Journal of Industrial Microbiology & Biotechnology (2002) 29, 259–263

www.nature.com/jim

Comparative studies on extracellular protease secretion and glucoamylase production by free and immobilized *Aspergillus niger* cultures

M Papagianni, N Joshi and M Moo-Young

Department of Chemical Engineering, Ohio University, Athens, OH 45701, USA

The effects of cell immobilization on the secretion of extracellular proteases and glucoamylase production by *Aspergillus niger* were investigated under a variety of immobilization techniques and culture conditions. Immobilization was achieved by means of cell attachment on metal surfaces or spore entrapment and subsequent growth on porous Celite beads. Free-suspension cultures were compared with immobilized mycelium under culture conditions that included growth in shake flasks and an airlift bioreactor. Cell attachment on metal surfaces minimized the secretion of proteases while enhancing glucoamylase production by the fungus. Growth on Celite beads in shake-flask cultures reduced the specific activity of the secreted proteases from 128 to 61 U g^{-1}, while glucoamylase specific activity increased from 205 to 350 U g^{-1}. The effect was more pronounced in bioreactor cultures. A reduction of six orders of magnitude in protease specific activities was observed when the fungus grew immobilized on a rolled metal screen, which served as the draft tube of an airlift bioreactor.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 259–263 doi:10.1038/sj.jim.7000289

Keywords: cell immobilization; proteases; glucoamylase; Celite; *Aspergillus niger*

Introduction

The aspergilli have a long history of use as producers of secreted proteins. These include glucoamylase, amylase, pectinases and proteases. Some of these enzymes are secreted at very high levels, often in excess of 20 g l^{-1}. Their ability to perform a series of posttranslational modifications required for eucaryotic protein secretion makes them attractive for use as expression–secretion hosts [12]. However, heterologous protein secretion levels are far lower compared to homologous levels [11]. In many cases, problems with heterologous protein production do not appear to be at the level of transcription but rather occur within the secretory pathway or following secretion [14]. Proteolytic degradation by fungal proteases is recognised as one of the major problems interfering with efficient heterologous protein production. These proteases may either be localized in the cell wall or secreted and may act intracellularly in lytic compartments or in the extracellular medium. Gouka *et al* [10], showed that intracellular or cell-bound proteases were the major cause of low yields of secreted human IL-6 by aspergillus.

Many strategies have been proposed for the improvement of heterologous protein production at both transcriptional and post-transcriptional levels. Concerning the posttranscriptional levels, fungal strains deficient in extracellular proteases have been isolated by random mutagenesis [13,19,21] or molecular genetic approaches [3], and the use of such strains resulted in significant improvement in the production of heterologous proteins [25]. Modifications of bioprocessing steps such as downstream processing at low temperatures, early separation of product and protease(s) or the use of protease inhibitors might also reduce proteolysis [14]. These steps will certainly not eliminate the problem because much of the degradation takes place *in vivo* during the production of protein.

In mycelial fermentations, fungal morphology plays a fundamental role in determining the overall process productivities. Manipulation of fungal morphology may result in increased yields of metabolites [20,21,23]. Work with the same organism used in this study showed that the formation and secretion of proteases was related to the morphological development of *Aspergillus niger*, with pelleted forms of growth being associated with lower extracellular protease activities compared to free filamentous forms [22]. Mycelial pellets are naturally aggregated cell populations, representing, therefore, an immobilized form of growth: the mycelium grows in a compact form around a core. Certain handicaps, however, such as the internal mass transport resistances imposed on the diffusion of oxygen and other nutrients around the pellets, lead to progressive cell lysis in the center of the pellets and loss of biosynthetic activity [27,29]. The induction of pellet formation is governed by complex interactions between biological and physical factors present in the culture, such as the genotype of the strain, inoculum level, medium composition, pH, and hydrodynamic shear forces [4]. Therefore, it is difficult in practice to manipulate these interacting factors to ensure reproducible conditions for pellet formation in mycelial cultures. Moreover, there are no realistic means to control the structure of the pellets to reduce the diffusional resistances in their internal structure, which often impair their biosynthetic efficiency.

