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Research review paper

# Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations

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## Abstract

Filamentous fungi have long been used for the production of metabolites and enzymes. With developments in genetic engineering and molecular biology, filamentous fungi have also achieved increased attention as hosts for recombinant DNA. However, the production levels of non-fungal proteins are usually low. Despite the achievements obtained using molecular tools, the heterologous protein loss caused by extracellular fungal protease degradation persists. This review provides an overview of the potential bioprocessing strategies that can be applied to inhibit protease activity thereby enhancing heterologous protein production. © 2004 Elsevier Inc. All rights reserved.

Keywords: Filamentous fungi; Heterologous proteins; Bioprocessing; Protease degradation; Fermentation process

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## 1. Introduction

Filamentous fungi are eukaryotic microorganisms that influence our everyday lives in areas as diverse as medicine, agriculture, and basic science. They have long been applied in the traditional food fermentation industry, such as the koji process (Hara et al., 1992). With the growth of the biotechnology industry, filamentous fungi have been widely employed in the fermentation industry, and become a principal source of enzymes and metabolites, and therefore have been widely investigated. Features such as low cost and high productivity have attracted many research efforts in both molecular-genetic techniques and bioprocess improvements (Finkelstein and Ball, 1992; Banerjee et al., 2003).

With developments in genetic engineering and molecular biology, filamentous fungi have also achieved increased attention as hosts for the production of heterologous proteins. The problem encountered is that the production levels by fungi of most non-fungal proteins of mammalian, bacterial or plant origin are much lower than those of homologous proteins, usually only reaching a few tens of milligrams per liter of culture medium (Gouka et al., 1997). Various efforts have been made in both strain and fermentation process improvements. From the aspect of strain improvement, Punt et al. (2002) reviewed filamentous fungi as hosts for heterologous protein production. This review illustrates the research efforts in bioprocessing used to enhance the production of heterologous proteins from filamentous fungi.

#### 2. Expression of foreign genes in recombinant filamentous fungi

Filamentous fungi are known to produce a broad range of enzymes in large quantities. Knowledge of fungal genetics and fungal biochemical pathways has accumulated from years of research and industrial applications. Many species have Generally Regarded As Safe (GRAS) status in the food and food processing industry. Filamentous fungi can secrete larger amounts of proteins, perform post-translational modifications such as glycosylation and disulfidation properly, and grow on a less expensive medium. Most filamentous fungi are transformed by plasmids that integrate into the fungal genome, suggesting potentially superior long-term stability of the fungal transformants. For these reasons, filamentous fungi have tremendous potential as hosts of recombinant DNA. In recent years, great improvements have been achieved (Wiebe, 2003). Several efficient transformation systems have been developed for a large number of fungal species. The genus *Aspergillus* in particular has been used with success as a

host for the production of recombinant proteins of both fungal and non-fungal origins, e.g., glucoamylase, bovine chymosin, human lactoferrin, hen egg-white lysozyme, human interleukin-6, and thaumatin. Other host strains include *Trichoderma reesei*, *Chrysosporium lucknowense*, *Mortierella alpinis*, etc. (Mach and Zeilinger, 2003; Gouka et al., 1997; Maras et al., 1999; Van den Hombergh et al., 1997). Punt et al. (2002) presented a rather detailed review.

Although the homologous protein production by filamentous fungi can reach the level of tens of grams per liter, the production levels of heterologous proteins can be several orders of magnitude lower. Several factors that negatively affect the production levels of non-fungal proteins have been reported, showing that the production can be limited at any level, i.e., transcription, translation, secretion, and extracellular degradation (Punt et al., 1994; Archer and Peberdy, 1997).

