The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*

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*Trichoderma reesei* was cultivated in chemostat cultures on lactose-containing medium. The cultures were characterized for growth, consumption of the carbon source and protein production. Secreted proteins were produced most efficiently at low specific growth rates, 0-022–0-033 h⁻¹, the highest specific rate of total protein production being 4·1 mg g⁻¹ h⁻¹ at the specific growth rate 0-031 h⁻¹. At low specific growth rates, up to 29% of the proteins produced were extracellular, in comparison to only 6–8% at high specific growth rates, 0-045–0-066 h⁻¹. To analyse protein synthesis and secretion in more detail, metabolic labelling of proteins was applied to analyse production of the major secreted protein, cellobiohydrolase I (CBHI, Cel7A). Intracellular and extracellular labelled CBHI was quantified and analysed for pI isoforms in two-dimensional gels, and the synthesis and secretion rates of the molecule were determined. Both the specific rates of CBHI synthesis and secretion were highest at low specific growth rates, the optimum being at 0-031 h⁻¹. However, at low specific growth rates the secretion rate/synthesis rate ratio was significantly lower than that at high specific growth rates, indicating that at low growth rates the capacity of cells to transport the protein becomes limiting. In accordance with the high level of protein production and limitation in the secretory capacity, the transcript levels of the unfolded protein response (UPR) target genes *pdi1* and *bip1* as well as the gene encoding the UPR transcription factor *hac1* were induced.

Regulation of the cellulase genes on various carbon sources has been studied in detail. The cellulase genes are to a large extent induced in a coordinate manner in the presence of cellulose, its hydrolytic products, or certain oligosaccharides, such as sophorose and lactose (Ilmén et al., 1997; Foreman et al., 2003). For the individual hemicellulase genes also, specific and partially overlapping induction mechanisms have been anticipated to exist based on differential expression of the genes on various carbon sources (Margolles-Clark et al., 1997). Wide-domain carbon catabolite repression has been shown to control the expression of both cellulase and hemicellulase genes in the presence of glucose (Ilmén et al., 1996, 1997; Margolles-Clark et al., 1997; Strauss et al., 1995; Takashima et al., 1996). Many of the transcription factors involved in regulation of the genes, CREI mediating carbon catabolite repression, the repressor ACEI, the activator ACEII, and the CCAAT binding complex Hap2/3/5, have been characterized in molecular detail (reviewed by Kubicek & Penttilä, 1998; Mach & Zeilinger, 2003; Schmoll & Kubicek, 2003).

INTRODUCTION

The filamentous fungus *Trichoderma reesei* produces a variety of extracellular cellulases and hemicellulases that hydrolyse plant-derived polysaccharides into monomeric sugars which in turn are used as a source of carbon and energy by the fungal cells (for reviews see Biely & Tenkanen, 1998; Penttilä, 1995). The cellulolytic system of the fungus consists of synergistically acting endoglucanases, cellobiohydrolases and β-glucosidases. The enzymes active on hemicellulose include xylanases and a mannanase as well as various enzymes cleaving hemicellulose side chains. The most abundant of these enzymes are the cellobiohydrolases and endoglucanases, with cellobiohydrolase I (CBHI) comprising the major part of the total extracellular protein produced by the fungus (Keranen & Penttilä, 1995).

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**Abbreviations:** CBHI or Cel7A, cellobiohydrolase I; D, dilution rate; 2D, two-dimensional; EGI or Cel7B, endoglucanase I; UPR, unfolded protein response.
states of the cells is scarce. Furthermore, very little information is available on the cellular responses to protein production. T. reesei has the potential to produce extracellular proteins in very large quantities, which sets a demand for the cells to adjust the capacity of protein synthesis and transport to the level required, and may also provoke stress responses within the cells. To obtain information on protein production and factors affecting the processes at different growth rates of the organism, we have analysed carbon-limited chemostat cultures of the strain RUT-C30 in detail. Specifically, the capacity of the cells to synthesize and secrete proteins has been studied using metabolic labelling of the proteins, a methodology previously set up for analysis of protein synthesis and secretion in defined culture conditions (Pakula et al., 2000). In addition, the expression levels of the major cellulase genes cbh1 and egf1, as well as genes involved in protein folding and transport, have been analysed under these conditions.