The recognition of the fundamental role of fungal morphology in determining performances in mycelial fermentations has led to the search for alternative ways to engineer the structure of these microorganisms into more desirable forms, e.g., cell immobilization on various surfaces. Concerning the protease problem during protein production from fungal cultures, the effects — if any — of cell immobilization on extracellular protease secretion have not

Correspondence: Dr Maria Papagianni, Department of Food Hygiene and Technology of Food of Animal Origin, School of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece
Received 29 October 2001; accepted 14 June 2002

been evaluated in the literature. In the present study, a wild-type *A. niger* strain producing homologous glucoamylase was used as a model system to investigate the effect of cell immobilization on protease secretion. To reduce mass-transfer limitations and for ease of immobilization, immobilization by passive cell attachment to surfaces as well as spore entrapment and growth on porous materials in suspension were investigated.

Materials and methods

Microorganism and media

A. niger ATCC 13496 was used throughout this study. The culture medium was Bacto YM broth (Difco) with the following composition (in g l^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; and dextrose, 10. The medium pH after sterilization at 121°C for 20 min was 5. Spores were harvested from 5-day-old plates (Bacto wort agar, Difco), incubated at 24°C. The spore inoculum used in all cases was in the order of 10^5 spores ml^{-1}.

Culture conditions

Shake flask cultures: Erlenmeyer flasks (250-ml) containing 50 ml of medium were inoculated from the same preculture plate and placed in a rotary shaker incubator at 25°C and 200 ×g. Shake-flask cultures were carried out with cells growing both in free suspension and immobilized on different materials.

A number of different materials were tested with regard to their affinity and degree of cell attachment to them. Best results (100% cell attachment) were obtained with an aluminium-coated pad of polyester latex felt (Pacific States Felt & Mfg., USA, ϕ 7.6 cm, one layer per flask) and a type of porous beads by Celite with a mean bead diameter of 550 μm (Product R-631, Celite, Wayne, NJ); these materials were used for cell immobilization in shake-flask cultures throughout this study. The technique used for treatment of Celite and entrapment of the mycelial spores onto it was based on the method developed by Gbewonyo and Wang [8]. Before use, the Celite material was treated with sulphuric acid to lower its pH from 9.5 to 5. The beads were then dried at 400°C for 3 h. Aliquots of the beads were weighed into the shake flasks and then autoclaved. The bead concentration used was 10% weight of Celite by volume of growth medium. The spore suspension was then added to the beads to give a concentration of 20% (w/v) beads. The mixture was allowed to remain static for a contact period of 1 h. After this period the supernatant liquid was decanted and the bead fraction was washed thoroughly with two volumes of sterile distilled water to remove the residual free spores remaining in the interstitial spaces between the beads. The wash water was decanted after allowing the beads to settle. Washing was repeated twice. Finally, the growth medium was added to the beads to start the fermentation. The number of spores entrapped in the beads was determined as the difference between the spore concentration in the initial inoculum suspension and the residual spore concentration in the supernatant suspension after entrapment. Samples of these suspensions were serially diluted and plated on agar plates (Bacto wort agar, Difco) to yield mycelial colonies from individual spores, which were counted to obtain the spore concentration.

Bubble column (BC) bioreactor cultures: The BC bioreactor used in this work consisted of a 10-cm-diameter glass pipe, 50 cm in length (Ace Glass, Vineland, NJ). The operating volume was 3 l. The bioreactor was sterilized by autoclaving it at 120°C for 20 min, and subsequently was supplied with sterile media. Temperature control was provided by warm water circulation through a jacket. Experiments in this bioreactor were performed with free-suspension cultures, as well as immobilized cultures, in which the reactor was operated as airlift. For airlift operation, a stainless steel screen was rolled up to form a scroll, 29 cm in length and 5 cm in diameter. The scroll was fitted inside the glass column to serve as the draft tube of the airlift. In both cases, air was provided through the bottom at a rate of 2.5 vvm. To avoid foaming, antifoam 289 (Sigma: Cat. No. A-5551) was used.