Protein secretion plays an important role in filamentous fungi and is an attractive feature of filamentous fungi as hosts of rDNA. However, few studies of protein secretion have been made with filamentous fungi; the molecular understanding of the protein secretion in fungi is still lacking, which led Peberdy (1994) to refer to it as "a highly productive black box". Some recent studies showed that gene expression and protein secretion in solid-state fermentation might be quite different from that in submerged fermentation (Iwashita, 2002). For example, in submerged cultures, some enzyme activities are found mainly in the cell wall of mycelia. However, in solidstate cultures, these enzyme activities are observed in the medium, while little activity is observed in the cell wall (Iwaihiia et al., 1998; Hashimoto et al., 1999). This suggests that the secretion of proteins is strongly affected by culture conditions. It is known that the secretory pathway performs the functions of protein folding, glycosylation, processing, etc. The cell wall acts as 'a barrier to all secreted proteins to some extent and many fungal enzymes are partially cell wall-associated' (Gordon et al., 2000; Archer and Peberdy, 1997). Seen from outside the 'black box', protein secretion in filamentous fungi is believed to occur mainly at the tips of growing hyphae, since the growing hyphal tips are more porous, making it easier for the exoenzymes to pass through the cell wall (Peberdy, 1994; Punt et al., 1994). Therefore, factors that increase the number of active tips, such as the properly controlled fungal morphology, may improve the protein yield (Pluschkell et al., 1996; Juge et al., 1998).

Even with successful secretion of heterologous proteins, a considerable fraction of the yield can be lost due to extracellular protease degradation. Heterologous proteins are more prone to proteolysis than homologous proteins. *Apergilli* can secrete a diversity of extracellular proteases, and it has been shown that proteases are responsible for the degradation of many heterologous proteins (Van den Hombergh et al., 1997). Proteolytic degradation by fungal proteases is recognized as one of the major problems interfering with efficient heterologous protein production.

To improve the non-fungal protein production, several strategies have been developed, both genetic and bioprocessing in nature. The genetic strategies include: the introduction of multicopies of the protein gene; the use of strong promoters and efficient secretion signals; gene fusion with a gene encoding all or part of a well-expressed and secreted protein; and the construction and use of protease-deficient host strains (Punt et al., 2002). So far, most researchers are concentrating their efforts on selecting and using proteasedeficient mutant strains as hosts for r-DNA to overcome or alleviate protease degradation problems. However, such strains are usually so debilitated that they are not desirable for large-scale bioreactor applications. Bioprocess strategies have also been shown to be of great help (Wang et al., 2003; O'Donnell et al., 2001; Xu et al., 2000). However, systematic research in these areas is inadequate. This will be the focus of the remainder of this review.

#### 3. Bioprocessing strategies to improve heterologous protein production

Fungal culture systems can be either solid-state or submerged. However, in industry, fermentation usually implies an emphasis on submerged liquid cultivation systems. This review will primarily focus on the progress in submerged cultures. A fermentation system is a complicated multi-phase, multi-component system. Growth and production are affected by a wide range of parameters, including cultivation medium, inoculum, pH, temperature, aeration agitation, shear stress, etc. Compared to many unicellular microbes, filamentous fungi fermentation processes present special challenges in optimization and scale-up because of the varying fungal morphological forms.

#### 3.1. Fungal morphology

Morphological variety is a typical feature of filamentous fungi. Fungal morphology has distinct effects on the rheological nature of a fermentation broth. The effects of broth rheology on mass, momentum, and heat transfer within a bioreactor have been well studied (Moo-Young et al., 1987; Charles, 1985; Funahashi et al., 1988).

In submerged fermentation, two extreme types of morphology are generally known, pellets and free filaments (also called dispersed hyphae). Between these two extremes lies an intermediate aggregated (but still dispersed) morphology termed clumps. For producing fungal metabolic products, the desired morphology varies from one product to another. For example, free mycelia are required for the production of penicillin from *Penicillium chrysogenum*, whereas pellets are required for the production of citric acid from *A. niger* (Vecht-Lifshitz et al., 1990). Different morphological forms result in different types of broth rheology. Cultures with filamentous growth usually exhibit a high apparent viscosity and non-Newtonian rheology. At moderate to high biomass levels, these broths display 'shear thinning or pseudoplasticity' (Harvey and McNeil, 1994). These effects can have a number of undesirable results, such as poor mass transfer, and as a result, decreased overall productivity. Image analysis is a powerful and accurate method of measuring morphological parameters. It is recommended for any study where the development of hyphae is important (Tucker et al., 1992).

