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ORIGINAL PAPER

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Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy

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Abstract A low-foaming hydrophobin II deletant of the *Trichoderma reesei* strain Rut-C30 was used for production of cellulases by continuous cultivation on lactose medium in a laboratory fermenter. The control paradigm of the addition of new medium to the continuous process was based on the growth dynamics of the fungus. A decrease in the rate of base addition to the cultivation for pH-minimum control was used as an indicator of imminent exhaustion of carbon source for growth and enzyme induction. When the amount of base added per 5 min computation cycle decreased below a given value, new medium was added to the fermenter. When base addition for pH control thereafter increased above the criterion value, due to increased growth, the medium feed was discontinued or decreased. The medium feeding protocol employed was successful in locking the fungus in the stage of imminent, but not actual, exhaustion of carbon source. According to the results of a batch cultivation of the same strain on the same medium, this is the phase of maximal enzyme productivity. The medium addition protocol used in this work resulted in a very stable continuous process, in which cellulase productivity was maintained for several hundred hours at the maximum level observed in a batch cultivation for only about 10 h. Despite a major technical disturbance after about 420 h, the process was restored to stability. When the cultivation was terminated after 650 h, the level of enzyme production was still maximal, with no signs of instability of the process.

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Introduction

A fundamental problem in the development of a continuous process for the production of fungal enzymes, such as cellulases by *Trichoderma reesei*, is the maintenance of conditions for maximal enzyme production. In a chemostat driven at a fixed dilution rate, the physiological state of the microorganism may not always be kept at its optimum for enzyme production, as a result of which the physiological balance may be set back from production to non-productive cell growth or forward to unwanted sporulation, leading in both cases to decreased enzyme productivity. The balance between these two undesirable physiological stages and the maximum productive stage on the basis of a fixed, time-dependent feeding rate over a long period of time is rather sensitive.

Many filamentous fungi, including *T. reesei*, show a strong tendency to decrease the pH of their culture medium during growth on carbohydrate substrates (e.g. Bailey and Viikari 1993; Bailey et al. 2002). After exhaustion of the carbon source, a marked increase in pH is observed. In batch cultivation for production of cellulases, the most efficient enzyme production by *T. reesei* occurs during decreasing substrate availability in the period immediately before and soon after exhaustion of carbon source from the medium. At the same time, the decreasing trend in pH weakens, ends and turns to an increasing trend.

The timing and dynamics of pH changes during growth of *T. reesei* on lactose lend themselves to their use as a control parameter in continuous fermenter cultivation for production of cellulases. Using the observed change in pH behaviour around the period of maximal cellulase production as the signal for medium addition, it should be possible to create a feeding program to maintain maximal productivity.

In an earlier, unpublished work to develop a continuous process with an industrially exploited *T. reesei* strain on lactose-based medium, a strategy of connecting a supply of new medium to the pump connected to upper-limit pH control was employed (M. Bailey, unpublished).

Thus, when the carbon source was exhausted, the pH of the culture increased and new medium (at the same pH, 4.8) was added instead of acid. Constant volume of the culture was maintained with the aid of a load cell and a harvest pump. After some time, the culture pH began to decrease spontaneously as a result of resumed growth on the carbon source in the added medium. Thus, the pH upper-limit pump was automatically switched off as the pH of the culture decreased. Continuing enzyme production was obtained by self-regulated repeating of this cycle of events.

In the present work, the procedure described above was refined and temporally adjusted using a computer control program. Rather than waiting for the increase in pH associated with exhaustion of substrate, the program was used to govern medium addition on the basis of a decrease in the rate of base addition for lower-limit pH control, at constant pH. Thus, the fungal biomass was to be maintained in the phase of imminent substrate exhaustion, equivalent to the period of maximal productivity on the macroscopic scale. The procedure described was also expected to be adaptable to other fungal enzyme production processes with similar pH behaviour patterns as that normally observed in *T. reesei*: a strong decreasing trend during growth, followed by an upward shift in pH after exhaustion of the carbon source.