All experiments were carried out in duplicate and mean values are presented.

Analytical methods

Glucose concentration was determined using an assay kit by Sigma (Cat. No. 315-100). Glucoamylase activity was assayed according to Lin *et al* [18]. Biomass concentration was determined by dry-weight measurements. For free-suspension cultures, 10 ml of broth was centrifuged at 6000 ×g for 10 min, washed with distilled water twice and dried at 80°C for 48 h. For immobilized cultures, the supernatant was removed by decanting it and the immobilized biomass was determined from the difference between total weight of the dried flask containing cells and of the flask without cells. For airlift cultures, the immobilized biomass was determined from the difference between the total weight of the stainless steel screen with the culture attached after drying it at 80°C for 48 h and the net weight of the screen.

Protease activity determination: 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) at 37°C. Sodium azide (5 mM) was added to the substrate solution to prevent growth of contaminants. At different time points, the reactions were terminated using 500 μl of 10% (w/v) trichloroacetic acid (TCA). After incubation at 0°C for 30 min, the precipitated proteins were removed by centrifugation at 6000 ×g 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. One unit of protease activity was defined as a change of one absorbance unit per hour at 280 nm for 1 ml reaction precipitation mixture. Extracellular protease activities were expressed in units per liter of culture broth. The specific activity of extracellular protease was defined as units per gram of CDW.

Results and discussion

The time courses for biomass, residual sugar concentrations, pH, extracellular protease and glucoamylase activities for free-suspension shake-flask cultures of *A. niger* are shown in Figure 1. Spores germinated within 10 h from inoculation and the mycelium grew in the pelleted form. The pH of the culture dropped within 40 h from an initial of 5.5 to 3.5 and remained unchanged thereafter. Glucose was depleted within 70 h from inoculation, while biomass levels reached a maximum of 7 g l^{-1} at 96 h to reduce slightly to 5.5 g l^{-1} for the remaining time. Extracellular protease activities were detected in the 48-h sample and their maximum concentration

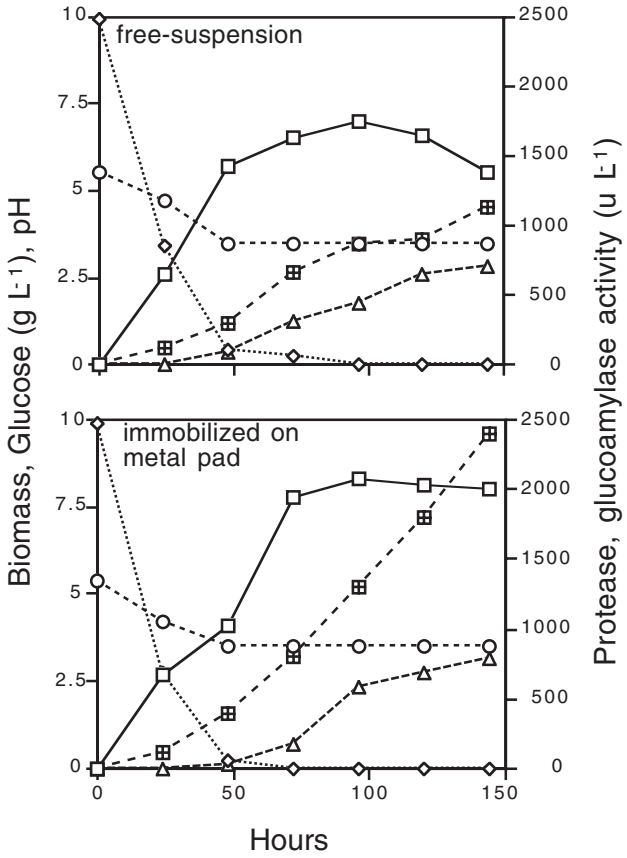

Figure 1 Time-courses of fermentation parameters in free-suspension and immobilized *A. niger* shake-flask cultures. (—□—) Biomass, (···◇···) glucose, (--○--) pH, (--△--) protease, (--⊞--) glucoamylase.

in terms of activity was noted at 145 h with the termination of the runs (705 U l^{-1}). In terms of specific activities, this corresponded to 128.2 U g^{-1}. The maximum glucoamylase specific activity in free-suspension shake-flask culture was 205 U g^{-1}.

