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Process technological effects of deletion and amplification of hydrophobins I and II in transformants of *Trichoderma reesei*

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Process Technological Effects of Deletion and Amplification of Hydrophobins I and II in Transformants of *Trichoderma Reesei*

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ABSTRACT

Transformants of the *Trichoderma reesei* strains QM9414 and Rut-C30 were constructed in which the genes for the two major hydrophobin proteins, hydrophobins I (HFBI) and II (HFBII), were deleted or amplified by molecular biological techniques. Growth parameters and foam production of the transformant strains were compared with the corresponding properties of the parent strains by cultivation in laboratory bioreactors under conditions of catabolite repression (glucose medium) or induction of cellulolytic enzymes and other secondary metabolites (cellulose and lactose media). All the transformed strains exhibited vegetative growth properties similar to those of their parent. The *Δhfb2* (but not the *Δhfb1*) transformant showed reduced tendency to foam, whereas both strains overproducing hydrophobins foamed extensively, particularly in the case of HFBII. Enzyme production on cellulose medium was unaltered in the *Δhfb2* transformant VTT D-99676, but both the *Δhfb2* and HFBII-overproducing transformants exhibited somewhat decreased enzyme

production properties on lactose medium. Production of HFBI by the multi-copy transformant VTT D-98692 was almost 3-fold that of the parent strain QM9414. Overproduction of HFBII by the transformant VTT D-99745, obtained by transformation with three additional copies of the *hfb2* gene under the *cbh1* promoter, was over 5-fold compared to production by the parent strain Rut-C30. The *Δhfb2* transformant VTT D-99676 produced a greatly increased number of spores on lactose medium compared with the parent strain, whereas the HFBII-overproducing transformant VTT D-99745 produced fewer spores.

INTRODUCTION

Trichoderma reesei, in common with many other fungi, produces extracellular hydrophobins. These low molecular weight proteins have a high cysteine content and high surface activity and amphiphilic properties (Wessels 1997, Wösten and Wessels 1997). Hydrophobin I (HFBI) of *T. reesei* is believed to be located on cell walls, whereas hydrophobin II (HFBII) is present on spore walls (Nakari-Setälä *et al.* 1996, 1997). Mature fungal spores generally have a hydrophobic surface that protects the spores from drying and aids their dispersal. In *T. reesei*, HFBI is produced in the presence of glucose in the growth medium. HFBII, along with extracellular enzymes such as cellulases and xylanases, is produced in enzyme-inducing conditions on complex plant-derived materials or *e.g.* lactose, along with extracellular enzymes such as cellulases and xylanases (Nakari-Setälä *et al.*, 1997). Whereas in liquid cultures HFBI is mainly cell wall-bound in liquid cultures (Askolin *et al.* 2001), HFBII is mainly secreted into the medium as an extracellular protein (Linder *et al.* 2001). Small microgram amounts of hydrophobins of, for example, *Fusarium* have been shown to cause gushing in beer (Kleemola *et al.* 2001). By analogy, foaming in fermentations for the production of extracellular enzymes by *T. reesei* under hydrolase-inducing conditions is probably due largely to production of HFBII.

Although hydrophobins may cause problems in both the brewing and the fermentation industries due to their foam-production properties, they also have interesting physico-chemical properties. They are able to self assemble on solid-liquid or solid-gas interphases, where they form highly stable amphipathic protein membranes (Wessels, 1994). Due to their interesting protein properties, a variety of different applications ranging from protein immobilization and surface

modification to tissue engineering have been suggested for hydrophobins (Wessels 1997, Scholtmeijer *et al.* 2001). Therefore there is interest in producing, on the one hand, fungal strains totally lacking a particular hydrophobin protein and on the other hand, strains overexpressing hydrophobin proteins for harvesting and utilisation in application studies.

In this work, transformants of *T. reesei* strains were produced in which either the *hfb1* or the *hfb2* gene was either deleted or amplified by molecular biological techniques. The transformed strains were cultivated in laboratory fermenters for assessment of the process technological effects of deletion and amplification and for harvesting of hydrophobin proteins for application experiments. The effects of deletion or amplification of the *hfb* genes were evaluated with respect to growth parameters, foaming and, in the case of the *hfb2* gene, production of extracellular enzymes.

MATERIALS AND METHODS

Strains

Trichoderma reesei QM9414 (VTT D-74075) (Mandels *et al*. 1975), its Δ*hfb1* transformant VTT D-99724 (Askolin *et al.*, MS in preparation) and the HFBI-overproducing transformant VTT D-98692 (Askolin *et al*. 2001) were used for investigation of the effects of deletion and amplification of the *hfb1* gene in a strain generally accepted as being rather similar to the wild-type isolate QM6a. The strain Rut-C30 (VTT D-86271) (Montenecourt and Eveleigh 1979), its Δ*hfb2* transformant VTT D-99676 (Askolin *et al.*, MS in preparation) and the HFBII-overproducing transformant VTT D-99745 were used to examine the effects of deletion and overexpression of HFBII in this efficient cellulase-producing strain.

