THESES OF THE PhD DISSERTATION

THE ROLE OF CHITINOLYTIC ENZYMES AND FREE RADICALS IN THE AUTOLYSIS OF *PENICILLIUM CHRYSOGENUM*

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1. INTRODUCTION

Since the discovery of penicillin V, *Penicillium chrysogenum* has become one of the most important filamentous fungi in biotechnology. Nevertheless, although serious problems may rise in the fermentation industry from the uncontrolled fragmentation, autolysis and ageing of the cultures – which will influence negatively the productivity and downstream processes – these events and their regulation, e.g. cell differentiation and redox changes during ageing, are only partially understood up to date. These researches may result in new strategies that could increase the effectiveness of biotechnological processes as well as the defence against dimorphic humanpathogen fungi.

Fungal autolysis is the natural, multistage process of self-digestion of aged hyphal cultures, involving partial permeabilization of the cellular envelope and leakage of intracellular material in its latter stage. Autolysis occurrs as a result of hydrolase (e.g. protease, glucanase and chitinase) activities, causing vacuolation and disruption of organelles and cell wall structure (White *et al.*, 2002). As a result, tolerance of hyphae against mechanical stress decreases and fragmentation increases (White *et al.*, 2002). The regulation of autolysis is only partially elucidated until now, but it has become clear that – in contrast to previous hypotheses – it should be considered as a dynamic, energy-dependent and highly controlled process that might be induced or effected by several intrinsic and extrinsic factors (McIntyre *et al.*, 1999), including nutrient limitations (Pusztahelyi *et al.*, 1997) or release of autolytic hydrolases (e.g. chitinases) into the culture medium (White *et al.*, 2002). In the bioprocessing industries, manufacturers usually want to prevent or delay autolysis, either to continue antibiotic production, or to prevent degradation of heterologous protein products by autolytic proteases.

Age-dependent chitinases are considered to play an important role in several fungal morphogenetic processes, including age-dependent autolysis and fragmentation (Gooday, 1992, 1997). To study the physiological role of these enzymes, using specific inhibitors (like allosamidin) in *in vitro* and *in vivo* inhibition experiments are convenient and reasonable tools.

The expression of genes responsible for morphological changes – involving autolysis and dimorphic switches – are most probably influenced by several stress-factors as well. For example, intracellular accumulation of reacive oxygen species (ROS) may induce spore germination (Lledías *et al.*, 1999) or even autolysis (Hansberg & Aguirre, 1990). According to recent findings, transient hyperoxidant states are important early signal-transduction events of these changes. Hansberg's "dioxygen avoidance theory of cell differentiation" (Hansberg & Aguirre, 1990) claims that the motive of morphological changes may be the microorganism's effort to eliminate molecular oxygen in unstable hyperoxidant states, resulting in a new, stable morphological and physiological status that

is adequate to environmental conditions. For instance, every stable state of the asexual cycle of *Neurospora crassa* was preceeded by a transient hyperoxidant state accompanied by GSH/GSSG redox imbalances (Hansberg *et al.*, 1993).

Fungal cells possess several defence systems to eliminate harmful free radicals. Antioxidant molecules, like glutathion or thioredoxin, and enzymes, like superoxid dismutase, catalase, glutathion-peroxidase are the first line of this defence system (Davis *et al.*, 2001). In higher eucaryotes the GSH/GSSG redox imbalances and prolonged oxidative stress are proved to play a crucial role in signal-transduction events of initiating apoptosis. Recent studies suggest that autolytic fungal cell death could be very similar, or even identical process to the apoptosis of higher eucaryotes (McIntyre *et al.*, 1999).

The researches seeking connection between oxidative stress and autolysis might be very important in submerged fermentation processes, since in most cases the microorganisms require high dissolved O_2 concentrations; under these conditions, the cells might be exposed to continous oxidative stress (White *et al.*, 2002). The side-chain precursors used in penicillin production may also generate oxidative stress (White *et al.*, 1999), and – in given concentration – they could deplete the intracellular glutathion pool (Emri *et al.*, 1997). Besides, GSH is structurally analogous to the key intermedier of β-lactam biosynthesis: ACV; hence, it inhibits penicillin production. Therefore it would be reasonable to decrease the intracellular GSH concentration without influencing negatively the physiological state of idiophase mycelia – which would hopefully increase industrial penicillin-production. According to previous experiments, intracellular GSH levels could not be decreased by changing quality and/or quantity of carbon, nitrogen and sulphur sources without influencing negatively β-lactam biosynthesis (Emri *et al.*, 1998). The only possibility to keep GSH levels low selectively is to take advantage of the GSH-dependent detoxification induced by the side-chain precursors (Emri *et al.*, 2000).