**METHODS**

**Strain and cultivation conditions.** *Trichoderma reesei* strain Rut-C30 (ATCC 56765) (MonteneCourt & Eveleigh, 1979) was used in this study. Composition of the culture medium was as follows: (NH₄)₂SO₄ 7.6 g, KH₂PO₄ 5.0 g, MgSO₄.7H₂O 0.5 g, CaCl₂.2H₂O 0.2 g, CoCl₂ 3.7 mg, FeSO₄.7H₂O 5 mg, ZnSO₄.7H₂O 1.4 mg, MnSO₄.7H₂O 1.6 mg l⁻¹ and lactose 20 g l⁻¹ (in batch culture) or 8 g l⁻¹ (in continuous culture). The inoculum was prepared by transferring 2 × 10⁷ spores into a 500 ml flask containing 200 ml growth medium (lactose 20 g l⁻¹). The preculture was grown for 4 days in a conical flask at 30 °C with shaking at 200 r.p.m., and finally transferred to the bioreactor (1:8 ratio of laboratory fermenter, Chemap) to the final volume of 1.5 l growth medium. The cultivations in the bioreactors were carried out at 30 °C, with aeration of 1:3 VVM (volumes of air per volume of liquid per minute) and stirring with impeller tip speed 2 m s⁻¹. The pH was kept at 4.8 ± 0.2 by the addition of 10% KOH. Occasionally Struktol SB 2032 (Schill & Seilacher) was added to prevent foaming. When performing the carbon-limited chemostat cultivations, the feed (growth medium containing 8 g lactose l⁻¹) was started at the time when the lactose in the batch phase was nearly consumed, about 40 h after transferring the preculture into the fermenter. The feed was supplied at a constant rate, specific for each dilution rate used in the study. For every dilution rate studied, a new culture was started.

Usually steady state in the cultures was achieved after cultivation of four to five residence times. Steady state of the cultures was monitored by measuring biomass dry weight and dissolved oxygen concentration in the culture, carbon dioxide content of the exhaust air, as well as lactose, phosphate and ammonium concentrations in the culture broth.

**Analysis of the chemostat cultures.** Dry weight was measured by filtering and drying mycelium samples at 105 °C to a constant weight (24 h). Residual lactose and glucose in the culture filtrate were measured using either HPLC or an enzymatic test kit for lactose (Boehringer Mannheim 176 303) and the GOD-Perid method for glucose (Boehringer Mannheim 124 036). The amount of phosphate was measured as described by Basset et al. (1987). An enzymatic test kit (Boehringer Mannheim 1112 732) was used for measuring ammonium concentration. Soluble protein was analysed using the Bio-Rad Protein Assay. The carbon content of the biomass was determined using a Carlo Erba C/N analyser, and the value for carbon content determined by Nielsen & Villadsen (1994) was used for extracellular proteins.

**Analysis of intracellular methionine.** Cells resuspended in double-distilled water to 13 mg biomass dry weight ml⁻¹ were disrupted by sonication (Pakula et al., 2000) and boiled for 15 min. The extracts were centrifuged at 14000 g for 10 min. Sulphosalicylic acid was added to the supernatant to a final concentration of 5% (w/v), and the samples were centrifuged at 14000 g for 10 min. The amino acids were analysed from the supernatant using HPLC.

**Analysis of intracellular protein.** Cell extracts were prepared and the protein amount in the extracts was measured as described previously (Pakula et al., 2000). Samples of the cultures were filtered through Millipore HVLP02500 filters to collect the mycelium, and the mycelium was washed with double-distilled water and resuspended in 20 mM N-ethylmaleimide, 10 mM NaN₃ (3–7 mg biomass dry weight ml⁻¹). The cells were disrupted by sonication (8 × 8 s with an MSE 150 W sonicator, 18 μm amplitude, 30 s cooling on ice between the sonication cycles). The cell lysates were first lyophilized, and then resuspended in two-dimensional (2D) lysis buffer [9 M urea, 2% (v/v) Triton X-100, 286 mM β-mercaptoethanol, 2% (v/v) Pharmalyte 3–10; 560 μg biomass dry weight per 25 μl buffer], 3 vols 2D sample buffer [the lysis buffer containing 0.5% (v/v) Triton X-100] was added, and the samples were incubated at room temperature for 1 h. Insoluble material was removed by centrifugation. Proteins were analysed using the Bio-Rad Protein Assay Kit.