Materials and methods

Fungal strain

The *T. reesei* strain chosen for this experiment was the Δ*hfb2* transformant VTT D-99676 (Nakari-Setälä et al. 2002; Bailey et al. 2002) of the hypercellulolytic mutant strain Rut-C30 (Montenecourt and Eveleigh 1979). Because the transformant does not produce hydrophobin II protein (HFBII), it does not induce foaming of the culture medium in aerated and agitated fermenter cultivations (Bailey et al. 2002).

Cultivation methods

The inoculum was cultivated starting from a stock spore suspension maintained in 15% glycerol at −80 °C. The buffered medium contained 20 g lactose l^{-1} and other components as described previously (Bailey et al. 2002) and was grown in a single stage of 3x 200 ml, 28 °C, 200 rpm, 2 days. The fermenter medium contained 40 g lactose l^{-1}, 4 g peptone l^{-1}, 1 g yeast extract l^{-1} and mineral salts as described previously (Bailey et al. 2002), along with 2x trace elements (Mandels and Weber 1969). The fermenter was a New Brunswick Scientific (N.J., USA) BioFlo IV, working volume 7.0 l. Cultivation conditions were: temperature 28 °C, pH>4.0 (correction with 7.5% NH_4OH), dissolved oxygen (DO)>30% (agitation 400–800 rpm), aeration 5 l min^{-1}. During the continuous phase of the cultivation, the volume of the culture was kept constant using a harvest pump operating according to the signal from a level probe, adjusted to a volume of 7.0 l. The fermenter was coupled to a computer running the fermenter control software AFS BioCommand version 2.62 (New Brunswick Scientific). This software was used to create a control diagram maintaining fungal growth in the required state of near-exhaustion of carbon source. After some empirical adjustments, the final version of the control diagram (Fig. 1) was as follows:

Line 1. Calculate BASETOT, the cumulative amount of base (ml) fed to the fermenter for pH lower limit control by the pump Feed 1 (100% output=3.4 ml $min^{-1}$with the tube size used). The parameter is automatically updated by the BioCommand program at intervals of 0.5 min.

Line 2. At intervals of 5 min (measurement over a 0.5 min period=one SAMPLE in the cyclic measurement protocol), the value BASPREV (=the previous value of Basetot, now 5 min old) is subtracted from the new value of BASETOT (which includes the volume of base added in the 5 min increment since the previous measurement=sample). This gives a new value to the output parameter DELTABAS, i.e. the increment in base addition (ml) during the past 5 min.

Line 3. At 5-min intervals, a new value of BASPREV is created by importing the current value of BASETOT (from line 1). This is the next new value of BASEPREV to be fed to the cyclic calculation on line 2.

Line 4. IF the calculated value of DELTABAS is less than 0.4 (low/no base consumption in the elapsed 5-min time sample) AND IF pH<4.1 (i.e. growth has not stopped), then the medium feed pump (Feed 2) will be given a value of 50% (=3.4 ml $min^{-1}$with the tube size used). IF either of these constraints is false, then the feed pump will be set to 15% (1.0 ml min^{-1}). Note that at the beginning of the continuous phase the lower setting was 0%.

Line 5. Calculate FEEDTOT, the cumulative amount of medium (ml) fed to the fermenter during the continuous phase by the addition pump Feed 2 (100% output=6.8 ml min^{-1}). The parameter is automatically updated at intervals of 0.5 min.

Analyses

Biomass dry weight was measured gravimetrically. Lactose was assayed enzymatically using the Boehringer-Mannheim (Germany) assay kit and following the manufacturer's instructions. Soluble extracellular protein was assayed by the method of Lowry et al. (1951) after precipitation of proteins with an equal volume of 10% trichloracetic acid and using bovine serum albumin as standard. The assays for overall cellulase activity (filter-paper units, FPU) and for β-1,4-endoglucanase with hydroxyethyl cellulose (HEC) as substrate were carried out at pH 5.0 using standard methods (IUPAC 1987).

