DOI: 10.1002/jctb.898

Effects of process parameters on heterologous protein production in *Aspergillus niger* fermentation

Liping Wang, Darin Ridgway, Tingyue Gu* and Murray Moo-Young†
Department of Chemical Engineering, Ohio University, Athens, OH 45701, USA

Abstract: Filamentous fungi such as *Aspergillus niger* are attractive hosts for recombinant DNA technology because of their ability to secrete bioactive proteins with post-translational processing such as glycosylation. Foreign genes can be incorporated into the chromosomes of the filamentous fungi, providing superior long-term genetic stability. However, heterologous protein production is often severely hampered by fungal proteases. In this work, a recombinant *Aspergillus niger* strain AB4.1[p*gpdA*GLAGFP]#11 which carries a glucoamylase (GLA)-green fluorescent protein (GFP) fusion gene was selected as a model system to study the effects of bioprocess parameters—agitation intensity, initial glucose concentration, initial yeast extract concentration, and dissolved oxygen tension (DO)—on extracellular protease inhibition and heterologous protein production. Based on previous experimental experience and results, a 2^{4-1} fractional factorial design was applied to the experiments. Each parameter was tested at two levels. It was found that agitation affected the GFP production most significantly. Higher agitation rate resulted in higher GFP production. Protease activity was most influenced by initial glucose concentration and DO. Fungal morphology was also affected by these parameters. The effects of these parameters on pellet size and pellet porosity are discussed.

Keywords: filamentous fungi; fermentation; protease inhibition; GFP; process parameters; fractional factorial design

1 INTRODUCTION

Filamentous fungi are widely used in the production of both homologous and heterologous proteins due to their desirable growth characteristics. However, the production of heterologous proteins is usually much lower than that of homologous proteins. Apart from the difficulties in improving the expression and secretion of heterologous proteins in filamentous fungi, the degradation of heterologous proteins by extracellular proteases is also an unsolved problem that hinders the development of filamentous fungal strains as hosts for recombinant proteins. For example, in Aspergilli, it has been found that proteases are responsible for the degradation of many heterologous proteins, such as human interleukin-6, bovine chymosin, and porcine pancreatic phospholipase A.[1,2]

A fungal fermentation system is widely recognized as a complicated multi-phase, multi-component process in which numerous problems can occur. Growth of the cultured microorganisms and product formation are determined by a wide range of parameters, including culture medium, fermentation pH, temperature, dissolved oxygen tension, shear stress, and fungal morphology. The problem becomes more complicated when the fermentation process involves a recombinant strain. Thus, more knowledge of the effects of morphology and operation parameters on fungi is needed to understand and perform the fermentation process properly and effectively. Unfortunately, this kind of information is rare in the literature, especially for the fermentation of recombinant strains.

In this work, a recombinant *Aspergillus niger* strain, AB4.1[p*gpdA*GLAGFP]#11, which carries a glucoamylase (GLA)-green fluorescent protein (GFP) fusion gene was selected as a model system to study the effects of bioprocess parameters—agitation intensity, initial glucose concentration, initial yeast extract concentration, and dissolved oxygen tension (DO)—on protease inhibition and heterologous protein production. Based on previous experimental experience and results, a 2^{4-1} fractional factorial design was applied to the experiments. Each parameter was tested at two levels.

* Correspondence to: Tingyue Gu, Department of Chemical Engineering, Ohio University, Athens, OH 45701, USA
E-mail: gu@ohio.edu
†Present address: Department of Chemical Engineering, University of Waterloo, Waterloo, ON, Canada N2L 3G1
Contract/grant sponsor: Russ Biomolecular Engineering Fund
(*Received 21 October 2002; revised version received 7 May 2003; accepted 10 June 2003*)
Published online 21 October 2003

2 MATERIALS AND METHODS

2.1 Fungal strain and medium

The recombinant *A niger* strain AB4.1[p*gpdA*GLA-GFP]#11 was kindly provided by Dr Peter Punt of Molecular Genetics & Gene Technology, TNO Nutrition and Food Research Institute, The Netherlands. It carries a plasmid containing the glucoamylase-GFP gene fusion driven by the constitutive *gpd*A promoter. This strain produces two types of glucoamylase, the plasmid encoded type that is part of the fusion protein, and a homologous type produced by the host. The strain AB4.1 is a *pyrG*1 derivative of N402 [3] and N402 is a *cspA*1 derivative of strain ATCC 9029.[4] The medium for *A niger* cultures was modified Vogel's medium.[5] The composition of the basic defined batch medium is listed in Table 1.

The trace element solution was stored as 10 000 times concentrated sterile-filtered stock solution. The salt solution was stored as 50 times concentrated sterile-filtered stock solution. The sugar carbon source (glucose, initial concentration 10 g dm^{-3}) and the antifoam reagent antifoam 289 (Sigma) were sterilized for 15 min at 121 °C, 15 psi prior to inoculation. Uridine solution was filter-sterilized and added into the medium to make an initial concentration of 2.44 g dm^{-3} before inoculation.

