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Response to Reviewers:	I am pleased to inform you that we corrected the manuscript according to the Editorial recommendations. (Alterations are indicated by blue color in the text.) We together with my colleagues hope that the manuscript is acceptable in its present form. Nevertheless, we will remain open and ready to make any further alterations on it if they will be required.

Autolytic hydrolases affect sexual and asexual development of *Aspergillus nidulans*

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Conflict of Interest

The authors declare that they have no conflict of interest.

Abstract

Radial growth, asexual sporulation, cleistothecia formation as well as extracellular chitinase and proteinase formation of *Aspergillus nidulans* were monitored in surface cultures in order to study the physiological role of extracellular hydrolase production in carbon stressed cultures. We set up carbon stressed and carbon overfed experimental conditions by varying the starting glucose concentration within the range of 2.5 and 40 g/L. Glucose starvation induced radial growth, hydrolase production and enhanced the maturation of cleistothecia meanwhile glucose rich conditions enhanced mycelial biomass, conidia and cleistothecia production. Double deletion of *chiB* and *engA* (encoding an extracellular endochitinase and a β-1,3-endoglucanase, respectively) decreased conidia production under carbon stressed conditions, suggesting that these autolytic hydrolases can support conidia formation by releasing nutrients from the cell wall polysaccharides of dead hyphae. Double deletion of *prtA* and *pepJ* (both genes encode extracellular proteases) reduced the number of cleistothecia even under carbon rich conditions except in the presence of casamino acids, which supports the view that sexual development and amino acid metabolism are tightly connected to each other in this fungus.

Keywords: *Aspergillus nidulans*; carbon stress; cleistothecia formation; conidiogenesis; extracellular hydrolases; radial growth

Introduction

Aspergillus species (Fungi, Ascomycota, Eurotiomycetes, Eurotiales, Trichocomaceae) - like any other microorganisms - have to cope with carbon stress frequently in Nature, coming from either carbon starvation or carbon limitation environmental conditions (van Munster et al. 2016). The quality and availability of carbon sources influence many aspects of their life including growth, asexual and sexual developments or even secondary metabolite production (Atoui et al. 2010; Cánovas et al. 2017; Han et al. 2003, 2009; Jeong et al. 2000; Matsuura 2002; van Munster et al. 2016). Carbon stress commonly induces the production of versatile extracellular hydrolases such as carbohydrate-active enzymes (CAZymes) and different peptidases (Emri et al. 2008; Katz et al 2008, 2013; van Munster et al. 2016) as well. These enzymes help the colony survive carbon stress *via* the utilization of weak carbon sources present in the media (*e.g.* plant cell wall materials) or by the degradation of various biopolymers released during autolysis (Emri et al. 2008; van Munster et al. 2016). Among the autolytic hydrolases of *Aspergillus nidulans*, which are produced by autolytic cultures, ChiB endochitinase, EngA β-1,3-endoglucanase, PrtA serine proteinase and PepJ metallo proteinase are notable and relatively well-characterized in terms of their physiological functions. For example, ChiB and EngA are responsible for autolytic cell wall degradation and, as a consequence, for fragmentation of hyphae, disorganization of pellets and autolytic loss of biomass observed in carbon-starved cultures (van Munster et al. 2016; Pócsi et al. 2009; Pusztahelyi et al. 2006; Pusztahelyi and Pócsi 2014; Szilágyi et al. 2010). Nevertheless, although PrtA and PepJ proteinases represent more than 50 % of extracellular protease activity in carbon starved cultures these hydrolases have no significant effect on autolytic cell wall degradation (van Munster et al. 2016; Szilágyi et al. 2011).

