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Stable production of recombinant proteins in filamentous fungi – problems and improvements

MARILYN G. WIEBE*

VTT Biotechnology, P.O. Box 1500, FIN-02044, VT Finland
Tel: +35 89 456-5139. Fax: + 35 89 455 2103

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Filamentous fungi such as *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei* are able to produce and secrete large concentrations of enzymes (e.g. amylases, proteases, cellulases) into the environment. Increasingly, these species and a few others are being used to produce recombinant proteins, particularly proteins of fungal origin (Table 1). For producing recombinant proteins (which may be a protein which the host organism already makes, i.e. an homologous protein, or a new, i.e. heterologous, protein), filamentous fungi offer the advantages of possessing an efficient secretion system, being able to glycosylate proteins and having higher specific growth rates than plant, insect or mammalian cells. Although the filamentous growth form causes more difficulties for mixing and aeration than does the unicellular growth form of yeast and bacteria, efficient fermentation technologies have been developed for antibiotic, organic acid and native enzyme production from filamentous fungi.

In order to produce a recombinant protein in a fungus, the gene encoding the protein is introduced to the fungal host on a plasmid which must integrate into the host genome. Self-replicating plasmids are not used for protein production in filamentous fungi. Since the recombinant DNA is integrated into the fungal genome it is not lost because of unequal partitioning, as may occur with the self-replicating plasmids used to produce recombinant proteins in bacteria or yeast (Fig 1). However, the ability to produce the recombinant protein may still be lost for a variety of reasons. Loss of the recombinant DNA from the host cell or loss of expression of this DNA leads to reduced productivity.

Heterokaryosis

Although unequal partitioning of plasmids does not occur when filamentous fungi are transformed with integrating plasmids, the multinucleate state of fungal hyphae means that nuclei which do not contain the integrated DNA may be present in the same compartment as nuclei containing the integrated DNA. These non-transformed nuclei may become isolated from the transformed nuclei within the mycelium to give rise to non-productive sectors or individuals. Regardless of whether transformants are derived from protoplasts, conidia or hyphal fragments, the possibility exists that more than one nucleus may have been present in the transformed propagule. As the recombinant DNA would be unlikely to integrate at the same site in two nuclei the resulting transformant will be heterokaryotic. After isolation of the initial transformant, isolation of single-conidium isolates should reduce the probability that stock cultures are heterokaryotic in species which produce homokaryotic conidia. Repeated isolations may be necessary for species which produce multinucleate conidia which may be heterokaryotic.

Heterokaryosis may also arise after isolation of a single-conidium isolate through loss or mutation of the recombinant DNA (see below). Repeated sub-culturing from the initial single-conidium stock will increase the probability of heterokaryosis occurring. Sporulation and hyphal fragmentation can result in the isolation of nuclei which have lost or fail to express the recombinant DNA and typically these strains increase in the population, displacing the original transformant (e.g. Mainwaring *et al*., 1999; Swift *et al*., 2000).

Fig 2 illustrates the variation between different single-conidium isolates, which may be seen in transformants which either were not taken through a single-conidium isolation process before storage of the stock culture or were maintained by subculturing. After isolation of the original transformant (*Fusarium venenatum* or *Aspergillus niger*), subcultures were made to obtain adequate spores to provide stock cultures. However, when single conidium isolates were obtained

Table 1. Examples of commercially available enzymes produced in recombinant filamentous fungi. Examples of specific applications are listed below the table. (data derived from the Association of Manufactures of Fermentation Enzyme Products, 2001)