2.2 Culture conditions

Fermentations were carried out in a QueueMouse (Queue System, Parkersburg, WV) stirred tank reactor with a 1.2 dm^3 working volume at pH 6. The agitation system consisted of two flat-bladed impellers, each one having six blades. Spores for inoculation were obtained from agar plates incubated at 27 °C for 5 days. The spores were harvested by adding 20 cm^3 of sterilized water to the plates. The standard inoculation level was 1×10^6 spores cm^{-3}. Two of the eight experimental runs were randomly chosen to be repeated once. Averages of the repeated experimental results were calculated.

Table 1. Composition of the basic defined batch medium

Medium ingredients	Concentration
Glucose	10 g dm^{-3}
Salts	
Na_3.citrate.2H_2O	3 g dm^{-3}
KH_2PO_4	5 g dm^{-3}
$(NH_4)_2SO_4$	3.33 g dm^{-3}
$CaCl_2$.2H_2O	0.1 g dm^{-3}
$MgSO_4$.7H_2O	0.2 g dm^{-3}
Trace elements	
Citric acid.H_2O	5 mg dm^{-3}
$ZnSO_4$.7H_2O	5 mg dm^{-3}
$Fe(NH_4)_2(SO_4)_2$.6H_2O	1 mg dm^{-3}
$CuSO_4$	0.25 mg dm^{-3}
$MnSO_4$.H_2O	0.05 mg dm^{-3}
H_3BO_3	0.05 mg dm^{-3}
Na_2MoO_4.2H_2O	0.05 mg dm^{-3}
Uridine	2.44 g dm^{-3}

3 SAMPLE ANALYSIS

3.1 Biomass

Biomass was determined by measuring dry cell weight. Samples (20 cm^3) were filtered through dried, preweighed filter paper, followed by washing with distilled water twice and then drying at 80 °C for 24 h.

3.2 Glucose assay

Glucose concentration was analyzed enzymatically with a glucose kit (Sigma, Catalog No 315-100).

3.3 Protease activity assay

Extracellular protease activity was determined according to the method of Van den Hombergh *et al*.[6] Each 450 mm^3 sample was incubated with 50 mm^3 1% (w/v) BSA (Fraction V, Sigma) in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C. At 30 min, 60 min and 90 min, the reactions were terminated with 500 mm^3 of 10% (w/v) trichloroacetic acid (TCA). After incubation at 0 °C for 30 min, 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. One unit of protease activity was defined as the change of one absorbency unit per hour at 280 nm for 1 cm^3 reaction precipitation mixture as described above. Extracellular protease activity was expressed as U dm^{-3}. The specific activity of extracellular protease was defined as activity per gram of dry cell weight, U g^{-1}.

3.4 GFP assay

The extracellular GFP was detected using a fluorescence assay. Measurements were performed with an HP 1000 series fluorescence detector (Hewlett Packard, Wilmington, DE) using the HP software package ChemStations. The conditions for fluorescence measurements were as follows: excitation at 488 nm; emission at 520 nm; temperature 25 °C. A 0.2 M phosphate buffer (pH 7) was pumped continuously into the flow cell of the fluorescence detector through an injector. A 25 cm^3 sample was injected each time for the measurement of Relative Fluorescence Units (RFU). The pH of the samples was adjusted to 7.0 before injections.

3.5 Pellet size, apparent wet pellet density and pellet porosity

In each experimental trial, a sample was withdrawn from the reactor 48 h after inoculation. Two hundred pellets were randomly chosen from the sample. The sizes of the pellets were measured under a microscope on a hemacytometer. The average diameter was calculated as:

$$D = \frac{1}{n}\sum d_i$$

where n is the number of pellets counted, and d_i is the diameter of each pellet.

The same group of pellets was used to measure the apparent density of the pellets. Pellets were dropped

into a long narrow tube of water at 25 °C and allowed to settle freely. The time for a pellet to pass a 15 cm length, in the zone where pellets were dropping at a uniform velocity, was recorded using a stopwatch. The average velocity (u) of the n total pellets was calculated. Then the apparent density of the wet pellets (ρ_{ap}) was then calculated according to Stokes's Law:[7]

$$u = \frac{D^2(\rho_{ap} - \rho_w)g}{18\mu}, \text{ for } 10^{-4} \leq Re \leq 1 \quad (1)$$

or

$$u = 0.27\sqrt{\frac{D(\rho_{ap} - \rho_w)g}{\rho_w}Re^{0.6}}, \text{ for } 1 \leq Re \leq 10^3 \quad (2)$$

where ρ_{ap} is the apparent wet pellet density, and ρ_w is the water density.

Knowing that water has a density of 997 kg m^{-3} at 25 °C, and the wet hyphae can be assumed to have a density of 1100 kg m^{-3} according to Cui *et al*,[8] thus, from the apparent density of wet pellets calculated above, the porosity of the pellets (ε) was estimated from:

$$\rho_{ap} = \rho_p(1 - \varepsilon) + \rho_w\varepsilon \quad (3)$$

where ρ_p is the wet hyphae skeleton density.

The calculated pellet porosity is not the real porosity of a pellet. It would represent the comprehensive features of a pellet's structure (dense or loose) and surface conditions (smooth or fluffy, hairs being long or short). Loose and fluffy pellets would have higher porosity values. It would give relative information of differences in the pellet structure under different fermentation conditions.

