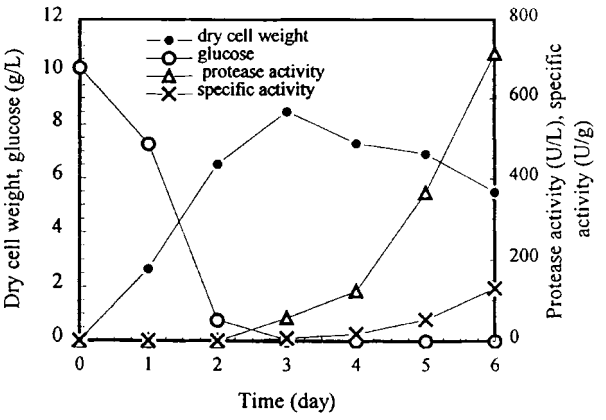

Figure 1 Free suspension culture of *A. niger* in shake flask. Initial spore size: 2.3 × 10^5 spores/mL.

through the end of the culture, reaching 714 units/L. The highest secretion rate of the extracellular protease was 15 units/Lh and was observed between 5 to 6 days of fermentation. Extracellular protease was secreted mainly after the cell growth approached the stationary phase. Degradation of heterologous proteins may also occur mostly in this phase. The results are consistent with the report that human interleukin-6 secreted by recombinant *A. nidulans* was degraded by extracellular proteases after the cell concentration reached its maximum at 48 h of fermentation (Yadwad *et al.*, 1996).

Comparison of cell growth on different immobilization materials

Six common materials with different structures were used to immobilize the cells in shake flasks. Table 1 summarizes the results. Maximum dry cell weight in Table 1 is for immobilized cells only.

The cells did not adhere to the glass beads or steel wire, and they did attach to the aluminum screen partially. The first two materials were hard, heavy, and smooth, and had large shear forces on the surfaces during fermentation. But the cells easily attached to the stainless steel scouring ball, cotton thread, and metal-coated pad because they were very puffy and light in weight. The stainless steel scouring ball used was in the globular shape, and did not completely submerge into the medium during incubation. The maximum cell concentration of 5.0 g/L obtained with this material was lower than that obtained with cotton thread (7.2 g/L) or metal-coated pad (7.5 g/L). Cotton threads have the potential problem of being utilized by the cells as a carbon source. Therefore, the metal-coated pad was selected as the material for cell immobilization of *A. niger* strain for further study.

Table 1 Comparison of cell growth on different immobilization materials. The initial spore concentration was 2.4×10^5 spores/mL

Material	*Immobilized percentage (% w/w)*	*Maximum dry cell weight (g/L)*
Glass bead (ϕ6 mm, 50/flask)	0	–
Aluminium screen [hole size 1.7 × 1.4 mm, 3 rectangular pieces (5 × 2.5 cm)/flask]	46	–
Stainless steel scouring ball (ϕ5 cm, 1/flask)	100	5.0
Helical steel wire (3 cm long, 8/flask)	0	–
Cotton thread (10 cm long, 12/flask)	100	7.2
Metal-coated pad of polyester latex felt (ϕ7.6 cm, 1 layer/flask)	100	7.5

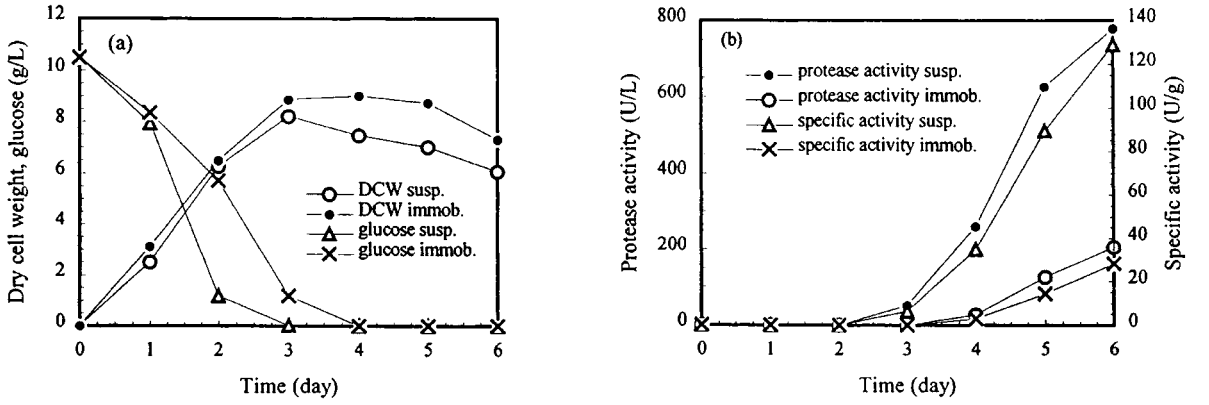

Figure 2 Comparison of free suspension and immobilization cultures of *A. niger*. (a) dry cell weight and glucose concentration; (b) extracellular protease activity and specific extracellular protease activity. Initial spores size: 2.6×10^5 spores/mL.

Effect of immobilization on extracellular proteases

Figure 2 shows the cell growth, glucose utilization and extracellular protease secretion in free suspension and immobilization cultures.

The maximum specific growth rate in immobilization was 0.03 h^{-1}, smaller than 0.04 h^{-1} in free suspension. But its biomass yield from glucose was 1.3 g cell/g glucose, much higher than 0.6 g cell/g glucose in free suspension. It was observed under microscopy that the cells grew in mycelia in immobilized culture. The cell concentration reached a maximum value after 3 days for free suspension, and after 4 days for immobilized culture [Figure 2(a)]. However, the maximum cell concentration in the immobilized system (9.0 g/L) was higher than that in free suspension (8.2 g/L). The secretion of the extracellular proteases was inhibited drastically by immobilization [Figure 2(b)]. The highest secretion rate and the maximum specific activity of the extracellular proteases were reduced from 15 units/Lh to 4 units/Lh and from 129 units/g to 28 units/g, respectively. The maximum extracellular protease activity in the immobilized system was 27% of the corresponding free suspension system. Our finding is consistent with Ramamurthy and Kothari's report (1993) on a loss of specific protease activity in surface culture compared to submerged culture in their effort to improve the harvest of fungal protease from *Rhizopus* SMC strain. Although immobilization reduced the secretion of proteases, its effect on the secretion of homologous glucoamylase by *A. niger* was the opposite. Experimental results show that the maximum specific activity of glucoamylase increased from 181 units/g to 244 units/g after immobilization. The exact mechanism of how immobilization affects protein secretion in this fungal system needs further investigations.

Conclusions

Among the six types of immobilization material used in this work, the metal-coated pad of polyester latex felt was found to be the best one for the *A. niger* culture in shake flasks at 24°C and 200 rpm. The experimental results showed that cell immobilization drastically reduced the secretion of extracellular proteases of *A. niger*.

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