DNA construction

The *hfb1* and *hfb2* genes were replaced with genes coding for *Aspergillus nidulans* acetamidase (*amdS*) and *Escherichia coli* hygromycin B phosphotransferase (*hph*) in the Δ*hfb1* and the Δ*hfb2* transformants, respectively. In pTNS24 (Δ*hfb1* construct) the *amdS* cassette is flanked by 2.8 and 2.0 kb of *hfb1* 5' and 3' non-

coding sequences, respectively, whereas in pTNS27 (Δ*hfb2* construct) the *hph* cassette is flanked with 1.2 kb and 1.75 kb of *hfb2* 5' and 3' flanking sequences (Askolin *et al.*, MS in preparation).

HFBII was overexpressed in strain Rut-C30 using the strong promoter of the cellobiohydrolase gene, *cbh1*. The *hfb2* coding region was amplified with PCR using the primers 5' AGA ACC GCG GAC TGC GCA TCA TGC AGT TCT TCG CCG TC (sense) and 5' TCA TTG GAT CCT TAG AAG GTG CCG ATG GC (antisense) and *phfb2* as a template (Nakari-Setälä et al. 1997). The purified PCR fragment was cut with KspI and BamHI and ligated with pMQ121 (Linder *et al.*, 2001), which was similarly digested to replace *hfb1* with *hfb2* in the vector. In the resulting expression vector pTNS31, *hfb2* is under the regulatory control of the *cbh1* promoter and terminator sequences. Prior to fungal transformation, the plasmid pTNS31 was digested with EcoRI and SphI to release the expression cassette.

Fungal transformation

T. reesei Rut-C30 was co-transformed according to Penttilä *et al.* (1987) using 10 µg of digested pTNS31 together with 3 µg of the hygromycin selection plasmid pARO21 (Aro *et al.* 2001). Transformants were streaked three times on selective medium and then transferred to potato dextrose agar for sporulation. Spore suspensions were plated out on selective medium to obtain single spore colonies for further analysis by Southern blotting.

Southern analysis

Southern blotting was used to screen fungal transformants for integration of the DNA constructs into the genome. Fungal DNA was isolated using the Easy-DNA Kit (Invitrogen). DNA of the HFBII-overproducing transformants was cut with XbaI and with NsiI, which have known recognition sequences within the *hfb2* flanking regions and within the *cbh1* promoter and terminator sequences. DNA fragments were size-fractionated by agarose gel electrophoresis and blotted onto nylon membranes using standard methods. Blots were hybridised with [α-^{32}P]dCTP labelled fragments of the *hfb2* coding region. Hybridisation was performed under stringent conditions according to Sambrook *et al.* (1989) and the blots were then exposed to Kodak XAR-5 X-ray film.

Cultivation methods

The cultivation media used contained, in g l^{-1}: (1) lactose (Valio Ltd, Finland) or glucose (Cerestar, Denmark) 40, peptone 4.0, yeast extract 1.0, KH_2PO_4 4.0, $(NH_4)_2SO_4$ 2.8, $MgSO_4x7H_2O$ 0.6, $CaCl_2{\cdot}2H_2O$ 0.8 (sterilised separately in distilled water), with 2x trace elements (Mandels and Weber 1969), or (2) Solka floc cellulose (James River, Hackensack, N.J.) 40, distiller´s spent grain 20, KH_2PO_4 5.0, $(NH_4)_2SO_4$ 5.0. Inocula (spore suspensions in 50% glycerol at -80°C) were grown on the same medium with only 20 g l^{-1} carbon source, buffered with 15 g l^{-1} KH_2PO_4, in two stages of 1x200 ml (2 days, 200 rpm, 30°C) and 5x200 ml (to 15 or 20 litres) or 3x200 ml (to 10 l) (2.5% v/v transfer, 1 day, 200 rpm, 30°C). The glucose concentration was kept within the limits of 10–25 g l^{-1} by addition of sterile 50% glucose solution when glucose was used as a carbon source.

The fermenters used were Chemap LF 20 (working volume 15 l) and CF 2000 (10 l) fermenters and in one cultivation of the *Δhfb2* transformant VTT D-99676 a Braun Biostat C30 (20 l). The LF 20 fermenter was not equipped with automatic control of dissolved oxygen (DO). DO control (>20%) in the CF 2000 fermenter was by agitation (500–800 rpm) and in the Braun Biostat by both agitation and enrichment of the incoming air with pure oxygen (0–25%). Oxygen enrichment reduces the need for agitation to maintain the setpoint DO level and therefore the tendency to foaming. Other cultivation conditions were: T=28°C, pH 4–5 (lower limit controlled by addition of NaOH in the cultivations with glucose feeding and by NH_4OH in cultivations in inducing conditions, upper limit by H_3PO_4), aeration 0.5 l l^{-1} min^{-1} overall gas flow. Foaming was controlled by automatic addition of Struktol J633 polyoleate antifoam agent (Schill & Seilacher, Germany). All media contained 0.2 ml l^{-1} antifoam agent (AF) to prevent foaming during sterilisation.

