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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

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Different control mechanisms regulate glucoamylase and protease gene transcription in *Aspergillus oryzae* in solid-state and submerged fermentation

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Abstract Solid-state fermentation (SSF) with *Aspergillus oryzae* results in high levels of secreted protein. However, control mechanisms of gene expression in SSF have been only poorly studied. In this study we show that both glucoamylase (*glaB*) and protease (*alpA*, *nptB*) genes are highly expressed during surface cultivation on wheat-based solid medium, and even higher during cultivation on wheat kernels. In wheat-based liquid medium, low levels of gene expression are observed. Typical SSF cultivation conditions, such as low water activity and the formation of aerial hyphae, did not contribute to the high-level gene expression on wheat-based solid medium. Analysis of wheat-based solid and liquid cultivations showed differences in carbon and nitrogen utilisation and external pH. The results presented show that the difference in regulation of transcription of the *alpA* and *nptB* genes in wheat-based liquid and solid medium could be pH dependent, involving a pH-dependent transcription regulator. The results obtained suggest that the difference in regulation of transcription of the *glaB* gene in wheat-based liquid and solid medium is caused by a difference in carbohydrate degradation and consumption under the different culture conditions.

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Introduction

Filamentous fungal cultivation on solid substrates differs in many aspects from growth in liquid culture. In particular, the extracellular localisation of secreted proteins differs greatly between liquid and solid cultures. On a solid substrate, the growing hyphae of filamentous fungi penetrate the enzymatically modified solid substrate by extension and branching of the growing hyphal tips (Trinci 1974; Prosser 1994, Dynesen and Nielsen 2003). In liquid fermentation, the secreted proteins are released into the culture medium, resulting in substrate degradation in the whole culture.

Numerous studies have described the presence of high level hydrolytic enzyme activities in extracts of filamentous fungi grown on different solid substrates (reviewed by Pandey et al. 1999). High protease activities are measured in extracts of wheat and rice kernel solid-state food fermentation by *Aspergillus* species (Su and Lee 2001; Tunga et al. 2001). Based on research with rice kernel solid-state fermentations (SSF) with *Aspergillus oryzae*, it was shown that high levels of glucoamylase B, an enzyme that is clearly different from glucoamylase A (Hata et al. 1998), are due mainly to induction of the glucoamylase B gene (Ishida et al. 1998). Until now, gene expression studies have focussed only on transcriptional regulation of *glaB* (Hata et al. 1998) and *pepA* (Gomi et al. 1993) in surface-grown filamentous fungi like *A. oryzae*. Transcription levels of the acid protease-encoding gene (*pepA*) have been shown to decrease due to high temperatures (>38°C) when *A. oryzae* was cultured on rice kernels (Kitano et al. 2002). Ishida et al. (2000) found that the promoter region of *glaB* mediates the induction of transcription by starch, high temperature, low Aw (water activity), and physical barriers to hyphal extension. The latter three are SSF-specific environmental conditions and

it has been suggested that induction of *glaB* transcription is due mainly to these conditions (Ishida et al. 2000).

N-terminal sequence analysis and enzyme activities of secreted proteins of *Aspergilli* grown on solid substrates were in agreement with preliminary northern analysis and suggested that the alkaline (*alpA*) and neutral (*nptB*) protease encoding genes are also highly expressed during surface growth (Malathi and Chakraborthy 1991; Te Biesebeke et al. 2002). However, it is unknown if, like the *glaB* gene, the *alpA* and *nptB* genes are also controlled by SSF-specific environmental conditions. To gain more insight into the different conditions that play a role in regulation of genes during submerged and surface growth conditions, we analysed the transcription of the *glaB*, *alpA* and *nptB* genes of *A. oryzae* during growth in wheat-based liquid and solid medium.

Materials and methods

Strains and media

A. oryzae strain ATCC16868 was used throughout this study. Ritmo wheat kernels (WK) (ACM, Meppel, The Netherlands) were pre-treated as described in Hoogschagen et al. (2002). Ground WK were prepared by pulverising non-pre-treated WK portions (20 g) with a Moulinex blender (SEB, Ecully, France) for 1 min. Wheat-based liquid medium (WLM) was prepared by shaking 4 g ground WK in 100 ml H_2O in shake flasks in a rotary shaker at room temperature for 5 min at 250 rpm. The suspension was poured through Miracloth, where 2 g of the ground WK (dry weight) remained in the filter resulting in 2% wheat-based liquid medium (2% WLM), which was sterilised for 15 min at 120°C. The 1% wheat based liquid medium (1% WLM) was prepared in a similar way using 2 g ground WK. Surface growth on wheat-based solid medium (WSM) was performed on 1% agar plates of 2% WLM (2% WSM).