In the presence of the aluminium-coated pad in the flasks, the mycelium grew completely attached on it. The mycelium grew densely interwoven and cell growth on the pad reached a maximum biomass of 8 g l^{-1}. The pH profile of the immobilized culture was similar to that of free-suspension culture. Figure 1 shows the time profile of the culture grown immobilized on the aluminium-coated pad. Secretion of protease was significantly lower under these conditions, while glucoamylase levels increased.

The immobilization technique of cell attachment on a porous metal material, as described above with the shake-flask culture, was tested under different culture conditions using an airlift bioreactor. The immobilization material, this time a stainless steel screen rolled up to form a scroll, served as the draft tube fitted in the original BC reactor for airlift operation. Growth on the BC bioreactor was not in the pelleted form as in the free-suspension culture in shake flasks, but in free filamentous form. The different mixing regime obviously affected the morphological development of the fungus. Biomass levels reached 6.5 g l^{-1}, while the secreted protease activity reached 2.548 U l^{-1}. The specific activity of extracellular protease was 606.67 U g^{-1} at the end of the run at 136 h. When the same bioreactor was operated as an airlift, the pH profile of the culture was similar to that in the BC, the biomass levels were almost the same as in the BC (6.8 g l^{-1}), while the extracellular protease specific activities were drastically reduced (102.5 U g^{-1}), as shown in Figure 2. Glucoamylase production, in terms of specific activities, was again significantly higher in the case of the immobilized culture.

The effects of cell immobilization on the secretion of extracellular proteases and glucoamylase production were also studied using a different immobilization technique, which involved the entrapment of spores onto a porous material. For this purpose, porous Celite beads were used and proved to be of high affinity for spore entrapment and growth of *A. niger*. Porous Celite beads and rods are used primarily as supports for the immobilization of whole cells [5] and fungi [6,9], and less frequently of isolated enzymes [2]. Gbewonyo and Wang [8] used Celite beads for spore entrapment and growth of *Penicillium chrysogenum* in an attempt to alter the morphology of non-Newtonian mycelial cultures. The criteria used for selection of the bead material included the affinity of the material for attachment of mycelial cells, being nontoxic to growth, and resistance to microbial degradation. Celite is an inexpensive natural product derived from the fossilized shells of diatoms. The main chemical constituent is silica, SiO_2, which constitutes 90% by weight of Celite, while smaller amounts of other inorganic oxides such as Al_2O_3, FeO_3 and CaO are also present. In the fermentation industry, Celite is commonly used as a filter aid in antibiotic recovery operations and for the adsorption of biocatalysts and it is commercially available in different forms, which vary in particle size, shape and porosity.

To initiate mycelial growth on Celite beads, a special technique described in the previous section was followed, based on a method developed by Gbewonyo and Wang [8] for *P. chrysogenum* spores. A very rapid rate of uptake of the spores occurred as soon as the spore suspension was added to the dry beads. Almost 50% of the spores were removed from suspension onto the beads within the first 5 min of contact. After 1 h, an equilibrium was attained with about 80% of the spores taken up onto the beads. About 90% retention of the entrapped spores was obtained after shaking the inoculated beads at 220 ×g on the shaker incubator. One hour of contact was found adequate to attain satisfactory inoculation of the