Analyses

Measurement of biomass on glucose and lactose media was performed gravimetrically after filtration of a known volume of culture through a tared Whatman GF/A filter, rinsing with the same volume of distilled water, drying at 103°C for 6 h or overnight and reweighing. Glucose and lactose were assayed using GOD-Perid (Roche, Germany) and lactose/β-D-galactose (Boehringer-

Mannheim, Germany) analysis kits, respectively, following the manufacturer´s instructions. The assays for overall cellulase activity (filter paper units, FPU) and β-1,4(1,3)-endoglucanase using hydroxyethyl cellulase (HEC) as substrate (IUPAC 1987) and for endo-β-1,4-xylanase (XYL, Bailey *et al.* 1992) were carried out at pH 5.0 using the standard methods. Soluble protein was assayed by the method of Lowry *et al.* (1951) after precipitation of proteins with an equal volume of 10% trichloracetic acid.

Hyphal thickness was determined as described by Askolin *et al.* (MS in preparation). The culture samples were fixed in 1% formaldehyde, stained with lactophenol blue (Fluka 61335, Switzerland), and examined with a light microscope and Analysis software imaging tool (Soft Imaging System).

Assay of HFBI and HFBII

HFBI and HFBII were quantified by analytic reversed phase high performance liquid chromatography (RP-HPLC). HFBI was assayed using a Resource RPC column (Pharmacia Biotech, Sweden) with a 0–60% acetonitrile gradient containing 0.1% trifluoroacetic acid and detection of HFBI at 215 nm (Askolin *et al.* 2001). HFBI was extracted from the mycelium three times with 100 mM sodium citrate / citric acid, pH 6.0 containing 1% SDS and the extracts were analysed by HPLC.

Prior to analytical HPLC, HFBII was extracted and purified from the culture supernatant using 2% of the non-ionic surfactant Berol 532 (Linder et al 2001). The extraction was made to avoid problems caused by closely eluting peaks. Berol 532 (20 mg; Akzo-Nobel, Sweden) was added to 10 ml of supernatant and the solution was mixed gently for 1 hour at 21°C on a laboratory shaker. The tubes were centrifuged for 10 minutes at 5000 *x* g and the lower phase was removed. One millilitre of 50 mM acetete buffer pH 5.5 and 150 µl isobutanol were added to the remaining surfactant phase and the centrifugation was repeated. HFBII was quantified from the lower aqueous phase using a 4.6 mm x 25 cm Vydac protein C4 analytical column (Vydac, Hesperia, Calif.) using a stepwise gradient of 0.1% trifluoroacetic acid in water (A) and in acetonitrile (B). The steps of the gradient were: 0–13 ml 0% B, 13–18 ml 0–20% B, 18–48 ml 20–70% B and 48–51 ml 70–100% B. Detection was by UV at 205 nm, 215 nm and 280 nm. Concentrations were calculated from the integrated peaks using a calibration curve.

RESULTS

Effect of HFBI on growth parameters, protein production and foaming of *T. reesei* QM9414 in fermenter cultivations

To investigate the effect of HFBI on growth parameters, protein production and foaming in the cultivation of *T. reesei*, the host strain QM9414, its Δ*hfb1* transformant VTT D-99724 and the HFBI-overproducing transformant VTT D-98692, carrying three copies of the native *hfb1* gene with its own promoter, were cultivated in the 10 l fermenter for 92 h. Since glucose induces the expression of HFBI, glucose medium was used in these fed-batch cultivations. The measured growth parameters of all three strains were very similar. The maximum value of biomass production (Table 1) had been reached by all the strains at 92 h and was within the normal range of variation observed for the host strain QM9414. Mycelial morphology was studied under a light microscope after fixation in 1% formaldehyde and lactophenol blue staining. No differences in morphology or in hyphal thickness were observed between the three strains. According to these results HFBI has no effect on growth or hyphal morphology of *T. reesei* in fermenter cultivations.

Table 1. Consumption of antifoam agent (AF) and maximum production of biomass, total soluble extracellular protein and HFBI by Trichoderma reesei QM9414, its Δhfb1 transformant VTT D-99724 (Δhfb1) and the HFBI-overproducing transformant VTT D-98692 (hfb1+++) in 92 h laboratory fermenter cultivations on glucose medium. The HFBI concentration includes both soluble and mycelium-bound HFBI. The assay of mycelium-bound HFBI was based on three sequential extractions with a buffer containing 1% SDS. nd, not determined.

Strain	Biomass g l^{-1}	AF ml l^{-1}	Soluble protein g l^{-1}	HFBI g l^{-1}
QM9414	32.9	2.3	0.2	0.5
QM9414 *Δhfb1*	37.0	2.7	0.1	nd
QM9414 *hfb1+++*	37.8	4.7	0.2	1.4

HFBI concentrations were assayed both from mycelial extracts and culture supernatants using HPLC (Askolin *et al.* 2001). Mycelium was extracted three times with 100 mM sodium citrate buffer, pH 6.0, containing 1% SDS. The HFBI-overproducing strain produced 1.4 g l^{-1} HFBI, which was almost three times higher than the amount produced by the host strain QM9414. Most of the HFBI was cell wall-bound in both strains (Fig. 1). The host strain secreted 0.2 g l^{-1} total soluble protein into the culture medium, half of which was HFBI. This explains the lower total protein concentration (0.1 g l^{-1}) in the culture medium of the *Δhfb1* transformant (Table 1).