4 HALF-FRACTIONAL FACTORIAL DESIGN

A fractional factorial design 2^{4-1} [9]was used to study the effects of the following process parameters: agitation rate (factor A), initial glucose level (factor B), initial yeast extract concentration (factor C), and dissolved oxygen (DO) level (factor D) (Tables 2 and 3). In our system, DO was measured in percentages of saturation amount of oxygen in fresh liquid medium under fermentation conditions. The 2^{4-1} design is a Resolution IV design. In this design, two-factor interactions are aliased with each other (AB = CD, AC = BD, and AD = BC). This design provides very good information about the main effects and also provides some information about two-factor interactions. In Table 3, the rows represent eight different experiments and Columns 2–5 represent different factors. For each factor, high (+) and low (−) levels were tested. Since the agitation rate was set constant for all experiments, the DO level was controlled by the air flow rate using cascade control. In choosing the factor levels, the ranges of these parameters typically used in fungal fermentation were considered. Dissolved oxygen is considered limiting when it is lower than 10% saturation;[10] while much a higher DO is hard to reach in this kind of stirred tank reactor during the exponential growth phase. Thus 15% saturation and 35% saturation were chosen for DO levels. An agitation rate of 250 rpm is necessary to prevent stagnant areas in the reactor; while the agitation rate over 500 rpm may cause serious damage to fungal hyphae. Thus 250 and 400 rpm were chose for the agitation rate levels.

5 RESULTS AND DISCUSSION

5.1 Cell growth and product formation

Typical time courses for cell growth and protein production are shown in Figs 1a and 1b. Figure 1a represents the trial *ab*, in which the specific GFP production was the highest. Figure 1b represents the trial *ad*, in which the specific protease activity was the highest. Extracellular protease secretion started after the cell growth approached the stationary phase around the end of Day 2, when the glucose in the medium was almost depleted. The protease

Table 2. Parameters and their values tested in the fractional factorial design experimental runs for the fermentation of *A niger*

	A (Agitation)	B (Glucose)	C (Yeast extract)	D (DO)
+	400 rpm	25 g dm^{-3}	3 g dm^{-3}	35%
−	250 rpm	10 g dm^{-3}	0 g dm^{-3}	15%

Table 3. Fractional factorial design experimental runs and results for the fermentation of *A niger*

	X_A	X_B	X_C	X_D	Biomass (g dm^{-3})	Biomass yield ($Y_{X/S}$)	Pellet diameter (mm)	Apparent wet pellet density (g cm^{-3})	Pellet porosity (%)	Specific Protease (U g^{-1})	Volumetric protease (U dm^{-3})	Specific GFP (×10^6 RFU g^{-1})	Volumetric GFP (×10^6 RFU dm^{-3})
(1)	−1	−1	−1	−1	5.64	0.56	1.0	1010.9	86.5	139	782	1.29	7.28
ad	+1	−1	−1	+1	3.61	0.36	0.4	1021.4	76.3	260	939	4.84	17.5
bd	−1	+1	−1	+1	8.55	0.34	1.0	1018.5	79.1	181	1544	1.25	10.7
ab	+1	+1	−1	−1	8.12	0.32	0.8	1014.5	83.0	18	143	5.02	40.8
cd	−1	−1	+1	+1	6.64	0.66	1.2	1007.2	90.1	241	1601	1.75	11.6
ac	+1	−1	+1	−1	5.75	0.58	0.7	1012.1	85.3	149	858	3.97	22.8
bc	−1	+1	+1	−1	9.97	0.35	1.4	1007.3	90.0	26	257	2.10	20.9
abcd	+1	+1	+1	+1	5.59	0.21	1.1	1013.4	84.1	61	343	3.53	19.7

A: Agitation rate; B: initial glucose concentration; C: initial yeast extract concentration; D: dissolved oxygen level.

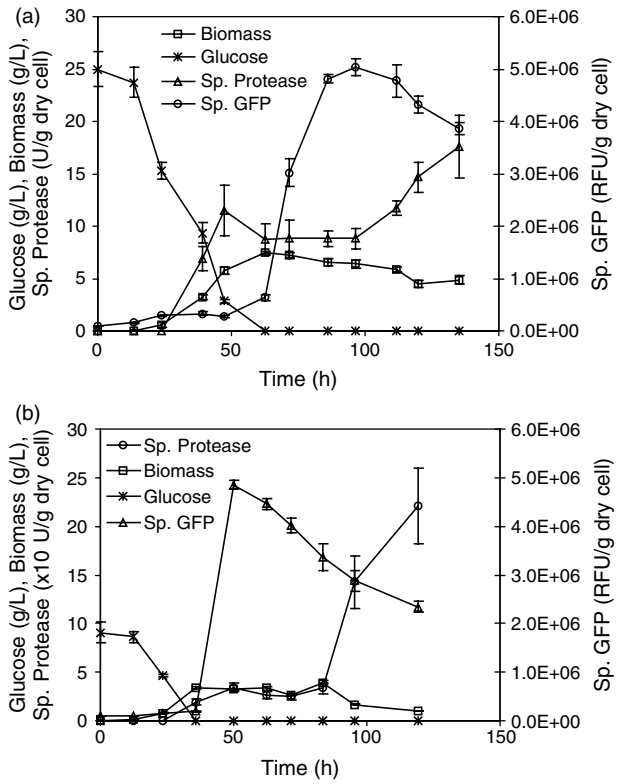

Figure 1. (a) Time course for cell growth and extracellular protein production in recombinant *A niger* culture (trial *ab*). The data points are the average values of two separate fermentation runs, and the vertical bars indicate the actual results from the two runs (b) Time course for cell growth and extracellular protein production in recombinant *A niger* culture (trial *ad*). The data points are the average values of two separate fermentation runs, and the vertical bars indicate the actual results from the two runs.

