Chitinase but N-acetyl-ß-D-glucosaminidase production correlates to the biomass decline in *Penicillium* and *Aspergillus* species

Tünde Pusztahelyi[a]*, István Pócsi[b]

[a]Central Laboratory, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, H-4032, Böszörményi út 138. Debrecen, Hungary

[b]Department of Microbial Biotechnology and Cell Biology, Faculty of Science and Technology, University of Debrecen, H-4032, Egyetem tér 1., Debrecen, Hungary

Running title: Role of chitinolytic enzymes in fungal biomass decrease

Abstract

Hydrolytic enzyme production is typical of the autolysis in filamentous fungi; however, less attention has been given to the physiological role of the enzymes. Here, the aim was to investigate the possible relation of the chitinolytic enzymes to the changes in the biomass in some filamentous fungi of high importance for pharmaceutical or food industry. In *Penicillium* and *Aspergillus* filamentous fungi, which showed different characteristics in submerged cultures, the growth and biomass decline rates were calculated and correlated to the chitinase and N-acetyl-ß-D-glucosaminidase enzyme productions. Correlation was found between the biomass decrease rate and the chitinase level at the stationary growth phase; while, chitinase production covariates negatively with N-acetyl-β-D-glucosaminidase activities. The chitinase production and the intensive autolysis hindered the production of N-acetyl-β-D-glucosaminidase and, therefore, could hinder the cell death in the cultures.

Keywords: *Penicillium*, *Aspergillus nidulans*, autolysis, chitinase, N-acetyl-ß-D-glucosaminidase.

1 Introduction

Autolysis has been generally used to describe hallmarks of aging cultures including vacuolization, hyphal fragmentation, increasing extracellular ammonia concentration and increase in extracellular hydrolase activities [1], which are followed by the degradation of cell wall constituents and cellular organelles, and finally the biomass decreases [1, 2]. The phenomenon contributes to the survival of filamentous fungi under harsh environmental conditions like carbon shortage [3] as its main function is to supply developmental processes with energy sources [4, 5].

Chitin hydrolytic (chitinolytic) enzymes are classified into two main groups: chitinases (E.C.3.2.1.14 according to IUBMB Enzyme Nomenclature) and N-acetyl-β-D-glucosaminidases (E.C.3.2.1.52), which are different in their splitting mechanisms. In filamentous fungi, *e.g. Aspergillus* [6] or *Trichoderma* [7], high number of chitinase genes (10-25) can be found. Contrarily, besides the high number of the chitinase genes, only some (2-3) N-acetyl-β-D-glucosaminidase genes were found in filamentous fungi. In *A. nidulans* (*nagA*, AN1502.3; [8, 9]) as well as in *P. chrysogenum* (*nagA*, AF056977; [10]) only one gene was proved to have N-acetyl-β-D-glucosaminidase coding function. As demonstrated by global transcriptome analyses performed in autolyzing cultures of *A. nidulans*, the onset of gross autolysis is preceded by the strong up-regulation of an array of genes encoding autolytic hydrolases [11] like glucanases (*e.g.* EngA 1,3-β-glucosidase) and chitin hydrolytic enzymes (ChiB, ChiC, NagA) [12-15]; however, some of the proteins have never been detected (*e.g.* ChiC) or the role of the proteins (*e.g.* NagA) was not proven in the process.

While the autolysis is an important process considering the secondary metabolite production [16, 17] or enzyme and heterologous protein production [18], the factors which influence the process have only been studied in yeast [19] and less attention has been given to the process in filamentous fungi. Therefore, the aim was to characterize biomass changes and

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concomitant chitinase and N-acetyl-β-D-glucosaminidase production, and investigate a possible correlation among them in *Penicillium* and *Aspergillus* species that possess high significance for pharmaceutical and food industry or food control.

