

Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity

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Abstract

The extracellular protease activity of *Aspergillus niger* AB4.1[pgpdAGLAGFP]#11, a recombinant strain producing a glucoamylase (GLA)–green fluorescent protein (GFP) fusion protein, was investigated in a 15 l stirred tank reactor and accordingly a pH control strategy was designed to minimize protease activity and increase recombinant yield. By maintaining pH at 6 recombinant protein production was enhanced over 10-fold to 21.0 mg/l compared to growth at acidic pH or without pH control. Protease activity was found to increase after 2 days of culture corresponding to the point where glucose in the culture medium had been completely utilized. When grown at pH 6, *A. niger* protease activity in the culture was decreased 6-fold to 560 U/l, compared to 3600 U/l under normal, acidic culture conditions. Protease activity at fermentation pH 6 was consistently lower than that at fermentation pH 3 regardless of assay pH, and results indicate that this decrease in activity was a combination of sub-optimal enzyme activity and variation in the spectrum of proteases secreted under the different pH conditions. A comparison of the concentrations of recombinant GLA and GFP demonstrated that high protease activity was responsible for GFP losses. More GFP was secreted in the pH 3 run, but less GFP remained in the broth because of the high protease activity. Although controlling pH at 6 did not completely inhibit the proteases, the GFP concentration in the fermentation broth was increased greatly. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Filamentous fungi; Fermentation; Protease inhibition; Glucoamylase (GLA); Green fluorescent protein (GFP)

1. Introduction

Filamentous fungi have been identified as promising hosts for the production of recombinant proteins due to their desirable growth characteristics. They can produce and secrete exceptionally large amounts of protein [9], process eukaryotic messenger ribonucleic acid (mRNA) and carry out post-translational modifications to proteins, such as glycosylation and disulfide bridge formation [11]. Since many plasmids used to transform filamentous fungi are integrated into the chromosome, the potential for genetic stability of recombinant strains is improved [7]. The main problem encountered is the expression of high levels of proteases, which cause yield reductions through degradation of the recombinant product [19]. As a result, the secreted yields of heterologous proteins are low compared to homologous proteins, and generally reach levels that do not exceed a few tens of milligrams per liter of culture medium [9]. Sev-

eral strategies have been developed to increase these yields. Those employed include the introduction of a large number of gene copies [22], the use of strong promoters and efficient fungal secretion signals [1] and the development of protease-deficient host strains [16]. One of the most successful strategies involves the construction of gene fusions of the target gene to genomic sequences encompassing the complete or partial coding region of a highly expressed fungal gene such as *Aspergillus niger* glucoamylase (GLA) [21]. In general, the fungal protein serves as a carrier to improve the translocation of the protein into the endoplasmic reticulum, aid the folding and protect the heterologous protein from degradation. Further along the secretory pathway, in most cases the fusion protein is cleaved either by autocatalytic processing of the heterologous protein by an unknown fungal protease, or by a KEX2-like protease, for which a recognition site has been introduced in the fusion protein, resulting in the secretion of separate proteins [9]. However, even with successful secretion of higher levels of heterologous proteins, the problem of extracellular proteases remains. The reduction of protease secretion through

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bioprocessing strategies such as pH control has not been investigated systematically.

Previous work carried out in this laboratory examined the effect of immobilization on protease and homologous GLA secretion in shake flask cultures of a wild-type *A. niger* [12]. It was found that immobilization significantly reduced the secretion of protease, with the specific activity of extracellular protease reduced from 129 U/g in free cell culture to 28 U/g in immobilized culture. The effect of morphology on proteases and recombinant protein productions in shake flask culture of *A. niger* AB4.1[*pgpdAGLAGFP*]#11 was also investigated. It was found that pelleted growth increased green fluorescent protein (GFP) production by 3-fold compared to free mycelial growth [10]. In this pH control study, recombinant protein production and yield improvement using pH control as a strategy to inhibit protease secretion in a recombinant strain of *A. niger* producing a GLA–GFP fusion protein was investigated. By keeping the culture pH at a level higher than the pH for the highest protease activity, it was expected that protease degradation would be decreased, resulting in higher product yields. Experiments were carried out in a bioreactor in the 3–7 pH range, and recombinant protein was detected by a GFP fluorescence assay.