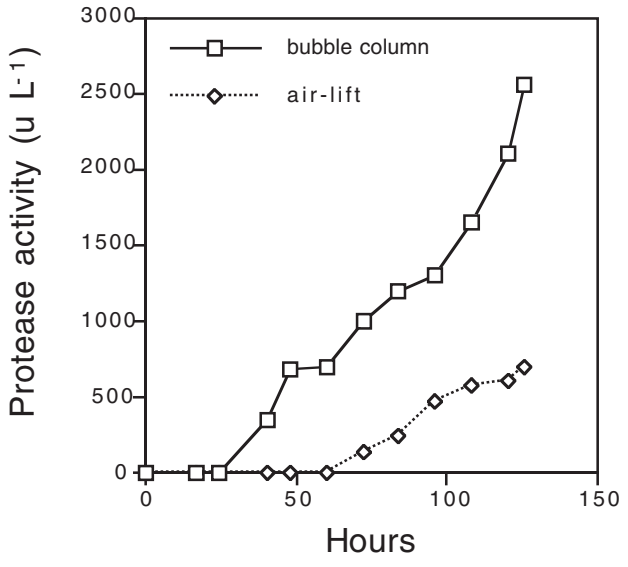

Figure 2 Time-courses of secreted protease activities in the BC bioreactor (free-suspension culture) and the airlift bioreactor (immobilized culture).

beads with the spores. The desired spore loading (10^5 spores ml^{-1}) was obtained by varying the initial concentration of spores in the inoculum suspension. Hyphal growth in this case was initiated within the internal matrix of the beads at the sites of spore entrapment. The filaments then proceeded to elongate along the interconnected pore paths in the bead eventually extending to the external surface of the beads. Progressively, large spherical particles were obtained with the beads entirely covered with the mycelium, which emerged from the pores of the beads. Biomass levels were lower compared to other cultures' (5.1 g l^{-1}), while protease secretion did not exceed 311 U l^{-1}, which corresponded to 61 U g^{-1} specific activities. Figure 3 shows the time profile of the immobilized culture on Celite beads.

The results in this study point out that under a variety of culture conditions the secretion of proteases was always drastically reduced when the fungus grew in immobilized form. Table 1 summarizes the effects of immobilization on specific activities of extracellular proteases and glucoamylase by *A. niger*. Although the pH was not controlled, its profile was similar in all experiments, a situation that ensures similar conditions for determination of enzyme activity. According to a recent review by van den Homberg *et al* [26], analysis of the proteolytic spectrum in *A. niger* revealed that four acid proteases, two aspartyl proteases and two serine carboxypeptidases, predominate and these extracellular proteases are probably involved in hydrolysis of nutrients to be used as carbon, nitrogen and sulfur sources. The low pH levels (pH 3.5) were expected, as *A. niger* is known for its strong acidification of culture media, which explains why acidic protease activities cause major problems during homologous and heterologous protein expression [14].

The exact mechanism that leads to decreased protease and increased glucoamylase secretion in immobilized cultures is not known and therefore needs further investigation. There are many studies that have investigated the effects of cell immobilization of a wide range of microorganisms and supporting materials [1,15–17,24,28]. These studies suggest that the reason that underlies the phenomena is in physiological differences between microbial cells growing at a solid–liquid interface as opposed to growing in liquid medium. Parameters such as altered gas diffusion, substrate concentration and pH gradients, biomass density, metabolite accumulation, surfactant production and accumulation of inorganic ions have been discussed as possible inducers of genetic responses or physiological changes at surfaces and within biofilms. Cell attachment and subsequent surface colonization is recognized as a major phenomenon in environmental and industrial habitats [7]. Naturally attached cultures, as well as those obtained by artificial immobilization *in vitro*, share a common feature: cell–solid surface contact. An essential question is whether cells use this physical contact for nutritional or protective purposes, or whether these interactions are also perceived as mechanophysiological stimuli modulating cellular structure or function. Comparisons between free and attached (immobilized) cells have demonstrated phenotypic differences, showing that the immobilization of cells may be a process of fundamental importance, affecting the cell wall [14] or the plasma membrane composition [13] and stimulating responses that may be important for expression of a diferentiated state.