Cultivation conditions

SSF was performed with 50 g pre-treated WK inoculated with 2 ml 5×10^7 conidia (1.0×10^6 conidia/g) in a sterile 1 l bottle, placed on a roller apparatus (Wheaton modular roller, Millville, N.J.) and kept rolling at maximum speed for 6 h at room temperature. The inoculated WK was transferred to sterile Petri dishes and incubated for 3, 4 or 5 days at 30°C at 98% water/air (v/v) content in a climatic incubator (VEA-Instruments, Houten, The Netherlands). Submerged cultivations were performed with 25 ml WLM inoculated with 10^6 spores/ml and incubated in shake flasks at 30°C in a rotary shaker at 250 rpm. Surface cultivation was performed after inoculation of nitrocellulose (NC) membranes (3 μm pore size; Millipore, Bedford, Mass.) placed on the agar plates of 2% WSM (25 ml), with 2.5×10^7 spores (10^6 spores/ml 2% WSM) followed by an incubation at 30°C at a relative humidity of 98% in a climatic incubator (VEA-Instruments). Liquid (LTC) and membrane (MTC) biomass transfer cultivations were performed as described by Ishida et al. (1998) after production of biomass in wheat-based medium (17 h in 2%WLM and 48 h in 2%WSM). After transfer to fresh wheat-based medium (2%WLM and 2%WSM, respectively) samples taken at time points 0, 2, 6, 8, 10 and 12 h in the case of MTC, and 0, 0.5, 1, 6, 8 and 24 h in the case of LTC, were used for transcription analysis. Low Aw in the culture medium was achieved by supplementing 2% WSM with maltose (5, 10 and 40%) or 5 M NaCl, resulting in Aw values of 0.98, 0.96, 0.93 and 0.93, respectively, measured using an electric hydrometer (Type EK 84/3H/63T, sensor type BSK-4; Novasina, Pfäffikon, Switzerland). The maltose- or NaCl-supplemented 2% WSM media were used in a 6 h MTC. A biomass layer without aerial hyphae—the so-called sandwich cultivation (SWC)—was prepared by covering the biomass on the NC membrane with another NC membrane. The SWC was transferred for 6 h to 2% WSM. Transfer experiments for 6 h to 1% WLM with and without 40% maltose were performed to determine the effects of maltose, pH and extracellular glucose on gene transcription.

Purification of total RNA and DNA

A. oryzae grown on WK was separated from the WK after 1 min vigorous shaking with a 0.7% NaCl solution, collected after filtration through Miracloth and rapidly frozen in liquid nitrogen. *A. oryzae* grown on NC membranes placed on WSM, and *A. oryzae* grown in WLM (collected after filtration through Miracloth) were rapidly frozen in liquid nitrogen. The collected *A. oryzae* biomass was ground in liquid nitrogen and total RNA

Table 1 Primers used to obtain probes for the *enoA*, *glaB*, *alpA* and *nptB* genes

Gene	Primers	Probe size (bp)	Reference
EnoA	5′-CGCTGCCCCCTCTTTCTCCG-3′ 5′-CCAACAATCTGGAAGTCAG-3′	420	Machida et al. (1996)
GlaB	5′-GAGCAATTCATCGGGGGA-3′ 5′-CCCTGATAGGTGTCCTCTGCG-3′	740	Gomi et al. (1993)
AlpA	5′-GAGCGCAACTACAAGATCAA-3′ 5′-CGGAATTCAGCCCAGTTGAAGCCGTC-3′	500	Cheevahanarak et al. (1991)
NptB	5′-GGCCGCCAAGGTCACCAAGG-3′ 5′-CGCCAGGGGCGTGAGTGAAC-3′	300	Tatsumi et al. (1991)

extracted using TRIzol reagent(Invitrogen, Carlsbad, Calif.) according to the suppliers instructions. Chromosomal DNA was isolated as described by Kolar et al. (1988) from *A. oryzae* after 30 h growth in 2% WLM.

Polymerase chain reaction and purification of probes

PCR was used to amplify probes for the *enoA*, *glaB*, *alpA* and the *nptB* genes from *A. oryzae* chromosomal DNA using 40 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C. Table 1 shows the primers used to obtain probes for the genes indicated. Probes were purified from 1% agarose gels using Qiaquick DNAeasy columns (Qiagen, Hilden, Germany).