2. Materials and methods

2.1. Fungal strains and medium

The recombinant *A. niger* strain AB4.1[*pgpdAGLAGFP*]#11 was kindly provided by Dr. Peter Punt, Molecular Genetics and Gene Technology, TNO Nutrition and Food Research Institute, The Netherlands. It carries a plasmid containing the GLA–GFP gene fusion driven by the constitutive *gpdA* promoter. This strain produces two types of GLA, 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 *pyrG1* derivative of N402 [17] and N402 is a *cspA1* derivative of strain ATCC 9029 [4]. The medium for *A. niger* cultures was YM broth (Difco), which consisted of yeast extract 3.0 g/l, malt extract 3.0 g/l, peptone 5.0 g/l and dextrose 10 g/l.

2.2. Culture conditions

Fermentations were carried out in a Biostat C (B. Braun Biotech.) stirred tank reactor with a 15 l working volume at pH 3, 5, 6, 7 and without pH control. The agitation system consisted of three disc impellers (Rushton turbine) with a stirring speed of 200 rpm. The cultures were processed at the aeration rate of 1 v.v.m. Spores for inoculation were obtained from wort agar plates incubated at 27°C for 5 days. The spores were harvested by adding 20 ml of sterilized water to the plates. The standard inoculation level was 2.5×10^5 spores/ml. Each experiment was repeated once or sometimes twice. Averages of the repeated experimental results were calculated. The errors were within 20%.

Fungal morphology can be greatly influenced by inoculum level, initial pH, agitation, polymer additives, etc. Morphology is determined within 1 day after spore inoculation [25–28]. In this work, fungal morphology was properly controlled to form optimum pellet size [10] by changing agitation rate in the first 12–18 h after spore inoculation. The final pellet size was between 1 and 2 mm.

3. Sample analysis

3.1. Biomass

Biomass was determined by measuring dry cell weight. Hundred milliliter samples were filtered through dried, pre-weighed 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 by a glucose kit (Sigma, Catalog No. 315-100).

3.3. GLA activity assay

Recombinant GLA was measured using two steps. First, total GLA was measured using PNPG (4-nitrophenyl- α -D-glucopyranoside) as substrate [22]. Freshly made 0.1% (w/v) PNPG in 0.1 M Na acetate buffer (pH 4.3) was equilibrated to 30°C. The reaction mixture, which was incubated at 30°C for 20 min, contained 250 μ l culture filtrate (or GLA standard) and 500 μ l PNPG solution. The reaction was terminated by the addition of 750 μ l borax (0.1M Na₂B₄O₇·10H₂O) solution and absorbance was read at 400 nm. Blank samples or GLA standards (250 μ l) were also set up with 750 μ l borax solution (added prior to the PNPG) and 500 μ l PNPG at room temperature, and the absorbance read at 400 nm. Total GLA concentration was determined by subtracting the absorbance value of the blank samples from the absorbance of the sample mixtures, and comparison with the standard curve. Second, the non-plasmid GLA was detected using starch as substrate [24]. Since the recombinant GLA has no starch binding site, only non-recombinant GLA is detected. A 0.2 ml aliquot of culture filtrate was mixed with 1.8 ml 2% soluble starch (Sigma, Catalog No. S-2630) in 0.1 M citrate buffer. The mixture was allowed to react at 37°C for 30 min. The reaction was stopped by boiling the reaction mixture at 100°C for 10 min, and glucose concentration in the solution was assayed using the Sigma glucose kit. Finally, recombinant GLA was determined by subtracting the value for non-plasmid GLA from the total GLA detected.

3.4. Protease activity assay

Extracellular protease activity was determined according to the method of van den Hombergh et al. [15]. Each 450 μ l

sample was incubated with 50 μ l 1% (w/v) BSA (Fraction V, Sigma) in 0.1 M sodium acetate buffer (pH 4.0) at 37°C. At 30, 60 and 90 min, the reactions were terminated with 500 μ l 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 (U) of protease activity was defined as a change of one absorbency unit per hour at 280 nm for 1 ml reaction precipitation mixture as described above. Extracellular protease activity was expressed as U/l. The specific activity of extracellular protease was defined as unit per gram of dry cell weight, U/g.

3.5. GFP assay

GFP was detected using a fluorescence assay. Measurements were performed with an HP 1000 series fluorescence detector (Hewlett Packard, Wilmington, DE) using the software package ChemStations (Hewlett Packard, Wilmington, DE). 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 ml sample was injected each time for the measurement of relative fluorescence units (RFU). Pure GFP (Clontech, Palo Alto, CA) was used for calibration. Before injection, the pH of samples was adjusted to 7.0.