2. AIMS OF THE PROJECT

The investigation of an industrial *P. chrysogenum* strain at the Department of Microbiology and Biotechnology of University of Debrecen in the last years aimed detailed mapping of the morphological and physiological changes (e.g. autolysis and hydrolase production) during senescence as well as the glutathion metabolism and its relation to β-lactam production. The aims of this work – based on the results of these previous experiments – can be summarized as follows:

I. Morphological and physiological characterisation of the industrial *P. chrysogenum* NCAIM 00237 strain's ageing cultures.

1. What are the roles of fungal chitinolythic enzymes in morphological changes of germinating, growing and carbon-limited, ageing cultures (with special emphasis on autolysis and fragmentation)? The consequences of *in vivo* inhibition of chitinases.

2. Changes of vitality, metabolic activity and respiration of ageing cultures under and after carbon limitation. The glucose utilisation of the fungus.

3. Changes in the redox status and GSH metabolism (on enzyme and metabolite levels) in different growth phases, with special regard to possible causal connections with the observed morphological changes.

4. Generation and protection against reactive oxygen species. Applicability of the "dioxygen avoidance theory of cell differentiation" to explain the morphological and physiological changes.

II. Investigating the connection between the GSH metabolism and β-lactam production of an industrial *P. chrysogenum* strain.

1. Could a transient, well defined pH drop at the beginning of the production phase induce a GSH-dependent detoxification of a side-chain precursor under industrial conditions? Could the β-lactam production be increased this way?

3. METHODS

The growth and the morphological and physiological changes of ageing cultures of the *P. chrysogenum* NCAIM 00237 strain was investigated using shake-flasks containing complex medium with 1% glucose as carbon source. At 35 h 9,6 μM allosamidin (chitinase-inhibitor) and/or at 115 h 57 mM glucose was added and the changes in dry cellular weight, several metabolit (GSH, GSSG, peroxid, superoxid, glucose, gluconate) concentrations as well as enzyme activities (GOX, AOX, GR, GPx, GST, γGT, catalase, SOD, chitinase, HexAm) were monitored. These were carried out with colorimetric rate assays, and in some cases (HexAm, chitinase, γGT), endpoint measurements. The GOX activities were detected by modified glucose-assay, while the gluconate determinations were measured by altering the gluconate-dehydrogenase assay. For detecting free radicals, fluorimetric assays were applied. The mycelial samples were disrupted using X-Press, acid or liquid nitrogen, while (in case of extracellular metabolite or enzyme samples) the filtered and centrifuged medium was used directly for the measurements. To remove the chitinase inhibitor the samples were dialyzed overnight. In isoenzyme purification experiments ammonium-sulphate precipitation and affinity cromatography, and for the determination of relative molecular weights non-reducing polyacrylamide gelelectrophoresis were applied. To prepare cell wall for elementary analysis of the alkali-insoluble residuals, the cells were boiled and hydrolized in alkali.

Morphological changes and alterations of metabolic activity was followed using light microscopy, semi-automated computerized image analysis combined with vitality staining, and viable counts on agar plates. The resulting data were evaluated with several statistical methods, including Student's T-test and "two-way analysis of variance". The vitality of the cultures was tested by transferring the mycelia to fresh medium, and the ability to revert to vegetative growth was monitored. The total and cyanide resistant respiration activities of the cultures were measured using a home-built oxygraphic cell and oxygen electrode. The β-lactam production and GSH-dependent detoxification of the industrial *P. chrysogenum* strain after a transient pH drop was studied using 5 liter fermentor vessels. In this case, the sugar (glucose and saccharose) contents of the medium was measured by a Cobas Mira robot. For estimating the β-lactam production, a penicillin-hypersensitive *Micrococcus* strain was used on agar plates.