activity barely changed during the stationary phase. It increased again when cell lysis occurred. GFP concentration reached the highest level after the depletion of glucose, followed by a decrease over the next days of culture. GFP is an extremely stable protein of 238 amino acids.[11] In our previous work, when GFP was incubated under room temperature in a protease-containing fermentation broth for 2 days, its concentration declined dramatically from 10.8 mg dm^{-3} to 2.6 mg dm^{-3}. When incubated in a fresh medium (protease-free), GFP concentration was only 6.5% lower after two days.[12] Thus, the decrease of GFP concentration in this experiment indicates that some GFP degradation by proteases present in the culture likely occurred. GFP degradation is affected by protease activity. In our previous published paper, degradation of GFP was found to be higher at a low pH (such as 3) than at a higher pH (such as 6) due to a higher protease activity at the higher pH.[13] The highest protease activity and GFP level in each experimental trial were considered to be the results and were analyzed statistically.

Figure 2 shows the growing phases of fungal pellets in the stirred tank bioreactor. The spores started germinating about 8 h after inoculation, and clumps were soon formed. Small pellets were formed one day after inoculation. The dense cores of the small pellets

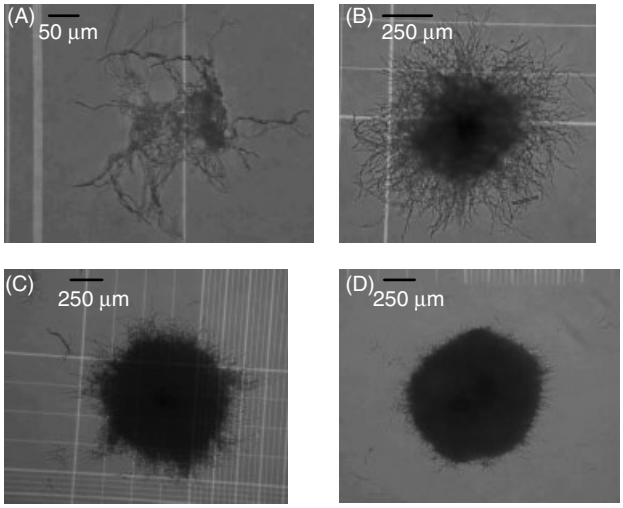

Figure 2. Growing phases of pellets in the fermentation of recombinant *A niger* in stirred tank bioreactor (trial *abcd*). (A) Clump at 12 h after inoculation; (B) small pellet with long hairs (24 h after inoculation); (C) pellets growing larger while hairs are becoming shorter (48 h after inoculation); (D) smoother pellet with short hairs (96 h after inoculation). In (C) and (D), the free filaments shaved off from the pellets can be seen around the pellets.

kept growing larger until the stationary phase, while the hairy length of the pellets decreased because of the presence of mechanical forces and the vacuole formation, which weakened the strength of hyphae.[14] The hairs continued to be shaved off. After 4 days of cultivation, the pellets became much smoother than they were on the second day. The shaved-off hairs—the free filaments—were observed in the broth. However, the fraction of free filament mass in total biomass was still quite small. No broken pellets were ever observed. In each experimental run, the pellets in phase (C) were withdrawn from the reactor to measure pellet size, apparent density and porosity.

5.2 Result of the fractional factorial design

The experimental results are listed in Table 3. The experimental data were analyzed using the software MINITAB. The output showed the results of fitting Multiple Linear Regression (MLR) models to describe how GFP production, protease activity and pellet porosity were related to the four parameters. The equations of the fitted models are:

$$\begin{aligned}\text{GFP} = {} & 2.97 \times 10^6 + 1.37 \times 10^6 \cdot X_A \\ & + 6250.0 \cdot X_B - 1.31 \times 10^5 \cdot X_C \\ & - 1.26 \times 10^5 \cdot X_D \qquad (4)\end{aligned}$$

$$\begin{aligned}\text{Protease} = {} & 134.0 - 12.2 \cdot X_A - 63.0 \cdot X_B \\ & - 14.9 \cdot X_C + 51.5 \cdot X_D \qquad (5)\end{aligned}$$

$$\begin{aligned}\text{Porosity} = {} & 84.3 - 2.13 \cdot X_A - 0.25 \cdot X_B \\ & + 3.07 \cdot X_C - 1.90 \cdot X_D \qquad (6)\end{aligned}$$

In eqns (4)–(6), X_A, X_B, X_C, and X_D are coded variables of factors A (agitation rate), B (initial glucose

concentration), C (initial yeast extract concentration), and D (dissolved oxygen tension). $X_I = 1$ (high level) or -1 (low level), I = A, B, C, or D.