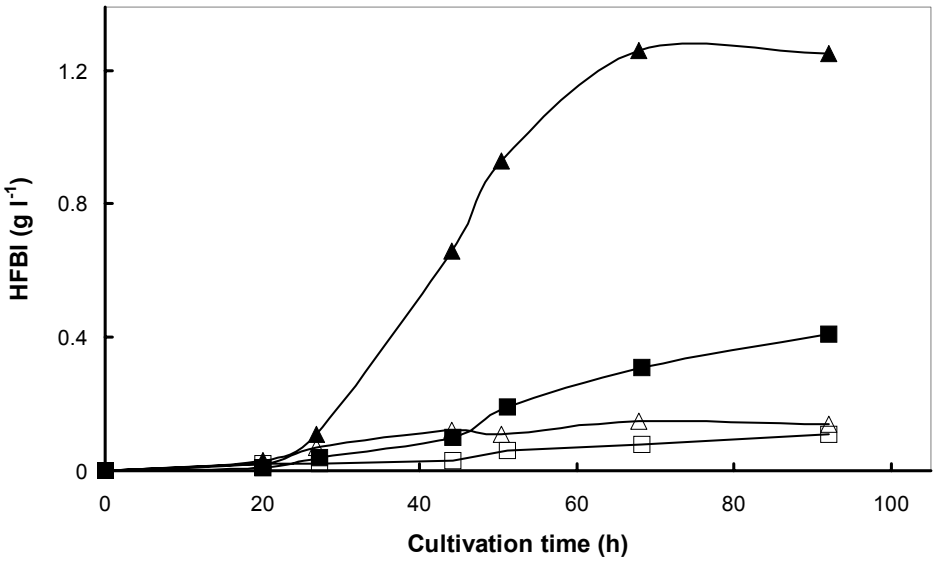

Figure 1. Distribution of HFBI between mycelium and growth medium. HFBI on mycelium (closed symbols) and in growth medium (open symbols) of Trichoderma reesei QM9414 (squares) and the HFBI-overproducing strain VTT D-98692 (triangles), respectively. The amount of HFBI on mycelium was estimated by three mycelial extractions with a buffer containing 1% SDS.

Foaming of bioreactor cultures was compared on the basis of consumption of antifoam agent (AF). The medium contained 0.2 ml l^{-1} AF prior to sterilisation. Addition of AF was automatically controlled during the cultivations. However, the fermentation broth was supplemented manually with 2 ml l^{-1} AF after 21 h of cultivation in order to minimize the possible effects of varying amounts of AF added automatically at different times. A clear difference in consumption of AF

was observed between the host strain QM9414 and the HFBI-overproducing strain (Table 1). The overproducing strain consumed almost twice the amount of antifoam agent (124 ml per mg dry weight) as that required by the host strain (70 ml per mg dry weight). However, deletion of the *hfb1* had no effect on antifoam consumption (73 ml per mg dry weight) compared to the host strain. These results show that the HFBI protein causes foaming in fermentation cultivations of *T. reesei* when produced in high amounts into the glucose-containing medium. Growth parameters and fungal morphology were not affected by HFBI overproduction.

Effects of deletion and amplification of the *hfb2* gene on growth parameters, sporulation, foaming and protein production

Growth

According to the results of dry weight measurements, carbon source consumption and culture pH, growth of the *Δhfb2* transformant VTT D-99676 on lactose medium in the 15 litre fermenter was similar to that of the parental strain Rut-C30 (Fig. 2). Maximum dry weights reached by Rut-C30 and VTT D-99676 were 16.5 (55 h) and 17.3 (47 h) g l^{-1}, respectively. As a result of manual adjustment of agitation (to 400, 600 or 800 rpm at different cultivation stages), the level of DO in both cultivations was above 20% at all times (results not shown). Growth of the HFBII-overproducing transformant VTT D-99745 on lactose medium in the 10 litre fermenter with DO control (by agitation only) was also similar to that of the control strain Rut-C30. The curves of lactose consumption and culture pH were almost identical to those of the control (Fig. 2). The apparently increased level of maximum biomass (22 g l^{-1} compared to 16 g l^{-1} for the control) was almost certainly an artefact caused by the observed incomplete rinsing of the considerable amount of AF present in the culture (see below) in the dry weight analysis. This conclusion is supported by the fact that the apparent increase in biomass between 50 and 72 h occurred after exhaustion of lactose from the medium (Fig 2). Maximum biomass production by the transformant VTT D-99745 in the repeat cultivation with oxygen enrichment and reduced addition of AF was 16.4 g l^{-1} (result not shown), i.e. very similar to the corresponding value for the control strain Rut-C30.