4. RESULTS AND DISCUSSION

4.1. Morphological and physiological changes of *P. chrysogenum* in the presence of allosamidin

The mycelial dry weight of the cultures increased rapidly in the exponential phase (20-34th h); the cells metabolised glucose quite effectively. This phase was characterised with the intensive elongation and branching of hyphae, resulting in typical pellet-morphology. After a short stationer phase (34-40th h) intensive vacuolisation, autolysis and fragmentation started; by the 115th hour of incubation the filamentous elements disappeared and mainly round-ended, cwo-celled, yeast-like fragments became dominant surviving forms of the cultures.

Chitinases are thought to participate in the morphogenesis of chitin-containing fungi in several ways, including the swelling and germination of spores, branching and apical extension of growing hyphae and age-related fragmentation and autolysis of hyphae (Gooday, 1997). In fact, most of the evidence supporting these roles has come from *in vivo* enzyme inhibition experiments by allosamidin (Gooday *et al.*, 1992; Sándor *et al.*, 1998), besides the disruption of genes coding for fungal chitinases (Takaya *et al.*, 1998). Allosamidin, and its semisynthetic derivatives have been regarded as potential antifungal agents since the end of the 80s'. Unfortunately, all attempts to inhibit the exponential growth of either yeasts or filamentous fungi by allosamidin have failed (Dickinson *et al.*, 1989; Sándor *et al.*, 1998). In my experiments, allosamidin also failed to affect the exponential growth of the studied *P. chrysogenum* strain. Although chitinases are always found in grownig fungal mycelia (Rast *et al.*, 1991), it is possible that they are protected from the

inhibitory effect of allosamidin (Gooday, 1995; Gooday *et al.*, 1997). Alternatively and according to the steady state model for hyphal growth proposed by Wessels (1984), these hydrolases may play no role in the apical wall extension. Accordingly, allosamidin is therefore unlikely to be a suitable tool to get get clear-cut evidence on the involvement of chitinases in the apical growth. On the other hand, chitinases are widely accepted to play an important role in branch formation and germination, where the local, temporary softening of the rigid cell wall is of primary importance (Gooday *et al.*, 1997; Wessels, 1984). Our data fully support this view. Allosamidin significantly hindered the autolytic loss of biomass in autolysing, ageing cultures. This effect was most apparent between 60-88th hours of incubation. The break-up of the allosamidin-treated mycelia resulted very similar forms, observed in control cultures, by the end of autolysis. Nevertheless, in contrast to the control cultures, where intensive germination and tip formation started after adding an extra dose of glucose at 115 h, in treated mycelia nor increase in dry weight, neither remarkable outgrowth could be observed. Thus, the chitinase-inhibitor hindered not only the autolysis, but also the cryptic growth and the ability to switch to vegetative growth very spectacularly. On the other hand, both the control and treated cultures utilised glucose quite effectively, though with slightly different rates.

The element-analysis of the alkali-insoluble (chitin-glucan) residues of the 165 hour cell wall residues revealed that in the presence of the inhibitor the chitin content of the cell wall increased remarkably (2,6 $\%_{control} \rightarrow$ 7,0 $\%_{allosamidin}$). On the other hand, the surviving fragments of the cryptic growth phase – despite the significantly different wall-composition (and most probably different structure) of the control and treated cultures showed very similar morphology in the microscope-studies. The increasing chitin content and the expected thickening of the chitin layer within the wall might be a consequence of two independent events. Namely, the equilibrium between cell wall biosynthesis and lysis (Bartnicki-Garcia, 1973) might be thrown out of balance, and/or the autolytic degradation of cell wall matrix polymers was hindered by the chitinase inhibitor (Reyes *et al.*, 1988). Nevertheless, the effective inhibition of extracellular chitinases and the increasing chitin content of the cell wall are most probably in causal connection.

