Microbiology (2009), **155**, 3430–3439 **DOI** 10.1099/mic.0.031062-0

The effect of environmental conditions on extracellular protease activity in controlled fermentations of *Aspergillus niger*

Machtelt Braaksma,[1,2] Age K. Smilde,[1,2]† Mariët J. van der Werf[1,2] and Peter J. Punt[1,2]

Correspondence
Machtelt Braaksma
machtelt.braaksma@tno.nl

[1]TNO Quality of Life, PO Box 360, 3700 AJ Zeist, The Netherlands

[2]Kluyver Centre for Genomics of Industrial Fermentation, PO Box 5057, 2600 GA Delft, The Netherlands

Proteolytic degradation by host proteases is one of the key issues in the application of filamentous fungi for non-fungal protein production. In this study the influence of several environmental factors on the production of extracellular proteases of *Aspergillus niger* was investigated systematically in controlled batch cultures. Of all factors investigated in a series of initial screening experiments, culture pH and nitrogen concentration in particular strongly affected extracellular protease activities. For instance, at a culture pH of 4, protease activity was higher than at pH 5, and protease activity increased with increasing concentrations of ammonium as nitrogen source. Interestingly, an interdependence was observed for several of the factors studied. These possible interaction effects were investigated further using a full factorial experimental design. Amongst others, the results showed a clear interaction effect between nitrogen source and nitrogen concentration. Based on the observed interactions, the selection of environmental factors to reduce protease activity is not straightforward, as unexpected antagonistic or synergistic effects occur. Furthermore, not only were the effects of the process parameters on maximum protease activity investigated, but five other protease-related phenotypes were studied as well, such as maximum specific protease activity and maximum protease productivity. There were significant differences in the effect of the environmental parameters on the various protease-related phenotypes. For instance, pH significantly affected final levels of protease activity, but not protease productivity. The results obtained in this study are important for the optimization of *A. niger* for protein production.

Received 20 May 2009
Revised 14 July 2009
Accepted 17 July 2009

INTRODUCTION

Aspergillus species such as *A. niger* and *A. oryzae* are known for their exceptional ability to secrete large amounts of homologous enzymes. For decades they have been commonly exploited as commercial production organisms for a variety of enzymes. With the development of transformation systems for these industrially important members of the genus (Buxton *et al.*, 1985; Kelly & Hynes, 1985; van Hartingsveldt *et al.*, 1987; Iimura *et al.*, 1987; Unkles *et al.*, 1989), the expression of large quantities of heterologous proteins seemed within reach as well. And indeed, nowadays *Aspergillus* species dominate the list of host organisms for the commercial production of enzymes from fungal origin (according to the Association of Manufacturers and Formulators of Enzyme Products at www.amfep.org). Also, proteins from non-fungal origin, such as chymosin, lysozyme, lactoferrin, interleukin-6 and antibody fragments, have been successfully expressed in several *Aspergillus* species (Yoder & Lembeck, 2004). However, thus far most of these products have only been produced at a laboratory scale, as the production levels, often not more than several tens of milligrams per litre, are too low to be commercially interesting.

The reason for the relatively poor production levels of non-fungal proteins in aspergilli is not completely understood. A combination of inefficient (post-)translational steps or proteolytic degradation by extracellular proteases probably affects secreted heterologous protein levels (Yoder & Lembeck, 2004). To date, this latter problem has mainly been approached by disruption or silencing of protease-encoding genes (Berka *et al.*, 1990; van den Hombergh

†Present address: Biosystems Data Analysis, Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands.

Abbreviations: ANOVA, analysis of variance; DWT, cell dry weight.

Comprehensive ANOVA data for each of the six protease-related phenotypes are available with the online version of this paper.

et al., 1997a; Zheng *et al.*, 1998; Moralejo *et al.*, 2002; Braaksma & Punt, 2008) or protease regulator genes (Punt *et al.*, 2008). With this approach, significant reduction of proteolytic activity is achieved with subsequent improvement of heterologous protein production levels (Moralejo *et al.*, 2000; Wang *et al.*, 2008). The recent sequencing of the genomes of several *Aspergillus* species, including *A. niger* (http://genome.jgi-psf.org/Aspni5/Aspni5.home.html; Pel *et al.*, 2007), has created the possibility of identification and disruption of new protease genes (Wang *et al.*, 2008). However, approximately 200 genes involved in proteolytic degradation have been identified in *A. niger* (Pel *et al.*, 2007). Due to this high number of putative proteases, the construction of production hosts essentially free of extracellular protease activities seems unrealistic. It is likely that in such strongly altered strains, other cellular processes will be affected as well, making these multiple protease-deficient mutants unsuitable for robust production conditions.

The role of extracellular proteases in fungi is to degrade proteins into small peptides or amino acids to supply the cell with nutrients when the preferred carbon or nitrogen sources are not available to the cell. Several wide-domain regulatory systems involved in the adaptation of the overall metabolism of nutrients in the cell are implicated in the regulation of extracellular protease expression. Complementary to strain improvement, manipulation of environmental conditions can help to reduce protease secretion and thus improve heterologous protein production, but this has not been investigated systematically (Braaksma & Punt, 2008). Examples of bioprocess parameters which have been investigated for their influence on extracellular protease activity include fungal morphology manipulation (Liu *et al.*, 1998; Xu *et al.*, 2000; Papagianni *et al.*, 2002; Papagianni & Moo-Young, 2002), pH control (O'Donnell *et al.*, 2001), oxygen enrichment and cultivation temperature (Li *et al.*, 2008). Studies on the effect of medium components have mainly focused on the derepression of protease genes when transferring mycelia to medium lacking either carbon or nitrogen sources (Cohen, 1981; Jarai & Buxton, 1994). In this study, the effect of pH and various medium components on extracellular protease activity levels in controlled batch cultures with *A. niger* N402 was investigated systematically.

METHODS

Strain and culture media. *Aspergillus niger* N402 used in this study is a *cspA1* (conferring short conidiophores) derivative of ATCC 9029 (Bos *et al.*, 1988). Stock cultures of this strain were maintained at −80 °C as conidial suspensions in 20 % (v/v) glycerol.

Minimal medium (MM) (Bennett & Lasure, 1991) contained 7 mM KCl, 11 mM KH_2PO_4, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4 and 134 nM EDTA. This medium was supplemented with the appropriate carbon or nitrogen source as indicated in Table 1. To prevent foaming, 1 % (v/v) antifoam (Struktol J 673) was added to the medium and, when necessary, additional antifoam was added during the cultivation.

Pre-cultivation. For inoculation of the batch cultures, baffled 500 ml Erlenmeyer flasks were inoculated with 10^6 spores ml^{-1}. The flasks were incubated at 30 °C in a rotary shaker at 125 r.p.m. until approximately half the amount of carbon source was consumed, which took 4–7 days. Each flask contained 100 ml MM (pH 6.5) supplemented with carbon source and nitrogen source, identical to the medium in the batch cultures.