Enzyme	Production (host) organism	Source organism	Industry in which applied
Aminopeptidase	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [a] and feed [b]
Catalase	*A. niger*	*Aspergillus* sp.	Food [c, d]
Cellulase	*T. reesei* or *T. longibrachiatum*	*Trichoderma* sp.	Textile [e] and feed [f]
	A. oryzae	*Humicola* sp., *Thielavia* sp., *Myceliopthora* sp.	Textile [d], detergent and pulp and paper
α-Galactosidase	*A. oryzae*	*Aspergillus* sp.	Feed [e]
ß-Glucanase	*T. reesei* or *T. longibrachiatum*	*Trichoderma* sp.	Textile [e, g] and feed [f]
Glucoamylase	*A. niger*	*Aspergillus* sp.	Food [h, i]
Glucose oxidase	*A. niger*	*Aspergillus* sp.	Food [d, j]
Laccase	*A. oryzae*	*Myceliopthora* sp. *Polyporus* sp.	Textile [k]
Lactase	*A. oryzae*	*Aspergillus* sp.	Food [l]
Lipase	*A. oryzae*	*Candida* sp., *Fusarium* sp., *Rhizomucor* sp. or *Thermomyces* sp.	Food [d, m], textile [n], detergent [o], leather [p], pulp and paper [q]
Mannanase	*T. reesei* or *T. longibrachiatum*	*Trichoderma* sp.	Feed [f]
Pectin lyase	*A. niger*, *T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [r], feed [f] and textile [s]
Pectinase	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [r], feed [f] and textile [s]
Pectinesterase	*A. niger*, *A. oryzae* or *T. reesei*	*Aspergillus* sp.	Food [r], feed [f] and textile [s]
Phospholipase A	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [d, t], feed [f] and textile
Phospholipase B	*T. reesei* or *T. longibrachiatum*	*Aspergillus* sp.	Food [d, t] and feed [f]
Phytase	*A. niger*, *A. oryzae*, *T. reesei* or *T. longibrachiatum*	*Aspergillus* sp. or *Peniophora* sp.	Feed [f]
Protease	*A. niger*, *A. oryzae*	calf stomach, *Aspergillus* sp. or *Rhizomucor* sp.	Food [m] and leather [p, u]
Pullulanase	*T. reesei* or *T. longibrachiatum*	*Hormoconis* sp.	Food [d] and feed [f]
Xylanase	*A. niger*, *A. oryzae* *T. reesei* or *T. longibrachiatum*	*Aspergillus* sp. *Thermomyces* sp. *Actinomadura* sp.	Food [d] and feed [f]

[a] reduce bitterness in protein digests, cheese, egg meat and milk industries
[b] pet food (to increase palatability)
[c] cheese, egg, starch, fat, sugar, industries, cold sterilisation of milk
[d] baking applications
[e] stone-washing of denim
[f] increasing the nutritional value of pig/chicken feed (e.g. cellulases and ß-glucanase for barley and oats, xylanase for rye, α-galactosidase & mannanase for legumes, phytase and phospholipases for phosphate release)
[g] treatment of hemp, jute, flax, etc. for textile and rope manufacture
[h] high fructose/glucose syrup production
[i] clarifying juices, wine or beer
[j] stabilizing foods (e.g. egg whites, mayonaise) and beverages (e.g. fruit juice, beer), improving flour
[k] bleaching of dyes such as indigo
[l] dairy products (e.g. yoghurt) and production of lactose syrup
[m] cheese ripening and flavour
[n] increase wettability
[o] stain removal
[p] during soaking and degreasing
[q] removal of pitch
[r] fruit processing
[s] bioscouring (removal of non-cellulose matter from cottons)
[t] production of lyso-lecithin (e.g. for margarine)
[u] liming, deliming and bating, softening

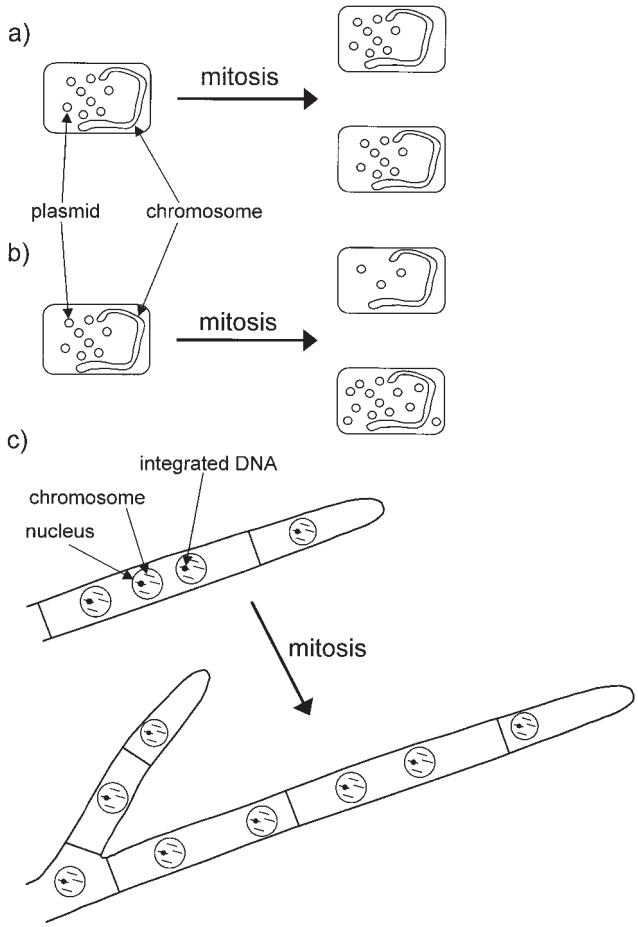

Fig 1 The fate of DNA introduced into cells as self-replicating plasmids or on integrating vectors. Self-replicating plasmids in bacteria and yeast may be a) equally partitioned between daughter cells or b) unequally partitioned between daughter cells. c) DNA integrated into the chromosome of a filamentous fungus will be equally partitioned during mitotic replication of the chromosomes.

from the stock conidia, some isolates were observed to produce more and others less recombinant protein (glucoamylase or hen egg white lysozyme) than cultures which were inoculated with a mixture of stock conidia. This indicates that the stock conidia were heterogeneous. All isolates produced the recombinant protein, so the differences between isolates may reflect differences in gene copy number, but not total loss of the recombinant DNA.