Regarding the autolytic hydrolases, both extracellular hexosaminidase (HexAm) and chitinase activities were negligible in the exponential growth phase, then in the stationary phase they started to increase, gaining a plateaut by 115th hour of incubation. Allosamidin didn't influence significantly the extracellular hydrolase production. On the other hand, in the treated cultures after glucose resupplementation the activity of these extracellular enzymes regulated by carbon catabolit repression decreased much more moderately, – although significantly – than in controls. The microsomal chitinase(s) of the studied *P. chrysogenum* strain have been demonstrated to be membrane-bound zymogens, at least during exponential growth (Sámi *et al.*, 2001b), which may lead to some form of cellular compartmentalization of the hydrolases. This may confer protection

against the effects of allosamidin *in vivo*. Later, during autolysis, no zymogen activation was observed, i.e. all the chitinases had been activated and might have been exposed to allosamidin. This may explain the age-dependent fungistatic effect of this chitinase inhibitor in the autolysing cultures.

In the *in vitro* enzyme-inhibition experiments allosamidin proved to be a potent inhibitor of the extracellular chitinases with an IC_{50}=1,4 μM value. This is at mid position within the range of IC_{50} values observed for fungal chitinases with allosamidin (Rast *et al.*, 2000). Thus, the 9,6 μM allosamidin concentration used in our experiments caused total inhibition. The isoenzyme profiles of 115 hour extracellular control and allosamidin-treated, then dialysed samples after purification with affinity-cromatography were very similar. The presence of several isoenzymes in the autolytic phase can be explained either by several active chitinase genes present in the cells, or by (partial) proteolytic digestion. In most cases the specific role of the isoenzymes are still unclear. Nevertheless, the similar isoenzyme profiles, *in vitro* and *in vivo* inhibition experiments and the observed morphological changes suggest that the chitinases of the studied *P. chrysogenum* strain were allosamidin-sensitive.

Based on the results of the statistical analysis of the data, both the total cell number and the amount of round-ended, two-celled fragments decreased significantly between 115-166th hour of incubantion; these changes were slightly, but significantly smaller in the presence of allosamidin. The number of hyphae (containing at least two septa) decreased as well, and of one-cell forms increased in the function of time; but these changes were usually insignificant. The number of these forms weren't influenced by allosamidin either. In ageing cultures, viable cell numbers were significantly lower than those of gained by microscopic cell count in every case. Allosamidin-treated and control samples showed no real differences in viable cell numbers. Accordingly, after transferring treated cells to fresh, allosamidin-free medium, very similar outgrowth rates were observed in every tested time. This demonstrated well the reversible effect of allosamidin on germination of the surviving forms.

The physiological effects of allosamidin were disadvantageous for the microorganism. For example, after glucose supplementation at 115 h, the decreases in the extracellular chitinase and HexAm activities, glucose utilisation and acidification rates were moderated in comparison with control cultures. In contrast to these, no essential differences were found between the metabolic activities of control and treated cultures using image analysis or vitality staining. Moreover, allosamidin didn't influence the GSH/GSSG redox ratios either. Although the specific GSH and GSSG concentrations were lower during autolysis and cryptic growth than in control cultures, these changes were due to the higher dry weight values, and were insignificant in all cases. These findings made any severe cell injuries as a result of allosamidin unlikely. After glucose-

resupplementation at 115 h the specific GSH concentrations didn't decrease in the presence of the inhibitor, while in control cultures a remarkable drop in the GSH levels was observable, as GSH was extensively used in controls as an easily available endogenous N and S source after the reinitiation of growth. Taking into consideration all these findings, we concluded that the overall effect of allosamidin on the autolysing *P. chrysogenum* mycelia was fungistatic and not fungicidal. The morphological and physiological effects of allosamidin on ageing *P. chrysogenum* cultures are summarized in Table 1.

PARAMETER	EFFECT*
Autolytic loss of biomass	**Decrease**
Number of hyphae & other morphological forms	**Slightly Decrease**
Size of morphological forms	**Slightly Increase**
Chitin-content of the cell wall	**Increase**
Autolytic hexosaminidase activities	**No change**
Autolytic chitinase activities	**(reversible) Inhibited**
Formation of new hyphal tips after glucose-resupplementation	**(reversible) Inhibited**
Vitality, viable cell counts	**No change**
Vitality, dry cellular weight & outgrowth frequency (after transfer)	**No change**
Vitality (by fluorescent staining)	**No change**
Intracellular GSH, GSSG levels and ratios	**No change**
Glucose utilisation rates after sugar resupplementation	**Slightly Decrease**
Acidification rates after sugar resupplementation	**Slightly Decrease**
Utilisation of intracellular GSH-reserves after resupplementation	**Decrease**

Table 1 *: Changes after addition of 9,6 μM allosamidin (35 h) and/or 57 mM glucose (115 h), between 80-167 h fermentation time.