66 The production of the aforementioned extracellular hydrolases is under complex regulation
67 which involves the following selected elements: i) The BrlA transcription factor, the first
68 member of the Central Regulatory Pathway of conidiogenesis (Adams et al. 1998), together
69 with its upstream regulator FluG are necessary for the initiation of extracellular autolytic
70 hydrolase production (Emri et al. 2008; van Munster et al. 2016; Pócsi et al. 2009; Szilágyi et
71 al. 2011). ii) Glucose represses extracellular hydrolase formation, which - at least in part -
72 depends on the CreA transcription factor (Emri et al. 2006; Katz et al. 2008), which mediates
73 carbon catabolite repression in this fungus (Ries et al. 2016; Shroff et al. 1997). iii) The XprG
74 transcription factor activates extracellular hydrolase production under carbon starvation and
75 influences the transcription of *brlA* (Katz et al. 2013). iv) Heterotrimeric G protein dependent
76 signaling pathways (*e.g.* FadA and GanB signalings), which are responsible for the
77 maintenance of vegetative growth (Krijgsheld et al. 2013a; Yu 2006), are known to inhibit
78 conidiogenesis (Krijgsheld et al. 2013a; Yu 2006) and can also influence hydrolase
79 production (Molnár et al. 2004, 2006). Importantly, deletion of *fluG* in *A. niger*, which do not
80 influence conidiogenesis in this species, enhanced enzyme secretion (Wang t al. 2015).
81 Meanwhile, deletion of *flbA,* an upper regulator of *brlA* of the same species, also resulted in
82 increased protein secretion (Krijgsheld et al. 2013b). These data emphasize that although
83 elements of the regulatory network of protein secretion seems to be conserved in aspergilli,
84 their effect on extracellular protein formation can be very different in various species.
85 Since the regulation of asexual sporulation and extracellular proteinase, chitinase and
86 β-1,3-glucanase production seems to be inherently coupled in *A. nidulans*, it is supposed that
87 these hydrolytic enzymes are likely to provide conidiogenic cells with sufficient nutrients
88 under carbon starvation (van Munster et al. 2016; Pócsi et al. 2009). Interestingly, the
89 verification of this hypothesis has remained yet to be done and the possible involvements of
90 these hydrolases neither in the maintenance of radial growth in surface cultures nor in the

formation of the sexual fruiting bodies cleistothecia have been studied until now. In this study, we present data on how radial growth, asexual sporulation and cleistothecia formation depend on the production of autolytic enzymes including autolytic cell wall hydrolases like ChiB endochitinase and EngA β-1,3-endoglucanase, and extracellular proteinases like PrtA and PepJ in surface cultures of *A. nidulans*.

Materials and methods

Strains and culturing conditions

In these experiments, the following *A. nidulans* strains were used; a control strain: tHS30.3 (*pyrG89; pyroA*$^{+}$*; veA*$^{+}$*; AfupyrG*$^{+}$) (Szilágyi et al. 2010); the double mutant *ΔchiBΔengA* strain: tNJ34.8 (*pyrG89; pyroA4; ΔchiB::AnpyroA*$^{+}$*; ΔengA::AfupyrG*$^{+}$*; veA*$^{+}$) (Szilágyi et al., 2010); the double mutant *ΔpepJΔprtA* strain: tNJ78.4 (*pyrG89; pyroA4; ΔpepJ::AfupyrG*$^{+}$*; ΔprtA::AnipyroA*$^{+}$*; veA*$^{+}$) (Szilágyi et al. 2011) and the loss-of-function *fluG* strain: FGSC A744 (*pabaA1; yA2; FluG1*) (McCluskey 2003). Strains were maintained at 37 ºC on Barratt's minimal-nitrate agar plates (pH 6.5) containing the appropriate supplements (Barratt et al. 1965) and only freshly made 6 d cultures were used for the experiments. In the case of the FGSC A744 strain, the incubation temperature was lowered to 24 ºC in order to induce asexual sporulation.

All strains were grown on Barratt's minimal-nitrate agar plates containing 2.5-40 g/L glucose as the sole carbon source, and selected plates were also supplemented with 2.5 g/L casamino acids. All plates were point inoculated with 5 μL freshly prepared spore suspension containing 1×10^5 conidia per mL and were incubated at 37 ºC for 7 d. To initiate cleistothecia formation certain plates were sealed with Parafilm on the 2 d and were incubated further for 5 d in dark (Kawasaki et al. 2002).

116 *Characterization of colonies*

117 Diameters of the colonies were recorded daily and these data sets were used to
118 calculate mycelial growth rates.

119 The densities of conidiophores (1/mm^2) and cleistothecia (mature+immature; 1/mm^2)
120 were determined with a stereomicroscope by counting these structures in nine areas of each
121 colony, among which three were chosen close to the outer edge of conidiophore/cleistothecia
122 forming area (Fig 1a section "II"), three were fixed at the center of the colony (Fig. 1a
123 section "IV") and three areas localized between them (Fig. 1a section "III"). The mean of
124 these nine values was calculated and used to characterize the colony. The total numbers of
125 conidiophores (1000 per colony) or cleistothecia (1000 per colony) were estimated by
126 multiplying conidiophore/cleistothecia density with the size of conidiophore/cleistothecia
127 forming part of the colony.