2 Materials and Methods

2.1 Culture media and cultivation conditions

Penicilli (Table 1) were maintained on malt extract agar (30 g l^{-1} malt extract, 5 g l^{-1} mycological peptone; 15 g l^{-1} agar; pH5.4) except *P. chrysogenum* NCAIM 00237 strain which was maintained on solid phase rice medium supplemented with 20% (v/w) Czapek-Dox medium (3 g l^{-1} NaNO$_3$, 1 g l^{-1} KH$_2$PO$_4$, 0.5 g l^{-1} MgSO$_4$×4H$_2$O, 0.5 g l^{-1} KCl, 0.01 g l^{-1} Fe$_2$SO$_4$×7H$_2$O, 30 g l^{-1} glucose, 15 g l^{-1} agar; pH 7.3) and 5% (w/w) peanut meal at 25 °C. *Aspergillus nidulans* strains (Table 1) were cultivated in solid minimal-nitrate medium {50 ml l^{-1} 20×nitrate salt solution (120 g l^{-1} NaNO$_3$, 10.4 g l^{-1} MgSO$_4$×4H$_2$O, 10.4 g l^{-1} KCl and 30.4 g l^{-1} KH$_2$PO$_4$), 1 ml l^{-1} 1000×trace element solution (22 g l^{-1} ZnSO$_4$×7H$_2$O, 11 g l^{-1} H$_3$BO$_3$, 5 g l^{-1} MgCl$_2$×4H$_2$O, 5 g l^{-1} Fe$_2$SO$_4$×7H$_2$O, 1.6 g l^{-1} CoSO$_4$×5H$_2$O, 1.6 g l^{-1} CuSO$_4$×5H$_2$O, 1.1 g l^{-1} (NH$_4$)$_6$Mo$_7$O$_{24}$×4H$_2$O, 50 g l^{-1} Na$_2$-EDTA) and 20 g l^{-1} agar} supplemented with 5 g l^{-1} yeast extract and 10 g l^{-1} glucose, which was also amended with 25 mg l^{-1} biotin, 200 mg l^{-1} *p*-aminobenzoic acid, and 200 mg l^{-1} pyridoxine (pH 6.5) at 37 °C.

For batch cultivation, 100 ml aliquots of YGL broth (0.4% K$_2$HPO$_4$, 0.2% KH$_2$PO$_4$, 0.05% MgSO$_4$, 1% glucose, 0.5% yeast extract) amended with 0.5% tryptone (Sigma-Aldrich Ltd., Budapest, Hungary) in 500 ml Erlenmeyer flasks were inoculated with 10^8 *Penicillium* spores and was incubated at 25°C on 3.66 Hz shaking frequency. For batch cultivation of *Aspergilli*, 100 ml aliquots of minimal nitrate medium amended with 5 g l^{-1} yeast extract, 10 g l^{-1} glucose and 25 mg l^{-1} biotin, 200 mg l^{-1} *p*-aminobenzoic acid, and 200 mg l^{-1} pyridoxine

supplements at pH 6.5 in 500 ml Erlenmeyer flasks were inoculated with 5×10^7 spores and were incubated with shaking at 37 °C and 3.66 Hz shaking frequency.

2.2 Determination of the growth and biomass decline rates

Mycelial dry weight was measured as described by Pócsi et al. [20]: 5 ml aliquots of the batch cultures were taken at 16, 24, 48, 72, 96, 120 and 144 h of cultivation times and were filtered through pre-weighted filter paper (3M, Whatman) placed on sintered glass. Mycelial mats were washed with distilled water and air-dried.

Growth rate was determined from dry cell mass increase between 24 h cultivation time and the time of maximal measured biomass production, while the biomass decline rate was calculated from the decrease of the dry cell mass between the cultivation time of the maximal measured biomass production and 120 h cultivation time.

2.3 Hydrolytic enzyme activity assays

To monitor chitinase production, to 100 μl aliquots of culture filtrates 100 μl volumes of 2 mg ml^{-1} carboxymethyl-chitin-Remazol Brilliant Violet substrate solution (Loewe Biochimica, Sauerlach, Germany) and also 400 μl aliquots of 0.1 mol l^{-1} citrate buffer (pH 5.0) were added and the reaction mixtures were incubated at 24 °C for 10 min [2]. After quenching the reaction with 100 μl 2 mol l^{-1} HCl, the samples were kept on ice for 10 min and centrifuged (16 000 g, 10 min). The released colored product was determined spectrophotometrically at $\lambda = 550$ nm.