4. Results and discussion

4.1. Effect of pH on protease activity

Initial experiments were carried out to determine the pH at which maximum protease activity is detected by assaying the same sample at different pH levels. Fig. 1 demonstrates that *A. niger* AB4.1[pgpdAGLAGFP]#11 protease activity

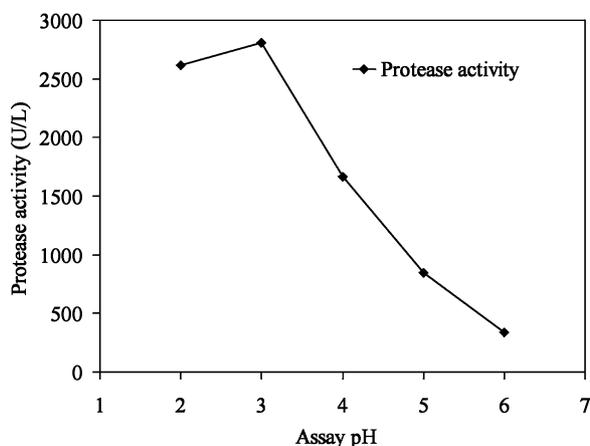


Fig. 1. *A. niger* protease activity under various assay pH levels. Sample is from a fifth day old batch culture without pH control.

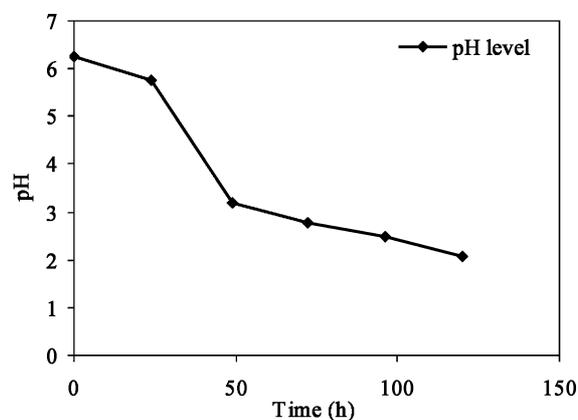


Fig. 2. pH profile of *A. niger* fermentation broth.

was found to be highest at pH 3 with a large decline in activity when the pH was raised to 6. A similar protease activity profile has been observed for an *A. niger* strain producing an *Erwinia* pectate lyase [2]. *A. niger* is known for its strong acidification of culture media [3,16] and analysis of the proteolytic spectrum in *A. niger* revealed that acid proteases predominate [8]. Without pH control, a typical pH profile of the fermentation of *A. niger* AB4.1[pgpdAGLAGFP]#11 is given in Fig. 2. It can be seen that by the end of the second day, the pH value of the fermentation broth dropped between 2 and 3, at which the protease activity was the highest. The broth pH stayed at 2–3 afterwards. Previous work in this lab showed that protease degradation contributed the most to the GFP loss [10]. Other researchers have also shown that proteases are responsible for the degradation of heterologous proteins such as human interleukin-6 [5], bovine chymosin [18] and porcine pancreatic phospholipase A [14]. Since protease activity is strongly affected by pH values, the recombinant protein production can increase if broth pH is properly controlled at a value at which protease activity is low, e.g., pH 5 or 6.

Fermentation experiments using *A. niger* AB4.1[pgpdAGLAGFP]#11 were carried out in the 151 bioreactor at pH 3, 5, 6, 7 and without pH control. The GFP concentration profile for each run is shown in Fig. 3. Biomass production is shown in Fig. 4.

4.2. Biomass development at various pH levels

The biomass profiles did not change much from pH 3 to pH 6, although the biomass concentrations were significantly lower at pH 7. At pH 3 biomass reached a maximum of 5.1 g/l, and at pH 6 it was 4.4 g/l. At pH 7, it only reached 1.9 g/l. The fact that acidic pH promotes development of *A. niger* biomass is not unexpected since *A. niger* normally tends to quickly acidify its medium when grown without pH control. This biomass yield is consistent with biomass levels found in wild-type *A. niger* shake flask cultures obtained previously in this laboratory [12]. The very low biomass

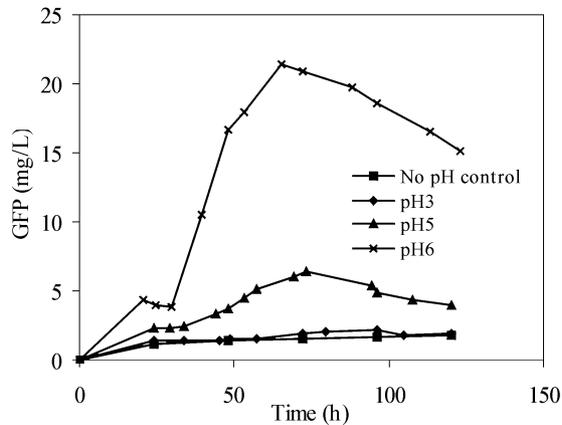


Fig. 3. *A. niger* GFP production at various pH levels.

level detected at pH 7 indicates that the strain was grown at a pH level too far away from its optimum pH for cell growth.