Southern and northern blot analysis

Southern blot analysis was performed according to standard procedures (Sambrook et al. 1989) after cutting chromosomal DNA with *Eco*RI and *Bam*HI. For northern analysis, total RNA (10 μg) was fractionated by formaldehyde-agarose (1%) gel electrophoresis and transferred to a Hybond-N nylon membrane (Amersham Pharmacia, Little Chalfont, UK). Southern and northern blots were hybridised with ^{32}P-labelled (Random Prime Labelling Kit, Pharmacia) PCR-amplified probes for the *enoA*, *glaB*, *alpA* and *nptB* genes (see Table 1) at 62°C in standard hybridisation buffer (Sambrook et al. 1989). Hybridisation signals were visualised after exposure to X-OMAT AR film (Kodak, cat. 1651512; Eastman Kodak, Rochester, N. Y.) and scanned with a Hewlett Packard 6200C Scanjet at 600 dpi (Hewlett Packard, Waldbronn, Germany). The signal intensities on the northern blots were quantified with gene tools from Syngene (Synoptics, Cambridge, UK) using the signal for *enoA* as a loading control. In Southern analysis, the probes for the *enoA*, *glaB* and *alpA* genes all showed single specific hybridising bands and the probe for *nptB* showed two hybridising bands.

Biomass, pH, glucose and amino acids determination

The wet weight biomass of *A. oryzae* grown in 2% WLM was determined after filtering the biomass through Miracloth. The wet weight biomass of *A. oryzae* grown on 2% WSM was determined after weighing the biomass grown on the filter. The extracellular growth medium (1 ml) of *A. oryzae* grown in 2% WLM was boiled for 5 min and used for determination of glucose and amino acid concentrations. Extracts of the growth medium of *A. oryzae* grown on 2% WSM were obtained after removing the membrane with biomass and freezing the 2% WSM at −20°C. Subsequently, the 2% WSM was thawed and, after addition of 10 ml milliQ water, vortexed for 2 min, centrifuged for 5 min at 3,500 rpm, boiled for 5 min and used for determination of glucose and amino acid concentrations. Glucose was analysed enzymatically using the glucose HK 125 method (ABX Diagnostics, Montpelier, France; cat. no. A11A00116) (Burrin and Price 1985). Amino acids were analysed using the TNBS (trinitrobenzenesulfonic acid) method described by Adler-Nissen (1979). The pH of the medium of samples of *A. oryzae* grown in 2% WLM and extracts of *A. oryzae* grown in 2% WSM were measured with a PHM83 autocal pH meter (Radiometer, Copenhagen, Denmark) at the time points indicated.

Results

Substrate conversion, biomass increase and external pH during surface and submerged cultivations

Surface and submerged cultivation in 2% WSM and 2% WLM, respectively, was carried out to determine biomass, glucose, amino acid concentrations, and pH, over time (Fig. 1). The final wet weight biomass produced under both cultivation conditions was equal but during surface cultivation it took around twice as long to produce the maximum amount of biomass on 2% WSM. During surface cultivation, glucose and amino acid concentrations liberated from the substrate were lower than 0.05 g/l and 0.05 mmol/l, respectively. In submerged cultivations, the glucose and amino acids concentrations were maximal (0.23 g/l and 0.22 mmol/l, respectively) at the beginning of the logarithmic phase and decreased towards the stationary phase. Apparently, during submerged cultivation, an excess of glucose was liberated from the substrate, whereas the glucose concentrations in surface cultivation remained low at all time points. The pH of the 2% WSM extract remained around 6.5 at all time points. In 2% WLM, the pH reached a minimum of about 5.5 after 30 h submerged cultivation. These results show that surface and submerged cultivations of *A. oryzae* in 2% WSM and 2% WLM differ in at least three environmental parameters.