Cultures exhibiting pelleted growth usually exhibit a low apparent viscosity and more or less Newtonian rheology. The fermentation broth is often assumed to be well mixed, and problems associated with poor bulk mixing and gas–liquid mass transfer are assumed to be slight in smaller, lab-scale fermenters. However, the centers of large pellets can suffer from oxygen starvation due to a mass transfer limitation, leading to autolysis. A 'critical radius' for pellets exists due to mass transfer (Metz and Kossen, 1977; Kobayashi et al., 1973).

Many parameters influence pellet formation, including inoculum level, initial pH, agitation, medium composition, and use of polymer additives or surface-active agents (Metz and Kossen, 1977). Among them, the inoculum level is generally recognized as one of the most important factors. When the medium is inoculated with spores, filamentous growth is observed at high initial spore levels, while pellets of increasing size are formed as the inoculum level is reduced (Metz and Kossen, 1977; Nielsen and Carlsen, 1996; Jimenez-Tobon et al., 1997). The initial medium pH also plays an important role in fungal morphology (Whitaker and Long, 1973). Higher pH values (5–6) produce pellets, while low pH values (2–3) lead to filamentous mycelium. The surface properties of the spores are influenced by pH and are responsible for this effect (Wainwright et al., 1993).

Fungal morphology to a large extent is affected by agitation in a stirred-tank bioreactor. Strong agitation will form free filaments. When pellets are formed, the pellet size, structure and survival are also affected by agitation. In general, more agitation yields smaller and more compact pellets (Lejeune and Braon, 1995; Mitard and Riba, 1988). Polymer additives can also influence the fungal morphology. Some polymer additives (sodium alginate, dextran, polyacylic acid, and carbopol) cause a more dispersed filamentous growth in comparison with the pronounced pellet growth under normal conditions (Elamayerhi, 1975; Elamayerhi and Scharer, 1973; Elamayerhi et al., 1973; Jones et al., 1988a,b). However, some researchers found an increase in pellet size with the addition of sodium-carboxymethycellulose (CMC) (Metz and Kossen, 1977).

To describe these fungal forms, microscopic morphology (micromorphology) and macroscopic morphology (macromorphology) are introduced. Micromorphology describes the shape and size of individual hyphal elements, e.g., the total hyphal length and number of tips, while macromorphology describes the shape and size of pellets (Nielsen, 1992; Nielsen and Krabben, 1995). So far, there are no standard methods of measurement and description of the mycelia clumps/pellets. Pellet size and structure vary greatly based on cultivation conditions. Measurements on hyphae within the three-dimensional structure of clumps or pellets are difficult. The parameters used vary by researcher. Pellet diameter is the most frequently used parameter. Others include pellet porosity, density, hair length, etc. (Cui et al., 1997a).

Several mathematical models have been presented in the literature to describe growth, given the large diversity of fungal morphology. For example, the *Tubular reactor analogy model* (Aynsley et al., 1990), *population model* (Nielsen and Krabben, 1995), *fractal model* (Bozhokin, 1996), etc. have been developed for micromorphology; and *cube-root* growth kinetics (Trinci, 1970), *pellet size distribution model* (Edelstein and Hadar, 1983), *fractal morphology* (Ryoo, 1999), etc. have been developed to explain macromorphology.

Although many models have been proposed to describe fungal morphology, few simultaneously deal with the influence of morphology on product formation. In fact, the relationship between fungal morphology and product formation is difficult to investigate, as there are many interrelated factors that exist in a fermentation system that affect both morphology and protein production.