Cellobiohydrolase I (CBHI), the main protein component of the cellulases produced by *T. reesei*, was assayed using a method based on hydrolysis of the chromophoric substrate 4-methyl umbelliferyl-β-D-lactoside (MUL, van Tilbeurgh et al. 1982). CBHI, endoglucanase I (EGI) and β-glucosidase (βG) all hydrolyse the MUL substrate, releasing the fluorescent component methyl umbelliferone (MU). MU can be measured spectrophotometrically in an alkaline environment. In the assay, the action of β-glucosidase is inhibited by making the reaction mixture 100 mM with respect to glucose. The reaction is carried out under two sets of conditions. In the first assay, the reaction mixture does not contain cellobiose in addition to 100 mM glucose. In the second assay, the reaction mixture also contains 5 mM cellobiose (CB). In the first assay, both CBHI and EGI are active, whereas in the second only EGI is active because the presence of 5 mM CB inhibits CBHI (van Tilbeurgh and Claeyssens, 1985). The contribution of CBHI to the result of the first assay can therefore be calculated by subtraction. According to available information, neither CBHII nor EGII of *T. reesei* react with MUL substrate (van Tilbeurgh et al. 1988; Reinikainen 1994). The accuracy of the subtraction method for estimation of CBHI activity was approximately 85% (5% residual activity of CBHI and 10% inhibition of EGI in the presence of 5 mM CB, results not shown).

The substrate was 1 mM MUL (Sigma M-2405, MW 500.5) in 50 mM Na-citrate buffer, pH 5.0. For the assay, 800 μl of substrate was added to two test tubes in a water bath at 50 °C. To one of the tubes was added 100 μl of a solution of 1.0 M glucose in citrate buffer. To the other tube was added 100 μl of a mixture containing 1.0 M glucose and 50 mM cellobiose. After temperating, 100 μl of