In N-acetyl-β-D-glucosaminidase activity measurements, 5 mmol l^{-1} p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich Ltd., Budapest, Hungary) substrate was used [20] in a microplate end-point assay ($V_{reaction\ mixture} = 100$ μl, $t_{reaction} = 10$ min, performed in 0.2 mol l^{-1} sodium acetate buffer, pH 5.0). After quenching the reaction with 200 μl of 0.2 mol l^{-1} sodium

borate buffer (pH 10.0), the quantity of the liberated 4-nitrophenolate ions were determined spectrophotometrically at $\lambda=400$ nm.

Protein contents were quantified by the Bradford method [21] using bovine serum albumin calibration and the data were used in specific enzyme activity calculations.

2.4 Statistical analysis

All measurements were done in triplicate. The means and the statistical deviations were calculated in Microsoft Excel 2010 software. Regressions at 95% confidence level and correlations were calculated using Analysis ToolPak of the Microsoft Office software.

3 Results

3.1 Characterization of biomass and morphology changes in submerged cultures

All strains reached the stationary growth phase around 40-50 h cultivation time (Fig. 1) except the wild type *P. chrysogenum* (Fig. 1A). The increase of the biomass of the wild type *P. chrysogenum* (Fig. 1A; Table 2) resulted in a maximum dry cell mass at 72 h cultivation time that was followed by the disintegration of the filaments and biomass decrease.

P. chrysogenum SMC 0514 strain (Fig. 1B) reached the highest biomass among studied *Penicilli*, while the *P. chrysogenum* NCAIM 00237 and ATCC 10.002 strains produced around 6-7 mg·ml^{-1} dry cell mass (Fig. 1C and D) with lower growth rates (Table 2). Some differences in the biomass decrease also could be observed as the biomass of the SMC 0514 strain decreased at higher rate and reached the lowest dry cell weight at the 120 h cultivation time (Table 2; Fig. 1B). Meanwhile, NCAIM 00237 (Fig. 1C) and ATCC 10.002 (Fig. 1D) strains showed slow decrease in the dry cell mass with a plateau of the dry cell mass curve from about the 120 h cultivation time (Fig. 1). While the pellet structure was characteristic for all investigated *P. chrysogenum* strains, the morphology of the cultures significantly changed

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under progressing autolysis. The filaments were strongly vacuolated in all aging cultures, and empty hyphal segments were observed in SMC 0514 and ATCC 10.002 strains as the cell wall remained seemingly untouched; while, an intensive fragmentation of the filaments was observable in NCAIM 00237 cultures resulting in one or two-celled fragments in the aging cultures.

P. glabrum ATCC 10.103 showed a slower growth in the exponential growth phase (Table 2) and the culture reached its maximal dry cell mass at the 24 h of the cultivation (Fig. 1E) and it also started the autolytic phase earlier than the *P. chrysogenum* strains. The autolyis was characterized by an intensive fragmentation and a reasonably high biomass decline rate (Table 2); however, after 120 h of cultivation a cryptic growth phase was started with the dry cell mass of about 5.3 mg ml^{-1}.

The dry cell mass of the *P. pinophylum* NRRL 1066 (Fig. 1F) increased with low rate and the maximum dry cell mass was measured only at 72 h cultivation time similarly to the wild type *P. chrysogenum* (Table 2). The autolysis that was characterized by a weak mycelial fragmentation resulted in slow decrease of the dry cell mass (Table 2) up to the steady cryptic phase with 4 mg ml^{-1} dry cell mass.