Since the *P*-values in the ANOVA tables (Tables 4a–4c) are less than 0.15 (0.076, 0.047 and 0.123), there is a statistically significant relationship between the variables at the 85% confidence level. For GFP and protease, it is higher than 90%. The R^2-values are all above 0.8 (0.898, 0.927 and 0.857), indicating acceptable regression models.

The significance of each parameter is listed in Table 5. This shows that not all the independent parameters are significant. For example, for the GFP production, the significance of initial glucose concentration (factor B) is 0.983, indicating that the production of GFP is minimally affected by the glucose concentration. The interactions of factors were not initially considered in the aforementioned models. Modifications of these models were made to include these.

Figures 3a–3c are the normal probability plots of effect estimates for GFP, protease, and pellet porosity. If none of the effects are significant, the estimates will behave like a random sample drawn from a normal distribution with zero mean, and the plotted effects will lie approximately along a straight line. Those effects that do not lie on the line are significant factors. For

Table 4a. Analysis of variance of regression on GFP production

Source	Sum-of -squares	df	Mean-square	*F*-ratio	*P*-value
Regression	1.531E13	4	3.827E12	6.63	0.076
Residual	1.721E12	3	5.769E11	—	—

Table 4b. Analysis of variance of regression on protease production

Source	Sum-of -squares	df	Mean-square	*F*-ratio	*P*-value
Regression	55925	4	13981	9.48	0.047
Residual	4425	3	1475	—	—

Table 4c. Analysis of variance of regression on pellet porosity

Source	Sum-of -squares	df	Mean-square	*F*-ratio	*P*-value
Regression	141.074	4	35.269	4.51	0.123
Residual	23.460	3	7.820	—	—

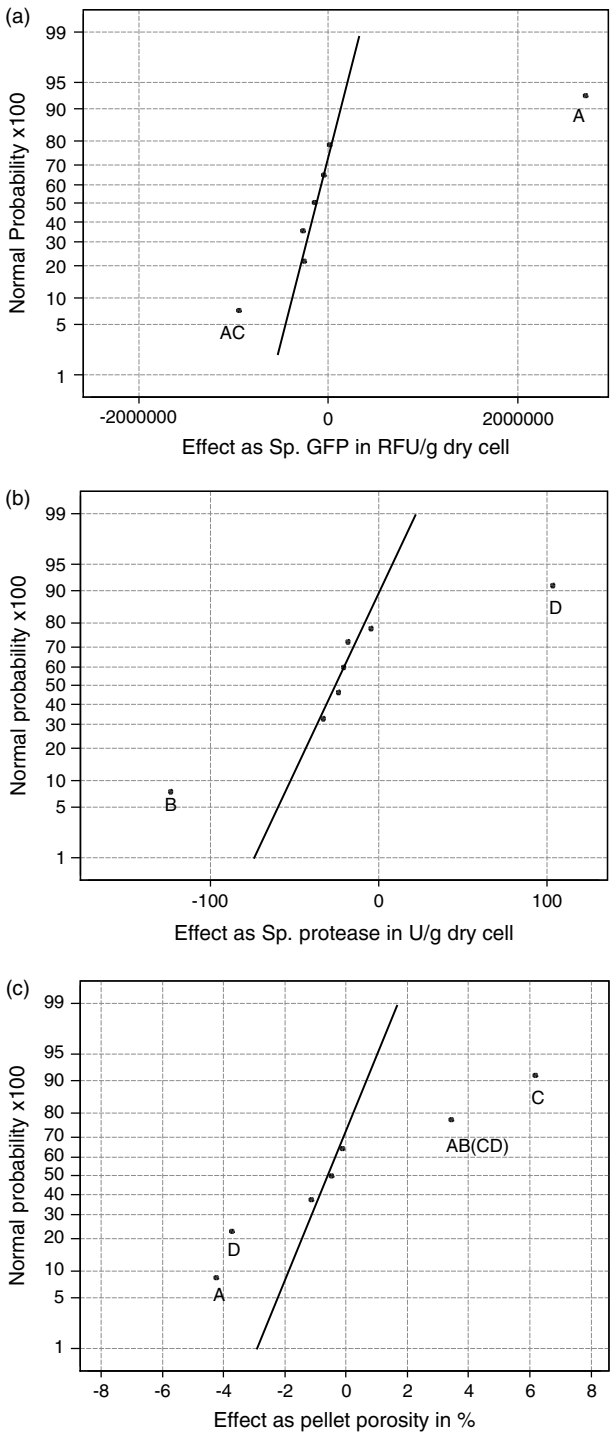

Figure 3. (a) Normal probability plot of effects for specific GFP production (b) Normal probability plot of effects for specific protease production (c) Normal probability plot of effects for pellet porosity.