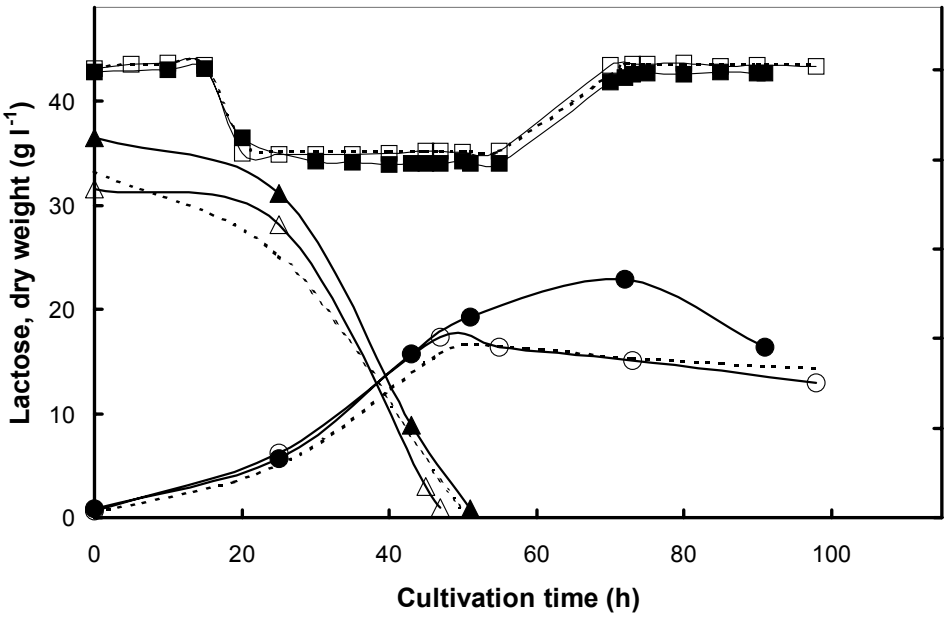

Figure 2. Lactose consumption (triangles), biomass formation (circles) and pH changes (squares) in fermenter cultivations of Trichoderma reesei Rut-C30 (dotted lines), its Δhfb2 transformant VTT D-99676 (open symbols) and its HFBII-overproducing transformant VTT D-99745 (filled symbols) on lactose medium in a laboratory fermenter.

On cellulose medium, estimation of growth was limited to measurement of DO and changes in culture pH. No attempt was made to analyse biomass on the complex lignocellulosic medium that contained solid particles interfering with the measurement. According to the on-line parameters, growth of the *Δhfb2* transformant VTT D-99676 was similar to that of the control strain Rut-C30 (results not shown).

Sporulation

On lactose medium, the culture of the *Δhfb2* transformant VTT D-99676 became intensely green very soon after consumption of the carbon source. The results of microscopy and of agar plating on Plate Count Agar confirmed that this was not due to contamination. Microscopical observation revealed that the reason for the observed green colour was extensive sporulation of the culture. In contrast, production of spores by the control strain Rut-C30 was less intense, although

some spore formation was clearly evident. In logical progression, the culture of the HFBII-overproducing strain VTT D-99745 produced no free spores visible under the microscope. Similar differences in sporulation behaviour compared with the control strain were observed in repeat cultivations of both transformants (results not shown). Further examination of the effects of deletion and amplification of the *hfb2* gene on sporulation was not made in this work.

Foaming

Deletion of the *hfb2* gene had a considerable effect on foaming in the fermenter cultivations. This was particularly true in the case of the cellulose medium: consumption of AF was 3.7 ml l^{-1} in the case of the control strain compared to only 0.4 ml l^{-1} for the *Δhfb2* transformant (Table 2). Higher consumption of AF in the cultivation of the control strain was not due to fouling of the foam probe: no over-feeding of antifoam agent occurred at any stage of the cultivation. Foaming was clearly less of a problem on the lactose medium, but the difference beween the control and *Δhfb2* strains was evident: whereas the control strain required 0.3 ml l^{-1} AF, the *Δhfb2* transformant did not consume any AF during the cultivation (Table 2).

Table 2. Maximum production of total soluble extracellular protein, enzyme activities and HFBII and consumption of antifoam agent (AF) by T. reesei Rut-C30, its Δhfb2 transformant VTT D-99676 (Δhfb2) and its HFBII-overproducing transformant VTT D-99745 (hfb2+++) on cellulose (7 day cultivations) and lactose media (4 days). HEC activity against hydroxyethyl cellulose, FPU filter paper units; XYL xylanase, nd not determined. The observed specific production rates of total soluble protein (g l^{-1} h^{-1}) and HEC activity (nkat ml^{-1} h^{-1}) during the linear production phase are also shown.