It may be necessary to periodically establish new single-conidium stock cultures after screening a range of isolates to ensure that the properties of the original strain have not been lost.

Proteolysis

Proteolysis, either within the hyphae or in the culture supernatant, has been recognised as a major problem associated with recombinant protein production, not only in fungal populations, but also in bacterial and yeast populations (e.g. Enfors 1992; Verdoes *et al*., 1995). Non-fungal recombinant proteins, such as hen egg white lysozyme and porcine pancreatic phospholipase A_2 (Archer *et al*., 1992), human interleukin 6 (Gouka *et al*., 1996), green fluorescent protein (Gordon *et al*., 2000) and tissue plasminogen activator (t-PA; Wiebe *et al*., 2001a), are more likely to be subject to proteolysis than fungal proteins. Proteolytic activity may also cause inactivation of active products, even when the product itself is not completely degraded. Where most of the degradation occurs extracellularly, the time of harvesting and post-harvest treatment of the supernatant will be important.

Several strategies have been employed for reducing proteolysis of recombinant proteins. The primary strategy has been to use a protease deficient host strain to generate the transformant. Protease deficient strains have been obtained both by mutagenesis (e.g. van den Hombergh *et al*., 1995) and by molecular methods (e.g. Zheng *et al*., 1998). There has also been increased recognition that strains deficient in different proteases are required for producing different proteins and that in some cases more than one protease needs to be eliminated (Archer *et al*., 1992; van den Hombergh *et al*., 1995).

In addition to the use of a protease deficient host, or when no such host is available, environmental parameters may be manipulated to reduce proteolysis. Environmental manipulation requires some knowledge of conditions which favour protease expression and repression. As many fungal proteases are inhibited by ammonium, addition of excess ammonium to the medium may result in reduced expression of proteases and thus a lower degree of proteolysis. For example, only 0.07 µg active t-PA l^{-1} was produced in batch cultures of recombinant *A. niger* in Vogel's medium, whereas 1.41 µg active t-PA l^{-1} was produced when the same medium was supplemented with a 10-fold excess of ammonium sulphate (Wiebe *et al*., 2001a). Similarly, it may be possible to grow the mycelia at a pH at which the extracellular protease responsible for most degradation is repressed or significantly less active, but at which the promoter for the recombinant protein is not repressed (Pitson *et al*., 1996; O'Donnell *et al*., 2001). A reduction in temperature will also lead to reduced protease activity (MacKenzie *et al*., 1994), but this may be countered by a general reduction in protein production. Addition of specific protease inhibitors, either during growth or after harvesting, is possible, but is generally limited to small scale processes.