Transcription of *enoA*, *glaB*, *alpA* and *nptB* genes

To examine the transcription of *enoA*, *glaB*, *alpA* and *nptB* under different cultivation conditions, northern blot analysis was performed. Total RNA was isolated from the mycelium after 17, 24, 30 and 48 h submerged cultivation in 2% WLM, after 2 and 3 days surface cultivation in 2% WSM and after 3, 4 and 5 days SSF with WK. The results of northern hybridisations with labelled probes for the *enoA*, *glaB*, *alpA* and *nptB* genes are shown in Fig. 2. The probe for the enolase (*enoA*) gene (Machida et al. 1996) was used as a loading control as its transcript levels are equal during cultivation on different carbon sources (Nakajima et al. 2000). The *glaB* gene was transcribed to a very low level at 17 h in 2% WLM (lane 1) compared to that on 2% WSM (lanes 5–8). Transcription of the *alpA* and *nptB* genes occurs on 2% WSM (lanes 5–8) and in 2% WLM at 17 h, and at lower transcript levels at 24 h (lanes 1, 2). Moreover, the transcript levels of the *glaB*, *alpA* and

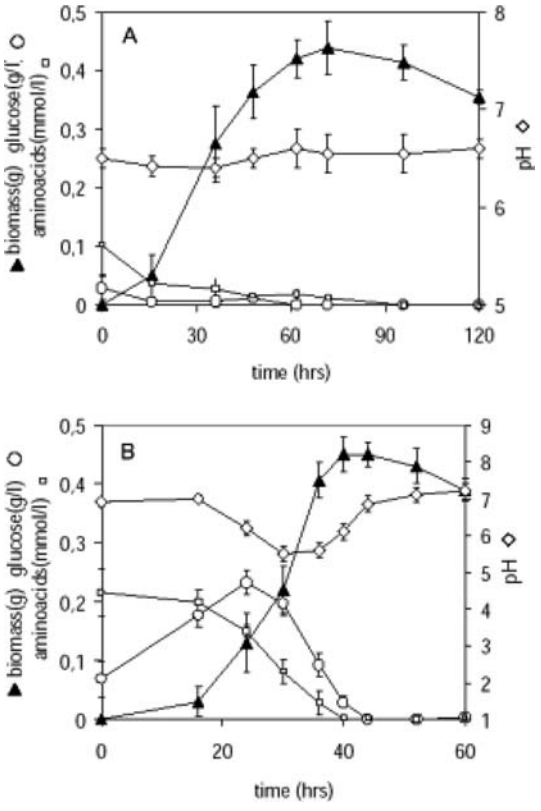

Fig. 1 Parameters measured in **a** surface and **b** submerged cultivation of *Aspergillus oryzae* grown in 2% wheat-based solid medium (WSM) and 2% wheat-based liquid medium (WLM), respectively. ▲ Wet weight biomass (g), ○ glucose (g/l), □ amino acids (mmol/l), ◊ pH measured in the growth medium of submerged cultivation and in the extracts of surface cultivation at the time points indicated

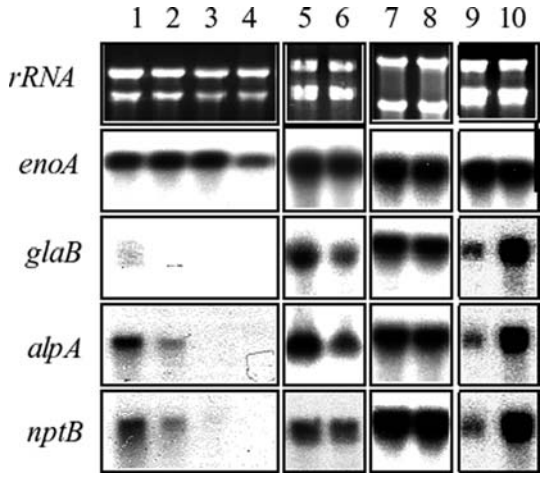

Fig. 2 Hybridisation of the probes for the *enoA*, *glaB*, *alpA* and *nptB* genes with RNA samples isolated from solid-state fermentations (SSF) on wheat kernels (WK) and surface cultivation on 2% WSM, and submerged cultivation in 2%WLM in northern analysis. Lanes: *1* 17 h WLM, *2* 24 h WLM, *3* 30 h WLM, *4* 48 h WLM, *5* 2 days WSM, *6* 3 days WSM, *7* 4 days WK, *8* 5 days WK, *9* 3 days WSM, *10* 3 days WK

nptB genes after 3 days on WK was about four times higher than on 2% WSM after 3 days as determined after quantification of signal intensity (lanes 9, 10). The transcriptional induction of the *glaB*, *alpA* and *nptB* genes during growth of *A. oryzae* was further investigated to understand the observed differences in transcript levels in submerged and surface cultivation.