4.2. Free radical accumulation

Derived from the propositions of the "dioxygen avoidance theory of cell differentiation" (Hansberg & Aguirre, 1990), both growing hyphae and the round-ended, yeast-like fragments in autolysing and cryptically growing *P. chrysogenum* cultures may represent physiologically stable, differentiated states of the fungus. According to this theory, every step of cell differentiation may be preceded by a transient, unstable hyperoxidant state, i.e. prior to autolysis, in the deceleration/stationery phases of growth. Our experimental data did not support this hypothesis. The exponential growth phase of the fungus was characterised with surprisingly high intracellular ROS and GSSG concetrations, and as a result, relatively low GSH/GSSG redox ratios. Due to this, prior to autolysis several antioxidative enzymes (GR, GPx, catalase) were active (or activated) in our case; but after the glucose was consumed, (in deceleration, stationary and early autolytic phases) the intracellular ROS concentration also decreased; the peroxid-levels reached their minimum at 44 h; the superoxid a little later, at 66 h. Thus, in the stationary (pre-autolytic) and early autolytic phases, the intracellular ROS levels – in contrast with the propositions of the dioxygen avoidance theory – clearly decreased, instead of transient increase. Later, as autolysis progressed, the intracellular peroxid and superoxid levels elevated considerably and steadily but the GSH/GSSG ratio was meanwhile improving as a consequence of decreasing intracellular GSSG levels. Therefore, real oxidative stress (which would be indicated by imbalance of the above mentioned redox ratio) was very unlikely in this case. Intracellular ROS levels could be influenced by many factors, including metabolic cell activity (as peroxide production was highest in the early exponential growth and after glucose readdition), age-dependent regulation of antioxidant enzymes and ageing of the surviving fragments. The specific GPx, catalase and GR activities that were very high in the exponential phase, decreased drastically during carbon limitation. The antioxidant enzyme activities also responded to various factors in *P. chrysogenum*. For example, GPx was regulated by intracellular peroxide levels (Emri *et al.*, 1997) as well as carbon starvation and supplementation; GR responded to any kind of oxidative stress (Emri *et al.*, 1997, 1998). Surprisingly, the specific catalase activity was found to be dependent on the growth phases but was independent of the actual intracellular peroxide levels, and the only factor that influenced SOD was the intracellular superoxide level.

In good accordance with the theory, after glucose readdition, new germination of the surviving fragments were accompanied by remarkable transient peroxid-accumulation (0,5→2,2 pmol/mg CDW) and the activation of several antioxidant enzymes (GR, GPx). On the other hand, the superoxid levels decreased significantly after glucose readdition. The specific SOD activities were always high in the post-autolytic cultures; nor this, neither the catalase activities were

influenced significantly by carbon resupplementation. The intracellular catalase activities didn't respond to the post-autolytic peroxid-accumulation either; from the 115th hour of incubation they remained at a low, constant level. Based on the changes of antioxidant enzyme activities, during autolysis GSH-independent, while in the exponential growth GSH-dependent enzymes were likely the main elements in defence against oxidative stress and elimination of free radicals.

GSH is well known to be a key metabolite in numerous physiological processes, including maintenance of the redox status in the cells, protection against ROS, detoxification and elimination of heavy metal ions, xenobiotics and harmful metabolites, uptake and intracellular transport of certain amino acids (Penninckx & Elskens, 1993). Besides, it is an easily available endogenous N and S source for the cells (Emri *et al.*, 1998). Thus, the intracellular GSH levels could be influenced by many factors. In my experiments, the specific intracellular GSH concentrations remained high (9-11 mmol/g CDW) in the whole incubation period, with the exception of 165 h samples, where – reflecting the declining vitality of the cultures – the GSH levels were also lower (6-9 mmol/g CDW). After the 115 h glucose addition the GSH reserves of the cells were rapidly depleted, and reached a low level at 165 h. The re-initialisation of vegetative growth involved the mobilisation of the endogenous N and S reserves; therefore, the profound shrinkage of the intracellular GSH pool together with the concomitant GSH/GSSG redox imbalance were most likely part of the complex metabolic stress-response provoked by the carbon source. The specific GSSG concentrations were relatively high in the exponential growth phase (0,4 mmol/g CDW) after which (34-66. h) decreased significantly, and reached a low, constans value in the later phases of autolysis (~0,17 mmol/g CDW). In fully autolysed cultures – despite ROS accumulation – further GSSG decrease was detected. As a result, the GSH/GSSG redox ratios increased considerably by 133 h (25→125).