Johansen et al. (1998) reported that morphological differences had only a limited effect on product formation, suggesting that the structural features such as hyphal length and the

number of tips are of less importance for product formation. Some studies indicate the primary effect of morphology on product formation is due to viscosity. When the fungal morphology is better controlled, the productivity can be increased. Bhargava et al. (2003a,b) found that when a fed-batch, process strategy using the pulsed addition of a limiting-carbon source, is used to control the fungal morphology and thus to reduce fungal broth viscosity, increased productivity of recombinant glucoamylase was observed. Many others, however, have reported productivity to be a function of morphological variation. Bocking et al. (1999) reported that in dissolved oxygen tension controlled fed-batch cultures of A. oryzae, producing  $\alpha$ -amylase and heterologous glucoamylase, one highly branched mutant made more total enzymes (24.3 g) than the parental strain (21.7 g). For pelleted growth, El-Enshasy et al. (1999) reported that the smaller fungal pellets formed during bioreactor cultivation were more efficient with respect to the production of an exocellular enzyme. It is generally believed that protein secretion in filamentous fungi mainly occurs at the tips of growing hyphae (Peberdy, 1994; Punt et al., 1994), and those factors that increase the number of active tips may improve yield (Pluschkell et al., 1996; Juge et al., 1998).

It is generally believed from the various studies that fungal morphology affects fermentation processes producing secreted proteins in two ways. First, it causes an apparent viscosity change of the broth; secondly, it may change the number of active tips of the mycelia. In the case where protein secretion acts as the bottleneck, the variation in the number of active of tips may cause a significant difference in final productivity (Peberdy, 1994; Allen and Robinson, 1990).

Researchers have seen a dramatic effect of morphology on protein production. The dependence of protein production on fungal morphology has been investigated in shake flasks cultures by employing the recombinant *A. niger* strain AB4.1[pgpdAGLAGFP]#11, which carries a gene for the glucoamylase-green fluorescence protein (GFP) fusion protein (Xu et al., 2000). Different inoculum levels were used to obtain different sizes of pellet or free mycelia. The extracellular protease activity of the cultures varied with the pellet size and decreased dramatically when the morphology was changed from free mycelia to pellets, resulting in a dramatic 3.4-fold increase in the GFP yield. The results indicate that a morphology control strategy can be effective in inhibiting protease activity in filamentous fungal fermentation, thereby enhancing heterologous protein production.

## 3.2. Fungal mycelia immobilization

Microbial cell immobilization refers to the systems or techniques in which "there is a physical confinement or localization of microorganisms that permits their economic reuse" (Anderson, 1975). Immobilized cells and enzymes have been attracting great attention since the 1970s. Initial interest in immobilized cells was directed towards their use as alternatives to certain immobilized enzymes, since whole cells obviated the need for enzyme isolation and purification. Initially, most work was carried out on the immobilization of bacterial cells. Eventually, yeasts and filamentous fungi received increasing attention (Anderson, 1975). Recently, there has been an appreciation of the potential of immobilized fungal cells for the production of metabolites such as various organic acids (Federici and Petruccioli, 1997; Vassilev and Vassileva, 1992; Garg and Sharma, 1992; Sakurai et al., 1997; Vassilev et al., 1993; Kwak and Rhee, 1992; Kautola et al., 1991) and enzymes such as glucose oxidase (Fiedurek and Ilczuk, 1991) and glucoamylase (Abraham et al., 1991).

Various immobilization methods and materials have been reported. These methods include adsorption, entrapment, cross-linking, and covalent bonding. Among them, adsorption and entrapment are most frequently used (Federici and Petruccioli, 1997; Vassilev and Vassileva, 1992; Arica et al., 1993; Ramakrishna and Prakasham, 1999; Lozinsky and Plieva, 1998; West and Strohfus, 1997; Seifert and Phillips, 1997). Pelleted fungal growth can also be viewed as a self-immobilization system (Anderson, 1975; Xu et al., 2000).