glaB, *alpA* and *nptB* transcription in LTC and MTC

To examine in more detail the observed differences in transcriptional regulation, we performed liquid and membrane biomass transfer assays (LTC and MTC, respectively) to 2% wheat-based medium after 17 h growth in 2%WLM and 48 h growth on 2%WSM, respectively. Similar to the results shown in Fig. 1, the concentration of glucose and amino acids is low over time during MTC (Fig. 3a). The pH remained between 7.1 and 6.7 (Fig. 3b). The transcription of the *glaB*, *alpA*, and *nptB* genes was induced at all time points in MTC (Fig. 3c; lanes 1–6) although transcript levels decreased more than two times over 12 h MTC (cf. lanes 1 and 6). In LTC, the amino acid concentrations decreased from 4 mmol/l to low levels in the first 6 h, and the maximum level of glucose was liberated from the substrate at 5.5 h (Fig. 3a). The presence of increasing amounts of glucose liberated from 2% WLM (Fig. 3a) during the first 5 h LTC suggested that within that time frame, sufficient levels of starch degradation products are available. In LTC, transcripts of the *glaB* gene disappeared at 0.5 h, when glucose levels increased, and re-appeared at 6 h (Fig. 3c; lanes 7–12).

The transcript level of the *alpA* and *nptB* genes decreased within the 1st hour of LTC (Fig. 3c; lanes 7–9) suggesting an overall degradation. Apparently, transcription of the *alpA* and *nptB* genes is not induced directly upon transfer. The results shown in Fig. 3(a, c) show that transcription of *alpA* and *nptB* genes occurs when glucose concentrations are low, suggesting that glucose could act as a repressor for *alpA* and *nptB* transcriptional induction. However, the observation that transcription of the *alpA* and *nptB* genes occurs at 17 and 24 h of submerged cultivation, when glucose concentrations are still high, suggests otherwise (Figs. 1b; 2, lanes 1–4). As shown in Fig. 3(b, c), the pH of the submerged cultivation also changed during fermentation. Directly after transfer of the growing *A. oryzae* to 2% WLM, the growth medium started to acidify (Fig. 3b), and at 6 and 8 h LTC the growth medium started to recover from acidity. At these time points, *alpA* and *nptB* gene transcript levels also increased (Fig. 3c; lanes 10, 11). However, besides pH effects, specific induction at 6–8 h may also play a role, since at 24 h LTC no transcription was observed although the pH was neutral.

Effect of low-Aw, maltose and pH on *glaB*, *alpA* and *nptB* transcription

Low-Aw is a characteristic of SSF (Ishida et al. 1998). It was postulated that transcriptional induction of the *glaB*