128 To determine the quantity of produced conidia and the protein content of the colony,
129 agar cubes (with 1× 0.5 cm^2 upper surface) were cut from the halfway point of the colony
130 radius (Fig. 1a section "III"). Altogether six agar cubes - three for conidia counting and three
131 for protein determination - were collected from each colony. Conidia were suspended in 0.5
132 mL 0.1 v/v % Tween 20 solution and the number of asexual spores was determined with a
133 hemocytometer. For protein determination, agar samples were lyophilized, were ground with
134 a sterile toothpick and the soluble proteins present in the samples were re-dissolved in 0.4 mL
135 sterile water. The protein concentrations of these solutions were quantified following the
136 procedure of Bradford (1976). Conidia and protein density were given in 1000/mm^2 and
137 ng/mm^2, respectively. The total number of conidia (10^6 per colony) and the total protein
138 content of the colony (mg per colony) were estimated by multiplying conidia/protein density
139 with the size of the colony.

To determine glucose concentrations in the agar plates, mycelia were removed from the surface of the plates with a sterile scalpel, and agar cubes (with 1 × 0.5 cm^2 upper side) were cut at selected areas of the plates (Fig. 1a sections "I-IV"). These samples were incubated in 0.5 mL sterile distilled water at room temperature for 1 h, and the glucose concentration of the liquid phase was determined according to Leary et al. (1992).

For measuring extracellular chitinase and proteinase activities, mycelia were removed from the surface of the plates and agar cubes (with 1 × 0.5 cm^2 upper side) cut from the halfway point of the colony radius (Fig. 1a section "III") were used. Samples were incubated in carboxymethyl-chitin-Remazol Brilliant Violet (chitinase assay; Loewe Biochimica GmbH, Sauerlach, Germany) or azocaseine (proteinase assay; 25 mg/mL azocaseine dissolved in 0.2 mol/L Na_2HPO_4 - 0.1 mol/L citric acid buffer, pH 6.5) solutions at 37 °C for two h. The liberated products of the enzyme reactions were recorded spectrophotometrically as described earlier (Szilágyi et al. 2010). One unit (U) was defined by the amount of enzyme causing 1.0 value increase in the A_{440} (proteinase) or A_{550} (chitinase) of the reaction mixtures. Enzyme activity values were expressed as mU/mm^2.

Results and discussion

Our primary aim was to study how extracellular hydrolases affect the formation of conidiophores with conidia and cleistothecia in surface cultures of the filamentous fungus model organism *A. nidulans*. Hydrolase production was modulated by altering the glucose content of the culturing media and by deletion of genes (*chiB*, *engA*, *prtA*, *pepJ*) encoding extracellular hydrolases.

When nitrate minimal medium (NMM) agar plates (Barratt et al. 1965) were supplemented with glucose at a concentration of 10 g/L, which can be regarded as the standard of carbon source supplementation in general laboratory practice, no glucose (<0.05

g/L) was detected beneath the colonies themselves and reduced level (5-6 g/L) of the monosaccharide was found at the outer edges of the mycelial mats after 120 h of incubation (Figs. 1a and 1b). Similar results were published by Levin et al. (2007) with *Aspergillus niger* where no carbon source was detected in the central zones of 7 d old sandwiched colonies on either xylose or maltose supplemented media. With these observations in our hands, we could set up both carbon stressed and carbon overfed culture conditions easily and in a highly reproducible manner by simply setting the starting glucose concentrations to 2.5 g/L or 40 g/L, respectively (Figs. 1c and 1d). After optimizing the culture conditions, we compared the growth, protein content and extracellular hydrolase production as well as asexual and sexual sporulation of our *A. nidulans* tHS30.3 control strain at various starting glucose concentrations chosen within the range of 2.5-40 g/L (Table 1). Please note that all physiological and sporulation data were compared to those measured at the standard 10 g/L glucose concentration.