Immobilized fungal cells have several advantages over dispersed cells. Immobilized cell systems make it easy to separate cells from the liquid medium, which makes repeated batch culture possible and simplifies the operation of both the continuous culture and subsequent downstream processes. Cell immobilization lowers the apparent broth viscosity and makes the rheological features more favorable for oxygen supply and mass transfer. (Thongchul and Yang, 2003). Moreover, cells are protected from shear damage by immobilization, resulting in higher cell loading, and higher volumetric productivities (Vassilev and Vassileva, 1992; Fiedurek and Ilczuk, 1991; Abraham et al., 1991). When applied to recombinant strains, cell immobilization can also alleviate strain genetic stability problems (Caunt et al., 1988; Dincbas et al., 1993).

Another advantage of cell immobilization is a reduction in the protease activity. Liu et al. (1998) studied the effect of fungal cell immobilization on the inhibition of protease secretion by a wild-type *A. niger* (ATCC 13496). In their work, cell immobilization was performed in shake flask culture on a stainless steel scouring ball, aluminum screen, cotton thread, metal-coated pad of polyester latex felt, etc. It was found that compared with the free suspension culture, the maximum specific activity of the extracellular proteases from immobilized cells was reduced from 129 to 28 units/g cell dry weight. A similar reduction in protease activity was also achieved with immobilized culture in a 3-L (working volume) airlift reactor (10 cm ID, 50 cm length) using a multi-layer rolled stainless steel screen in the center as both a draft tube and the immobilization substrate. These results suggest that immobilized cell culture has a high potential for increasing heterologous protein production from recombinant filamentous fungi.

Successful applications of immobilized fungal cells in industry remains limited due to its disadvantages. The process of immobilization may be uneconomical. Mass transfer limitations have an important effect in altering the physiology and kinetics of cells. The product of interest has to be secreted from the cell. The immobilizing matrix might be disrupted because of cell growth and gas evolution due to cell metabolism. Oxygen mass transfer limitations are more severe in an immobilized cell culture as compared to a suspended cell culture. Due to the oxygen supply problems, immobilization techniques have been mainly confined to anaerobic processes (Anderson, 1975). Pelleted growth can overcome this problem if the pellet size is maintained below the critical diameter mentioned earlier.

In conclusion, immobilized cell culture systems offer a great potential for industrial applications, but with certain unresolved technical difficulties. Additional research investigating their proper application is necessary.

#### 3.3. Fermentation pH effects

Many genes in fungi are regulated by ambient pH. A detailed review on pH regulation of gene expression in fungi has been provided by Denison (2000). It was found that, in A. niger, production of extracellular proteases and phosphatases are regulated by ambient pH, e.g., acid proteases are only expressed at an acidic pH (Denison, 2000). A. niger is well known for acidifying the medium as it grows. A pH regulatory system may be especially important. Apart from the regulatory effect on gene expression, cultivation pH can also affect fungal morphology greatly (Whitaker and Long, 1973). Thus, development of an optimal pH control strategy is helpful in obtaining higher protein productivity. Swift et al. (1998) reported that, in the pH auxostat culture of A. niger B1, pH values had effects on morphological mutants formation and the recombinant glucoamylase production. O'Donnell et al. (2001) investigated a pH control strategy to inhibit extracellular protease activity and to enhance the heterologous protein (GFP) production from the recombinant A. niger AB4.1(PgpdAGLAGFP)#11. Cultures grown at pH 6 demonstrated an 85% decrease in extracellular protease activity and a 10-fold increase in GFP yield as compared to either growth at pH 3 or without pH control. This work illustrated that implementing a pH control strategy could be a viable method for decreasing extracellular protease activity, thereby increasing recombinant protein yields.