Batch culture. For the screening of the environmental parameters involved in extracellular protease production, as listed in Table 1, cultivation was carried out in 3.3 l BioFlo 3000 bioreactors (New Brunswick Scientific) with a working volume of 2 l. The cultivations of the full two-level factorial design (Table 2) were carried out in 6.6 l BioFlo 3000 bioreactors with a working volume of 5 l. The bioreactors were equipped with two six-blade Rushton turbines and one pitched blade impeller between both Rushton turbines rotating at 400 r.p.m. at the start of the cultivation. When the dissolved oxygen tension dropped below 20 %, the agitation was automatically increased to a maximum of 1000 r.p.m., maintaining the dissolved oxygen tension at 20 %. Air was used for sparging the bioreactor at a constant flow of 0.25 VVM [vol. gas (vol. liquid)$^{-1}$ min^{-1}]. The pH was controlled at the set value (Tables 1 and 2) by automatic addition of 8 M KOH and 1.5 M H_3PO_4, and the temperature was maintained at 30 °C. The controlled batch cultures were inoculated with 4 % (v/v) pre-culture.

Cell dry weight determination. For the quantification of cell dry weight (DWT), a known volume of cell culture was filtered though a dried, pre-weighed filter paper, followed by washing with distilled water twice and then drying at 110 °C for 24 h.

Analysis of carbon source concentration. Enzymic kits were used to analyse glucose (ABX Pentra), sucrose and fructose (Sigma). Lactose concentration was analysed by incubating 4 × -diluted culture samples with an equal volume of 10 % (v/v) β-galactosidase (Roche) in 0.1 M citrate buffer, pH 6.6, at 37 °C for 10 min, to convert lactose to free glucose and galactose. Glucose concentration was determined as a measure of lactose concentration, with correction for free glucose present before incubation with β-galactosidase. All these assays were automated on a COBAS MIRA Plus autoanalyser (Roche Diagnostic Systems). Xylose concentration was measured by using the dinitrosalicylic method for quantification of reducing sugars (Sumner & Somers, 1949). The culture sample was 10–50 × diluted and 1 ml of this sample was incubated with 1.5 ml DNS reagent (1 % 3,5-dinitrosalicylic acid, 1.6 % NaOH, 30 % potassium sodium tartrate) at 100 °C for 5 min, cooled to room temperature and the absorbance was measured at 540 nm.

Analysis of ammonium concentration. Ammonium was assayed by the phenol-hypochlorite colorimetric assay according to Weatherburn (1967). This assay was automated on a COBAS MIRA Plus autoanalyser.

Preparation of dimethyl BSA for protease assay. *N,N*-Dimethyl BSA was prepared by a modification of the procedure described by Lin *et al.* (1969). BSA fraction V (20 g) was dissolved in 2 l 0.1 M borate buffer, pH 9.0, and then cooled to 0 °C. The solution was rapidly stirred, and 4 g sodium borohydride was added. Formaldehyde (40 ml) was then added in 1.3 ml increments over a period of 30 min. A few minutes after the last addition of formaldehyde, the solution was acidified to pH 6.0 by the addition of 50 % acetic acid and dialysed against deionized water. The desalted protein was lyophilized and stored at −20 °C as a fluffy white powder.

Protease assay. Extracellular proteolytic activities were measured according to a modified procedure as described by Holm (1980) using *N,N*-dimethylated BSA as substrate. The procedure was fully automated using a COBAS MIRA Plus autoanalyser. Proteolytic

Table 1. Fermentation results obtained under the different environmental conditions used in the screening design

Experiment name	Carbon source	Carbon source level (mM)*	pH	Nitrogen source	Nitrogen source level (mM)	Max. biomass, DWT_{max} (g l^{-1})	Max. protease activity at pH 4 (U l^{-1})	Specific protease activity (U g^{-1})†
Glucose/ $NaNO_3$‡	Glucose	277.5	4	$NaNO_3$	70.6	12.2 (±11.7 %)	239 (±15 %)	26.3 (±20.7 %)
Sucrose	Sucrose	138.8	4	$NaNO_3$	70.6	14.3	171	18.5
Xylose	Xylose	333.0	4	$NaNO_3$	70.6	13.0	177	15.8
Citric acid	Citric acid	277.5	4	$NaNO_3$	70.6	2.2§	17§	7.9§
Lactose	Lactose	138.8	4	$NaNO_3$	70.6	9.6§	37§	3.8§
Proline	Proline	333.0	4	$NaNO_3$	70.6	13.4§	56§	4.2§
Glucose/proline	Glucose	277.5	4	Proline	70.6	14.4	150	26.6
Glucose/ NH_4Cl‡	Glucose	277.5	4	NH_4Cl	70.6	12.9 (±17.6 %)	199 (±16 %)	19.4 (±8.1 %)
0.5NH_4Cl	Glucose	277.5	4	NH_4Cl	35.3	8.6	77	10.3
2NH_4Cl	Glucose	277.5	4	NH_4Cl	141.2	17.9	258	19.5
4NH_4Cl	Glucose	277.5	4	NH_4Cl	282.4	11.4	369	32.3
8NH_4Cl	Glucose	277.5	4	NH_4Cl	564.8	10.9	469	43.9
4$NaNO_3$	Glucose	277.5	4	$NaNO_3$	282.4	11.4	366	32.2
8$NaNO_3$	Glucose	277.5	4	$NaNO_3$	564.8	13.5	389	28.9
pH 6/$NaNO_3$	Glucose	277.5	6	$NaNO_3$	70.6	1.1	13	12.8
pH 5/$NaNO_3$	Glucose	277.5	5	$NaNO_3$	70.6	4.2	49	25.0
pH 5/NH_4Cl	Glucose	277.5	5	NH_4Cl	70.6	8.4	93	11.1

*The concentration of carbon was equal under all conditions.

†Specific protease activity was calculated as the maximum protease activity (in U l^{-1}) divided by the dry weight concentration (in g l^{-1}) at the time point that the maximum activity was reached.

‡These experiments were performed in quadruplicate. Results are presented as means (±RSD).