Strain	**Medium**	**Protein** **g l^{-1}**	**g l^{-1} h^{-1}**	**HEC** **nkat ml^{-1}**	**nkat ml^{-1} h^{-1}**	**FPU** **u ml^{-1}**	**XYL** **nkat ml^{-1}**	**HFBII** **mg l^{-1}**	**AF** **ml l^{-1}**
Rut-C30	cellulose	13.9	0.16	1,600	21.6	10.9	1,870	nd	3.7
Rut-C30 *Δhfb2*	cellulose	16.4	0.16	1,715	18.7	9.0	2,240	nd	0.4
Rut-C30	lactose	7.6	0.23	890	23.1	4.5	640	30	0.3
Rut-C30 *Δhfb2*	lactose	6.5	0.21	690	21.1	4.1	500	0	0.0
Rut-C30 *hfb2+++*	lactose	4.0	0.08	290	5.9	1.3	180	100[a]	24.0
Rut-C30 *hfb2+++*	lactose[b]	6.8	0.13	580	11.0	2.9	530	240	2.5

[a]Result uncertain due to interference by the high content of surface-active antifoam agent
[b]Repeat cultivation in a fermenter with DO control by agitation and oxygen enrichment, see text.

Due to the severe problems anticipated in the cultivation of an HFBII-overproducing strain on cellulose medium, the transformant VTT D-99745 was grown only on the lactose medium. Even on this medium, in the fermenter equipped with DO control by agitation (but not by oxygen enrichment, see Materials and Methods), consumption of AF was as high as 24 ml l^{-1} (Table 2). On the basis of frequent inspection of the condition of the headspace and of the probe, there was never any evidence of unnecessary addition of AF due to fouling of the probe. Evidently, the observed phenomenon was due to overproduction of HFBII.

A repeat cultivation of the strain VTT D-99745 overproducing HFBII was performed in the 20 litre fermenter equipped with DO control by agitation and oxygen enrichment. In this experiment the maximum agitation required for DO control to >20% was limited to 450 rpm (cf. 800 rpm in the previous cultivation) by using oxygen enrichment up to a maximum of 25% of the incoming air. For the greater part of this cultivation, the agitation was below 400 rpm (min 250 rpm). Although the culture evidently had a strong tendency to foam formation, the low agitation sufficed to ensure that addition of AF could be kept within an acceptable range. The total addition of AF to the culture was 2.5 ml l^{-1} (Table 2), approximately one tenth of that in the first cultivation with this transformant.

Enzyme production

Production of cellulases (soluble protein, HEC) by the *Δhfb2* transformant VTT D-99676 on cellulose medium was similar to that by the control strain Rut-C30 (Fig. 3). The maximum values of all the enzyme activities assayed, along with soluble protein, are displayed in Table 2. The level of xylanase production in the *Δhfb2* transformant was slightly elevated, although the result for FPU (a rather imprecise assay with an accuracy of only ca. ± 20%) was somewhat below that obtained with the control strain Rut-C30. The observed differences in production of enzyme activities and soluble protein by the control and transformant strains probably represented normal between-batch variation rather than any significant differences in enzyme production properties.

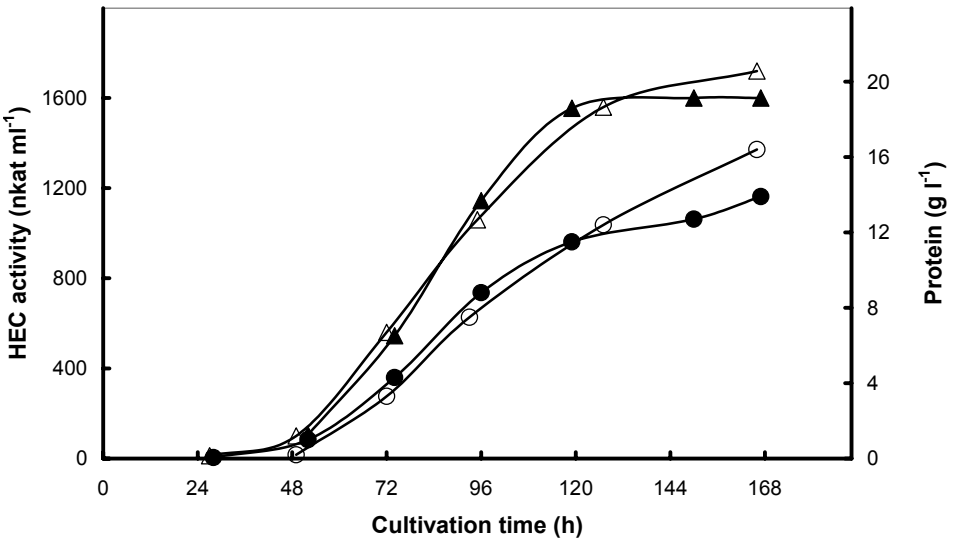

Figure 3. Production of cellulase (HEC, triangles) and total soluble protein (circles) by Trichoderma reesei Rut-C30 (open symbols) and its Δhfb2 transformant (filled symbols) on cellulose-spent grain medium in a laboratory fermenter.

On the lactose medium, enzyme production by the *Δhfb2* transformant was slightly poorer than by the control strain (Table 2). This result was confirmed in repeat cultivations of both strains on the same medium (data not shown). Maximum specific production rates HEC and protein by the transformant strain were similar to those for Rut-C30 (see Table 2), but the length of the linear production phase was shorter (results not shown). Note that whereas the cultivations on cellulose medium were continued for 7 days, a cultivation time of 4 days was sufficient for the attainment of maximum activities on lactose in all cases.