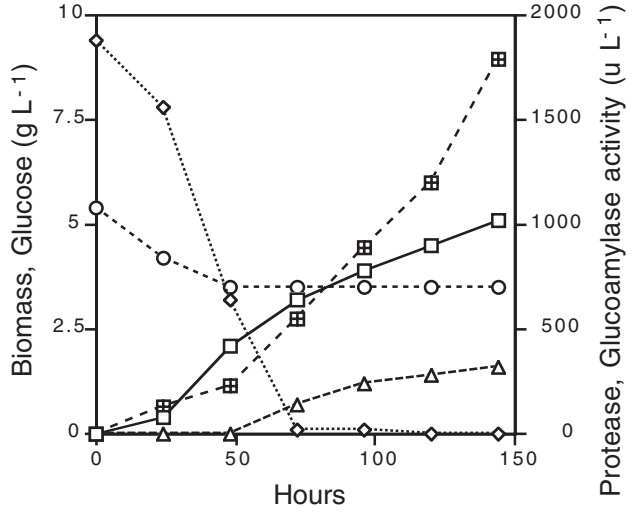

Figure 3 Time-courses of fermentation parameters in *A. niger* shake-flask cultures immobilized on Celite beads. Legends as in Fig. 1.

Table 1 Cell immobilization effects on specific activities of secreted protease and glucoamylase in shake-flask and bioreactor cultures

A. niger culture	Specific activities of secreted protease (U g^{-1})	Specific activities of glucoamylase (U g^{-1})
Free-suspension (shake flasks)	128±20	205±35
Growth on metal coated pad (immobilized shake flasks)	98±10	300±25
Growth on Celite beads (immobilized shake flasks)	61±10	350±20
Free suspension in BC	606±50	150±30
Growth on metal screen (immobilized airlift)	102±10	402±20

In an earlier study on the same production system [22] we showed that pelleted morphologies are associated with lower levels of secreted preoteases compared to filamentous morphologies. The results reported here show that attachment of cells to solid surfaces further enhances this trend. It is interesting, however, that natural mycelial aggregation in the form of pellets and attachment of cells on solid surfaces or growth on porous materials appear to have the same effect on protease secretion and glucoamylase production by *A. niger*. Obviously, the high level of cell-to-cell interaction and signalling resulting from short diffusional distances leads to a state of differentiation quantitatively different from that of freely dispersed mycelia.

References

1 Araujo MLGC, RP Oliveira, RC Giordano and CO Hokka. 1996. Comparative studies on cephalosporin C production process with free and immobilized cells of *Cephalosporium acremonium* ATCC 48272. *Chem Eng Sci* 51: 2835–2840.
2 Basso A, L Martin, C Ebert, L Gardossi and P Linda. 2000. Controlling the hydration of covalently immobilized penicillin G amidase in low-water medium: properties and use of Celite R-640. *J Mol Catal B Enzym* 8: 245–253.
3 Berka RM, M Ward, LJ Wilson, KJ Hayenga, KH Kodama, LP Carlomagno and SA Thomson. 1990. Molecular cloning and deletion of the aspergillopepsin A gene from *Aspergillus awamori*. *Gene* 86: 153–162.
4 Braun S and SE Vecht-Lifshitz. 1991. Mycelial morphology and metabolite production. *TIBTECH* 9: 63–68.
5 Chun GT and SN Agathos. 1991. Comparative studies of physiological and environmental effects on the production of cyclosporin A in suspended and immobilized cells of *Tolypocladium inflatum*. *Biotechnol Bioeng* 37: 256–265.