**Metabolic labelling of the proteins.** Metabolic labelling of the proteins was carried out essentially as described previously (Pakula et al., 2000). Aliquots (25 ml) of the chemostat culture were gently transferred into shake flasks on a rotary shaker (210 r.p.m., 30 °C), and the labelling was started immediately after the transfer. To avoid changes in concentration of the nutrients, fresh medium was added into the shake flasks. Medium was added at intervals of 15 s, the amount of the added medium corresponding to the amount of medium fed into the chemostat culture during the same time period. [³⁵S]Methionine (Amersham SJ 1015, in vivo cell labelling grade, 1000 Ci mmol⁻¹, 10 μCi ml⁻¹) was added to the shake flasks, either 5-5 or 12-5 μCi (mg biomass dry weight)—1 in the labelling experiment (1 Ci = 3.7 × 10⁹ Bq), and samples of 1 ml were collected at short intervals. The samples were rapidly filtered through Millipore HVLP2500 filters to separate the culture medium and mycelium. The mycelium on the filters was washed with 10 ml double-distilled water and frozen immediately in liquid nitrogen.

**Analysis of the labelled proteins.** The cells were disrupted by sonication and the cell extracts were prepared as previously described (Pakula et al., 2000). Protein samples prepared from the cell extracts and culture medium were subjected to 2D gel electrophoresis (Pakula et al., 2000) for analysis of specific proteins. Equal amounts of total protein (measured using the Bio-Rad Protein Assay Kit as described above) were loaded in the gels. The amount of protein loaded was confirmed to be in a range giving a linear response between the signal analysed and the sample volume. Typically, 20 μg intracellular protein or 3 μg extracellular protein was loaded. The 2D gels were analysed using a phosphorimager (Molecular Dynamics). The intracellular labelled protein per culture volume and per biomass amount was calculated by taking into account the protein content of the biomass and the biomass amount under each of the culture conditions. For analysis of total labelled protein, the proteins in the cell extracts and in culture supernatant were precipitated using TCA, and the radioactive in the TCA-insoluble material was measured by scintillation counting (Pakula et al., 2000).

**Parameters describing protein synthesis and secretion.** The parameters, protein synthesis and secretion rate, the mean synthesis time of specific proteins, and the minimum secretion time of the molecules were determined essentially as described by Pakula et al.
(2000). The amount of labelled protein per biomass and culture volume in cell extract and in culture supernatant was plotted against time (scintillation counting of TCA-insoluble material was used for quantification of total labelled protein, and specific labelled proteins were quantified in 2D gels using a phosphorimager). The specific rate of protein synthesis was determined as the slope of the linear part of the curve representing intracellular labelled protein at the early time points where protein secretion was not yet detectable. The specific rate of protein secretion was determined as the slope of the linear part of the curve representing extracellular labelled protein. The specific rates of protein synthesis and secretion per biomass and time unit were normalized with the ratio of $^{35}$S methionine taken up by the cells to the total intracellular methionine. The mean synthesis time for a specific protein was determined by extrapolating the linear part of the curve representing the amount of the labelled protein in cell extract to the abscissa. The intercept of the curve and the abscissa corresponds to half the synthesis time of the protein (see also Braakman et al., 1991; Horwitz et al., 1969; Löffeld & Eigner, 1958). The minimum secretion time of the protein was determined as the distance of the intercepts of the intracellular and extracellular protein curves extrapolated to the abscissa.

**Northern analysis.** Mycelium samples of 50 ml were filtered, washed with equal volume of 0-7% NaCl, frozen immediately in liquid nitrogen, and stored at $-80\degree C$. Total RNA was isolated using the Trizol Reagent (Gibco-BRL), essentially according to the manufacturer’s instructions. Northern blotting and hybridization on nitrocellulose filters were carried out according to standard procedures (Sambrook et al., 1989). Five micrograms of total RNA isolated from each sample was used in the Northern analysis. cDNAs of the genes (cbh1, E00389; egli, M15665; pdli, AJ222773; sar1, Y08636; ypt1, AJ277108) were used as probes. The signals were normalized against the signals of act1 encoding actin.