§With these carbon sources growth was very slow and therefore these cultivations were stopped while biomass concentrations and protease activity were still increasing.

activity of cleared culture supernatants was determined by incubating 2 or 8 µl samples with 75 µl 0.5 % (w/v) *N*,*N*-dimethylated BSA in 0.25 M sodium acetate buffer, pH 4.0, for 17.5 min at 37 °C. As a blank, samples were incubated with sodium acetate buffer without *N*,*N*-dimethylated BSA. The reaction was stopped by the addition of 185 µl 0.1 M borate buffer, pH 9.3, with 0.5 g Na_2SO_3 l^{-1}. Simultaneously, 5 µl 1 × -diluted 2,4,6,-trinitrobenzene sulfonic acid (TNBSA; Pierce) was added. TNBSA reacts with the free amino acid groups, resulting in a yellow colour, which was measured at 405 nm after 3 min. Glycine was used as standard. One unit of protease activity was defined as the amount of enzyme which in 1 min under the given standard conditions produces a hydrolysate of which the absorption at 405 nm is equal to 1 µmol glycine l^{-1}. Proteolytic activities were determined at pH 6 (0.25 M MES buffer, pH 6.0) and pH 8 (0.25 M MOPS buffer, pH 8.0) as well, but protease activities were very low (results not shown).

Statistical analysis. Before statistical analysis, the curves for DWT and protease activity were corrected for noise and possible outliers by using a smoothing algorithm based on penalized least-squares (Eilers, 2003). The degree of smoothing depended on the value of the penalty (λ) and the derivative that was used. Several combinations of restrictions and derivatives (first- and second-order) were considered in order to find the most appropriate smoothing. For the analysis of full two-level factorial design experiments, six different phenotypes were defined to express protease activity. With analysis of variance (ANOVA) the effect of an environmental factor or a combination of factors on protease activity levels was evaluated for each of the six protease-related phenotypes. Each ANOVA model contained all main effects (i.e. the effect of pH, carbon source, nitrogen source or nitrogen concentration on the protease-related phenotype) and the interaction effects of two and three environmental factors. Interaction between all four environmental factors was not included in the models, for this effect was not significant for any of the six individual protease-related phenotypes. An effect was considered significant when the *P*-value was below 0.05. As a measure for the relative contribution of each effect to variation in protease activity, η^2 was calculated as the sum of squares of each effect relative to the total sum of squares. Both smoothing and ANOVA were performed using Matlab Version 7.5.0.342 R2007b (The Mathworks).

RESULTS

Screening of environmental parameters involved in extracellular protease production by *A. niger*

The effect of various environmental factors (Table 1) on extracellular protease production by *A. niger* N402 was investigated in controlled batch cultures with a change-one-factor-at-a-time approach. Tested variables included carbon source, nitrogen source, nitrogen concentration and pH.

To investigate the effect of carbon sources on production of extracellular protease, six different carbon sources were tested at a culture pH of 4 in a minimal medium containing 70.6 mM sodium nitrate as nitrogen source.

Table 2. Conditions of the full factorial design used in this study

Experiment name	pH	Carbon source*	Nitrogen source	Nitrogen level†
4 G 4NO_3	4	Glucose	$NaNO_3$	Low
4 G 8NO_3	4	Glucose	$NaNO_3$	High
4 G 4NH_4‡	4	Glucose	NH_4Cl	Low
4 G 8NH_4‡	4	Glucose	NH_4Cl	High
4 X 4NO_3	4	Xylose	$NaNO_3$	Low
4 X 8NO_3‡	4	Xylose	$NaNO_3$	High
4 X 4NH_4‡	4	Xylose	NH_4Cl	Low
4 X 8NH_4	4	Xylose	NH_4Cl	High
5 G 4NO_3‡	5	Glucose	$NaNO_3$	Low
5 G 8NO_3	5	Glucose	$NaNO_3$	High
5 G 4NH_4	5	Glucose	NH_4Cl	Low
5 G 8NH_4	5	Glucose	NH_4Cl	High
5 X 4NO_3‡	5	Xylose	$NaNO_3$	Low
5 X 8NO_3‡	5	Xylose	$NaNO_3$	High
5 X 4NH_4‡	5	Xylose	NH_4Cl	Low
5 X 8NH_4	5	Xylose	NH_4Cl	High

*Glucose and xylose were used at 277.5 and 333.0 mM, respectively. The concentration of carbon was equal under both conditions.
†$NaNO_3$ and NH_4Cl were used at 282.4 (low) or 564.8 mM (high).
‡These cultivations were performed in duplicate.

Fig. 1 depicts the concentration profiles of carbon source and biomass as well as protease activity as assayed at pH 4 for a controlled batch culture with glucose as carbon source. Protease activity was assayed at pH 6 and 8 as well. However, at these pH values hardly any or no proteolytic activity was detected, as reported by van Noort *et al.* (1991) (results not shown). *A. niger* N402 grew exponentially until the carbon source was completely consumed at approximately 96 h; after this, biomass concentration started to decline. Before glucose depletion, extracellular protease activity had already started to rise and increased rapidly until approximately 18 h after the carbon source in the medium was completely utilized. Near the end of the culture period, the rate of increase in extracellular protease activity decreased. This cultivation with glucose as carbon source was carried out in quadruplicate and used as a reference culture in this study. Physiological parameters for growth and protease activity under this culture condition and all other environmental conditions of this screening design are summarized in Table 1.

With xylose as carbon source, profiles for growth and protease secretion (data not shown) showed similar trends as for glucose, although the maximum protease activity and specific protease activity were significantly lower with xylose (Table 1). When sucrose is used as carbon source, it is first converted into glucose and fructose, after which glucose is consumed prior to fructose. Growth did not stall during the switch from glucose to fructose and was comparable to the cultures with glucose or xylose. Both maximum protease activity and specific protease activity were considerably lower compared to growth with glucose (Table 1).

With citric acid, lactose and proline the lag phase was long (4–5 days) and subsequent growth was slow. Therefore, these cultivations were stopped before the stationary phase was reached, suggesting that the carbon source was not yet completely consumed. This was confirmed for lactose, where 40 % of the carbon source was not consumed at the time point when the cultivation was stopped. Protease activity appeared much earlier in cultures with less preferred carbon sources (data not shown), at a time point

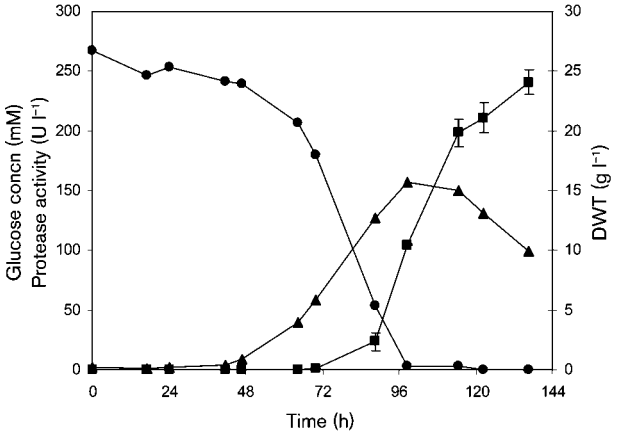

Fig. 1. Profile for extracellular protease production by *A. niger* during controlled batch culture with glucose, sodium nitrate and at pH 4. ●, Glucose; ■, protease activity assayed at pH 4; ▲, biomass concentration. Protease activity was measured in duplicate and the results are expressed as means ± SD.