Growth rate and biomass production

Low glucose concentrations (2.5 or 5 g/L) stimulated the growth rates and, as a consequence, increased the colony diameters but concomitantly reduced the protein contents of the colonies (Table 1), as it was also described earlier by Matsuura (2002) and more recently also by Cánovas et al. (2017). Similar tendencies were found when biomass production was characterized with dry cell mass instead of protein content of colonies (data not shown). The fast radial growth of *A. nidulans* hyphae observed at low starting glucose concentrations were not only the mere consequence of the glucose concentration gradients typically evolving in NMM agar beneath the growing colonies because such glucose gradients between the center and the edges of the colonies also formed even at starting glucose concentrations as high as 40 g/L (Fig. 1c). It is reasonable to assume that the acceleration of radial growth

("exploratory growth"; Matsuura 1998) can be an adequate stress response of filamentous fungi to carbon stress. In the baker's yeast *Saccharomyces cerevisiae*, glucose depletion was reported to stimulate pseudohyphal growth and invasion of agar in surface cultures (Broach 2012; Cullen and Sprague 2012; Palecek et al. 2002). In addition, nitrogen starvation promotes the hypha/pseudohypha formation and "nosing" for nutrients by the yeasts *S. cerevisiae* (Pan and Heitman 1999), *Schizosaccharomyces pombe* (Amoah-Buahin et al. 2005) and *Schizosaccharomyces japonicus* (Sipiczki et al. 1998). In this regard, various starvation conditions seem to trigger similar morphological/growth responses in either filamentous fungi or yeasts.

Production of conidiophores, conidia and cleistothecia

Sexual and asexual developments were studied and compared under both carbon starved and glucose overfed conditions, and the densities of conidiophores, produced conidia and cleistothecia as well as the total number of conidia and cleistothecia produced by single colonies were found to be depended on the starting glucose concentration (Table 1). High density production of asexual sporulation structures, asexual spores and sexual fruiting bodies on fungal colonies were recorded at high glucose levels, which is in good accordance with the high energy and nutrient requirements of the asexual and sexual development processes, and which is in line with previous observations published by other research groups (Adams et al. 1998; Atoui et al. 2010; Han et al. 1990, 2001, 2003, 2009; Jeon et al. 2000). Interestingly, we could not find any correlation between the starting glucose concentration and the total number of conidiophores counted on single colonies, *i.e.* higher conidiophore density was accompanied by smaller colonies under glucose overfed conditions (Table 1).

Initiation of asexual and sexual developments

Carbon stress dependent induction of conidiogenesis was observed in submerged cultures in previous studies (Emri et al. 2004; Lee and Adams 1996). Interestingly, starting glucose concentration did not have any effect on the time needed for the rise of conidiophores in our surface cultures, and it was always within the 16-18 h incubation time range. Nevertheless, carbon stress induced conidiogenesis in surface cultures of the loss-of-function *fluG* (*fluG1)* mutant, which does not produce any conidia at all when it is grown at 10 g/L starting glucose concentration (Adams et al. 1998), or even in the cultures of wild type *veA*$^{+}$ strains cultivated in the dark (Han et al. 2003). In our experiments, the *fluG1* strain produced 43 ± 5, 6 ± 1 and <0.1 conidiophores per mm^2 and 20000 ± 3000, 9000 ± 1000 and <1000 conidia per mm^2 on solid agar media supplemented with 2.5, 10 and 40 g/L glucose, respectively. All these data suggest that carbon stress can stimulate conidiogenesis, however, this effect is difficult to detect under conditions beneficial for asexual development. Glucose limitation also induced the maturation of cleistothecia because the first mature sexual fruiting bodies emerged after 5, 6 and 7 d (or even longer) of incubation, when the colonies were grown in the presence of 2.5, 10-20 or 40 g/L glucose, respectively.

Production of extracellular chitinase and proteinase

In a previous set of experiments, we found that the asexual sporulation transcription factor BrlA and its upstream regulator FluG were also activators of extracellular hydrolase production in this model organism in addition to the initiation of conidiogenesis (Emri et al. 2005; Pócsi et al. 2009; van Munster et al. 2016). Not surprisingly, loss-of-function *fluG* mutants showed aconidiogenic and non-autolytic ("fluffy") phenotypes in surface cultures (Lee and Adams 1996; Adam et al. 1998), however, it does not necessarily mean that the formation of spores and autolytic hydrolases are always tightly co-regulated processes. Glucose-repressed chitinase and proteinase formation were observed in our experiments on