Comparing to the other investigated strains, the increase of the dry cell mass of *A. nidulans* FGSC A26 was moderate, while the decrease in the biomass was quite intensive (Table 2). Here, the autolytic phase was characterized by an intensive vacuolation and moderate fragmentation. In FGSC A1079 strain *brlA* (main regulator transcription factor of asexual development) gene was deleted [4], which resulted in weak fragmentation; while, in FGSC A744 the FluG protein was inactive [22]. Deletion of the *brlA* gene resulted in moderate growth that was similar to FGSC A26; however, the biomass decline rate (Table 2) was much lower than that of FGSC A26. Meanwhile, the inactive FluG protein expression

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caused intensive growth and a high biomass decline rate in the autolytic phase of growth (Table 2).

3.2 Characterization of chitinase and *N*-acetyl-β-D-glucosaminidase production

The chitinase production of the wild-type *P. chrysogenum* (Fig. 1A), SMC 0514 (Fig. 1B), NCAIM 00237 (Fig. 1C), *P. pinophilum* NRRL 1066 (Fig. 1F) and *A. nidulans* FGSC A26 (Fig. 1G) reached its maximum at the stationary phase of growth. The highest activity was reached by the NRRL 1066 (Fig. 1F) strain (Table 2). Age related chitinase production was observed in the exponential phase of *P. chrysogenum* ATCC 10.002 (Fig. 1D); *P. glabrum* ATCC 10.103 (Fig. 1E), *A. nidulans* FGSC A1079 (Fig. 1H) and FGSC A744 (Fig. 1I) strains, where the highest activities were measured in the exponential phase of growth that indicated different regulation of the chitinase genes. For these strains low chitinase activities were characteristic at the autolytic phase except *A. nidulans* mutant strains (Fig. 1H and Fig. 1I), where the exponential phase and the autolysis were characterized by a clearly separately activated set of chitinases.

The *N*-acetyl-β-D-glucosaminidase expression and the extracellular production were also different in the investigated strains. The enzyme was produced either constitutively, like in the wild type *P. chrysogenum* (Fig. 1A), or was induced throughout the stationary phase of growth as it was usual in the other investigated strains (Fig. 1). The *N*-acetyl-β-D-glucosaminidase production of the *A. nidulans* mutant strains was also interesting as two activity peaks were detected, one in the exponential and one in the autolytic phase of growth (Fig. 1H-1I), which phenomenon needs further investigation.

3.3 Statistical analysis

In the correlation analyses the enzyme activities detected at 24 h cultivation time (exponential growth phase) and at the maximum dry cell mass and at 96 h cultivation time (autolytic phase) were included in the statistical analysis to investigate correlation with the

decline of the biomass and/or the growth rates (Table 2). Significant correlations ($p<0.05$) were found between the hydrolytic activities in the stationary growth phase and during autolyis, and the chitinase production *versus* the biomass decline rate also showed a significant but weak correlation at 95% confidence level (Table 3). Interestingly, negative covariance (-7.55 at the maximum dry cell mass and -37.58 in the autolytic phase) was calculated for the chitinase and the *N*-acetyl-β-D-glucosaminidase activities. While investigating data from the exponential growth phase (24 h cultivation time) any significant correlations were determined (data not shown).

4 Discussion

4.1 Chitinase activity correlates with the rate of the biomass decline

Under carbon shortage highly vacuolated cytoplasmic structure and empty hyphal walls were observed in submerged cultures of filamentous fungi [1, 23]. Specific chitinase activities significantly correlated with the rate of the biomass decline in aging cultures (Table 3). In several fungal strains specific inhibition of chitinase activity inhibited the fragmentation and the autolysis [24-25] indicating also the significance of chitinase activities in the autolytic process. In *A. nidulans* FGSC 26 wild-type strain, the expression of the ChiB chitinase is predominant giving more than 90 % of the detectable extracellular chitinase activity during the early autolytic phase of growth [26]. The deletion of the *chiB* gene resulted in decreased autolytic cell wall degradation [13] and the gene was described as inevitable for the progress of fragmentation [5]. Cell wall degradation process in these fungal cultures is under glucose repression, nutrition sensing and BrlA regulation [5, 27]. The deletion of *brlA* gene and the inactivation of the upstream FluG protein resulted in similar chitinase and *N*-acetyl-β-D-glucosaminidase production patterns (Fig. 1H and 1I), which were different from that of the wild type *A. nidulans* A26 strain (Fig. 1G) and showed clearly the age-related production of the proteins. The modulation of the gene expression of *brlA* had role in the repression of the

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autolysis directly and/or indirectly, *e.g.* through the regulation of MpkB mitogen activated protein kinase, as it down-regulates the expression of chitinase production in *A. nidulans* [28].