Although the biomass profiles are similar, there is however a difference in the length of the initial lag period between the higher pH cultures (pH 5 and 6) and lower pH cultures (pH 3 and 4), most likely due to an inhibitory effect on sporulation at lower culture pH. A similar inhibitory effect at pH 2 and 3 on germination of spores of *Phanerochaete chrysosporium* has been reported in the literature [20]. Once the culture had started to grow, it exhibited a regular growth profile. Experiments carried out in this laboratory showed that spores inoculated at pH 3 germinated much slower than those inoculated at pH 6, resulting in a longer period before significant glucose consumption. Once germination had occurred in the acidic culture, glucose was quickly used up and biomass was quickly developed to the highest in the following 24 h.

4.3. GFP production at various pH levels

GFP productions in fermentation runs at pH 3 and no pH control are similar, with a total GFP detected reaching

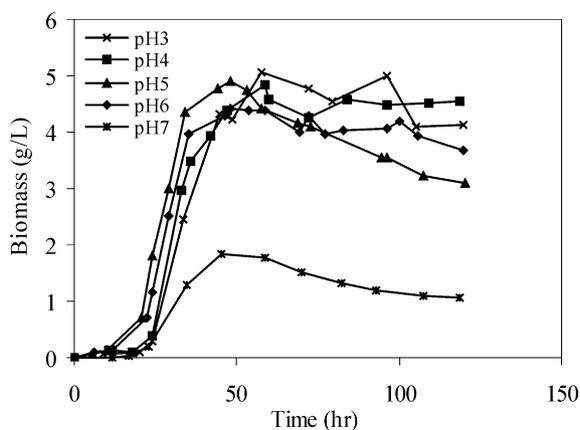


Fig. 4. *A. niger* biomass development at various pH levels.

maximum concentrations of 2.0 and 1.8 mg/l, respectively. Raising pH to 6 resulted in a significant increase in recombinant protein production. Recombinant protein production increased to a maximum at pH 6 and decreased beyond this point. Maximum GFP detected at pH 6 was 21.0 mg/l, 10 times higher than at pH 3. Also of interest is the fact that at pH 6 GFP concentration increased to a maximum after 3 days, followed by a decrease over the next 2 days of culture, suggesting that some GFP degradation by proteases present in the culture occurred.

4.4. Protease activity in pH controlled fermentations

Protease activities in culture filtrates of the various fermentations were determined to investigate its correlation with the GFP production. Protease activity changes in relation to pH, so assays were carried out at the pH specific to the fermentation. This was done so that the potency of the secreted protease in the specific environment of the culture would be determined rather than assaying all samples at a single pH and detecting protease activity that might not occur at higher pH levels. *A. niger* AB4.1[p*gpdAGLAGFP*]#11 protease activities at the various pH levels are shown in Fig. 5.

Protease activities at the various pH levels generally started to increase on the second day of culture, corresponding to the time at which glucose concentration in the medium decreased to zero. The exhaustion of glucose in the medium seems to be the trigger for production of proteases. The results are consistent with the report that human interleukin-6 secreted by recombinant *Aspergillus nidulans* was degraded by extracellular proteases after the cell concentration reached its maximum at 48 h of fermentation [23].