6 Chun GT and SN Agathos. 1993. Dynamic response of immobilized cells to pulse addition of L-valine in cyclosporin A biosynthesis. *J Biotechnol* 27: 283–294.
7 Costerton JW, KJ Cheng, GG Geesey, TI Lodd, JC Nickel, M Dasupta and TJ Marrie. 1987. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 41: 435–467.
8 Gbewonyo K and D Wang. 1983. Confining mycelial growth to porous microbeads: a novel technique to alter the morphology of non-Newtonian mycelial cultures. *Biotechnol Bioeng* 25: 967–983.
9 Gikas P and AG Livingston. 1997. Specific ATP and specific oxygen uptake rate in immobilized cell aggregates: experimental and theoretical analysis using a structured model of immobilized cell growth. *Biotechnol Bioeng* 55(4): 660–673.
10 Gouka RJ, PJ Punt and JGM Hessing. 1996. Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. *Appl Environ Microbiol* 62: 1951–1957.
11 Gouka RJ, PJ Punt and CAMJJ van den Hondel. 1997. Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl Microbiol Biotechnol* 47: 1–11.
12 Gwynne D and M Devchand. 1992. Expression of foreign proteins in the genus *Aspergillus*. In: Bennett JW and MA Klich (Eds), *Aspergillus*: Biology and Industrial Applications. Butterworth Heinemann, Boston, MA, pp. 202–214.
13 Jirku V. 1992. Permeabilization of covalently immobilized *Saccharomyces cerevisiae*. *Acta Biotechnol* 12: 333–336.
14 Jirku V. 1996. The use of covalently immobilized mycelia to modify the b-glucanase system of *Aspergillus vesicolor*. *J Chem Technol Biotechnol* 65: 179–185.
15 Jirku V. 1997. Changes in the starvation response through covalent cell attachment. *Antonie van Leeuwenhoek* 71: 369–373.
16 Laca A, C Quiros, LA Garcia and M Diaz. 1998. Modelling and description of internal profiles in immobilized cell systems. *Biochem Eng J* 1: 225–232.
17 Lee TH, GT Chun and YK Chang. 1997. Development of sporulation/immobilization method and its application for the continuous production of cyclosporin A by *Tolypocladium inflatum*. *Biotechnol Prog* 13: 546–550.
18 Lin W, RS Feldberg and E Clark. 1993. Kinetics of cell growth and heterologous glucoamylase production in recombinant *Aspergillus nidulans*. *Biotechnol Bioeng* 41: 273–279.
19 Mattern IE, JM van Noort, P Berg, DB Archer, IN Roberts and CAMJJ van den Hondel. 1992. Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Mol Gen Genet* 234: 332–336.
20 Papagianni M. 1995. Morphology and citric acid production in *Aspergillus niger* submerged fermentation. PhD thesis. University of Strathclyde, Glasgow, UK.
21 Papagianni M. 1999. Fungal morphology. In: Kristiansen B, M Mattey and J Linden (Eds), Citric Acid Biotechnology. Taylor and Francis, London, pp. 69–84.
22 Papagianni M and M Moo-Young. 2002. Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochem* 37: 1271–1278.
23 Schügerl K, SR Gerlach and D Siedenberg. 1997. Influence of the process parameters on the morphology and enzyme production of *Aspergilli*. *Adv Biochem Eng Biotechnol* 60: 195–267.
24 Shuler ML and F Kargi. 1992. Bioprocess Engineering. Prentice Hall, NJ.
25 van den Homberg JPTW, PJI van de Vondervoort, NCBA van der Heijden and J Visser. 1995. New protease mutants in *Aspergillus niger* result in strongly reduced *in vitro* degradation of target proteins; genetical and biochemical characterization of seven complementation groups. *Curr Genet* 28: 299–308.
26 van den Homberg JPTW, PJI van de Vondervoort, L Fraissinet-Tachet and J Visser. 1997. *Aspergillus* as a host for heterologous protein production: the problem of proteases. *TIBTECH* 15: 256–263.
27 van Suijdam JC, NWF Kossen and PG Paul. 1980. An inoculum technique for the production of fungal pellets. *Eur J Appl Microbiol Biotechnol* 10: 211–221.
28 Willaert RG, GV Baron and L De Backer. 1996. Immobilized living cell systems. Wiley, Chichester.
29 Wittler R, H Baumgardi, DW Lübbers and K Schügerl. 1986. Investigations of oxygen transfer into *Penicillium chysogenum* pellets by microprobe measurements. *Biotechnol Bioeng* 28: 1024–1036.