4.2 N-acetyl-β-D-glucosaminidase and chitinase activity show negative covariance

In relation of N-acetyl-β-D-glucosaminidase and chitinase production a negative covariance and significant correlation was calculated. Similarly to that of *chiB* chitinase regulation in *A. nidulans* [13, 26], glucose repression of the N-acetyl-β-D-glucosaminidase gene is characteristic [9, 20, 26]. However, while the accumulation of the N-acetyl-β-D-glucosaminidase activity was detected in the soluble intracellular samples [20], it was not characteristic to the production of the autolytic chitinase of *P. chrysogenum* [24] or to *A. nidulans* ChiB [26]. Distinct regulation of chitinase and *N*-acetyl-β-D-glucosaminidase gene expressions and negative covariance of the activities suggested that these proteins can be connected to different physiological function [5, 13, 29]. *N*-acetyl-β-D-glucosaminidase was not essential for growth on easily metabolizable carbon sources [9] and it is activity was not essential in autolysis, too; however, it was shown to play a role in morphogenesis and nutrient utilization during autolysis [26]. Changes in the N-acetyl-β-D-glucosaminidase production of the asexual development signal transduction cascade mutant *A. nidulans* strains were observed for the first time. Until now the only suggestion on possible regulation of N-acetyl-β-D-glucosaminidases through stress related signal transduction cascades was done by Shin *et al.* [30], as they suggested that the enzyme had importance in cell death, but it needs further research. We demonstrated recently that the levels of *nagA* and *chiB* mRNA were high during the stationary and autolytic phases [26], and ChiB and NagA accumulated at high levels during autolysis and/or cell death in *A. nidulans* and thereby coordinately conferred cell death and degradation of fungal cell walls.

We described regularity and correlation of the enzyme productions in closely related fungal species. It can be concluded that high chitinase activity under carbon shortage speeded up the

autolysis and the intensive autolysis and/or the changes in the regulation hinder the intracellular accumulation and release of *N*-acetyl-β-D-glucosaminidase. By the investigation of *N*-acetyl-β-D-glucosaminidase and chitinase we can get a picture on the physiological state of *Aspergillus* and *Penicillium* cultures and estimate the intensity of the autolysis in submerged cultures.

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* - Author for correspondence:

Tünde Pusztahelyi, Ph.D.; Central Laboratory, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, H-4032, Böszörményi út 138. Debrecen, Hungary. tel.: 36-52-512900 ext. 88514. e-mail: pusztahelyi@agr.unideb.hu.

Table 1 Organisms in the experiments

Strain	Code	Genotype/ origin
P. chrysogenum	-	wild type (G. Winkelman, University of Tübingen, Germany)
P. chrysogenum	SMC[1] 0514	wild type (University of Szeged, Szeged, Hungary)
P. chrysogenum	NCAIM[2] 00237	industrial strain
P. chrysogenum	ATCC[3] 10.002	industrial strain (alias Wisconsin Q-176)
P. glabrum	ATCC 10.103	type strain
P. pinophylum	NRRL[4] 1066	alias *P. purpurogenum var. rubrisclerotium* Thom.
A. nidulans	FGSC[5] A26	*biA1*
A. nidulans	FGSC A1079	*biA1; pabaA1; pyroA4; ΔbrlA*
A. nidulans	FGSC 744	*pabaA1; yA2; fluG1*

[1]SMC, Mycological Collection of University of Szeged, Hungary;

[2]NCAIM, National Collection of Agricultural and Industrial Microorganisms, Hungary;

[3]ATCC, American Type Culture Collection, USA;

[4]NRRL, Northern Regional Research Laboratory, now ARS Culture Collection, USA;

[5]FGSC, Fungal Genetics Stock Center, USA.