**RESULTS**

**Growth parameters and protein production in chemostat cultures of T. reesei Rut-C30**

Growth characteristics and protein production of T. reesei strain Rut-C30 were studied in carbon-limited chemostat cultures on lactose-containing minimal medium at dilution rates of 0.021–0.076 h$^{-1}$. At steady state, reached typically after four to five residence times, the levels of produced biomass and extracellular protein and residual amount of lactose were constant (Fig. 1a). Between dilution rates of 0.021 and 0.033 h$^{-1}$, the amount of biomass increased...
significantly with increasing dilution rate (the dilution rate in the cultures corresponds to the specific growth rate), after which the biomass amount remained constant until the maximal specific growth rate of the organism was exceeded (between 0·066 h\(^{-1}\) and 0·076 h\(^{-1}\)), leading to washout of the biomass (Fig. 1a). The growth yield (grams of biomass formed per gram of lactose consumed) at the different dilution rates displayed a similar trend (Fig. 1d).

By applying the Monod equation \[1/\mu = (1/S)(K_S/\mu_{\text{max}}) + 1/\mu_{\text{max}}\] where \(\mu\) is the specific growth rate, \(S\) the substrate concentration, \(K_S\) the substrate saturation constant and \(\mu_{\text{max}}\) the maximal specific growth rate), and plotting \(1/\mu\) vs \(1/S\) (Fig. 1b), the values for \(\mu_{\text{max}}\) and \(K_S\) were deduced. The obtained value, \(\mu_{\text{max}} 0·068 \text{ h}^{-1}\), was close to the maximal specific growth rate measured in batch cultures on the same culture medium, which was 0·073 h\(^{-1}\) (data not shown). The value obtained for \(K_S\) was 0·025 g l\(^{-1}\).

The amounts of residual lactose and its cleavage product, glucose, in the cultures were low. The concentration of lactose varied between 0·012 and 0·150 g l\(^{-1}\) (Fig. 1a) and the amount of glucose was below the detection limit. The specific consumption rate of lactose increased with increasing specific growth rate from 0·068 g g\(^{-1}\) h\(^{-1}\) to 0·137 g g\(^{-1}\) h\(^{-1}\) (Fig. 1c). The equation introduced by Pirt (1965) \((q_S = \mu Y_{\text{max}} + m_0\), where \(q\) is the specific rate of substrate consumption, \(Y\) the molar growth yield and \(m\) the maintenance coefficient) has often been used for estimation of the amount of energy source required for the maintenance of the cells \((m_0)\), assuming that the energy requirement for the maintenance and the maximal growth yield are constant over the different growth rates and that no metabolites other than carbon dioxide and water are produced. A fairly good linear regression was obtained for the values of the specific lactose consumption rate plotted against the specific growth rate, giving the values 0·027 g g\(^{-1}\) h\(^{-1}\) and 0·60 g g\(^{-1}\) for the maintenance coefficient and the maximal growth yield, respectively.

The highest specific production rate of extracellular proteins was obtained at low specific growth rates (0·022–0·033 h\(^{-1}\)), the maximal value being 4·1 mg g\(^{-1}\) h\(^{-1}\) at the specific growth rate 0·031 h\(^{-1}\) (Fig. 1c). However, at the lowest specific growth rate studied (0·021 h\(^{-1}\)), the specific protein production rate was reduced close to the level measured at high specific growth rates of 0·045–0·066 h\(^{-1}\) (1·4–1·6 mg g\(^{-1}\) h\(^{-1}\)). Similarly, the yield of extracellular protein produced per the amount of carbon source consumed was the highest at the specific growth rate 0·031 h\(^{-1}\) (Fig. 1d). At low specific growth rates (0·021–0·031 h\(^{-1}\)), both the biomass yield and the yield of protein produced increased with increasing specific growth rate, after which much less protein was produced per amount of lactose consumed, whereas the production of biomass related to lactose consumption remained at a high level (Fig. 1d). Carbon balances accounting for biomass, secreted protein and CO\(_2\) produced in the cultures showed a mean closure of 90 ± 7% total carbon consumed.