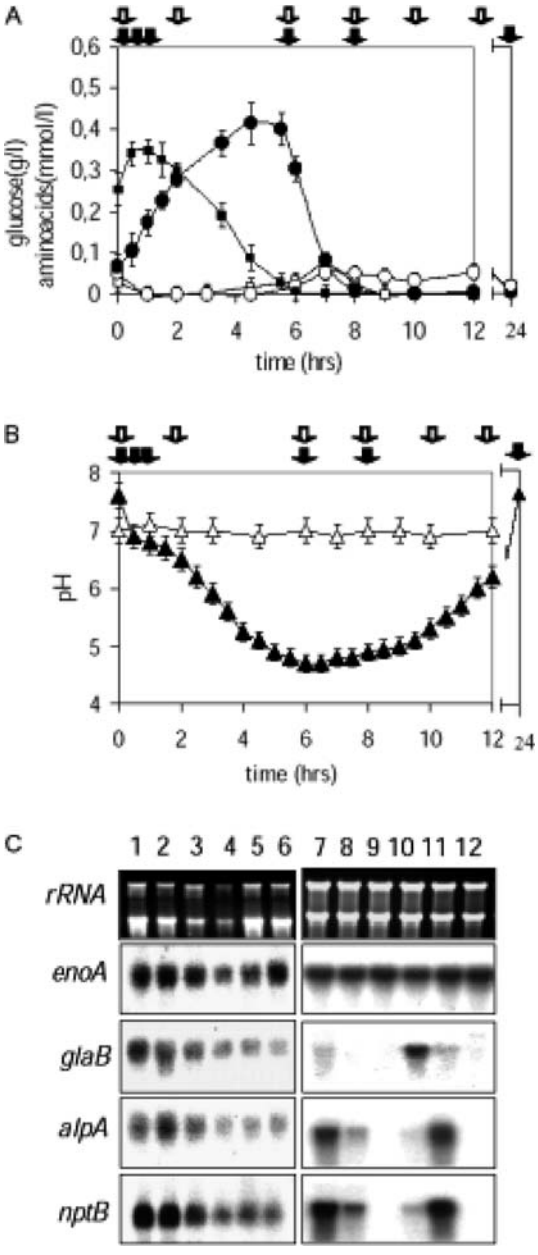

Fig. 3 **a** Glucose (○, ●; g/l) and amino acid (□, ■; mmol/l) concentrations, and **b** pH (▵,▲) during membrane biomass transfer (MTC, *open symbols*) or liquid biomass transfer (LTC, *closed symbols*) over time. *Open* and *closed arrows* MTC and LTC samples, respectively, taken for **c**. **c** Northern hybridisations with probes for the *enoA*, *glaB*, *alpA* and *nptB* genes with RNA time samples isolated from MTC and LTC. Lanes: *1–6* MTC, *7–12* LTC: *1* 0 h, *2* 2 h, *3* 6 h, *4* 8 h, *5* 10 h, *6* 12 h, *7* 0 h, *8* 0.5 h, *9* 1 h, *10* 6 h, *11* 8 h, *12* 24 h

gene is enhanced by low-Aw (Ishida et al. 2000). As not only the *glaB* gene but also the *alpA* and *nptB* genes were transcribed strongly during surface growth on 2% WSM, we examined the effect of low-Aw on transcription of these genes using MTC. We chose to study the effect of low-Aw at 6 h MTC, as transcript levels of the *glaB*, *alpA* and *nptB* genes after 6 h MTC were representative of induction during surface growth on 2% WSM (Fig. 3c). Low-Aw was achieved by supplementing 2% WSM with 5, 10 and 40% maltose or 5 M NaCl, resulting in Aw values of 0.98, 0.96, 0.93 and 0.93, respectively. The results of the MTCs are presented in Fig. 4. The *glaB* transcript level was about two and four times higher in 2% WSM with 5% (lane 2) and 10% (lane 3) maltose, respectively, and the *glaB* transcript level in 2% WSM with 5 M NaCl (lane 5) was the same as in unsupplemented 2% WSM (lane 1) as determined after quantification of transcript levels. From these results we concluded that the *glaB* gene was induced not by the Aw lowering effect of maltose during surface cultivation, but by maltose directly. We cannot formally exclude that the lack of effect of NaCl on gene expression is due to a combination of counteracting effects, including Aw. Transcription of the *alpA* and *nptB* genes was not induced by 5 and 10% maltose in MTC. Surprisingly, MTC to 2% WSM with 40% maltose showed equal transcript levels for the *glaB* gene, and five and six times lower transcript levels of the *alpA* and *nptB* genes (cf. lanes 3 and 4). These lower *alpA* and *nptB* transcript levels were not due to the effects of low Aw (cf. lanes 1, 4, 5).

Further analysis of the environmental conditions of the maltose-supplemented surface cultivation revealed that the amount of sugar available in the 5, 10 and 40% maltose was high, and that in the 40% maltose cultures the pH was clearly lower than in the other cultures (Fig. 4). To analyse whether the observed decrease in *alpA* and *nptB* gene transcript level was due to pH regulation, LTC to 1%