Table 3. The six protease-related phenotypes evaluated in this study (see also Fig. 2)

Protease-related phenotype	Description
Maximum protease activity (Max. act.)	Maximum extracellular protease activity measured during cultivation
Maximum specific protease activity – 1 (Max. spec. act. – 1)	Maximum activity divided by the DWT at the time point that the maximum activity was reached
Maximum specific protease activity – 2 (Max. spec. act. – 2)	Maximum activity divided by DWT_{max}
Maximum protease productivity (Max. prod.)	Maximum increase of extracellular protease activity per unit of time
Maximum specific protease productivity – 1 (Max. spec. prod. – 1)	Maximum productivity divided by the DWT at the time point that the maximum productivity was reached
Maximum specific protease productivity – 2 (Max. spec. prod. – 2)	Maximum productivity divided by DWT_{max}

when excess carbon source was still present, whereas with glucose, protease activity increased after glucose depletion.

We further investigated the effects of different nitrogen sources, i.e. proline, ammonium chloride and sodium nitrate, on extracellular protease levels at pH 4 with glucose as carbon source. In addition to cultivation with nitrate, cultivation with ammonium was performed in quadruplicate and used as a reference culture as well. Although DWT_{max} was in the same range for cultures grown with any of the three nitrogen sources, maximum protease activity was especially lower with proline, whereas with ammonium the specific protease activity was lower (Table 1).

Based on our analysis, ammonium was limiting for at least a period of time during cultivation in medium containing 70.6 mM ammonium chloride (result not shown). To analyse the effect of the ammonium concentration, we tested a variety of initial ammonium chloride concentrations. From this analysis it was clear that ammonium was limiting only at an initial ammonium chloride concentration of 141.2 mM or below (results not shown). Regarding the relationship to protease activity, with an increasing concentration of ammonium chloride maximum protease activity increased as well (Table 1).

Based on the results obtained with ammonium chloride, the effect of an increase in the nitrogen concentration by a factor of 4–8 was also studied for sodium nitrate. Nitrate is not expected to be limiting in these cultures, although its concentration was not actually determined. In comparison to the reference culture with sodium nitrate, protease activities increased considerably at elevated nitrate concentrations (Table 1). However, the highest concentration tested did not result in a further increase in protease activity, as was the case with ammonium.

The last process parameter tested in the screening design was culture pH. Using a minimal medium containing 277.5 mM glucose and 70.6 mM sodium nitrate, an increase in the culture pH from 4 to 5 resulted in a fivefold decrease in maximum protease activity to 49 U l^{-1}, and DWT_{max} decreased threefold to 4.2 g l^{-1} (see Table 1). The specific protease activity, however, was equal at both culture pH values. At pH 6, maximum protease activity and DWT_{max} were even more severely affected (13 U l^{-1} and 1.1 g l^{-1}, respectively) than at pH 5. Notwithstanding the concomitant decrease of biomass formation, the specific protease activity at pH 6 was approximately 50 % lower compared to cultures controlled at pH 4. It was striking that biomass formation was so severely affected by an increase in pH, although the rate of carbon consumption was normal. This suggests increased production of carbon dioxide or other carbon metabolites, such as organic acids, at elevated pH.

The effect of an increase in culture pH varied for different nitrogen sources. When pH was increased from pH 4 to 5, maximum protease activity and DWT_{max} were significantly lower when nitrate was used instead of ammonium (see Table 1). At pH 4 the differences between the two nitrogen sources were marginal. This discrepancy in response to pH suggests that, for growth and protease activity, pH and nitrogen source may be interdependent.

To regulate pH at the indicated values, KOH was added to the various cultures. The final concentration of K^+ varied between 288 and 820 mM. However, no clear correlation was found between $[K^+]$, pH and protease activity at the time of maximum protease activity.

Analysis of the interaction effects between environmental factors on protease production

In the screening experiments, large variations in both maximum protease activity and specific protease activity were observed for the different culture conditions. There were also indications that the effects of some of the environmental factors were dependent on a combination of factors, e.g. nitrogen source and nitrogen concentration, or nitrogen source and pH. However, the screening approach applied, in which a single factor was changed while keeping all other factors constant, is unsuitable for identifying interactions among environmental factors. A full factorial design, on the other hand, is effective in assessing the contribution of a single environmental factor on the response studied as well as possible interaction effects between these factors (Lundstedt *et al.*, 1998; Kennedy & Krouse, 1999). In this type of design, each factor is considered at two or more levels and the experiments are

carried out at each possible combination of these levels. A full two-level factorial design was applied with four environmental factors from the screening experiments. For each factor, two levels were selected (Table 2). The resulting 2^4 full factorial design was performed with eight replicates that were randomly selected.

As in the screening experiments, the response to the various experimental factors was sometimes different for protease activity and specific protease activity. Therefore, six phenotypes to express protease activity (Table 3) were evaluated in the analysis of the experiments of the full 2^4 factorial design. In addition to maximum protease activity (see A in Fig. 2), the maximum rate of protease production, i.e. the maximum protease productivity (see B in Fig. 2), was also considered. However, for secreted products, the concentration (or activity) and rate of production also depend on the biomass concentration. Therefore, maximum specific protease activity and maximum specific protease productivity were included as well. These two phenotypes can be calculated using the DWT at the time point of maximum protease activity (see A1 in Fig. 2) or maximum protease productivity (see B1 in Fig. 2), respectively. However, these phenotypes were reached while biomass concentration was declining, thus making maximum specific protease activity and maximum specific protease productivity strongly dependent on the degree of lysis. Therefore, both phenotypes were also calculated in relation to the maximum biomass concentration reached, DWT_{max} (see A2 and B2 in Fig. 2).

For each of these six protease-related phenotypes, similar trends of protease activity or protease productivity were observed under the different environmental conditions (Table 4). In general, trends showed that protease activity and protease productivity were high in cultures at pH 4 and low in cultures at pH 5. An exception to this were cultures performed at pH 5 in the presence of high ammonium concentrations, which resulted in relative high values. A closer look at the six protease-related phenotypes revealed subtle differences in reaction to the different process conditions, as illustrated for maximum specific protease activity and maximum specific protease productivity (Fig. 3). For both phenotypes, the experiments were displayed in the same order, showing a clear difference in response. More specifically, in the experiments with high ammonium concentrations, maximum specific protease activities were lower at pH 5 compared to pH 4, while for the same experiments the maximum specific protease productivities were clearly highest at pH 5.