It is important to emphasize, that although intracellular ROS and GSSG levels as well as the activities of the antioxidant enzymes were highest in the exponential and deceleration phases, autolysis wasn't preceded by ROS accumulation and hyperoxidant state. Consequently, ROS are unlikely to play any role in the signal transduction events of autolysis in this case. Nevertheless, this is not exclusive of an inherent physiological analogy between apoptosis and autolysis (McIntyre *et al.*, 1999) because an oxidative stress independent physiological pathway of apoptosis is also present in more complex eukaryotes in addition to the damage-induced mitochondrial path (Coppola & Ghibelli, 2000). To sum it up, in *P. chrysogenum* cultures both ROS generation and GSH metabolism were influenced by several intrinsic and extrinsic factors during the whole period of incubation. The remarkable complexity of this system may explain the failure of the "dioxygen avoidance theory of cell differentiation" to give a coherent explanation for the physiological changes leading to cell differentiation during autolysis.

4.3. GOX activities and respiration rates

It is well known that the two major ROS generating metabolic pathways in *P. chrysogenum* are the direct oxidation of glucose by glucose oxidase (GOX) and the regeneration of NAD^+ and FAD through the cytochrome-dependent mitochondrial respiratory chain. The latter can be reduced by the alternative (cyanide resistant) oxidases (AOX), where – during respiration – no ROS is generated. Thus, AOX may play a crucial role in the antioxidative defence of the fungus, especially when the total respiration is intensive. Therefore – in the knowledge of the ROS accumulation tendencies – the GOX and AOX activities as well as total respiration rates of the cultures as a function of time and carbon source were studied.

The total GOX activity (total cell-bound + extracellular) was high, constant in the exponential and deceleration growth phases, while the specific activities decreased, due to the increasing biomass. The enzyme was secreted into the medium actively; this was shown by the syncronous intracellular decrease and extracellular increase of the specific activity. Meanwhile, the wall-bound activities remained unchanged. While GOX is located mainly in the cell wall in the major industrial GOX producer *A. niger*, in the studied *P. chrysogenum* strain GOX was predominantly a soluble enzyme (either extracellular or cell-bound), and only a minute amount of enzyme was trapped within the cell wall. The precise location of the soluble cell-bound enzyme fraction has remained yet to be elucidated, but a periplasmic location seems to be probable (Nielsen, 1995). In the early exponential phase of growth (up to 20 h of incubation), the cells metabolised approximately 30% of the starting concentration of glucose, without any significant conversion to gluconate; besides, at 20 h only a small increase in CDW was recorded. Later, between 20-26 h, half of the remaining (36 mM) glucose was converted to gluconate. Thus, the exponentially growing cultures converted the starting glucose to gluconate quite effectively, as a consequence of the high GOX production and secretion. By the 34^{th} h all the starting glucose, and 2 hours later all the generated gluconate was completely consumed from the medium; paralelly, the CDW increased further (8 mg/ml). In good agreement with the results of Nielsen (1995) we also found that gluconate was not utilised prior to the total consumption of all the starting glucose from the culture medium; after that, however, it was rapidly utilised.

The high or increasing antioxidative enzyme activities (catalase, GR, GPx) recorded in later exponential phase of growth (24-28 h) were likely the consequences of the oxidative stress generated by the earlier H_2O_2 formation by GOX. The induction of antioxidative enzymes in the early exponential phase of growth may explain satisfactorily the remarkable oxidative stress tolerance of the late exponential phase *P. chrysogenum* mycelia (Emri *et al.*, 1997). It is noteworthy that H_2O_2 has been shown to play a crucial role in the induction of *A. niger* glucose oxidising

system (GOX, lactonases and catalases; Witteveen *et al.*, 1993) and to stimulate fungal cyanide resistant respiration (Karaffa *et al.*, 2001).