Production of cellulases by the HFBII-overproducing strain VTT D-99745 in the first cultivation on lactose medium was considerably poorer than by the control strain (Table 2). In the repeat cultivation in the fermenter with DO control by oxygen enrichment and lower addition of antifoam agent, the production was clearly improved. However, some depression of enzyme production was still observed (Table 2).

Production of HFBII

Production of HFBII was investigated on the lactose medium. In the first cultivation of the HFBII-overproducing transformant VTT D-99745, with heavy addition of AF (see above), the observed production of about 100 mg l^{-1} was approximately 3-fold that of the control cultivation (30 mg l^{-1}, see Table 2), despite the lower level of enzyme production. When the cultivation was repeated with lower agitation and reduced addition of AF agent, the production level of HFBII was still higher, at 240 mg l^{-1}. The HFBII-overproducing transformant contains both endogenous *hfb2* and the three extra copies of *hfb2* introduced to the genome under the *cbh1* promoter (data not shown).

DISCUSSION

Hydrophobins are highly surface active proteins of filamentous fungi (Wösten and de Vocht 2000), which were first isolated and characterised in the 1990s (Wessels 1997). Foaming can cause serious problems in fermentation processes, for example, by blocking the air outlet filter and by displacing biomass from the fermenter. Repeated additions of AF decrease the availability of DO, due to the collapse of bubble structures within the culture broth, necessitating increased agitation and thereby increasing the tendency to foam. AF in the culture filtrate may also cause severe problems in the various unit operations of downstream processing, *e.g.* in membrane filtration. Our results show that in the case of *T. reesei*, the hydrophobins HFBI and especially HFBII play a major role in foaming of the culture broth. However, in this study we showed that foaming can be controlled even in the case of overproduction of hydrophobin protein for harvesting. The extensive foaming of the HFBII-overproducing transformant was effectively reduced by lower agitation achieved with oxygen enrichment, a technique already adaptable to production scale.

Deletion of *hfb2* resulted in a clear decrease in consumption of AF, whereas amplification of the gene caused very high consumption. In the experiment with the highest consumption of AF, massive addition occurred during the phase of enzyme production, after the growth phase (result not shown). This was logical because the excess production of HFBII was governed by the *cbh1* promoter, the homologous product of which normally appears in the culture filtrate after the

phase of maximum growth. The impact of HFBI on foaming was not as pronounced as in the case of HFBII. This may be due to the fact that most of the HFBI produced was cell wall-bound. In addition, the foam stabilisation characteristics of these two proteins may differ. HFBI was observed to affect foaming only when it was produced in high amounts by the HFBI-overproducing transformant, leading to increased secretion of HFBI into the culture medium. The AF consumption of the *Δhfb1* transformant and the host strain were similar. It is possible that this low level of foaming was in fact caused mainly by factors other than HFBI, and that the effect of *hfb1* deletion was therefore difficult to detect within the limits of the accuracy of the measurement method used.

No major differences were observed in growth parameters, biomass formation or hyphal thickness between the Δ*hfb1* transformant, the HFBI-overproducing transformant and the host strain in the controlled fermentation cultivation. The deletion of *hfb1* was previously shown to decrease biomass formation and hyphal thickness in the early growth phase of the fungus in shake flask cultivations (Askolin *et al.*, MS in preparation). This effect was possibly not observed in the fermenter cultivations because the early growth phase had already been superseded during the inoculation cultivations and the growth was effectively controlled in the fermenter, in contrast to shake flasks.

On the basis of the experiments reported in this work, neither deletion nor amplification of the *hfb2* gene had a significant effect on the rate or final level of biomass accumulation on lactose medium in bioreactor cultivation. However, a clear difference was observed in sporulation. Deletion of *hfb2* caused early and massive sporulation, whereas amplification of the gene appeared to result in decreased ability of the fungus to produce free spores in the liquid culture medium. On agar plates, the outward appearance of colonies of all three strains was rather similar, including spore production.

Production of cellulases was apparently unchanged on cellulose medium when the *hfb2* gene was deleted, but a slight decrease in enzyme activities was observed on lactose medium. The maximum rate of enzyme production on lactose medium was approximately the same in the *Δhfb2* transformant VTT D-99676 as in the control strain Rut-C30 (Table 2) but the length of the linear production phase was shorter, probably due to the observed early onset of sporulation. Improved and considerably prolonged production of cellulases by

the *Δhfb2* strain VTT D-99676 in continuous cultivation with process control to prevent the onset of sporulation will be reported in a later work. Interestingly, amplification of the *hfb2* gene also caused a slight decrease in enzyme production on lactose medium, even in the repeat cultivation in which AF consumption was successfully minimised by oxygen enrichment of the incoming air. This may have been due to the depressing effect of overproduction of HFBII protein under the *cbh1* promoter on homologous enzyme production. It has been suggested that several copies of the *cbh1* promoter may be sufficient to titrate out regulatory or transcription factors needed for high enzyme production (Karhunen *et al.*, 1993, Paloheimo *et al.*, 1993, Margolles-Clark *et al.*, 1996). The transformant VTT D-99745 carries three extra copies of the *cbh1* promoter in addition to that contained in the endogenous *cbh1* gene.