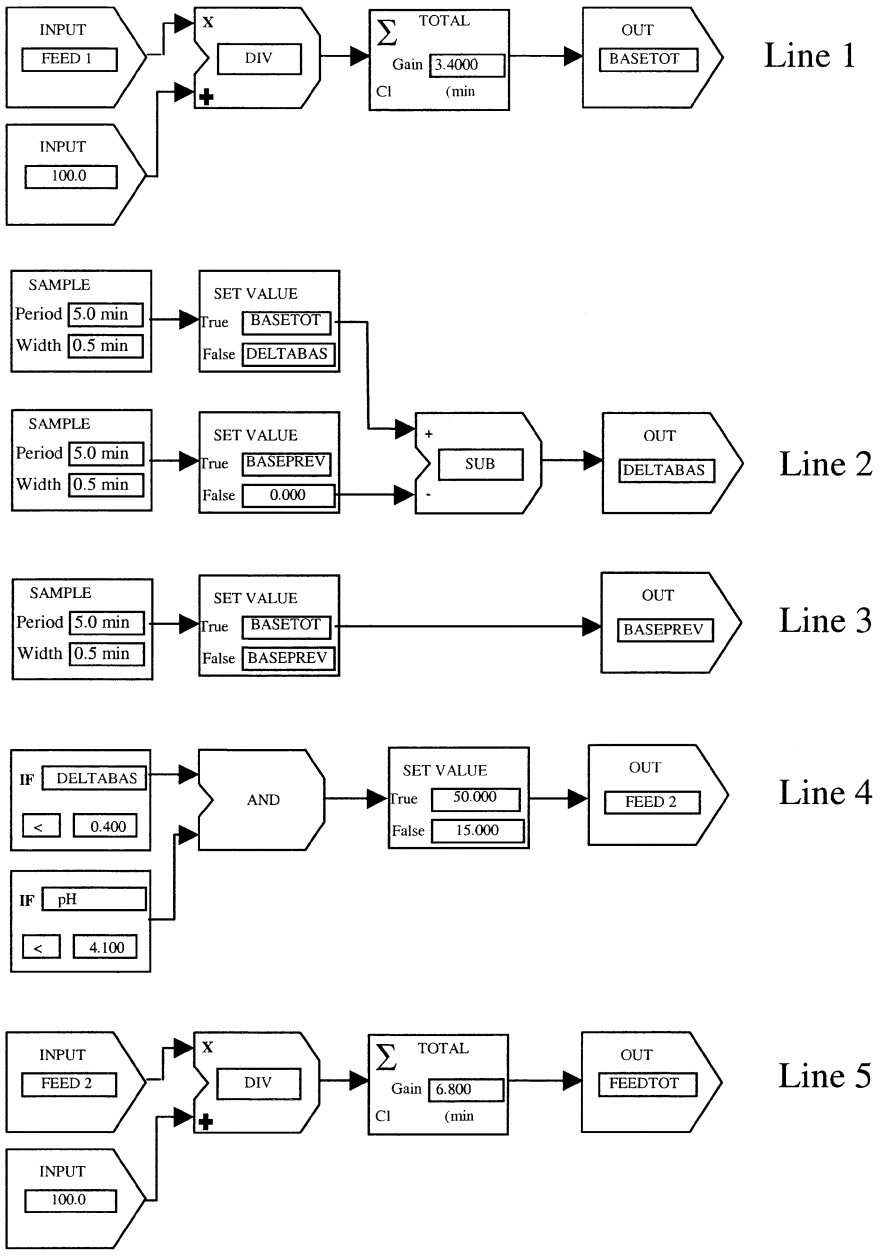

Fig. 1 Representation of the control diagram used in the fermenter control computer for monitoring and adjustment of feeding of new medium to the continuous cultivation

sample (dilutions in citrate buffer) was added to each tube and the mixture was vortexed and incubated at 50 °C for 600 s (10 min). The reaction was terminated by addition of 1.0 ml 1.0 M Na_2CO_3 and mixing. After cooling, the fluorescence produced in the reaction by release of MU was measured at 370 nm against a reagent blank containing substrate, glucose solution and Na_2CO_3. When necessary, relevant enzyme blanks were also used. The absorbance values obtained were converted to enzyme activity units (nkat ml^{-1}) using a standard line prepared with 1.0-ml aliquots of dilutions of a stock solution of the standard, 0.1 mM MU (Aldrich 12,872-4, MW 176.2) in 1.0 M Na_2CO_3 mixed with 800 μl substrate, 100 μl 1.0 M glucose solution and 100 μl citrate buffer. The standard dilutions used were between 1:1 (undiluted) and 1:10. In the calculation of activity, the fact that the volume of standard solution was 1.0 ml (100 μl of enzyme) was taken into account. The reason for the higher volume of standard solution was the limited solubility of MU. A detailed method protocol (method VTT-3712-2000) is available from one of the authors (MJB).

Results

The continuous process developed in this work was based on earlier experimentation using medium addition in place of acid for pH upper-limit control. Initially, this strategy did lead to continuing production. However, after about 150 h of continuous operation, the production efficiency of the system decreased, probably due to repeated oscillation of the fungal metabolism between growth and starvation. The activities assayed in the culture removed from the fermenter remained at a high level, but the volume of culture removed per unit time decreased steadily due to weakening growth (decrease in pH reduction after addition of new medium). On the basis of overall fermenter productivity, taking into account the

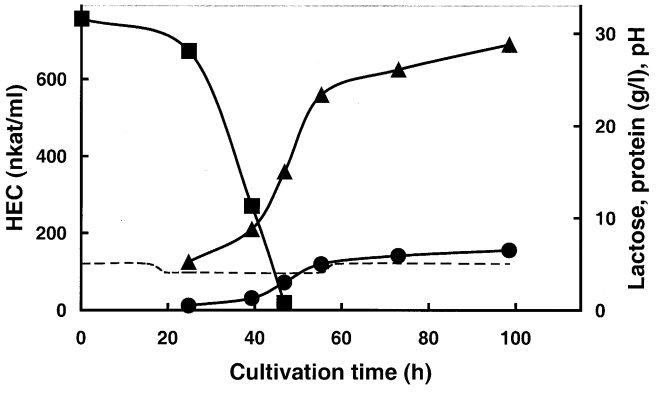

Fig. 2 Profile of a batch cultivation of *Trichoderma reesei* VTT D-99676 on lactose-peptone medium in a 7-l laboratory fermenter (Bailey et al. 2002). Growth parameters were represented by lactose consumption (*filled squares*) and changes in culture pH (*dotted line*). Production of cellulases was represented by measurement of total soluble extracellular protein in the culture filtrate (*filled circles*) and by assays of endo-1,4-β-glucanase using hydroxyethyl cellulose (HEC) as substrate (*filled triangles*)