In the stationary and autolytic growth phases GOX activities decreased rapidly in every cell-fraction, and completely disappeared by the 98th hour of incubation. In autolytic cultures no apparent gluconate conversion was detectable after glucose readdition, and GOX activities remained negligible as well – in spite of the effective utilisation of the new carbon source. Unexpectedly, the generation of free radicals after glucose supplementation in autolysing cultures could not be attributed to GOX as indicated by the low specific enzyme activities and the very low glucose→gluconate conversion rates. It is very important to emphasize the fact that although the intracellular peroxide level was high and glucose was present in the culture medium neither GOX nor catalase induction was observed after glucose re-addition. We can therefore conclude that the synchronous upregulation of the glucose oxidizing system in the presence of glucose and H_2O_2 is age-dependent in *P. chrysogenum.*

In good correlation with the glucose consumption, the intensity of both total and cyanide resistant respiration increased significantly in the exponential phase, reaching their maxima at 28th hour of incubation, then started to decrease in the deceleration phase. The total respiration remained at a relatively low constant value after 88 h incubation time, while the ratio of cyanide resistant respiration increased continuously in stationary, autolytic and cryptic growth phases. After glucose re-addition, both total and cyanide resistant respiration increased significantly in every cases. The intensity of total respiration reached 26-44 % of that observed in the exponential phase, 57-70 % of which was due to cyanide resistant respiration. Similarly to exponential growth, after consumption of the extra dose of glucose both total and cyanide resistant respiration decreased significantly; but the latter started to increase again, as the cultures reached a new, second autolytic phase. Under these circumstances, disintegrating mitochondria with partly disorganised cristae were the most likely sources of ROS. The loss of mitochondrial function was clearly demonstrated by significantly reduced total respiration rates after glucose re-addition. In this case, increased cyanide resistant respiration together with increased GPx and GR activities were the most important elements of the primary defence against oxidative stress. Intracellular ROS accumulation during cryptic growth, declined ability of the surviving fragments to revert to hyphal growth after glucose supplementation as a function of time, and decreasing mitochondrial functions are in good accordance with the propositions of the free radical theory of ageing (Harman, 1993) and, therefore, the use of the term "ageing" to describe the physiological changes taking place in post-autolytic *P. chrysogenum* cultures can be recommended. In contrast to exponentially growing hyphae, ageing yeast-like fragments were most probably exposed to oxidative-stress induced apoptosis, and therefore, they seem to be suitable objects to further studies of ageing in fungi.

4.4. Investigation of GSH-dependent detoxification during β-lactam production

Although several substrates of GST (epoxides and non-specifically hydroxiled, GSH conjugated toxic intermediers; Emri *et al.*, 2000) are still hypothetical in *P. chrysogenum*, the proposal to take advantage of the GSH-dependent detoxification of POA to keep the intracellular GSH levels low (Emri *et al.*, 2000) was tested in fed-batch cultures of the *P. chrysogenum* NN-08 strain. Keeping the GSH level low (e.g. by applying a transient, well-controlled pH drop in the beginning of the production phase) would be beneficial for the penicillin production of the fungus (Van de Kamp *et al.*, 1999). When the pH dropped, POA poured into the cells. After this, as expected, the specific activities of the GSH-dependent detoxification's two key enzymes, GST and γGT increased 2-3 times between 45-69th hours of fermentation. In spite of this, the intracellular GSH levels remained unchanged – even at pH=5,0. Furthermore, GSSG levels remained negligible in every case. Thus, altering pH did not result in changes of GSH/GSSG ratio; accordingly, the activity of the specific GR activity (which is sensitive to this ratio) remained unchanged. When the *P. chrysogenum* NCAIM 00237 strain was grown in a complex medium and was subsequently transferred into a defined medium containing 10 g l^{-1} Na-glutamate as a nitrogen source the GSH pool was significantly decreased depending on the buffer capacity of the medium (EMRI *et al.* 1997, 2000). The substitution of 15 g l^{-1} $(NH_4)_2HPO_4$ for Na-glutamate stabilised the intracellular GSH levels by hindering considerably the induction of the γGT activity by POA (EMRI *et al.* 1997). The nitrogen repression of this key enzyme of the GSH catabolism seemed to be a crucial element in the maintenance of high intracellular GSH levels even in the presence of POA.