The highest protease activity and specific protease activity (based on dry cell mass) were found to occur at pH 3. Protease activity reached a maximum of 3600 U/l at pH 3, compared to a maximum of 560 U/l at pH 6, thus protease activity for the acidic culture was 6.4 times higher than the

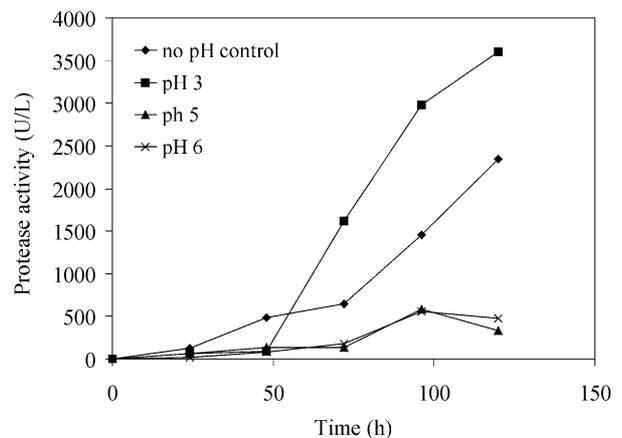


Fig. 5. *A. niger* protease activity at various pH levels.

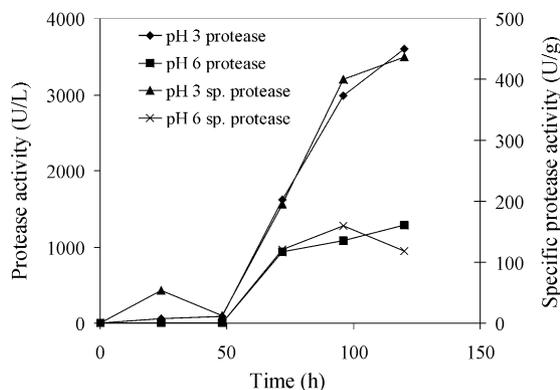


Fig. 6. Protease activity and specific protease activity for *A. niger*. Fermentations carried out at pH 3 and pH 6, assayed at pH 3.

more neutral pH 6 culture. The fact that these assays were carried out at the particular fermentation pH of the sample suggested that the decrease in protease activity might only be due to inability of the enzyme to work outside of its optimal pH. By comparing protease activities determined at the same pH the relative potencies of the samples could be assessed.

To determine whether the difference in protease activities at pH 3 and 6 was due to an actual decrease in secreted proteases or due to the fact that the secreted proteases were functioning in a sub-optimal environment, protease assays were carried out on samples of the pH 3 and pH 6 runs at the same assay pH. The results for both runs assayed at pH 3 and pH 6 are shown in Figs. 6 and 7, respectively.

The assay carried out at pH 3 determined that the maximum extracellular protease activity at the pH 3 fermentation was 3600 U/l, 2.8 times larger than the maximum protease activity for the pH 6 fermentation (1288 U/l) at the same assay pH. When the protease assay was carried out at pH 6, the maximum extracellular protease activity detected for the pH 3 fermentation was 1134 U/l, two times higher than the maximum protease activity for the pH 6 fermenta-

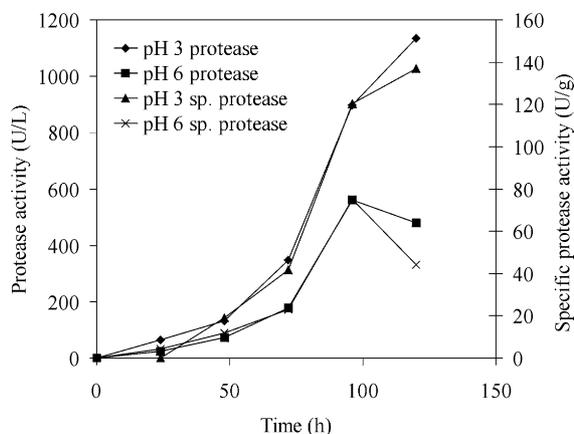


Fig. 7. Protease activity and specific protease activity for *A. niger*. Fermentations carried out at pH 3 and pH 6, assayed at pH 6.

tion (560 U/l). pH 6 cultures gave consistently lower protease activities than acidic (pH 2, 3, 4, 5) cultures, regardless of assay pH. The fact that the difference in activities decreased from 6.4 times when assayed at their respective fermentation broth pH (pH 3 and 6) to 2.8 times when assayed at the same pH, suggests that a certain proportion of the observed decrease is a protease activity effect due to the fact that the proteases simply did not work as well without a rather acidic pH environment. There may also be an actual reduction in secreted proteases at the higher pH value or the protease secreted at lower pH may be active over a wider pH range. Taken in tandem, the results of protease assays suggest that the decrease in protease activity is a combination of sub-optimal protease activity and variation in the spectrum of proteases secreted under different pH conditions.