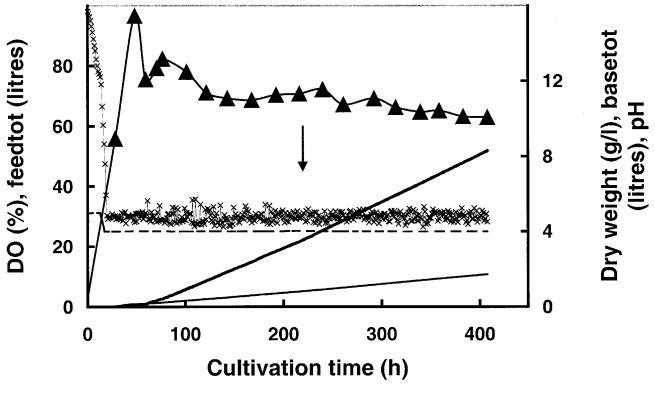

Fig. 3 Growth parameters measured during start-up of the continuous cultivation and the uninterrupted period of continuous operation (0–407 h, see text for details): *filled triangles* biomass dry weight, *dotted line* culture pH, *hatched line with crosses* dissolved oxygen (DO), *thicker solid line* cumulative medium feed volume, feedtot, *thinner solid line* cumulative base volume for pH control, basetot. The minimum setpoint value of the feeding pump was increased from 0 to 15% at 215 h (*arrowed*, see text for details)

inevitable down-time associated with batch cultivation, the system was considered to be economically advantageous for a period equivalent to approximately four batch cultivations

Typical production of cellulase (endoglucanase, HEC) and soluble protein by the *T. reesei* Δ*hfb2* transformant VTT D-99676 in batch cultivation (described in Bailey et al. 2002) on lactose medium is presented in Fig. 2. Note that high productivity of cellulases begins before exhaustion of carbon source (lactose) from the medium. Lactose, although a soluble disaccharide, is not a readily available carbon source for *T. reesei*, due apparently to the growth-limiting level of β-galactosidase produced by this fungus (unpublished observations), and does not cause catabolite repression.

In the continuous-production experiment described in this work, the initial batch phase was run for 45 h. The culture pH decreased to 4.0 (at 18 h), DO to approximately 30% and biomass dry weight reached a maximum of 15.5 g l^{-1}, which is typical for this strain and medium (Bailey et al. 2002). Due to addition of medium in the continuous phase, starting at 45 h, the lactose concentration of the culture was close to the detection level of the assay (0–0.1 g l^{-1}) for the entire duration of the continuous operation (results not shown). After 45 h, consumption of base for lower-limit pH control began to decrease and the control paradigm DELTABAS was activated in the computer. The final version of the control diagram (Fig. 1) was adopted at 216 h. Whenever base consumption for lower-limit pH control decreased below the assigned criterion value, new medium (at pH 4) was added to the fermenter. This caused an increase in base consumption for pH control, a consequent increase in the value of DELTABAS above the criterion value, and halting or slowing of the addition of new medium according to the False criterion in line 4 of the control diagram. The culture level (7.0 l) was maintained on the basis of the signal from the level probe. Note that because the parameter DELTABAS was based on temporal variation in consumption of pH correctant, it was important that the base used to replenish the NH_4OH supply was always diluted to exactly the same concentration (7.5%).

Due to the feeding paradigm used, the growing culture was successfully maintained in a state of near-exhaustion of carbon source. This is also the phase of maximal enzyme production in batch cultivations (see Fig. 2). The level of biomass in the culture remained approximately constant, and utilisation of new medium and of base for pH control increased linearly (Fig. 3). Maximal enzyme productivity was successfully maintained for the uninterrupted period of continuous cultivation between sampling at 101 and 407 h, with no indication of decreased productivity. This is clearly indicated in Fig. 4, in which the levels of HEC, CBHI and FPU activity and soluble protein are displayed along with the overall increase in total feed volume.