Foaming in fermentation processes based on *T. reesei* Rut-C30 was clearly demonstrated to be due mainly to production of HFBII, rather than to the much greater amount of other extracellular proteins (cellulases and hemicellulases) produced by this organism on inducing media. Traditionally, cellulase production has generally been considered to be the prime cause of foaming, rather than the only relatively recently identified hydrophobins. However, despite the strong foaming tendency of HFBII, this protein can be successfully overproduced (by even 8-fold) in a bioreactor equipped with oxygen enrichment to avoid excessive agitation and therefore minimise foaming. The HFBII produced in this work using the overproducing strain VTT D-99745 will be purified for use in application studies.

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REFERENCES

Aro, N., Saloheimo, A., Ilmén, M., Penttilä, M. (2001) ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J Biol Chem 276: 24309–24314.

Askolin, S., Nakari-Setälä, N., Tenkanen, M. (2001) Overproduction, purification, and characterization of the *Trichoderma reesei* hydrophobin HFBI. Appl Microbiol Biotechnol 57: 124–130.

Bailey, M.J., Biely, P., Poutanen, K. (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23: 257–270.

IUPAC (International Union of Pure and Applied Chemistry) (1987) Measurement of cellulase activities. Pure Appl Chem 59: 257–268.

Karhunen, T., Mäntylä, A., Nevalainen, K.M.H., Suominen, P.L. (1993) High frequency one-step gene replacement in *Trichoderma reesei*. I: Endoglucanase overproduction. Mol Gen Genet 241: 515–522.

Kleemola, T., Nakari-Setälä, T., Linder, M., Penttilä, M., Kotaviita, E., Olkku, J., Haikara, A. (2001) Characterisation and detection of the gushing factors produced by fungi. Proceedings of the European Brewing Convention 28th Congress, Budapest 2001, contribution no. 12, 129–138, CD-ROM, ISBN 90-70143-21-6.

Linder, M., Selber, K., Nakari-Setälä, T., Qiao, M., Kula, M.-R., Penttilä, M. (2001) The hydrophobins HFBI and HFBII from *Trichoderma reesei* showing efficient interactions with nonionic surfactants in aqueous two-phase systems. Biomacromolecules 2: 511–517.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Mandels, M., Weber, J. (1969) The production of cellulases. Adv Chem Ser 95: 391–414.

Mandels M.M., Sternberg D., Andreotti R.E. (1975) Growth and cellulase production by *Trichoderma*. In: Bailey M.J., Enari T.-M., Linko M. (eds) Symposium on enzymatic hydrolysis of cellulose. Finnish National Fund for Research and Development (SITRA), Helsinki, Finland, 1975. Pp. 81–109.

Margolles-Clark, E., Hayes, C.K., Harman, G.E., Penttilä, M. (1996) Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. Appl Environ Microbiol 62: 2145–2151.

Montenecourt, B.S., Eveleigh, D.E. (1979) Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei.* Adv Chem Ser 181: 289–301.

Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E., Penttilä, M. (1996) Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFBI. Eur J Biochem 235:248–255.

Nakari-Setälä, T., Aro, N., Ilmén, M., Muñoz, G., Kalkkinen, N., Penttilä, M. (1997) Differential expression of the vegetative and spore-bound hydrophobins of *Trichoderma reesei*: cloning and characterization of the *hfb2* gene. Eur J Biochem 248: 415–423.

Paloheimo, M., Miettinen-Oinonen, A., Torkkeli, H., Nevalainen, H., Suominen, P. (1993) Enzyme production by *Trichoderma reesei* using the *cbh1* promoter. In: Suominen, P., Reinikainen, T. (eds) Proceedings of the 2nd TRICEL Symposium on *Trichoderma reesei* cellulases and other hydrolases. Foundation for Biotechnical and Industrial Fermentation Research 8, Espoo, Finland. Pp. 229–238.

Penttilä, M., Nevalainen, H., Rättö, M., Salminen, E., Knowles J. (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61: 155–164.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) Molecular cloning: a Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scholtmeijer, K., Wessels, J.G.H., Wösten, H.A.B. (2001) Fungal hydrophobins in medical and technical applications. Appl Microbiol Biotechnol 56:1–8.

Wessels, J.G.H. (1994) Developmental regulation of fungal cell wall formation. Annu Rev of Phytopathol 32: 413–437.

Wessels, J.G.H. (1997) Hydrophobins: Proteins that change the nature of the fungal surface. Adv Microb Physiol 38: 1–45.

Wösten, H.A.B., de Vocht, M.L. (2000) Hydrophobins, the fungal coat unravelled. Biochim Biophys Acta 1469: 79–86.

Wösten, H.A.B., Wessels, J.G.H. (1997) Hydrophobins, from molecular structure to multiple functions in fungal development. Mycoscience 38: 363–374.