For the determination of the balances, the carbon content of the mycelial samples was measured, and the elemental composition CH\(_{172}\)O\(_{63}\)N\(_{27}\)S\(_{6}\)O\(_{4}\) described by Nielsen & Villadsen (1994) was used for secreted proteins.

To elucidate how the capacity of protein production was directed to production of intracellular and extracellular proteins, the specific synthesis rates of intracellular and extracellular protein were compared at the different specific growth rates. The amount of intracellular protein in the cell extracts was determined, and the specific synthesis rate of intracellular proteins (grams of intracellular protein per gram of biomass dry weight per hour) was deduced. The specific synthesis rate of intracellular and extracellular protein as well as the specific rate of total protein synthesis (combined synthesis of extracellular and intracellular protein) plotted against the dilution rate in the culture are shown in Fig. 2(a). The specific rate of total protein synthesis increased with increasing specific growth rate until a constant level was reached at the specific growth rate 0·045 h\(^{-1}\) (Fig. 2a). At low specific growth rates, between 0·022 and 0·033 h\(^{-1}\), a markedly higher percentage of the total protein synthesis was directed to

![Fig. 2. The synthesis of intracellular and extracellular proteins in the chemostat cultures. (a) The specific rate of total protein synthesis (●), the specific rate of synthesis of intracellular proteins (□) and the specific rate of synthesis of extracellular proteins (■) (g protein per g biomass dry weight per hour) at different growth rates in the chemostat cultures. (b) Synthesis of intracellular proteins (□) and extracellular (■) proteins as a percentage of the total proteins produced.](image)
production of extracellular proteins compared to that of cultures with the high specific growth rates of 0.045–0.066 h\(^{-1}\) (Fig. 2b). At most, at the specific growth rate 0.022 h\(^{-1}\), 29% of the synthesized proteins were extracellular, whereas at high specific growth rates (0.045–0.066 h\(^{-1}\)), only 6–8% of the synthesized proteins were those transported into the culture medium.

**Synthesis and secretion of CBHI at different specific growth rates**

To be able to study in more detail the synthesis and transport of the extracellular proteins at different specific growth rates, a series of metabolic labelling experiments were set up using the methodology described by Pakula *et al.* (2000). For the labelling, aliquots of the chemostat cultures were withdrawn into shake flasks on a rotary shaker. A fed of fresh medium was supplied to maintain the cells under conditions resembling those in the bioreactor. Newly synthesized proteins were metabolically labelled by the addition of \(^{35}\)S-methionine to the cultures, and the synthesis and secretion of the major cellulase Cel7A (CBHI) was analysed as a model system. Frequent samples were collected from the labelled cultures and intracellular and extracellular protein extracts were subjected to 2D gel analysis. The amount of labelled CBHI in the samples was quantified and plotted against time to determine the parameters which indicated the efficiency of protein synthesis and secretion. The specific synthesis rate of the protein was measured at the early time points of the labelling experiment, before any secretion of the protein into the culture medium was detectable. At these early time points, the most prominent pl isoforms of the protein were those representing the early, nascent forms of the protein, whereas the extracellular CBHI was represented by multiple pl isoforms forming a pattern typical for secreted CBHI under these conditions (Pakula *et al.*, 2000).

The specific rates of CBHI synthesis and secretion (the amount of protein synthesized or produced in the culture medium per biomass and time unit) at different specific growth rates are shown in Fig. 3. The highest specific synthesis and secretion rates of labelled CBHI were both obtained at the specific growth rate 0.031 h\(^{-1}\), which is in accordance with the result of total protein production into the culture medium. However, at low specific growth rates (0.022–0.045 h\(^{-1}\)), under conditions where the specific CBHI synthesis rate was high, the ratio between the secretion rate and the synthesis rate was much lower than the ratio at high specific growth rates. At 0.031 h\(^{-1}\), the secretion rate was 57% of the synthesis rate, whereas at 0.066 h\(^{-1}\), the secretion rate was 62% of the synthesis rate. The result indicates that although CBHI was efficiently synthesized at the low specific growth rates, the protein secretion capacity limits protein production under these conditions. The mean time of synthesis of CBHI molecules and the minimum time of secretion of the protein did not differ significantly at the different specific growth rates. The mean synthesis time of CBHI was 3.9 ± 0.3 min and the minimum secretion time of the molecules was 12.6 ± 0.2 min. The values are close to the ones obtained previously for cultures carried out at the specific growth rate 0.07 h\(^{-1}\) (Pakula *et al.*, 2000).