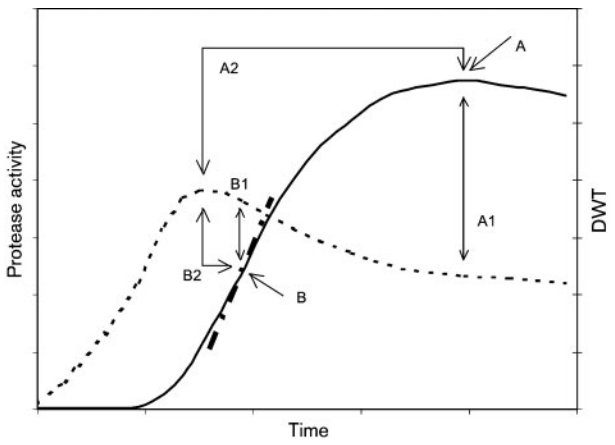

Fig. 2. Schematic representation of extracellular protease production by *A. niger* during controlled batch culture to explain the various protease-related phenotypes as described in Table 3. Solid line, protease activity; dashed line, biomass concentration. A, Maximum protease activity; A1, maximum specific protease activity – 1; A2, maximum specific protease activity – 2; B, maximum protease productivity; B1, maximum specific protease productivity – 1; B2, maximum specific protease productivity – 2.

To establish the contribution of the various environmental factors to protease activity and possible interaction effects between these factors, analysis of variance (ANOVA) was performed for each of the six individual protease-related phenotypes (Table 5; for a complete overview of the results and the interaction plots, see the online supplementary data). In the main, nitrogen source and nitrogen concentration had a large relative contribution (η^2) to protease-related phenotypes (Table 5). The contribution of pH was substantial as well, but only to maximum protease activity and maximum specific protease activity.

However, caution is necessary in the interpretation of these main effects, for they cannot be interpreted without taking interaction effects into account. For instance, from Table 5 nitrogen concentration comes up as a significant main effect for all protease-related phenotypes. When only looking at this main effect, comparing the mean protease activities of experiments with either high or low nitrogen concentrations, one might conclude that an increase in nitrogen concentration affects protease activity in all cases. However, this was only true for ammonium, while nitrogen concentration had little effect on protease-related phenotypes with nitrate as the nitrogen source. This difference in response to nitrogen concentration for the two nitrogen sources tested points towards an interaction effect of nitrogen source and nitrogen concentration. Similarly, for nitrogen source the main effect cannot be viewed without taking into account that the effect is dependent on pH. With the exception of maximum protease activity, protease-related phenotypes were more affected by a change in culture pH when nitrate was used as a nitrogen source instead of ammonium. However, the contribution of the two-way interaction effect between nitrogen source and nitrogen concentration was considerably higher than that for pH in combination with nitrogen source (see Table 5).

Additionally, significant three-way interaction effects were detected (Table 5). For example, the combination of pH, nitrogen source and nitrogen concentration showed a significant interaction effect for maximum protease

Table 4. Values of the six protease-related phenotypes (Table 3) under the experimental conditions of the full factorial design

Experiment name	Max. biomass, DWT_{max} (g l^{-1})	Max. act. (U l^{-1})	Max. spec. act. – 1 (U g^{-1})	Max. spec. act. – 2 (U g^{-1})	Max. prod. (U l^{-1} h^{-1})	Max. spec. prod. – 1 (U g^{-1} h^{-1})	Max. spec. prod. – 2 (U g^{-1} h^{-1})
4 G $4NO_3$	15.4	300	30.0	19.4	5.5	0.39	0.36
4 G $8NO_3$	13.5	326	34.6	24.1	6.1	0.46	0.45
4 G $4NH_4$*	19.1 (±0.5 %)	312 (±5 %)	25.1 (±12.5 %)	16.3 (±4.8 %)	9.5 (±7.5 %)	0.51 (±5.01 %)	0.50 (±7.23 %)
4 G $8NH_4$*	19.4 (±3.5 %)	652 (±0 %)	51.9 (±3.5 %)	33.7 (±3.9 %)	13.7 (±16.5 %)	0.71 (±19.93 %)	0.71 (±19.93 %)
4 X $4NO_3$	12.8	225	23.2	17.7	6.2	0.50	0.49
4 X $8NO_3$*	14.9 (±9.7 %)	249 (±1 %)	23.0 (±5.7 %)	16.8 (±8.6 %)	6.3 (±24.8 %)	0.44 (±35.70 %)	0.43 (±34.02 %)
4 X $4NH_4$*	17.4 (±7.5 %)	286 (±7 %)	25.7 (±14.1 %)	16.5 (±14.6 %)	6.3 (±12.9 %)	0.37 (±17.02 %)	0.36 (±20.31 %)
4 X $8NH_4$	19.4	613	53.9	31.7	13.5	0.70	0.70
5 G $4NO_3$*	9.1 (±41.4 %)	70 (±39 %)	12.4 (±19.2 %)	7.7 (±2.9 %)	1.7 (±46.4 %)	0.21 (±7.94 %)	0.18 (±5.49 %)
5 G $8NO_3$	11.4	93	12.0	8.1	1.7	0.15	0.15
5 G $4NH_4$	15.5	170	20.4	11.0	3.8	0.25	0.25
5 G $8NH_4$	16.5	472	43.9	28.5	16.7	1.08	1.01
5 X $4NO_3$*	10.7 (±5.0 %)	117 (±4 %)	14.9 (±3.4 %)	10.9 (±9.3 %)	3.3 (±36.2 %)	0.32 (±42.22 %)	0.31 (±40.80 %)
5 X $8NO_3$*	11.5 (±1.9 %)	100 (±21 %)	11.5 (±28.0 %)	8.7 (±22.7 %)	2.6 (±48.7 %)	0.23 (±50.68 %)	0.23 (±50.38 %)
5 X $4NH_4$*	17.2 (±3.6 %)	187 (±1 %)	16.2 (±17.0 %)	10.8 (±4.1 %)	4.5 (±1.4 %)	0.27 (±0.01 %)	0.26 (±2.19 %)
5 X $8NH_4$	16.7	381	40.6	22.9	14.7	0.89	0.88

*These cultivations were performed in duplicate. Results are presented as means (±RSD).

productivity and maximum specific protease productivity. For instance, in the case of ammonium as nitrogen source, the effect of nitrogen concentration on maximum protease productivity was larger at pH 5 than at pH 4. On the other hand, the effect of nitrogen concentration was negligible at both pH values with nitrate as nitrogen source. However, in general the contribution of the interaction effects between three environmental factors to variation in protease activity was small.