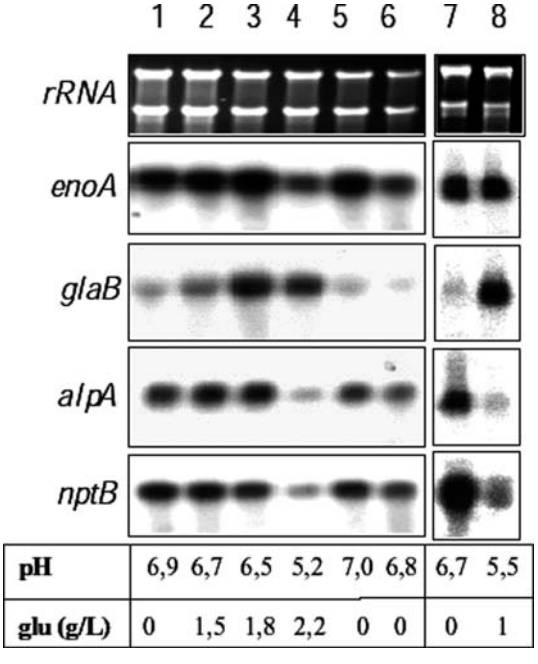

Fig. 4 Effect of pH, external glucose, absence of aerial hyphae, and low water activity (Aw) on transcription of the *enoA*, *glaB*, *alpA* and *nptB* genes after 6 h MTC or LTC. Addition of maltose or NaCl resulted in low Aw. Lanes: *1* MTC to 2% WSM (Aw =0.99), *2* MTC to 2% WSM +5%maltose (Aw =0.98), *3* MTC to 2% WSM +10% maltose (Aw =0.96), *4* MTC to 2% WSM +40% maltose (Aw =0.93), *5* MTC to 2%WSM +5 M NaCl (Aw =0.93), *6* MTC with sandwich cultivation (SWC—without aerial hyphae) to 2% WSM, *7* LTC to 1% WLM, *8* LTC to 1% WLM and 40% maltose

WLM was also carried out in the presence of 40% maltose. As shown in Fig. 4 (lanes 7, 8) similar effects were observed also in liquid cultivation regarding increased *glaB* and decreased *alpA* and *nptB* transcript levels, suggesting that *glaB* is induced by maltose while induction of the *alpA* and *nptB* genes is pH-mediated.

Effect of aerial hyphae on *glaB, alpA* and *nptB* transcription

Filamentous fungal morphology during surface cultivation is different from that in submerged cultivation. A strongly characteristic feature is the presence of aerial hyphae during surface cultivation. The role of the aerial hyphae in gene transcription during surface cultivation was investigated by suppression of aerial hyphae grown with the aid of an oxygen permeable membrane in a 6 h MTC with sandwich cultivation (SWC). Northern analysis with mRNA samples from 6 h SWC showed no difference in transcript levels of the *glaB*, *alpA* and *nptB* genes compared to 6 h MTC (cf. lanes 1 and 6 in Fig. 4), indicating that the presence of aerial hyphae does not affect *glaB*, *alpA* and *nptB* gene transcription during surface cultivation.

Discussion

In solid-state and submerged fermentation, the environmental conditions for growth of *A. oryzae* differ in many aspects. In contrast to submerged fermentation, SSF is characterised by gradients of nutrients, oxygen, temperature and water availability (Iwashita 2002, Te Biesebeke et al. 2002) and, as a consequence, differences in transcriptional regulation are expected. The only well studied example of a gene that shows high transcription in rice kernel SSF is the glucoamylase-B-encoding gene (Ishida et al. 2000). In the study presented here, we have carried out northern analysis showing that transcription of *A. oryzae* genes encoding *glaB*, alkaline protease (*alpA*) and neutral protease (*nptB*) is induced during growth in 2% WSM, and that the level of transcription is 4-fold higher in *A. oryzae* during WK SSF. In submerged cultivation with 2% WLM, the *alpA* and *nptB* genes were only transiently transcribed after 17 and 20 h growth. We have investigated some possible mechanisms for transcription of the *glaB*, *alpA* and *nptB* genes during surface cultivation on 2% WSM by comparing it to transcriptional induction in submerged cultivation in 2% WLM.

Southern analysis with a probe for the *nptB* gene (Table 1) showed two bands. Neutral proteases I (GenBank accession number AF099904), II (E05048) and III (AB041338) are isolated from *A. oryzae*. The sequence of a single gene fragment from the *nptB* gene obtained by PCR with the primers shown in Table 1 showed 100% identity to neutral protease II, while gene sequence similarities to the neutral protease I and III genes were low (results not shown). A high degree of identity was observed between the neutral protease II protein (E05048) and a recently identified fourth neutral protease-like protein (CAE11758) of *A. oryzae* (*E*-value E−98, results not shown) indicating that two closely related copies of the neutral protease II gene could be present in the genome of our *A. oryzae* strain. Northern results obtained with a probe for the *nptB* gene showed one hybridising band in all analysis.

The glucose and amino acid concentrations available during surface cultivation in 2% WSM are low in comparison to those measured in submerged cultivation in 2% WLM (Figs. 1, 3a). An explanation for this difference could be that secreted enzymes are immobilised in the substrate during surface cultivation. Consequently, their hydrolytic activity is limited to substrate in the close vicinity of the penetrating hyphae. The low levels of glucose and amino acids liberated from 2% WSM are probably directly consumed by the filamentous fungal mycelium, also explaining the slower biomass formation in surface cultivation, as seen in Fig. 1. The absence of free glucose in 2% WSM may result in increased levels of gene expression due to the absence of carbon catabolite repression. In contrast to *glaB*, the transcriptional induction of *alpA* and *nptB* was observed even in the presence of high concentrations of sugar (Figs. 2, 4) suggesting that the *alpA* and *nptB* genes are not regulated by carbon catabolite repression like that mediated by the negatively acting transcriptional regulator CREA (Dowzer and Kelly 1991).