During the first hours of the continuous phase (45–101 h), different criterion values of DELTABAS for Line 4 of the control diagram were tested, starting from 0.1. The value eventually chosen for this parameter was 0.4 (from 77 h), i.e. new medium was added to the fermenter if consumption of base for pH control was below 0.4 ml in 5 min (0.01 ml min^{-1} per l of fermenter volume). Until 215 h, addition of new medium was at a rate of 3.4 ml min^{-1} (dilution rate D=0.029 h^{-1}) or 0 according to the value of DELTABAS. After 215 h, the lower alternative was changed to 1.0 ml min^{-1} (minimum feed pump setting 15%, D=0.0087 h^{-1}), in order to avoid excessive oscillation of substrate availability. The overall productivity of raw culture during the period of uninterrupted continuous cultivation was 45.9 l in 306 h or 0.15 l h^{-1} (D=0.02 h^{-1}), i.e. the fermenter produced approximately one working volume (7 l) in 2 days. The change in the feeding

Table 1 Volumetric productivities of cellulase (HEC) activity and soluble protein in batch and continuous cultivations of *Trichoderma reesei* VTT D-99676 on lactose medium. Values of HEC and protein assayed during the various time spans are presented, along with the calculated productivities for the time period in question. Mean values expressed for the harvest from the continuous cultivation were obtained by averaging the assay results obtained from the analysis points presented in the curves in Fig. 4 between 101 and 407 h. Because the time elapse and therefore feed volumes between samplings varied, the mean values presented are not exact, but they represent a good approximation. The formula used for calculation of volumetric productivities was: {units/l produced}×{harvest volume}/{time period}×{fermenter volume}. Note that in the case of HEC activity, nkat ml^{-1} is numerically equivalent to μkat l^{-1}

Cultivation mode	Sampling period (h)	Time span (h)	Harvest volume (l)	HEC produced		Protein produced	
				nkat ml^{-1}	μkat l^{-1} h^{-1}	g l^{-1}	g l^{-1} h^{-1}
Batch	98.5	98.5	7	690	7.0	6.5	0.07
Batch	73	73	7	625	8.6	5.9	0.08
Batch	46.8→56.3	9.5	7	360→560=200	21.1	3.0→5.0=2.0	0.21
Continuous	101→407	306	45.9	903 (mean)	19.4	10.3 (mean)	0.22

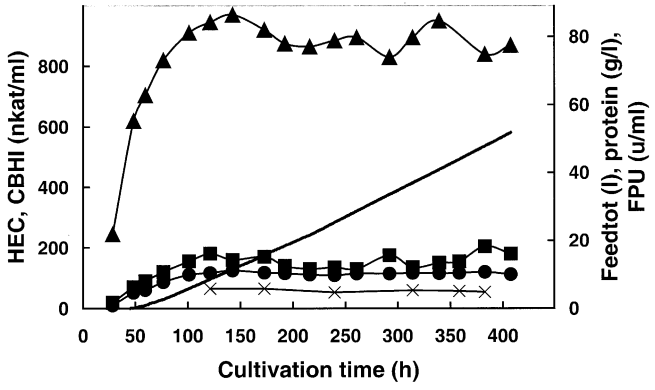

Fig. 4 Enzyme production during start-up of the continuous cultivation and the uninterrupted period of continuous operation (0–407 h, see text for details): endo-β-1,4-glucanase using hydroxyethyl cellulose (HEC) as substrate (*filled triangles*); *filled squares* cellobiohydrolase I(CBHI), *crosses* activity against filter paper (FPU), *filled circles* total soluble extracellular protein. The cumulative medium feed volume during the continuous phase, feedtot (*solid line*) is also shown

programme at 215 h caused a small but discernable increase in the overall rate of medium addition to the cultivation (arrowed in Fig. 3). Although the throughput increased, the enzyme activity levels in the culture removed from the fermenter remained approximately constant (Fig. 4) and therefore the productivity of the fermenter increased slightly. This small change was not taken into account in the calculation of volumetric enzyme/protein productivity in the following.