4.5. Correlation between the recombinant GLA and GFP productions

It is expected there is some relationship between the concentrations of GFP and recombinant GLA in the broth. They are coded by the fusion protein gene with a 1:1 molar ratio. The fusion protein is cleaved in the secretion pathway. GLA (84 kDa) is around three times larger than GFP (27 kDa). If there was no degradation of both proteins, the concentration (mg/l) ratio of the recombinant GLA/GFP should be constant at about 3 in the fermentation broth.

The recombinant GLA/GFP ratio was not constant during the fermentation (see Fig. 8). In the pH 6 run, the ratio of recombinant GLA to GFP was between 3 and 4 on the third day when recombinant GLA and GFP began to be secreted. Then the ratio eventually increased, and by the end of the fifth day, the ratio becomes 9.2. In the pH 3 run, this ratio was quite high (about 18) even at the beginning, and by the end of the fifth day, it reached 127, which hints that most of the GFP secreted had been degraded since the two proteins had a stoichiometric relationship in the fusion gene. The most likely explanation lies in protease degradation of

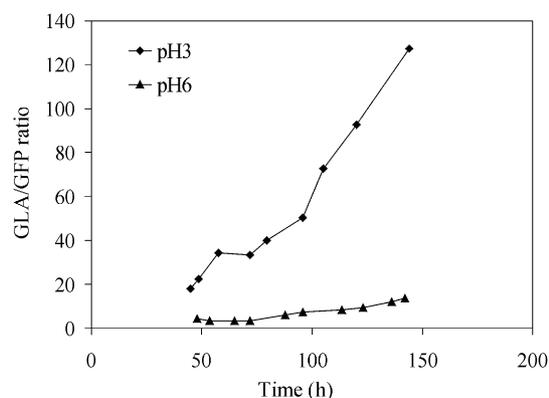


Fig. 8. Recombinant GLA/GFP ratios in the fermentations carried out at pH 3 and pH 6.

the two different proteins. GFP was completely “foreign” to *A. niger* and therefore was quickly degraded. However, the GLA section of the fusion protein originated in *A. niger*, thus the recombinant GLA was less likely degraded, since homologous proteins tend to be less prone to degradation than heterologous proteins [16]. Since the recombinant GLA was not sensitive to the protease degradation, it served as a signal of the expression of the fusion gene. Controlling pH at 6 did not completely inhibit the protease expression, but the surviving GFP had a much higher concentration benefiting much from the lowered protease activity at pH 6.

This work showed that pH control maximized recombinant protein production in *A. niger* AB4.1[pgpdAGLAGFP]#11 at pH 6. However a pH control strategy will be species-specific, dependent on the particular spectrum of proteases produced by the strain. For instance, for extracellular proteolytic activities in *A. niger* acidic proteases predominate [13,15] but for *A. nidulans* at least three neutral or alkaline proteases, have been reported [6,10]. Obviously if the culture produces more neutral or alkaline proteases then a different pH regime to decrease protease activities in that culture will have to be determined and there can be no general guidelines laid down covering optimal pH control in all cases. However, investing time in an investigation of the optimal pH control strategy is a relatively easy and inexpensive method to improve yields through control of extracellular protease activity.

5. Conclusion

The aim of this work was to enhance heterologous protein production by *A. niger* AB4.1[pgpdAGLAGFP]#11, using pH control to minimize extracellular protease degradation of the recombinant product. It was found that pH 3 was the optimum pH for protease activity. Without pH control *A. niger* quickly acidified the culture medium to a pH between 2 and 3 during the first two days of fermentation and remained there afterwards. Thus the majority of the process was carried out under conditions which were optimal for extracellular protease activity. By maintaining the culture pH at an artificially high level, it was aimed to take away this optimal activity period.

Results obtained show that pH control is a viable method of decreasing extracellular protease activity and increasing recombinant protein yields. The optimal pH identified for GFP in *A. niger* AB4.1[pgpdAGLAGFP]#11 was 6. A corresponding amount of recombinant GLA was detected at this pH, suggesting that protease activity at this pH was quite low and degradation of GFP compared to recombinant GLA was relatively slow. At this pH, protease activity was 6.4 times lower than at pH 3, while specific protease activity was four times lower. The scope of this work did not allow determination of whether the molar amount of protease secreted at pH 3 decreased or whether the spectrum of proteases secreted at pH 3 included more powerful or more

widely active proteases than those secreted at pH 6. A more detailed analysis, which includes separation and purification of the various enzymes, would be required. Nonetheless, this work highlights the effectiveness of a pH control strategy in decreasing protease degradation of recombinant proteins to give increased yield.

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