Table 5. Significance of factors represented by *t*-values and *P*-values

	Porosity			Yields ($Y_{X/S}$)			Specific GFP			Specific protease		
Factor	Coeff	*t*-value	*P*-value	Coeff	*t*-value	*P*-value	Coeff	*t*-value	*P*-value	Coeff	*t*-value	*P*-value
Constant	84.3000	85.26	0.0000	0.4225	13.40	0.001	2.969E6	11.05	0.002	134.34	9.89	0.002
Agitation	−2.1250	−2.15	0.120	−0.055	−1.74	0.179	1.371E6	5.11	0.015	−12.21	−0.90	0.435
Glucose	−0.2500	−0.25	0.817	−0.1175	−3.73	0.034	6250.0	0.02	0.983	−62.99	−4.64	0.019
Yeast extract	3.0740	3.11	0.053	0.0275	0.87	0.447	−1.313E5	−0.49	0.659	−14.94	−1.10	0.352
DO	−1.9000	−1.92	0.150	−0.03	−0.95	0.411	−1.263E5	−0.47	0.670	51.49	3.79	0.032

specific GFP production, the only significant effects are factor A (agitation) and AC interaction (agitation and yeast extract). For specific protease, the significant effects are B (glucose) and D (dissolved oxygen). For porosity, the major effects are A (agitation), C (yeast extract), D (DO), and AB (CD). Thus the models can be modified to be:

$$\text{GFP} = 2.97\text{E}6 + 1.37 \times 10^6 \cdot X_\text{A} - 4.59 \times 10^5 \cdot X_\text{A} \cdot X_\text{C} \quad (7)$$

$$\text{Protease} = 134.0 - 63.0 \cdot X_\text{B} + 51.5 \cdot X_\text{D} \quad (8)$$

$$\text{Porosity} = 84.3 - 2.13 \cdot X_\text{A} + 3.07 \cdot X_\text{C} - 1.90 \cdot X_\text{D} + 1.62 \cdot X_\text{A} \cdot X_\text{B} \quad (9)$$

5.3 Morphology and biomass development

It is well known that filamentous fungal cells exhibit two extreme types of morphology in submerged cultures: pelleted and free filamentous forms. Fungal morphology can be influenced by inoculum level, initial pH, agitation, polymer additives, etc.[15–18] Filamentous growth is common in industrial fermentation. However, reduced extracellular protease secretion was found in pelleted growth and it is beneficial for heterologous protein production.[12] On the other hand, pelleted growth may result in reduced cell mass due to substrate and oxygen limitation in the dense core of the pellet when the pellets exceed a 'critical radius'.[8,15] Oxygen depletion in the center of the pellets will cause autolysis of the cells and eventually the formation of a hollow center.

Under the inoculum level and experimental conditions adopted in this work, fungal pellets with the diameter of 0.4–1.4 mm were formed (Table 3). No hollow pellets were observed. Our previous work showed that in shake flask culture, the maximum biomass accumulation was almost the same among the cultures with 10 g dm^{-3} initial glucose concentration with pellet diameters ranging from 0.5 mm to 2.1 mm.[19] Thus, in these experiments, biomass development should not be oxygen controlled when the initial glucose concentration was 10 g dm^{-3}.

In the eight experimental runs, the biomass yield on glucose ($Y_{X/S}$) varied from 0.21 to 0.66. It was greatly affected by the initial glucose concentration, initial yeast extract concentration, and agitation intensity. Statistically, initial glucose concentration and agitation intensity affected the biomass development the most (Table 5). When the initial glucose concentration was 30 g dm^{-3}, the biomass concentration was lower than expected. Since the carbon to nitrogen ratio in the medium was kept at 8, and the other nutrients were in excess, the only nutrient that could be limiting was oxygen. It has been shown that high biomass concentration in a fungal culture system might greatly decrease the oxygen mass-transfer coefficient, $k_\text{L}a$.[20]

The porosity of the pellets varied from 79% to 93%. It was most affected by initial yeast extract concentration in the broth (Table 5). The initial yeast extract concentration was the only factor that caused a positive change in the porosity. It was observed that the existence of yeast extract in the medium made the spores more likely to aggregate together and finally fluffier and looser pellets were formed. The broth became more viscous, and poor mixing was observed at the lower agitation rate (250 rpm). Agitation intensity and dissolved oxygen tension also affected the pellet porosity significantly. Higher agitation and DO level resulted in lower pellet porosity.

5.4 Factors influencing the GFP production and protease activity

Specific GFP production and protease activity are shown in Table 3. The significance values for the factors are listed in Table 5. The specific GFP production was most significantly influenced by agitation intensity (P-value = 0.015), less significantly affected by initial yeast extract concentration and DO level, and barely affected by initial glucose concentration (P-value = 0.983). The normal probability plot of effects (Fig 3a) showed that GFP production was also significantly affected by the interaction AC (BD). Specific protease activity was significantly affected by initial glucose concentration (P-value = 0.019) and DO level (P-value = 0.032), and less significantly affected by agitation intensity and initial yeast extract concentration. The highest GFP production and lowest protease activity were obtained in trial *ab*, ie the run with higher levels of agitation and DO and lower levels of initial glucose and yeast extract concentrations (Table 3).

5.4.1 Effect of agitation intensity

The effect of agitation on fungal morphology has been widely discussed.[21] Increasing agitation intensity enhanced the mechanical forces on the fungal cells as well as the gas–liquid mass transfer. Pellet size and hair length in a submerged fermentation were controlled by mechanical forces.[22] Previous work showed that fungal morphology has a dramatic effect on GFP production.[12] Agitation also determines the mixing conditions in the reactor which greatly influence the substrate mass transfer. It is therefore not surprising that agitation rate was found to be the most important factor affecting GFP production in these experiments. Higher agitation rates resulted in higher GFP production. However, it was observed that with the agitation rate higher than 400 rpm, fungal hyphae were severely damaged by shear stress which resulted in poor biomass development. Thus, an agitation rate of 400 rpm is considered to be the optimal rate for GFP production. Although agitation was not the strongest parameter affecting protease activity, the protease activity was lowered when the agitation rate was increased (Table 5). The increase in GFP production could also be a benefit resulting from the decrease in protease activity.