The calculated volumetric productivity of the 7-l fermenter during the period of uninterrupted continuous cultivation (101–407 h) is presented in Table 1. For simplicity, the results are presented as single (averaged) figures over the specified time period because the assayed levels were rather similar throughout this period and the amount of medium added to the fermenter increased almost linearly with time (see Fig. 4). The calculated productivities of HEC activity and soluble extracellular protein in the continuous phase were very similar to the maximum productivities observed during batch cultivation (46.8–56.3 h). Compared with the overall batch cultivation (0–73 or 0–98 h), the productivity of the fermenter in the continuous cultivation was very high. Taking into account the inevitable down-time associated with repeated batch cultivations, the advantage of the continuous mode would of course be even greater. Calculated volumetric productivities of CBHI and FPU activity in the continuous cultivation (101–407 h) were 3.4 μkat l^{-1} h^{-1} and 96.4 u l^{-1} h^{-1}, respectively. For the overall batch run (0→98.5 h), the corresponding figures were 1.1 μkat l^{-1} h^{-1} and 41.6 u l^{-1} h^{-1} (primary data not shown).

Specific production of extracellular soluble protein was calculated for the batch and continuous cultivations. Protein production in the batch process (at 98 h) was 6.5 g l^{-1} and the maximum production of biomass (at 47 h) was 17.3 g l^{-1}. This gives a protein productivity of 0.38 g g^{-1} biomass dry weight. In the continuous cultivation (101–407), the mean protein and biomass concentrations assayed were 10.3 and 11.1 g l^{-1}, respectively, giving a specific protein productivity of 0.93 g g^{-1}. Specific productivity of CBHI in the batch process (final activity 110 nkat ml^{-1}) was 6.4 μkat g^{-1} and in the continuous run 14.1 μkat g^{-1} (mean activity 157 nkat ml^{-1}). Thus, as in the case of the volumetric productivities, specific production of soluble protein and of cellulase activity was more than twofold higher in the continuous cultivation than in the batch process.

Although the Δ*hfb2* transformant strain used in this work was known to sporulate heavily in batch cultivations very soon after exhaustion of carbon source from the medium (Bailey et al. 2002), no sporulation was observed during examination of the culture by phase-contrast microscopy. A 50-ml sample of the culture was removed at 216 h and transferred to a sterile 250-ml shake flask. This flask was incubated at 200 rpm and 28 °C for 1 day (to 239 h overall cultivation). The pH of the flask culture increased during this period to 6.5 and the culture became characteristically green due to the formation of large numbers of spores, clearly visible under the microscope. By contrast, no sporulation was observed in microscopy of the culture in the fermenter at 239 h. A similar observation was again made after a run time of 360 h (shake cultivation to 382 h). Clearly, although the

medium feeding program used resulted in a state of limited substrate availability, actual exhaustion of growth substrate (with consequent sporulation) did not occur.

At approximately 420 h, a major process disturbance occurred. The fungus grew into the headspace of the fermenter and covered the level probe. This resulted in a false-positive signal (level high at all times), the level pump was therefore continuously activated and the fermenter was emptied to the level of the sample valve used for harvesting (approximately 1.5 l). At 445 h, the fermenter was filled to volume with sterilised medium and the run was, in effect, restarted in batch mode. At 480 h, the DELTABAS control paradigm was reinstated and continuous operation was commenced as before. Despite this disturbance, with dramatic decrease in the levels of all activities at the beginning of the new batch phase due to dilution with new medium, the continuous running was again successful (results not shown). Addition of medium increased linearly for a further period of 1 week (485–650 h), with enzyme activities even very slightly higher than those before the disturbance. After 650 h, when the run was ended, there were still no signs of decreased enzyme productivity.

Discussion

Indication of the stability problems associated with continuous enzyme production was clearly observed in the preliminary, unreported experiment in which upper limit pH control was used as the control parameter for medium addition. In this case, fungal metabolism progressed from imminent to actual exhaustion of carbon source (indicated by increase in pH). This led to the situation in which at least part of the biomass started to sporulate, after which a return to the productive phase no longer occurred. After a period of about 150 h, the productivity of this system began to decrease.