## 3.4. Cultivation medium

The carbon and nitrogen source are two important factors affecting cell growth and product formation of microorganisms. For example, it was reported that the production of  $\beta$ -fructofuranosidase by A. japonicus could vary by 100 times on different carbon sources, i.e., corn starch, maltose, fructose, glucose, galactose, sorbital, and sucrose, the latter of which was found to be the best for  $\beta$ -fructofuranosidase production (Chen and Liu, 1996). Carbon and nitrogen sources may have either repressing or inducing effects on enzyme productions. Glucoamylase, amylase, and  $\alpha$ -glucosidase are all 'upregulated' (induced) by starch and 'down-regulated' (repressed) by glucose (Archer and Peberdy, 1997). When the expression of recombinant genes is under the control of A. niger glucoamylase promoter (glaA), similar inducing and repressing profiles were found for protein production, i.e., starch is better than maltose, while maltose is better than glucose as the substrate (inducer) (Siedenberg et al., 1999; Mackenzie et al., 1994). Most extracellular proteases are repressed under the conditions of high glucose and ammonium levels in the medium. For example, the extracellular proteases of A. nidulans are subjected to carbon, nitrogen and sulfur metabolite reprocessing. The depletion of low molecular weight sources of nitrogen, carbon, phosphorus, and sulfur elevates protease activity (Van den Hombergh et al., 1997; Katz et al., 1996). Since proteases were found to be responsible for the loss of many heterologous proteins, the regulation of proteases by carbon and nitrogen sources would benefit the heterologous protein production. Some researchers applied statistical factorial designs for the culture media optimization (Ooijkaas et al., 1999; Gomes et al., 2000; Manimekalai and Swaminathan, 1999).

#### 3.5. Agitation intensity and dissolved oxygen tension

Agitation intensity may affect both fungal morphology (Amanullah et al., 1999, 2002; Cui et al., 1998a,b) and mass transfer in the bioreactor (Badino et al., 2000; Li et al., 2002). In many fungal fermentations, a high agitation rate is necessary to provide adequate mixing and mass transfer, especially when the fungal cells grow in a freely dispersed form which results in a non-Newtonian broth and a high apparent viscosity. However, mechanical forces can cause mycelia damages. Thus, the agitation rate is limited to a range that avoids exerting high shear stresses on fungal mycelia.

In a chemostat culture of a recombinant A. oryzae, Amanullah et al. (1999) investigated the effects of agitation intensity on mycelial morphology and protein production ( $\alpha$ -amylase and amyloglucosidase). Fungal morphology was measured by mycelia fragmentation (for both clumps and freely dispersed forms) and by tip number (for the freely dispersed form). It was found that mycelia morphology varied significantly when the agitation rate changed in the range 550–1000 rpm. However, protein productions were not affected by the significant variations in mycelia morphology, as long as the specific growth rate held constant. The same result was obtained in a fed-batch culture (Amanullah et al., 2002). Their conclusion was that the agitation intensity needed to be manipulated to meet process requirements in terms of the dissolved oxygen level and bulk mixing. Although mycelia morphology could change greatly with agitation, the recombinant amyloglucosidase production was not comprised as long as the broth was well mixed and oxygen supply was adequate. Conditions may be strain specific. In a certain way this conclusion supports the result obtained by Johansen et al. (1998), which stated that morphological differences had only a limited effect on product formation; the primary effect of morphology on product formation is due to viscosity.

Oxygen is often a limiting component in fermentations because of its low solubility and the low volumetric mass transfer in bioreactors. Some researchers used oxygen instead of air to maintain the dissolved oxygen (DO) at a higher level. The dissolved oxygen tension may affect productivity, cell autolysis, fungal morphology, etc. (Cui et al., 1998a,b). The effect of DO on fungal fermentation can be either positive or negative. In the culture of aerobic microorganisms, oxygen serves as a substrate for energy generation. It may enhance the cell growth and protein production directly or through changes in morphology. The negative effect can be caused by oxygen toxicity or undesirable morphological changes (Wongwicharn et al., 1999; Rothberg et al., 1999; Kreiner et al., 2000).