5.4.2 Effect of dissolved oxygen level

Dissolved oxygen tension affects productivity, cell autolysis, the rigidness of the cell wall, and many

other features of fungal fermentation.[21] The effect of DO on protein production is profound, as reported in the literature. Oxygen can potentially impact fungal cultures in a number of ways. It may enhance growth and protein secretion directly. It may also indirectly influence these processes by altering the morphology of the culture, either positively or negatively. It has been shown that oxygen enrichment in the gas supply resulted in a higher percent of 'active lengths' (cytoplasm filled compartment, production and/or secretion region) in the hyphal elements.[23] Since protein synthesis occurs in the cytoplasmic regions [24] and protein secretion takes place mainly at the hyphal tips, an increase in the activity of native glucoamylase was obtained.[25,26] However, most of these studies were based on filamentous growth. In the pelleted growth, mass transfer in the pellets might exert critical effects on the protein production as well.

In this study, protease activity was significantly affected by the DO level in the broth (P-value = 0.032). Higher DO tension resulted in higher protease activity. The dissolved oxygen level did not significantly affect the pellet size. However, pellet structure was considerably influenced by DO—a higher DO level resulted in denser pellets (Table 3). The same results have been reported by Cui *et al*.[8] The result that GFP production was slightly decreased when DO was at a higher level was probably due to the formation of denser pellets and higher protease activities.

5.4.3 Effect of initial glucose concentration

GFP production was only slightly affected by the initial glucose concentration according to the statistical results. However, a high initial glucose concentration showed a great effect in inhibiting protease activities (P-value = 0.019), even though in all eight experimental runs, complete consumption of glucose was observed and the highest protease activities were obtained after the complete consumption of glucose. In *A niger*, extracellular proteases are regulated by carbon and nitrogen sources. All the extracellular proteases are repressed at the conditions of high levels of glucose and ammonium in the medium. This is because of the so-called wide-domain regulatory genes, which are involved in carbon or nitrogen metabolite repression.[27] The GLA-GFP fusion gene in this work is under the control of a *gpdA* promoter, which is a constitutive promoter. Therefore GFP production is not directly regulated by the carbon source.

5.4.4 Effect of adding yeast extract to the medium

It was found that in shake flask cultures adding yeast extract to the medium greatly increased the GFP production (data not shown). However, in this group of experiments in the stirred tank reactor, yeast extract did not show any good effect in GFP production. Yeast extract increased the yield of biomass upon glucose and exerted an important effect on fungal morphology. Changes in rheological conditions caused by the fluffier and looser pellets might be the reason for the decreased GFP production and protease activity.

6 CONCLUSIONS

Among the four parameters tested, agitation had the greatest effect on GFP production in the stirred tank reactor. A higher initial glucose concentration was beneficial for GFP production because it significantly repressed protease activity. The factor AC interaction (agitation intensity and yeast extract concentration) was significant for GFP production. Since its influence was negative (−) (eqn (7)), and agitation (A) was positive (+), maintaining the yeast extract concentration (C) at a low level would be good for GFP production. Within the range tested (15–35%), DO tension affected GFP production negatively since protease activity was affected positively. The great effect of yeast extract on fungal morphology counteracted its positive effect on GFP production. In summary, a set of recommended cultivation conditions for this reactor can be as follows: agitation intensity 400 rpm, initial glucose concentration 25 g dm^{-3}, no yeast extract, and DO tension of 15%. Under these conditions, the specific GFP production was the highest. The volumetric GFP production was also the highest, which should not be surprising because higher biomass was also obtained under these conditions, and better biomass development is the basis of better protein production.

The effects of agitation intensity and yeast extract concentration were prominent. It was obvious that a higher agitation rate led to good mixing and a higher mass transfer rate in the reactor. The more viscous broth formed by adding yeast extract to the medium caused poor mixing and difficulties in mass transfer and oxygen supply. Thus it is likely that the rheological conditions in the reactor might be critical to GFP production. However, changes in rheological conditions caused by fungal morphology in the reactor have not yet been studied in detail. Further research might be necessary.

ACKNOWLEDGEMENTS

The extensive help of Dr Peter Punt, Molecular Genetics & Gene Technology, TNO Nutrition and Food Research Institute, The Netherlands, in donating the strain and providing related information for research is gratefully acknowledged. The support from the Russ Biomolecular Engineering Fund is greatly appreciated.

REFERENCES

1 Gouka RJ, Punt PJ and van den Hondel CAMJJ, Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects, *App Microbiol Biotechnol* **47**:1–11 (1997).

2 Gouka RJ, Punt PJ, Hessing JGM and van den Hondel CAMJJ, Analysis of heterologous protein in defined recombinant, *A awamori strains Appl Environ Microb* **62**:1951–1957 (1996).