The medium feeding regime used in the continuous cultivation described in the present work was dynamic, governed by the physiological state of the growing fungus. As a result of the dependence of feeding rate on the rate of uptake of base for pH control at constant pH, the amount of medium added to the cultivation per unit time varied according to the availability of lactose, as measured indirectly by the rate of uptake of base. Although this variation was true over short periods of time, the system as a whole was very stable for several hundred hours, and the culture was maintained in the phase of imminent carbon-source exhaustion, which was also the most productive phase in a batch cultivation. Importantly, however, the carbon source was never completely exhausted, as was indicated by the observed non-sporulation of the culture throughout the period of continuous operation. This fact led to the observed stability of production over a period of more than 300 h of uninterrupted operation.

In this work, no attempt was made to optimise either the medium (compostion or concentration) or the cultivation conditions. Higher productivities than those recorded in this work have been obtained using more highly developed fungal strains and higher medium concentrations (for a review see Tolan and Foody 1999). Nonetheless, the cultivation method described would lend itself very well to investigations of parameter variation for improved productivity with any strain of *T. reesei* growing on a soluble production medium. Furthermore, the same method could be applied for optimisation of the production of secondary metabolites by other fungi (e.g. *Aspergillus, Penicillium*) and other microorganisms with a similar pH behaviour, i.e. maximum productivity during the phase of imminent carbon-source exhaustion as indicated by decreasing base consumption for pH-minimum control. The method described in this work has also been used successfully to control medium addition to a fed-batch process for production of a double cellulose-binding-domain fusion protein in *T. reesei* (Bailey, unpublished).

The advantages of efficient continuous production of a secondary metabolite over batch production are considerable. In the case of the process studied, a batch process required approximately 3 days to reach the economically maximum level of activity (see Fig. 2).

The measured maximum levels of enzyme activities obtained in batch cultivation were clearly exceeded in the continuous cultivation (see Table 1), but, more importantly, the length of the phase of maximum productivity was extended from only 10 h to over 300 h in the uninterrupted continuous cultivation. In conclusion, the cultivation method described in this work is a dynamic and stable, self-adjusting protocol for continuous production of cellulases or other proteins by the fungus *T. reesei* for an apparently indefinite period.

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References

Bailey MJ, Viikari L (1993) Production of xylanases by *Aspergillus fumigatus* and *Aspergillus oryzae* on xylan-based media. World J Microbiol Biotechnol 9:80–84

Bailey MJ, Askolin S, Hörhammer N, Tenkanen M, Linder M, Penttilä M, Nakari-Setälä T (2002) Process technological effects of deletion and amplification of hydrophobins I and II in transformants of *Trichoderma reesei.* Appl Microbiol Biotechnol 58:721–727

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

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (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

Montenecourt BS, Eveleigh DE (1979) Selective screening methods for the isolation of high-yielding cellulase mutants of Trichoderma reesei. Adv Chem Ser 181:289–301

Nakari-Setälä T, Penttilä M, Bailey M, Tenkanen M (2002) Improved biotechnical production method (Parannettu biotekninen tuotantomenetelmä). Finnish Patent 108863 (in Finnish)

Reinikainen T (1994) The cellulose-binding domain of cellobiohydrolase I from *Trichoderma reesei*. VTT Publications 206, VTT, PO Box 2000, 02044 VTT, Finland

van Tilbeurgh H, Claeyssens M (1985) Detection and differentiation of cellulase components using low molecular mass fluorogenic substrates. FEBS Lett 187:283–288

van Tilbeurgh H, Claeyssens M, De Bruyne CK (1982) The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes. FEBS Lett 149:152–156

van Tilbeurgh H, Loontiens FG, De Bruyne CK, Claeyssens M (1988) Fluorogenic and chromogenic glycosides as substrates and ligands of carbohydrases. Methods Enzymol 160:45–59

Tolan JS, Foody B (1999) Cellulase from submerged fermentations. Adv Biochem Eng Biotechnol 65:41–67