The effect of DO on fungal morphology has been reported in the literature. Cui et al. (1997a,b) found that pellets were denser when DO was close to the saturation level. While in the case of very low DO levels, pellets were rather weak and fluffy. The biomass per wet pellet volume and the porosity of the pellets were all functions of DO and the pellet size. Smaller pellets formed under higher DO levels had a higher intrinsic strength. Wongwicharn et al. (1999) reported that two distinct morphologies were observed when varying the oxygen enrichment in the gas supply. Under oxygen limiting conditions, long, sparsely branched hyphae with a low percentage of 'active' length were formed. While under higher oxygen enrichment levels (e.g., 30–50%), shorter hyphae with more

branching and a higher percentage of 'active' length were formed. The production of both the native and foreign enzymes was correlated well with the 'active' length, or tip numbers.

Bai et al. (2004) found that the enrichment of oxygen could result in a change in the respiratory pathway, and thus affect protein synthesis greatly. However, the fungal morphology changed simultaneously, and the decreased intracellular protein content correlated with the shortened means of both the main hyphal length and total hyphal length, as well as with oxygen enrichment.

Up to now, it is difficult to draw a general conclusion. It is still unclear whether the variation in final productivity is caused by the change of cell physiology or the change of the fungal morphology. Further investigation is definitely needed.

## 4. Batch, fed-batch, and continuous cultures

In contrast to the wide application of continuous processes in the large-scale chemical industry, most commercial bioprocesses are based on batch reactors, although the advantages of continuous operation appear to be overwhelming if regulatory concerns are not factors. Continuous processes generally have high productivity. This is due to a combination of favorable features, including long runs with a low proportion of downtime for start-up and shutdown, steady-state conditions resulting in easier process control, an even demand on utilities, and consistency of product quality. Batch processing, however, also provides a number of advantages. It is better for non-growth associated product formation and genetic stability, and has a lower risk of contamination (Shuler and Kargi, 1992).

Apart from batch and continuous systems, fed-batch culture has also been employed for the production of various bioproducts, including primary and secondary metabolites, proteins, and other biopolymers. During fed-batch cultivation, one or more nutrients are supplied to the bioreactor, while cells and products remain in the bioreactor until the end of the operation. Fed-batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the productivity and yield of the desired product, as in the case of substrate inhibition.

Fed-batch culture was reported to be successful in alleviating catabolite repression effects (Szabo et al., 1996; Spohr et al., 1998). Many enzymes are subject to catabolite repression where enzyme synthesis is prevented by the presence of a rapidly utilized carbon source. It is obvious that this should be avoided, and fed-batch is therefore appropriate. The substrate (usually the carbon source) that causes catabolite repression is fed to the culture in a concentrated solution at a rate that prevents the substrate from reaching the threshold value for catabolite repression. For example, the cellulase system of *Phanerochaete chrysosporium* is adaptive and thus sensitive to carbon catabolite repression. Szabo et al. (1996) reported that specific yields (cellulase produced per carbon source) in batch cultivations on cellulose were 46–60 mg/g, while in fed-batch cultivation, the production was increased to 120 mg/g when cellulose was the substrate. Spohr et al. (1998) reported that the  $\alpha$ -amylase production by recombinant *A. oryzae* was increased in fed-batch culture compared to batch culture in which the cells were exposed to a high glucose concentration. Fed-batch has also been applied to control

fungal morphology in order to obtain a lower broth viscosity and higher enzyme productivity (Bhargava et al., 2003a,b).

## 5. Concluding remarks

Filamentous fungi have long been employed in the fermentation industry and continue to be the principal source of antibiotics and enzymes, but surprisingly, recombinant mycelial fungi are not currently used in producing heterologous protein biopharmaceuticals. Understanding of the molecular genetics of filamentous fungi has always lagged behind that of yeast and bacteria, but advances within the last decade have provided commercially promising recombinant fungal strains, thus ushering in a new era in fermentation biotechnology. Punt et al. (2002) noted that "it has been process development that has driven the final breakthrough to achieving commercially relevant quantities of proteins". Along with the molecular genetic developments, the research on bioprocessing technologies may have a competitiveness-enhancing role for production of enzymes and healthcare products, especially for generic biopharmaceuticals.

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