The β-lactam production of the cultures were similar in every cases. The cells – despite the high concentration (5 g/l) of the toxic side-chain precursor – utilised the carbon source (saccharose) quite effectively. Nevertheless, as pH decreased, the increase in biomass slowed down. When pH was dropped under 5,0 (4,6-4,8) the GSH pool of the cells depleted rapidly, and the antibiotic-production observed at higher pH also stopped. In this case, specific γGT and GST activities increased suddenly; but even these changes were insufficient to prevent the following intensive autolysis and cell death. Thus, the very narrow (4,8-5,0) pH range (with the given experimental setup) seemed to be critical – concerning not only β-lactam production, but the mere survival of the fungus. Taking into consideration all these data, and the rheological properties of high-volume industrial fermentor vessels, the exploitation of GSH-dependent detoxification of the penicillin side-chain precursors seems to be rather unlikely in penicillin fermentation technology.

5. NEW RESULTS (SUMMARY)

1. The *in vivo* enzyme inhibitions justified the role of age-related chitinases in the process of autolysis and fragmentation in carbon-limited, ageing *P. chrysogenum* cultures. The substrate-analogue inhibitor *allosamidin* did not only hinder the breakup of pellets and onset of autolysis, but also prevented the surviving fragments from new germination after glucose re-supplementation.

2. In the presence of this chitinase inhibitor the chitin content of the fungal cell wall increased, while the metabolic activity and vitality of the mycelia reamined unaffected. The antifungal effect of allosamidin on autolysing *P. chrysogenum* cultures was fungistatic rather than fungicidal. The young, exponentially growing and ageing cultures showed remarkably different sensitivity to allosamidin, which could be indicative of chitinase isoenzymes with different localisation and/or enzymological status as a function of time.

3. Changes in the redox status and glutathion (GSH) metabolism of ageing *P. chrysogenum* strains as a function of time have been registered. During the different growth phases both the GSH levels and the formation of reactive oxygen species (ROS) were influenced by several exogenous and endogenous factors. Hence, the dioxygen avoidance theory was appliacble only with certain restrictions, and no strict, causal correlation was found between the observed redox changes, ROS levels and morphology of the cultures.

4. The exponential growth phase of *P. chrysogenum* was characterised by intensive respiration, high intracellular ROS levels and remarkable antioxidative enzyme-activities, where the main source of ROS generation was most probably the high glucose oxidase (GOX) activity. On the other hand, in senescent, carbon-supplemented cultures, the increase of intracellular ROS was due to the cytochrome-dependent mitochondrial respiration. The inducibility of both GOX and catalase activities appeared to be age-dependent.

5. In the exponential growth phase high catalase, glutathion reductase and peroxidase activities, while in the cell death and cryptic growth phases, high superoxid dismutase and alternative oxidase activities could be key elements of the antioxidative defense system.

6. The events observed in post-autolytic *P. chrysogenum* cultures were in good accordance with Harman's free radical theory of ageing; accordingly, we were the first recommending to use the term "ageing" to describe these physiological changes in post-autolytic cultures.

7. In large-scale, industrial fermentations, the applicability of the depletion of the intracellular GSH pool *via* GSH-dependent detoxification of the penicillin-precursor phenoxyacetic acid (POA) in order to increase penicillin yield and productivity seemed rather unlikely.

6. ABBREVIATIONS

ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valin
AOX	alternative (cyanide resistant) oxidase
CDW	cellular dry weight
GOX	glucose oxidase
GPx	glutathion peroxidase
GR	glutathion reductase
GSH	glutathion (γ-L-glutamyl-L-cysteinyl-glycin), reduced form
GSSG	glutathion, oxidised form
GST	glutathion S-transferase
γGT	γ-glutamyltranspeptidase
HexAm	*N*-acetyl-β-D-hexosaminidase
NCAIM	National Collection of Agricultural and Industrial Microorganisms
POA	phenoxyacetic acid
ROS	reactive oxygen species
SOD	superoxide dismutase

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