3 Van Hartingsveldt W, Mattern IE, van Zeijl CMJ, Pouwels PH and van den Hondel CAMJJ, Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene, *Mol Gen Genet* **206**:71–75 (1987).

4 Bos CJ, Debets AJM, Swart K, Huybers A, Kobus G and Slakhorst SM, Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*, *Curr Genet* **14**:437–443 (1988).

5 Vogel HJ, A convenient growth medium for *Neurospora* (Medium N), *Microb Genet Bull* **13**:42–44 (1956).

6 Van den Hombergh JPTW, van de Vondevoort PJI, van derHeijden NCBA and Visser J, New protease mutants in *Aspergillus niger* result in strongly reduced *in vitro* degradation of target proteins: genetic and biochemical characterization of seven complementation groups, *Curr Genet* **28**:299–308 (1995).

7 Bird RB, Stewart WE and Lightfoot EN, *Transport Phenomena*. John Wiley & Sons: New York, pp 190–194 (1960).

8 Cui YQ, van der Lans RGJM and Luyben KChAM, Effects of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentation, *Biotechnol Bioeng* **57**:409–419 (1997).

9 Montgomery DC, *Introduction to Statistical Quality Control*, 3rd edn. John Wiley & Sons: New York, p 555 (1995).

10 Wongwicharn A, Harvey LM and McNeil B, Secretion of heterologous and native proteins, growth and morphology in batch cultures of *Aspergillus niger* B1-D at varying agitation rates, *J Chem Technol Biotechnol* **74**:821–828 (1999).

11 Prasher DC, Eckenrode VK, Ward WW, Prendergast FG and Cormier MJ, Primary structure of the *Aequorea victoria* green fluorescent protein, *Gene* **111**:229–233 (1992).

12 Xu J, Wang L, Ridgway D, Gu T and Moo-Young M, Increased heterologous protein production in *Aspergillus niger* fermentation through extracellular proteases inhibition by pelleted growth, *Biotechnol Prog* **16**:222–227 (2000).

13 O'Donnell D, Wang L, Xu J, Ridgway D, Gu T and Moo-Young M, Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity, *Bichem Eng J* **8**:187–193 (2001).

14 Cui YQ, van der Lans RGJM, Giuseppin MLF and Luyben KChAM, Influence of fermentation conditions and scale on the submerged fermentation of *Aspergillus awamori*, *Enzyme Microb Technol* **23**:157–167 (1998).

15 Metz B and Kossen NWF, Biotechnology review: the growth of molds in the form of pellets—a literature review, *Biotechnol Bioeng* **19**:781–799 (1977).

16 Mitard A and Riba JB, Morphology and growth of *Aspergillus niger* ATCC 26036 cultivated at several shear rates, *Biotechnol Bioeng* **32**:835–840 (1988).

17 Lejeune R and Braon GV, Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation, *Appl Microbiol Biotechnol* **43**:249–258 (1995).

18 Pazouki M and Panda T, Understanding the morphology of fungi, *Bioprocess Eng* **22**:127–143 (2000).

19 Kobayashi T, van Dedem G and Moo-Young M, Oxygen transfer into mycelial pellets, *Biotechnol Bioeng* **15**:27–45 (1973).

20 Bai F, Wang L, Huang H, Xu J, Caesar J, Ridgway D, Gu T and Moo-Young M, Oxygen mass-transfer performance of low viscosity gas–liquid–solid system in a split-cylinder airlift bioreactor, *Biotechnol Lett* **23**:1109–1113 (2001).

21 Cui YQ, van der Lans RGJM and Luyben KChAM, Effect of agitation intensities on fungal morphology of submerged fermentation, *Biotechnol Bioeng* **55**:715–726 (1997).

22 Cui YQ, Okkerse WJ, van der Lans RGJM and Luyben KChAM, Modeling and measuring of fungal growth and morphology in submerged fermentations, *Biotechnol Bioeng* **60**:216–229 (1998).

23 Wongwicharn A, McNeil B and Harvey LM, Effect of oxygen enrichment on morphology, growth, and heterologous protein production in chemostat cultures of *Aspergillus niger* B1-D, *Biotechnol Bioeng* **65**:416–424 (1999).

24 Freudenberg S, Fasold KI, Muller SR, Siedenberg D, Kretzmer G, Schugerl K and Giuseppin M, Fluorescence microscopic investigation of *Aspergillus awamori* growing on synthetic and complex media and producing xylanase, *J Biotechnol* **46**:265–273 (1996).

25 Nykanen M, Raudaskoski R, Nevalainen KMH and Mikkonen A, Expression and secretion of barley cysteine endopeptidase B and cellobiohydrolase I in *Trichoderma reesei*, *Appl Environ Microbiol* **63**:4929–4937 (1997).

26 Wollsten HAB, Moukha SM, Sietsma JH and Wessels JGH, Localization of growth and secretion of proteins in *Aspergillus niger*, *J Gen Microbiol* **137**:2017–2023 (1991).

27 Van den Hombergh JPTW, van de Vondervoort PJI, Fraissinet-Tachet L and Visser J, *Aspergillus* as a host for heterologous protein production: the problem of proteases, *Trends Biotechnol* **15**:256–263 (1997).