240 both unsealed and sealed nutrient agar plates (Table 1), which were in line with previous
241 experimental data recorded in submerged cultures (Brown et al. 2013; Emri et al. 2006; Katz
242 et al. 2008; Yamazaki et al. 2007). The transcription factor CreA, which mediates glucose
243 repression in *A. nidulans* (Ries et al. 2016; Shroff et al. 1997), is thought to be responsible -
244 at least in part - for the down-regulated extracellular hydrolase production in the presence of
245 high glucose concentrations (Brown et al. 2013; Emri et al. 2006; Katz et al. 2008; Yamazaki
246 et al. 2007). Importantly, deletion of the *creA* gene also reduced conidia production (Shroff et
247 al. 1997). Therefore, CreA-dependent regulation of proteinase and glycohydrolase production
248 can be an important factor which switches off conidiogenesis and extracellular hydrolase
249 formation in the presence of glucose. It is worth noting that the deletion of *flbA* encoding an
250 RGS (regulator of G protein signaling) domain protein, which is a negative regulator of the
251 FadA heterotrimer G protein signaling (maintaining vegetative growth) and also an activator
252 of *brlA* (initiating conidiophore development) (Lee and Adams 1996; Adam et al. 1998), had
253 only minor effects on extracellular chitinase or proteinase formation (Molnár et al. 2004).
254 This gene-deletion-mutant strain showed an autolytic phenotype without the production of
255 any conidia in surface cultures (Lee and Adams 1996; Adam et al. 1998). The different
256 effects of FlbA on conidiogenesis and hydrolase production demonstrate that the balance
257 between the two BrlA-dependent processes, *i.e.* conidiogenesis and extracellular hydrolase
258 production, is under a complex and sophisticated regulation. It was also emphasized by the
259 results of Krijgsheld et al. (2013b) with *A. niger* where deletion of *flbA* even enhanced
260 protein secretion.
261
262 *Consequence of ΔchiBΔengA and ΔprtAΔpepJ deletions*
263 To test how autolytic hydrolases may influence the growth, asexual and sexual
264 developments taking place in surface cultures, the *ΔchiBΔengA* (both genes code for endo-

265 acting glycosidases) and the *ΔprtAΔpepJ* (both genes encode proteinases) double mutant
266 strains were studied. It is worth noting that the effects of single gene deletions were also
267 tested but their impacts were typically smaller or even insignificant and, hence, these
268 experiments are not presented here for clarity. The *ΔprtAΔpepJ* double deletion reduced the
269 extracellular proteinase activities with approximately 50 % in submerged cultures (Szilágyi et
270 al. 2011). In surface cultures this reduction was at least 60 % (data not shown). As an
271 example, the extracellular proteinase activity of the double mutant was only 1 ± 0.2 U/mm^2
272 (on 2.5 g/L glucose), 0.8 ± 0.4 U/mm^2 (on 10 g/L glucose) and < 0.2 U/mm^2 (on 40 g/L
273 glucose) in sealed cultures. Deletion of *chiB* completely eliminated the extracellular chitinase
274 activities in both submerged (Pócsi et al. 2009) and surface cultures: The chitinase activities
275 were always less than 0.2 U/mm^2 in the cultures of the *ΔchiBΔengA* strain. The applied
276 double gene deletions had the following developmental consequences on surface cultures:
277 i) Neither the *ΔprtAΔpepJ* nor the *ΔchiBΔengA* double mutations influenced the radial
278 growth of colonies (Table 2) and, as a consequence, it is highly unlikely that any degradation
279 of biopolymers by either ChiB/EngA or PrtA/PepJ in the central, carbon-starved and
280 autolysing part of the colony would support efficiently the growth of the younger mycelial
281 mats extending at the outer parts. In contrast, carbon starvation induced macroautophagy,
282 which significantly supports the radial growth of different *Aspergillus* species (Kikuma et al.
283 2006; Nitsche et al. 2013; Richie et al. 2007).
284 ii) On the other hand, conidia (but not conidiophore) production was significantly
285 lower in the *ΔchiBΔengA* strain in comparison to the control when grown at low starting
286 glucose concentrations meanwhile no significant differences were observed in glucose rich
287 media (Table 2). This observation underlines that - besides macroautophagy (Kikuma et al.
288 2006; Nitsche et al. 2013; Richie et al. 2007) - autolytic cell wall degradation (Emri et al.
289 2008; van Munster et al. 2016) is also important to support conidia production with nutrients

under prolonged carbon starving conditions. The ongoing autolytic cell wall degradation in starving surface cultures suggests that mechanisms, which protect living cells like foot cells in conidiopohres against cell wall hydrolyzing enzymes (*e.g.* cell wall melanization; Szilágyi et al. 2018) can have primary importance in the maintenance of efficient conidiogenesis. Importantly, the double *ΔchiBΔengA* deletions had no effect on cleistothecia formation even under carbon limitations (Table 2).