**Northern analysis of genes encoding cellulases, factors involved in protein transport and folding, and ribosomal proteins in the chemostat cultures**

Northern analysis was carried out to elucidate whether or not the cellulase genes were differentially expressed at different specific growth rates and whether or not the proposed limitation in the secretion capacity was manifested at the expression level of genes involved in protein transport and folding. The steady-state transcript levels of *cbhl* and *egl1* (encoding CBHI/Cel7A and EGI/Cel7B, respectively) were the highest at the specific growth rate 0.031 h\(^{-1}\) (Fig. 4a, b). Thus, the high synthesis rate of labelled CBHI can, at least in part, be explained by the high transcript levels of the genes, as well as the high specific production rate of total extracellular proteins at this growth rate, since CBHI and EGI form the major part of extracellular proteins produced by *T. reesei*. However, by comparing the protein production rates and the transcript levels over the full range of dilution rates studied, it is obvious that the transcript levels do not fully explain the differences in protein production rates at different dilution rates, but that other factors are involved as well. Especially at dilution rates above 0.06 h\(^{-1}\), the mRNA levels of *cbhl* and *egl1* were relatively high compared to the synthesis rate of CBHI (Fig. 3) and the specific production rate of total extracellular protein into the culture medium (Fig. 1).

The mRNA levels of the chaperon/foldase genes *bip1* and *pdi1* were higher at low specific growth rates (0.022–0.033 h\(^{-1}\)) than at high specific growth rates (0.045–0.066 h\(^{-1}\)) (Fig. 4c). This is in accordance with the
observation that at these growth rates a high amount of secreted protein was synthesized; however, the secretion capacity was postulated to be a limiting factor under these conditions, which would necessitate an increased capacity of the folding machinery. The genes $bip1$ and $pdi1$ are known to be induced by the unfolded protein response (UPR) pathway, which is mediated by the $hac1$ gene. As the UPR pathway is activated, a shorter form of the $hac1$ transcript is generated via splicing of an unconventional intron and via truncation of the transcript at the 5' flanking region. The shortened form of the transcript then produces the transcription factor HACI (Saloheimo et al., 2003). The $hac1$ transcript levels were slightly induced at low growth rates (Fig. 4d), the presence of the induced form being more pronounced in the conditions corresponding to the highest rate of synthesis and secretion of extracellular proteins, at the dilution rate 0·031 h$^{-1}$. In contrast, the transcript levels of $ypt1$ and $sar1$, encoding proteins involved in protein transport, were not induced at low specific growth rates (Fig. 4e). For comparison, the transcript levels of $rps11$ and $rpl4$, encoding ribosomal components, were also analysed (Fig. 4f). The Northern analysis showed that the signals of $rps11$ and $rpl4$ increased with increasing growth rate.

**DISCUSSION**

A detailed analysis of carbon-limited chemostat cultures of the strain RUT-C30 was carried out to elucidate the capacity of *T. reesei* to synthesize and secrete proteins under different physiological conditions and under the influence of different factors affecting protein production by the fungus. The cultures were characterized for growth, consumption of the carbon source, and production of extracellular proteins. Metabolic labelling of newly synthesized proteins was used for analysis of the synthesis and secretion of the major secreted protein produced by the fungus CBH1, and Northern analysis was carried out to analyse cellulase gene expression and selected cellular stress responses to protein production under these conditions.