DISCUSSION

Degradation of secreted proteins by native extracellular proteases is one of the key factors hindering the successful application of filamentous fungi in non-fungal protein production. Approaches to overcome this problem have mainly focussed on strain improvement (Berka *et al.*, 1990; Mattern *et al.*, 1992; van den Hombergh *et al.*, 1995, 1997b; Zheng *et al.*, 1998; Moralejo *et al.*, 2000; Wiebe *et al.*, 2001; Moralejo *et al.*, 2002). In addition, the use of fungal strains with growth characteristics (e.g. optimal pH) more favourable to the stability of these non-fungal proteins has been evaluated (e.g. Heerikhuisen *et al.*, 2005). However, unlike *A. niger*, these strains were shown to produce high levels of protease at pH values higher than pH 4. Reduction of protease secretion by means of manipulation of the environmental conditions has obtained relatively little attention. In this study, the influence of several environmental factors on the extracellular protease activity levels of *A. niger* N402 was systematically investigated in batch cultures. After an initial screening designed to select important environmental parameters that influence protease activity, a full factorial design was applied to determine the contribution of each environmental factor to the induction of protease activity. An important additional advantage of a full factorial design is that it can be used to identify possible interaction effects between the environmental factors tested (Lundstedt *et al.*, 1998; Kennedy & Krouse, 1999). In this study, the existence of significant interaction effects between several of the environmental factors was established. To our knowledge, interaction effects between environmental factors in relation to protease secretion of *A. niger* have not been reported before.

One of the most prominent interaction effects identified was between nitrogen source and nitrogen concentration, as the effect of concentration is dependent on the nitrogen source. Both individual factors have been reported to affect extracellular protease production in *A. niger* and other aspergilli. Several extracellular protease encoding genes in *A. nidulans* (Katz *et al.*, 1996, 2008) and *A. niger* (van den Hombergh *et al.*, 1994; Jarai & Buxton, 1994) are derepressed when nitrogen source limitation occurs. In the presence of low-molecular-mass nitrogen sources, such as ammonium and nitrate, the induction of extracellular proteases is repressed (Cohen, 1972). In our research we found that in the presence of ammonium, extracellular protease activity only appeared as soon as the carbon source was depleted (results not shown). However, with nitrate as nitrogen source, protease activity levels started to increase before the carbon source depleted, as shown in Fig. 1. This is in agreement with the findings of van den Hombergh *et al.* (1997b), who found nitrate to be less repressive than ammonium and urea. Based on the

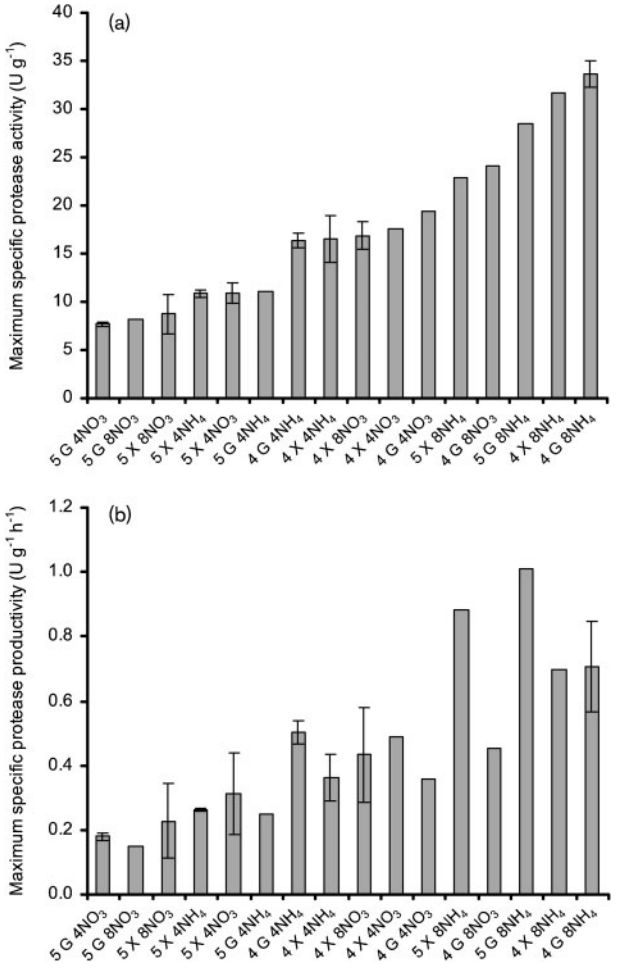

Fig. 3. (a) Maximum specific protease activity and (b) maximum specific protease productivity under the experimental conditions of the full factorial design. To illustrate the differences between the two protease-related phenotypes under identical environmental conditions, the ordering of the experiments is identical in both figures.

above-mentioned findings, ammonium seems to be the preferred nitrogen source to repress protease activity. Excess ammonium has been suggested as a means to reduce proteolytic degradation of heterologous proteins (Wiebe *et al.*, 2001; Wiebe, 2003). However, the effect of ammonium as the nitrogen source was less advantageous as soon as derepression of extracellular proteases occurred, for instance due to carbon source depletion. We found that final protease activity levels were higher with ammonium than with nitrate. The existing interaction between nitrogen source and concentration was demonstrated by the increase in maximum protease activity with increasing initial ammonium concentrations, while protease activities remained unchanged when nitrate concentrations were further elevated.

Less prominent interaction effects were observed involving culture pH. Culture pH itself has often been indicated as an important environmental parameter in controlling extracellular protease activity. It affects both the activity of the secreted proteases (O'Donnell *et al.*, 2001) as well as the expression of protease (Jarai & Buxton, 1994; van den Hombergh *et al.*, 1997b). Also, in our experiments we observed that extracellular protease activities were higher at a culture pH of 4 than at pH 5 or 6 (Table 1). Most of the extracellular proteases previously purified from culture filtrates of *A. niger* have acidic pH optima (van den Hombergh *et al.*, 1997b; van Noort *et al.*, 1991; de Vries *et al.*, 2004), which is consistent with the acidifying properties of this fungus. Also, the recent sequencing of the *A. niger* genome revealed the presence of an abundance of genes encoding secreted proteases that are expected to be mostly active at low pH (such as aspartic proteases and carboxypeptidases) (Pel *et al.*, 2007).

Due to the observed interactions, the selection of culture conditions to reduce protease activity levels is not straightforward, as several factors are dependent on each other and might have unexpected antagonistic or synergistic effects. Moreover, the environmental parameters affect the biomass levels and protein secretion. For instance, in general protease activity levels are higher at pH 4 and with ammonium as nitrogen source, but so are biomass levels and, for example, glucoamylase levels (data not shown). The selection of the most optimal protein production conditions will therefore require a balance between reduction of protease activity on the one hand and optimization of growth and the level of production of the desired protein on the other. On top of this, a deliberate choice of the phenotype of interest is crucial before the start of an optimization route. In this research, we have illustrated that the effect of an environmental parameter on the six studied protease-related phenotypes is not always the same. It is likely that this is also the case for other fermentation products, both undesired – in this case protease – as well as desired products. When, for instance, a short process time is important, productivity can be the phenotype to be optimized, while in other cases time is less relevant and final product levels are crucial. One might also consider the optimal yield in relation to an expensive substrate or medium component.