iii) The double deletion of *prtA* and *pepJ* had no significant effect on asexual sporulation but significantly decreased the production of cleistothecia even on 40 g/L glucose (Table 2). In accordance with our findings, Han (2009) demonstrated that the presence of amino acids enhance cleistothecia formation meanwhile amino acid starvation can block this process (Hoffmann et al. 2000). It is worth mentioning that deletion of *ggtA* encoding an extracellular γ-glutamyl transpeptidase, which is most likely involved in the utilization of peptides and amino acids, also significantly interfered with the maturation of cleistothecia (Spitzmüller et al. 2015). Importantly, the addition of casamino acids to the culture media eliminated the observed phenotypic differences between the control and the *ΔprtAΔpepJ* strains (Table 2).

Conclusions

Summing it up, carbon stress had a dual effect on the growth and development of *A. nidulans* colonies in surface cultures because it induced radial growth and extracellular hydrolase production, enhanced the maturation of cleistothecia, however its effect on the rise of conidiophores was not significant. On the other hand, carbon stress reduced the quantity of mycelial biomass and also the number of conidia and cleistothecia produced. Autolytic hydrolases significantly influenced both asexual and sexual development taking place in surface agar cultures. Namely, the endoglycosidases ChiB and EngA supported conidia

formation under carbon stressed conditions most likely *via* releasing metabolizable building blocks from the cell wall polysaccharides of autolysing dead hyphae. Furthermore, PrtA and PepJ proteinases had a positive effect on the sexual development of the fungus even under carbon rich conditions by liberating free amino acids.

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520 **Figure legend**

521

522 **Fig. 1** Changes in the glucose concentration in surface cultures of *A. nidulans*.

523 Agar plates were point-inoculated with conidia of *A. nidulans* tHS30.3 (control) strain and

524 were incubated at 37 °C for 168 h. Mean ± S.D. calculated from 3 independent experiments

525 are presented.

526 Part a – Spatial distribution of samples. (I) near the edge of the colonies, (II) at the edge of

527 the colonies, where the first conidiophores developed, (III) at the halfway point of the colony

528 radius and (IV) at the center of the colony. (A representative photo is presented.)

529 Part b - Effect of cultivation time on glucose consumption. The starting glucose concentration

530 was set to 10 g/L and samples were taken at 24 h (green), 72 h (yellow) and at 120 h (blue)

531 incubation time. Because the size of the colonies was too small at 24 h only one sample was

532 taken from under each colony at this time point.

533 Part c - Effect of starting glucose concentration on glucose consumption. The starting glucose

534 concentrations were 2.5 g/L (white), 5 g/L (yellow), 10 g/L (green), 20 g/L (orange) and 40

535 g/L (blue), and samples were taken at 120 h incubation.

536 Part d - Changes in the glucose concentrations under cleistothecia formation. The culture

537 conditions were the same as described in Part d with the exception that all nutrient agar plates

538 were kept in the dark and sealed with Parafilm on the 2nd d of incubation to induce

539 cleistothecia formation.

540

Table 1 Glucose concentration dependence of growth, sporulation, cleistothecia formation and extracellular hydrolase production in surface agar cultures of *A. nidulans* tHS30.3 strain.