Table 2 Summary of the growth and biomass decline rates and specific enzyme activities of the investigated species.

Strain	Strain code	Growth rate[†] (mg*ml^{-1}*h^{-1})	Biomass decline rate[†] (mg*ml^{-1}*h^{-1})	Chitinase activity[††] (A$_{550\,nm}$*10^3*µg^{-1})			N-acetyl-β-D-glucosaminidase activity[††] (kat*kg^{-1})		
				Exp.	Max.	Auto.	Exp.	Max.	Auto.
P. chrysogenum	-	0.125±0.015	0.075±0.005	7.04±0.35	10.25±0.35	9.24±0.55	0.14±0.01	0.15±0.002	0.17±0.11
P. chrysogenum	SMC 0514	0.166±0.024	0.061±0.004	2.08±0.11	10.92±0.56	4.53±0.21	0.51±0.03	2.1±0.03	2.32±0.13
P. chrysogenum	NCAIM 00237	0.135±0.014	0.029±0.005	0.67±0.04	8.47±0.44	5.36±0.32	1.19±0.05	1.77±0.05	83.3±2.3
P. chrysogenum	ATCC 10.002	0.150±0.008	0.016±0.007	17.32±0.21	12.28±0.21	4.91±0.18	1.81±0.02	11.1±0.08	91.0±2.4
P. glabrum	ATCC 10.103	0.215±0.011	0.034±0.002	8.38±0.28	3.45±0.08	3.61±0.20	0.11±0.01	0.08±0.01	1.03±0.10
P. pinophylum	NRRL 1066	0.079±0.007	0.028±0.001	3.63±0.25	16.19±0.75	12.90±0.43	0.1±0.01	0.33±0.02	1.05±0.12
A. nidulans	FGSC A26	0.113±0.031	0.053±0.003	0	1.48±0.05	3.67±0.11	4.64±0.09	12.89±0.8	29.55±0.32
A. nidulans	FGSC A1079	0.115±0.025	0.035±0.006	28.72±0.7	4.27±0.07	1.58±0.12	13.74±0.15	6.43±0.05	22.62±0.32
A. nidulans	FGSC A744	0.250±0.021	0.046±0.002	32.65±0.41	11.3±0.01	18.34±0.71	8.98±0.24	2.98±0.04	9.00±0.61

[†]Growth rate was determined from dry cell mass increase between 24 h cultivation time and the time of the maximum measured biomass production, while the biomass decline rate was calculated from the decrease of the dry cell mass between the 72 and 120 h cultivation times.

[††]Measured from the samples of 24 h cultures (Exp. - exponential growth phase), at the maximum dry cell mass (Max.) and of 96 h cultivation time at the autolysis (Auto.).

Table 3 Summary of the significant correlations.

Correlated data[†]	Correlation coefficient (r)	r^2	t test	p value[††]
Chitinase *vs*. N-acetyl-β-D-glucosaminidase at maximum dry cell mass	0.3674	0.1349	4.7672	0.002
Biomass decline rate *vs*. chitinase at maximum dry cell mass	0.1481	0.0219	2.3600	0.050
Chitinase *vs*. N-acetyl-β-D-glucosaminidase at autolytic phase	0.2663	0.0709	3.6259	0.008

[†] Specific chitinase and N-acetyl-β-D-glucosaminidase activities.

[††]Calculated at 95% confidence level.

Captions to the Figures

Figure 1 Changes in dry cell mass (■), chitinase (○) and N-acetyl-β-D-glucosaminidase (●) hydrolytic enzyme production of the *Penicilli* and *Aspergilli:* (A) wild-type *P. chrysogenum;* (B) *P. chrysogenum* SMC 0514; (C) *P. chrysogenum* NCAIM 00237; (D) *P. chrysogenum* ATCC 10.002; (E) *P. glabrum* ATCC 10.103; (F) *P. pinophilum* NRRL 1066; (G) *A. nidulans* FGSC A26; (H) *A. nidulans* FGSC A1079; (I) *A. nidulans* FGSC A744.

Figure 1