In our study, the combination of controlled batch cultures with sampling at regular time intervals enabled us to express protease activity in various ways. Differences were observed for pH with a large contribution to protease activity and a small or insignificant contribution to protease productivity.

This study is part of an effort to investigate the regulation of protease production in *A. niger*. The experiments of the full factorial design show large and evenly distributed variation of protease activity, and are therefore a suitable starting point for a full-scale systems biology approach. Samples from these experiments will be used for metabo-

Table 5. ANOVA analyses of the main and interaction effects of the full factorial design

An effect with a *P*-value <0.05 is considered significant (shown in bold).

Source of variation*	Max. act.		Max. spec. act. – 1		Max. spec. act. – 2		Max. prod.		Max. spec. prod. – 1		Max. spec. prod. – 2	
	η^2	*P*-value	η^2	*P*-value	η^2	*P*-value	η^2	*P*-value	η^2	*P*-value	η^2	*P*-value
Main effects												
pH	**0.22**	**<0.0001**	**0.18**	**<0.0001**	**0.24**	**<0.0001**	**0.05**	**0.0034**	0.02	0.1242	0.03	0.0801
C	**0.01**	**0.0015**	**0.01**	**0.0296**	**0.01**	**0.0243**	0.00	0.7208	0.00	0.8125	0.00	0.9159
N	**0.30**	**<0.0001**	**0.26**	**<0.0001**	**0.18**	**<0.0001**	**0.42**	**<0.0001**	**0.27**	**0.0003**	**0.28**	**0.0002**
[N]	**0.17**	**<0.0001**	**0.21**	**<0.0001**	**0.21**	**<0.0001**	**0.20**	**<0.0001**	**0.20**	**0.0007**	**0.22**	**0.0005**
Two-way interaction effects												
pH × C	**0.00**	**0.0045**	0.00	0.2348	0.00	0.0897	0.00	0.3673	0.00	0.7992	0.00	0.6387
pH × N	0.00	0.2362	**0.01**	**0.0171**	**0.02**	**0.0063**	**0.02**	**0.0273**	**0.07**	**0.0146**	**0.07**	**0.0176**
pH × [N]	**0.01**	**0.0034**	0.00	0.1037	0.00	0.1403	**0.02**	**0.0376**	0.04	0.0506	0.03	0.0663
C × N	0.00	0.5292	0.00	0.1953	0.00	0.7232	0.01	0.1155	0.02	0.1439	0.02	0.1419
C × [N]	**0.00**	**0.0175**	0.00	0.5587	**0.01**	**0.0151**	0.00	0.9660	0.00	0.6652	0.00	0.6661
N × [N]	**0.14**	**<0.0001**	**0.20**	**<0.0001**	**0.19**	**<0.0001**	**0.20**	**<0.0001**	**0.27**	**0.0003**	**0.26**	**0.0003**
Three-way interaction effects												
pH × C × N	**0.01**	**0.0035**	**0.02**	**0.0056**	**0.01**	**0.0094**	0.00	0.8227	0.00	0.8447	0.00	0.9268
pH × C × [N]	**0.00**	**0.0373**	0.00	0.8798	0.00	0.9339	0.01	0.2031	0.00	0.5394	0.00	0.6177
pH × N × [N]	**0.00**	**0.0358**	0.00	0.9171	0.00	0.6553	**0.03**	**0.0197**	**0.07**	**0.0171**	**0.06**	**0.0206**
C × N × [N]	0.00	0.1753	0.00	0.2583	0.00	0.9265	0.00	0.7193	0.00	0.8017	0.00	0.5989

*C, Carbon source; N, nitrogen source; [N], nitrogen concentration.

lomics and transcriptomics analyses with the objective to disclose the regulatory pathways of the proteolytic system of *A. niger*.

ACKNOWLEDGEMENTS

The authors thank Wout Kroes for performing some of the cultivations, Carina M. Rubingh for the smoothing algorithm analyses and calculating the six protease-related phenotypes, Peter M. van de Ven for the ANOVA analyses, and Robert A. van den Berg (Katholieke Universiteit Leuven) for fruitful discussions. This project was carried out within the research programme of the Kluyver Centre for Genomics of Industrial Fermentation, which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

REFERENCES

Bennett, J. W. & Lasure, L. L. (1991). Growth media. In *More Gene Manipulations in Fungi*, pp. 441–447. Edited by J. W. Bennett & L. L. Lasure. San Diego, CA: Academic Press.

Berka, R. M., Ward, M., Wilson, L. J., Hayenga, K. J., Kodama, K. H., Carlomagno, L. P. & Thompson, S. A. (1990). Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori*. *Gene* **86**, 153–162.

Bos, C. J., Debets, A. J. M., Swart, K., Huybers, A., Kobus, G. & Slakhorst, S. M. (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Curr Genet* **14**, 437–443.

Braaksma, M. & Punt, P. J. (2008). *Aspergillus* as a cell factory for protein production: controlling protease activity in fungal production. In *The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods*, pp. 441–455. Edited by G. H. Goldman & S. A. Osmani. Boca Raton, FL: CRC Press.

Buxton, F. P., Gwynne, D. I. & Davies, R. W. (1985). Transformation of *Aspergillus niger* using the *argB* gene of *Aspergillus nidulans*. *Gene* **37**, 207–214.

Cohen, B. L. (1972). Ammonium repression of extracellular protease in *Aspergillus nidulans*. *J Gen Microbiol* **71**, 293–299.

Cohen, B. L. (1981). Regulation of protease production in *Aspergillus*. *Trans Br Mycol Soc* **76**, 447–450.

de Vries, R. P., Burgers, K., van de Vondervoort, P. J., Frisvad, J. C., Samson, R. A. & Visser, J. (2004). A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. *Appl Environ Microbiol* **70**, 3954–3959.

Eilers, P. H. C. (2003). A perfect smoother. *Anal Chem* **75**, 3631–3636.

Heerikhuisen, M., van den Hondel, C. A. M. J. J. & Punt, P. J. (2005). *Aspergillus sojae*. In *Production of Recombinant Proteins. Novel Microbial and Eucaryotic Expression Systems*, pp. 191–214. Edited by G. Gelissen. Weinheim: Wiley.

Holm, K. A. (1980). Automated colorimetric determination of acid proteinase activity in fermentation samples using a trinitrobenzene-sulphonic acid reagent. *Analyst* **105**, 18–24.

Iimura, Y., Gomi, K., Uzu, H. & Hara, S. (1987). Transformation of *Aspergillus oryzae* through plasmid-mediated complementation of the methionine-auxotrophic mutation. *Agric Biol Chem* **51**, 323–328.