The dilution rates studied (0·021–0·076 h$^{-1}$) covered the
range from a very low specific growth rate to a rate exceeding the maximal specific growth rate of the fungus. The maximal specific growth rate estimated from the chemostat data was 0·068 h⁻¹, which is close to the value 0·073 h⁻¹, determined in batch bioreactor cultures for the strain on the same medium, and to the value previously obtained for T. reesei strain C5 on lactose-containing minimal medium, 0·07 h⁻¹ (Chaudhuri & Sahai, 1994). At low specific growth rates (0·021–0·033 h⁻¹), the fungal biomass increased significantly with increasing growth rate, but at higher specific growth rates (0·033–0·066 h⁻¹), the biomass amount remained approximately constant. Cultivation at the dilution rate 0·076 h⁻¹ resulted in washout of the biomass, as predicted. The specific consumption rate of the carbon source lactose increased until the critical dilution rate was reached. Assuming that the amount of energy source used for maintenance as well as the maximal growth yield would be constant over the range of growth rates studied, the maintenance coefficient (0·027 g g⁻¹ h⁻¹) and maximal growth yield (0·60 g g⁻¹) were determined (Pirt, 1965). A good linear regression was obtained for the specific lactose consumption rate over the dilution-rate range studied, indicating that the assumptions of the Pirt equation were valid under these conditions.

Protein production into the culture medium was most efficient at low specific growth rates (below 0·033 h⁻¹). The specific production rate of extracellular proteins increased first with increasing dilution rate, reaching the maximal level, 4·1 mg g⁻¹ h⁻¹, at the specific growth rate 0·031 h⁻¹, after which the production rate decreased significantly. Similar trends for specific protein production rates at different dilution rates have previously been reported for lactase production by Rut-C30 from continuous cultures on lactose medium (Castillo et al., 1984) and for production of cellulase activity by T. reesei strain C5 (Chaudhuri & Sahai, 1994) and have been modelled for cellulase productivity on medium containing xylose and sorbose by T. reesei strain QM9414 (Schafner & Toledo, 1992). In accordance with the other data on production of biomass and extracellular protein, at low specific growth rates (0·022–0·033 h⁻¹), a higher proportion of the total protein synthesis was also directed to production of extracellular proteins compared to the cultures at higher specific growth rates (0·045–0·066 h⁻¹). At low specific growth rates, up to 29% of the proteins synthesized were extracellular and at the high growth rates only 6–8% were extracellular.

Production of extracellular proteins by filamentous fungi, such as x-amylase in Aspergillus oryzae (Carlsen et al., 1996; Spohr et al., 1998) and glucoamylase in Aspergillus niger (Pedersen et al., 2000; Schrickx et al., 1993; Wethers et al., 1998), have been shown to be growth-associated in many cases. However, examples of growth-rate-disassociated production are also known, such as recombinant protein production by Fusarium venenatum (Wiebe et al., 2000). As active growth of the cells and the fungal hyphae requires efficient transportation of, for example, cell wall material, protein transport is expected to take place efficiently in the growing hyphae. However, a majority of the extracellular enzymes produced by T. reesei are needed for degradation of polymeric compounds derived from plant material to provide the fungus with a source of carbon and energy. Therefore, production of hydrolytic enzymes would be beneficial for the fungus under low nutrient conditions in which easily metabolized carbon sources, such as glucose, are not available and growth might be slow.

Taking into account the data from the wide range of growth rates studied, the results in our study as well as those obtained by other groups (Castillo et al., 1984; Chaudhuri & Sahai, 1994; Schafner & Toledo, 1992) indicate that production of the hydrolytic enzymes that constitute the major part of the extracellular proteins produced by T. reesei under these conditions is not directly growth-rate associated. In terms of specific protein production rate as well as the yield of extracellular protein per amount of carbon source consumed, the production of extracellular proteins was maximal at the specific growth rate 0·031 h⁻¹, but at higher growth rates a significant reduction in production was observed. However, in the range of the low dilution rates 0·021–0·031 h⁻¹, both protein and biomass production are positively correlated with the specific growth rate. The production of extracellular protein production at very low specific growth rates is due to the maintenance requirement of the cells. At low specific growth rates, the specific consumption rate of the carbon and energy source lactose was very low. At the dilution rate 0·021 h⁻¹, 0·068 g lactose g⁻¹ h⁻¹ was consumed, of which the estimated maintenance coefficient 0·027 g g⁻¹ h⁻¹ forms 40%. The high substrate consumption for maintenance under these conditions would limit production of both biomass and extracellular protein.