Studied parameter[a]	**Starting glucose concentration**				
	2.5 g/L	**5 g/L**	**10 g/L**	**20 g/L**	**40 g/L**
Growth rate (mm/h)	0.45 ± 0.03*	0.40 ± 0.02*	0.35 ± 0.02	0.32 ± 0.02	0.30 ± 0.02*
Colony diameter (mm)	53 ± 3*	49 ± 2*	43 ± 3	40 ± 1	38 ± 1*
Protein density (ng/mm^2)	86 ± 9*	115 ± 26*	381 ± 58	584 ± 84*	1213 ± 80*
Protein content (mg per colony)	0.19 ± 0.02*	0.22 ± 0.03*	0.55 ± 0.06	0.73 ± 0.08*	1.5 ± 0.1*
Conidiophore density (1/mm^2)	90 ± 10*	110 ± 12*	140 ± 12	150 ± 16	170 ± 20*
Conidiophore number (1000 per colony)	135 ± 20	145 ± 18	150 ± 20	140 ± 25	145 ± 15
Conidia density (1000/mm^2)	22 ± 3*	32 ± 3	39 ± 5	51 ± 6*	93 ± 10*
Conidia number (10^6 per colony)	31 ± 4*	40 ± 5	43 ± 5	45 ± 6	77 ± 9*
Cleistothecia density (1/mm^2)[b]	2 ± 0.6*	3 ± 0.6*	8 ± 2	12 ± 2*	14 ± 2*
Cleistothecia number (1000 per colony)[b]	6 ± 1*	8 ± 2*	19 ± 2	25 ± 3*	25 ± 4*
Proteinase activity (U/mm^2)	8 ± 1*	n.d.	4.5 ± 1	n.d.	1.3 ± 0.4*
Proteinase activity (U/mm^2)[b]	5.8 ± 0.6*	n.d.	3.7 ± 1	n.d.	0.5 ± 0.15*
Chitinase activity (U/mm^2)	1.2 ± 0.2	n.d.	1.5 ± 0.4	n.d.	< 0.2
Chitinase activity (U/mm^2)[b]	0.6 ± 0.3	n.d.	< 0.2	n.d.	< 0.2

[a] - The growth and sporulation parameters were assessed at 120 h while cleistothecia were counted at 168 h incubation time. Extracellular chitinase and proteinase activities were recorded at 120 h (unsealed cultures) and at 168 h (sealed cultures). Means ± S.D. values calculated from 4 independent experiments are presented. "n.d." - not determined.

[b] - Plates were sealed with Parafilm on 2 d of incubation to induce cleistothecia formation.

* - Significant difference (Dunnett's test, $p < 0.05$) in comparison to control cultures, which were always supplemented with glucose at 10 g/L starting concentration.

Table 2 Characterization of the growth, sporulation and cleistothecia formation of certain *A. nidulans* mutants defected in autolytic hydrolase production.

Studied parameter and starting glucose concentration[a]	**tHS30.3 (control)**	**tNJ34.8 (*ΔchiBΔengA*)**	**tNJ78.4 (*ΔprtAΔpepJ*)**
Colony diameter (mm); 2.5 g/L[c]	53 ± 3	54 ± 4	54 ± 4
Protein density (ng/mm^2); 2.5 g/L[c]	86 ± 9	78 ± 7	80 ± 7
Conidiophore density (1/mm^2); 2.5 g/L[c]	90 ± 10	96 ± 10	99 ± 9
Conidia density (1000/mm^2); 2.5 g/L	22 ± 3	16 ± 3*	20 ± 3
Conidia density (1000/mm^2); 10 g/L	39 ± 5	29 ± 4*	34 ± 4
Conidia density (1000/mm^2); 40 g/L	170 ± 20	180 ± 20	160 ± 20
Cleistothecia density (1/mm^2); 2.5 g/L[b]	2 ± 0.5	1.8 ± 0.6	0.9 ± 0.5*
Cleistothecia density (1/mm^2); 2.5 g/L[b,d]	3.9 ± 0.8	4.1 ± 0.9	3.8 ± 0.8
Cleistothecia density (1/mm^2); 10 g/L[b]	8 ± 2	7 ± 2	4 ± 1*
Cleistothecia density (1/mm^2); 40 g/L[b]	14 ± 3	11 ± 2	8 ± 3*

[a] - The growth and sporulation parameters were determined at 120 h, while cleistothecia were counted at 168 h incubation time. Means ± S.D. values calculated from 4 independent experiments are presented.

[b] - Plates were sealed with Parafilm on 2 d of incubation to induce cleistothecia formation.

[c] - No significant differences were found among the strains at 10 and 40 g/L starting glucose concentrations.

[d] - Plates were supplemented with 2.5 g/L casamino acids.

* - Significant differences (Dunnett's test, $p < 0.05$) in comparison to the tHS30.3 control strain.

Figure 1

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