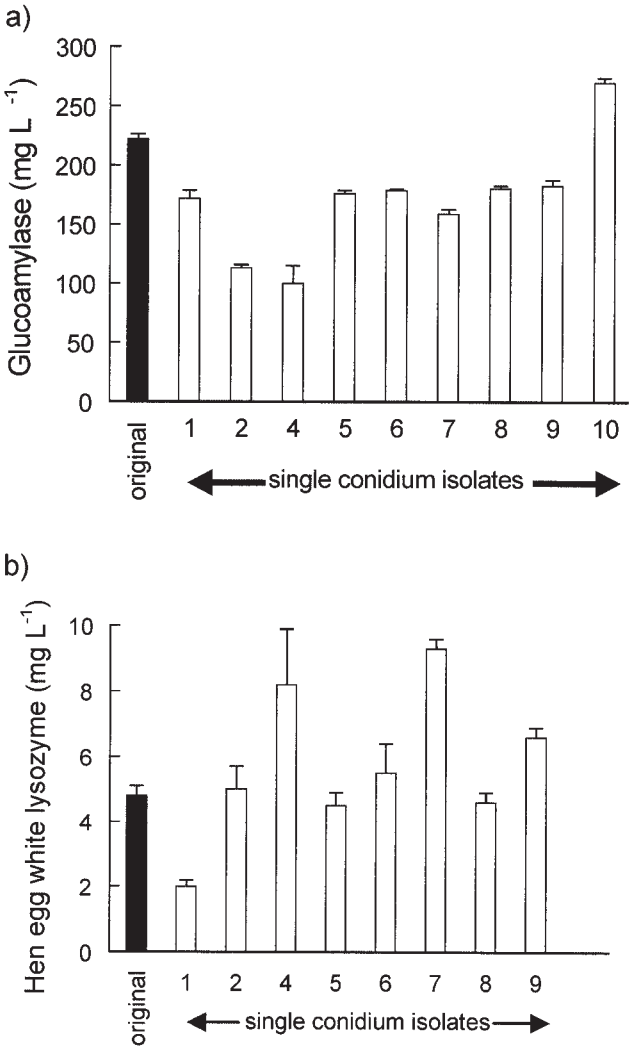

Fig 2 Production of a) recombinant glucoamylase by a transformant of *Fusarium venenatum* (solid bar) and 9 single conidium isolates of the original transformant and of b) recombinant hen egg white lysozyme by a transformant of *Aspergillus niger* (solid bar) and 8 single conidium isolates of the original transformant.

Excision or modification of recombinant DNA

Numerous reports have now demonstrated that recombinant DNA can be excised from the genome (e.g. Mainwaring *et al*., 1999; Wiebe *et al*., 2001b). Rearrangements within the genome may also occur, as has been observed in low-producing mutants of recombinant *F. venenatum* producing *A. niger* glucoamylase and *A. nidulans* acetamidase (M. G. Wiebe, unpublished data).

Incorporation of multiple gene copies, whether homologous or heterologous, into the genome of some fungi may itself lead to gene silencing (in which expression of a gene is turned off without removal of the gene from the chromosome) through a variety of mechanisms which are collectively known as homology-dependent gene silencing (Faugeron, 2000). Mechanisms which occur only during the sexual cycle (e.g. methylation induced premeiotically, MIP, and repeat-induced point mutation, RIP) are unlikely to result in gene silencing in industrially important fungi. However, quelling, which may involve production of aberrant RNA or methylation of heterologous DNA (Selker, 1997), occurs in vegetative hyphae. While studied in most depth in *Neurospora crassa*, quelling has been observed in other fungi and has been exploited as a means to down-regulate specific genes such as proteases (e.g. Zheng *et al*., 1998), as an alternative to making gene deletions. Methylated recombinant DNA has been observed in *A. niger* (Swift *et al*., 2000), but the role of DNA methylation in reducing gene expression in *A. niger*, *A. orzyae* or *T. reesei* has not been extensively investigated.

Selection pressure

Strains producing a recombinant protein are generally displaced by strains which produce little or no recombinant protein. Production of recombinant proteins is thought to impose a metabolic burden or load on the producing cells, although this is not always measurable (Withers *et al*., 1998). Low- and non-producing strains may have higher specific growth rates than producing strains because they use less energy and fewer metabolic resources in the production of proteins which provide no growth benefit to the fungus (Wiebe *et al*., 2001b).

Environmental conditions affect the rate at which producing strains are displaced by non-producing or low-producing strains, and thus contribute to the stability of enzyme production. Both pH and medium composition have been shown to enhance or reduce stable production of recombinant proteins in filamentous fungi, although the reasons for this are as yet unknown. Recombinant glucoamylase can be produced at high concentrations in complex media at pH 4.0, whereas production ability is quickly lost in the same media at pH 5.4 (Swift *et al*., 2000). Similarly, growth with both gelatine and yeast extract resulted in more stable production than growth with peptone or casamino acids. Changes in the environment affect the selection pressures to which the population is subjected, but a reduction in the metabolic burden of producing the recombinant protein cannot account for the improved stability since the same amount of glucoamylase was initially produced, regardless of the culture pH or source of complex nitrogen. It should also be noted that the same environmental parameters will not enhance stable recombinant protein production for

all transformants. Production of hen egg white lysozyme by recombinant *A. niger* was very unstable, regardless of whether the strain was cultured at high or low pH, in defined or complex media (Mainwaring *et al*., 1999).

Summary

Reductions in recombinant protein production may occur because of heterokaryosis in the original inoculum, internal or external proteolysis and modifications to the genome (including excision or modification of the recombinant DNA). Strain instability is most obvious when recombinant strains are grown in continuous flow cultures, but is also observed in batch or fed-batch cultures (Dunn-Coleman *et al*., 1992). Strain stability and stability of protein production can be enhanced by manipulating the environment. Environmental conditions can also be used to reduce protease activity, however choice of the right protease deficient host is of greater importance.

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