Ishida et al. (2000) postulated that *glaB* transcription is induced by the low Aw that results from addition of maltose to MTC. Northern analysis (Fig. 4) confirmed that transcription of *glaB* is also induced in fungal mycelium during MTC to 2% WSM supplemented with 5, 10 and 40% maltose (Aw =0.98, 0.96 and 0.93, respectively) compared to MTC to 2% WSM (Aw =0.99). Although 2% WSM with 40% maltose has the same low Aw as 2% WSM with 5 M NaCl, *glaB* transcript levels in MTC to 2% WSM with 5 M NaCl were equal to those in MTC to 2% WSM. Therefore, we conclude that transcriptional induction of *glaB* in the fungal mycelium during MTC to 2% WSM supplemented with maltose is due to an effect of maltose other than the induction of low Aw.

Small oligosaccharides, such as maltose, isomaltose, maltotriose and dextrin, have been shown to induce *glaB* gene transcription (Ishida et al. 1998). As expected, transcription of the *glaB* gene was induced in LTC to 1% WLM with 40% maltose (Fig. 4; lane 8) and in MTC to 2% WSM with 40% maltose (Fig. 4; lane 4), at glucose concentrations of 1 and 2.2 g/l, respectively. In agreement with a deletion analysis of the *glaB* promoter (Ishida et al. 2000), these results show that glucose repression did not overrule maltose induction (Fig. 4; lanes 2–4, 8). The results obtained with LTC to 2% WLM suggest that starch cannot induce *glaB* transcription (Fig. 3c; lanes 7–9), but that the breakdown products of starch that appeared in the culture medium after 6 h of LTC (Fig. 3c; lane 10) induced *glaB* gene expression. This proposed induction of *glaB* transcription by starch breakdown products suggests that

induction of *glaB* gene expression in surface cultivation on 2% WSM (Fig. 3; lanes 1–6) is due to the continuous presence of inducer. Therefore, we propose that the processes of carbohydrate breakdown and consumption differ in 2% WLM submerged and 2% WSM surface cultivation.

As described previously, the promoter region of the *glaB* gene contains cis-acting elements mediating maltose induction, and two heat shock elements (f-HSE) that have been shown to be essential for the high level transcriptional induction in rice kernel SSF. Interestingly, in the promoter region of the *A. oryzae alpA* gene (accession no. D10062), three potential f-HSE motifs (Mary et al. 1994) in the stretch from −149 to −123 were also found, suggesting that the high level transcriptional induction of the *glaB* and *alpA* genes during WK SSF is mediated by these potential f-HSE motifs.

Under aerobic conditions, the cellular metabolism of filamentous fungi involves a net intracellular production of protons that takes place by the formation of tricarboxylic acid cycle acids, CO_2/H_2CO_3 and protein synthesis (Sanders and Slayman 1982). Consequently, proton fluxes occur across cellular membranes to maintain intracellular pH homeostasis (Hesse et al. 2002). Upon transfer of growing *A. oryzae* biomass to 2% WLM, the intracellular pH decreases due to metabolic activity and, as a consequence of proton fluxes across the extra cellular membrane, the growth medium started to acidify. The extracellular medium no longer acidifies at 6 h LTC when glucose concentrations are low, and subsequently recovers from acidity. In surface cultivation (Figs. 1a; 3a, b), less sugar is available, *A. oryzae* grows more slowly, and the pH of the growth medium remains between 6.5 and 7.1, indicating a balance between growth and metabolism.

Transcription of the *alpA* and *nptB* genes was induced in surface cultivation when the extracellular pH was about 6.8 and in LTC when the pH recovered from acidity. This suggested that *alpA* and *nptB* gene transcription is regulated by pH. As described previously, pH regulation is mediated by the pH-responsive wide domain regulator PACC (Panelva and Arst 2002). The possible involvement of PACC is sustained by the presence of a potential PACC binding site (GCCAAG) at position −685 in the promoter sequence of the *alpA* gene (accession no. D10062). Unfortunately, the sequence upstream of *nptB* is not available. The pH regulatory mechanism of PACC (Panelva and Arst 2002) in *alpA* and possibly *nptB* transcriptional induction may explain in part the observed differences in transcript levels in submerged and surface cultivation in wheat-based medium presented in this paper.

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