As cellulase gene expression by T. reesei is known to be regulated by the carbon source, one explanation for the differences in the protein production levels at different growth rates could be differences in the concentrations of the residual carbon sources. However, the concentration of the repressing carbon source glucose in the cultures in this study was very low, and thus unlikely to cause repression of cellulase production. In addition, the strain Rut-C30 is defective in the cre1-mediated carbon catabolite mechanism (Ilmén et al., 1996) and therefore cellulase production by the strain is partially derepressed even in a glucose medium. Further, the residual levels of lactose were low in the cultures and no positive correlation between cellulase expression and the residual lactose concentration in the cultures was observed. Thus the residual lactose in the cultures was unlikely to act as an inducer for cellulase production. Interestingly, under high protein production conditions at low specific growth rates, the specific consumption rate of lactose was low. Previous studies have shown that cellulase gene expression is induced in batch growth.
cultures after the carbon source is exhausted (Ilmén et al., 1997). It is possible that the low consumption rate of the carbon source might trigger a similar type of induction as does carbon and energy source starvation. In natural habitats of the fungus, this type of mechanism might be important for activation of synthesis of hydrolytic enzymes to enable sequestering of easily metabolizable carbon sources from complex plant polymers.

To address the question of transcriptional regulation of cellulase genes at different specific growth rates, we carried out a Northern analysis of the genes cbh1 and egl1. The transcript levels of both genes were slightly increased at low specific growth rates, thus being in accordance with the results on production of total extracellular proteins. In addition, metabolic labelling of the cultures was used for analysis of the synthesis and secretion of CBHI specifically. The results showed that both the specific synthesis and secretion rate of labelled CBHI were the highest at the low specific growth rate 0.031 h\(^{-1}\). Thus, the differences in transcript levels at the different growth rates could, at least in part, explain the differences in the amounts of the corresponding proteins synthesized per time unit. However, further inspection of the full range of dilution rates studied indicated that the transcript levels alone do not fully explain the differences in protein production. Especially at high specific growth rates, the specific protein production rate (Fig. 1) and the specific synthesis rate of CBHI (Fig. 3) were lower than that which could have been anticipated based on the transcript levels of the major cellulase genes cbh1 and egl1.

Interestingly, the metabolic labelling studies also showed that the ratio between the specific secretion rate and the synthesis rate of labelled CBHI was much lower at the low specific growth rates than at the high specific growth rates. The result indicates that at the low growth rates the capacity of the cells to transport the proteins would become limiting as the amount of secreted protein synthesized increases. Accumulation of unfolded proteins in the endoplasmic reticulum is known to activate the UPR, in both lower and higher eukaryotes. In filamentous fungi, UPR induction has been reported to take place under conditions in which protein transport or folding is impaired by treatment of the cultures with different chemical agents or under conditions where a heterologous protein is produced (Mulder et al., 2004; Ngiam et al., 1997; Pakula et al., 2003; Punt et al., 1998; Saloheimo et al., 1999, 2003; van Gemeren et al., 1997). In addition, we have recently shown that the UPR pathway is activated along with cellulase gene induction when the cultures are shifted from a glucose repressed state to an induced state (Collén et al., 2004). In the present study, the transcript levels of the UPR transcription factor gene hac1 and the UPR target genes pdi1 and bip1 were more abundant at low specific growth rates than at high growth rates. The result indicates that the UPR pathway was activated in response to increased production of secreted proteins, such as CBHI, and also in response to the postulated limitation in the transport process under those conditions. For comparison, no such increase at low specific growth rates was observed in the transcript levels of ypt1 and sar1, which encode proteins involved in other functions in protein transport. It has been previously shown that ypt1 and sar1 transcript levels are not induced by endoplasmic reticulum stress caused by treatment of the cultures with DTT, an agent known to induce UPR via inhibition of protein folding and transport (Saloheimo et al., 2004).

In conclusion, our study offers new insight into the protein production characteristics of the industrially important host organism *Trichoderma reesei*. Production of extracellular proteins was favoured at low specific growth rates, which is beneficial for fed-batch processes where efficient protein production without extensive biomass formation is required. However, our study indicated that the capacity of the cells to transport the newly synthesized proteins is a limiting factor in the process under these conditions, and, for optimal protein production, strategies to improve the transport step should be designed. In addition, information on cellulase gene regulation at different growth rates and on consumption of the carbon and energy source was obtained to form a basis for further studies, especially in the field of starvation signalling for cellulase expression.

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REFERENCES


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