Jarai, G. & Buxton, F. (1994). Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. *Curr Genet* **26**, 238–244.

Katz, M. E., Flynn, P. K., vanKuyk, P. A. & Cheetham, B. F. (1996). Mutations affecting extracellular protease production in the filamentous fungus *Aspergillus nidulans*. *Mol Gen Genet* **250**, 715–724.

Katz, M. E., Bernardo, S. M. & Cheetham, B. F. (2008). The interaction of induction, repression and starvation in the regulation of extracellular proteases in *Aspergillus nidulans*: Evidence for a role for CreA in the response to carbon starvation. *Curr Genet* **54**, 47–55.

Kelly, J. M. & Hynes, M. J. (1985). Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. *EMBO J* **4**, 475–479.

Kennedy, M. & Krouse, D. (1999). Strategies for improving fermentation medium performance: a review. *J Ind Microbiol Biotechnol* **23**, 456–475.

Li, Q., Harvey, L. M. & McNeil, B. (2008). The effects of bioprocess parameters on extracellular proteases in a recombinant *Aspergillus niger* B1-D. *Appl Microbiol Biotechnol* **78**, 333–341.

Lin, Y., Means, G. E. & Feeney, R. E. (1969). The action of proteolytic enzymes on *N,N*-dimethyl proteins. Basis for a microassay for proteolytic enzymes. *J Biol Chem* **244**, 789–793.

Liu, F., Li, W., Ridgway, D., Gu, T. & Moo-Young, M. (1998). Inhibition of extracellular protease secretion by *Aspergillus niger* using cell immobilization. *Biotechnol Lett* **20**, 539–542.

Lundstedt, T., Seifert, E., Abramo, L., Thelin, B., Nyström, Å., Pettersen, J. & Bergman, R. (1998). Experimental design and optimization. *Chemom Intell Lab Syst* **42**, 3–40.

Mattern, I. E., van Noort, J. M., van den Berg, P., Archer, D. B., Roberts, I. N. & van den Hondel, C. A. (1992). Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Mol Gen Genet* **234**, 332–336.

Moralejo, F. J., Cardoza, R. E., Gutiérrez, S., Sisniega, H., Faus, I. & Martín, J. F. (2000). Overexpression and lack of degradation of thaumatin in an aspergillopepsin A-defective mutant of *Aspergillus awamori* containing an insertion in the *pepA* gene. *Appl Microbiol Biotechnol* **54**, 772–777.

Moralejo, F. J., Cardoza, R. E., Gutiérrez, S., Lombraña, M., Fierro, F. & Martín, J. F. (2002). Silencing of the aspergillopepsin B (*pepB*) gene of *Aspergillus awamori* by antisense RNA expression or protease removal by gene disruption results in a large increase in thaumatin production. *Appl Environ Microbiol* **68**, 3550–3559.

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

Papagianni, M. & Moo-Young, M. (2002). Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochem* **37**, 1271–1278.

Papagianni, M., Joshi, N. & Moo-Young, M. (2002). Comparative studies on extracellular protease secretion and glucoamylase production by free and immobilized *Aspergillus niger* cultures. *J Ind Microbiol Biotechnol* **29**, 259–263.

Pel, H. J., de Winde, J. H., Archer, D. B., Dyer, P. S., Hofmann, G., Schaap, P. J., Turner, G., de Vries, R. P., Albang, R. & other authors (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol* **25**, 221–231.

Punt, P. J., Schuren, F. H. J., Lehmbeck, J., Christensen, T., Hjort, C. & van den Hondel, C. A. M. J. J. (2008). Characterization of the *Aspergillus niger prtT*, a unique regulator of extracellular protease encoding genes. *Fungal Genet Biol* **45**, 1591–1599.

Sumner, J. B. & Somers, G. F. (1949). Dinitrosalicylic method for glucose. In *Laboratory Experiments in Biological Chemistry*, pp. 38–39. Edited by J. B. Sumner & G. F. Somers. New York: Academic Press.

Unkles, S. E., Campbell, E. I., de Ruiter-Jacobs, Y. M. J. T., Broekhuijsen, M., Macro, J. A., Carrez, D., Contreras, R., van den Hondel, C. A. M. J. J. & Kinghorn, J. R. (1989). The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: a convenient and general selection system for filamentous fungal transformation. *Mol Gen Genet* **218**, 99–104.

van den Hombergh, J. P. T. W., Jarai, G., Buxton, F. P. & Visser, J. (1994). Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus niger*. *Gene* **151**, 73–79.

van den Hombergh, J. P. T. W., van de Vondervoort, P. J. I., van der Heijden, N. C. B. A. & Visser, J. (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.

van den Hombergh, J. P. T. W., Sollewijn-Gelpke, M. D., van de Vondervoort, P. J. I., Buxton, F. P. & Visser, J. (1997a). Disruption of three acid proteases in *Aspergillus niger*: effects on protease spectrum, intracellular proteolysis, and degradation of target proteins. *Eur J Biochem* **247**, 605–613.

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

van Hartingsveldt, W., Mattern, I. E., van Zeijl, C. M. J., Pouwels, P. H. & van den Hondel, C. A. M. J. J. (1987). Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Mol Gen Genet* **206**, 71–75.

van Noort, J. M., van den Berg, P. & Mattern, I. E. (1991). Visualization of proteases within a complex sample following their selective retention on immobilized bacitracin, a peptide antibiotic. *Anal Biochem* **198**, 385–390.

Wang, Y., Xue, W., Sims, A. H., Zhao, C., Wang, A., Tang, G., Qin, J. & Wang, H. (2008). Isolation of four pepsin-like protease genes from *Aspergillus niger* and analysis of the effect of disruptions on heterologous laccase expression. *Fungal Genet Biol* **45**, 17–27.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem* **39**, 971–974.

Wiebe, M. G. (2003). Stable production of recombinant proteins in filamentous fungi – problems and improvements. *Mycologist* **17**, 140–144.

Wiebe, M. G., Karandikar, A., Robson, G. D., Trinci, A. P. J., Candia, J.-L. F., Trappe, S., Wallis, G., Rinas, U., Derkx, P. M. F. & other authors (2001). Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. *Biotechnol Bioeng* **76**, 164–174.

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

Yoder, W. T. & Lembeck, J. (2004). Heterologous expression and protein secretion in filamentous fungi. In *Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine*, pp. 201–219. Edited by J. S. Tkacz & L. Lange. New York: Kluwer Academic/Plenum Publishers.

Zheng, X. F., Kobayashi, Y. & Takeuchi, M. (1998). Construction of a low-serine-type-carboxypeptidase-producing mutant of *Aspergillus oryzae* by the expression of antisense RNA and its use as a host for heterologous protein secretion. *Appl Microbiol Biotechnol* **49**, 39–44.

Edited by: C. J. Hewitt