Growth and product formation in chemostat and recycling cultures by *Aspergillus niger* N402 and a glucoamylase overproducing transformant, provided with multiple copies of the *glaA* gene

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Continuous and recycling cultures were carried out with *Aspergillus niger* N402 wild-type and a glucoamylase overproducing transformant to investigate growth and product formation characteristics. In shake flask cultures, the amount of glucoamylase produced by the transformant was about five times more than by the wild-type strain. In contrast with these results, a twofold overproduction was found in glucose-limited continuous cultures, while no overproduction was found under maltodextrin-limitation. Two regions of specific growth rates could be distinguished, one at specific growth rates lower (domain I) and one at specific growth rates higher than 0.12 h^{-1} (domain II). In domain I changes in mycelium morphology and conidia formation were observed. It has been concluded that maintenance requirements are dependent on the specific growth rate over the whole range of measured growth rates. The deviation in linearity in the linear equation of substrate utilization, caused by this phenomenon, should be considered when continuous cultures with filamentous fungi are performed. In recycling cultures, xylose as limiting carbon source repressed glucoamylase production very strongly. Under maltodextrin-limitation a fivefold overproduction was found. After about 150 h, the total amount of glucoamylase produced was still increasing, while total amount of product, measured as carbon, remained constant. After this time no increase in the amount of biomass formed was observed. These results suggest autolysis and cryptic growth taking place in a recycling fermenter and cell death rate equalling growth rate.

Introduction

Filamentous fungi represent a physiologically diverse group of micro-organisms. They grow slowly, compared with bacteria. Their growth-form can be long, thin, branched threads of mycelium, but also compact mycelial pellets. These morphologically different growth-forms make them rather difficult to handle and introduce problems of heterogeneity.

On a microscopic scale, for example, pellets are subject to internal substrate and oxygen limitations (Braun &

Vecht-Lifshitz, 1991). The filamentous growth-form, however, poses problems, especially in continuous culture fermentations, due to the tendency of the organism to accumulate on the walls and probes of the culture vessel. Furthermore, accumulation in the outflow system acts as a filter, allowing medium to flow through, while the mycelium partially remains (Pirt & Callow, 1960; Righelato & Pirt, 1967). The growth form of the mycelium can also influence the characteristics of product formation (Pirt & Callow, 1959; Whitaker, 1992).

The economic and social importance of fungi as sources of food and biologically active metabolites has stimulated considerable interest in their growth and physiology (Berry, 1975; Righelato, 1975).

The use of filamentous fungi and bacteria for commercial enzyme production has been developed in

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this century. The filamentous fungi of the genus *Aspergillus* are organisms of considerable importance for a variety of biotechnological industries. In addition to their well documented use in starch processing industries (Norman, 1979), several *Aspergillus* species are used for the production of secondary metabolites and hydrolytic enzymes (Blain, 1975; Bhella & Altesaar, 1988).

One of these enzymes is glucoamylase (GLA; α -1,4-glucan glucohydrolase, EC 3.2.1.3), which can be used for the conversion of starch, glycogen and other polyglucose dextrans to D-glucose and malto-oligosaccharides. Syrups containing high levels of glucose and maltose, industrially produced from starch by using glucoamylase, are widely used in the soft-candy, brewing, baking, soft drinks and canning industries (Norman, 1979; Manjunath *et al.*, 1983).

There is considerable interest in methods to improve the production of glucoamylase by *Aspergillus*. One way to achieve this is by increasing the number of copies of the glucoamylase gene. In general, an increase in the number of copies results in an increased production of the enzyme encoded by the multiplied gene.

For the application of genetically engineered strains in industrial processes, it is important that the newly introduced gene copies should be expressed under various conditions used in production processes. Detailed studies on the physiology of protein overproduction by transformant fungi under different fermentation conditions have not been performed to date. This paper reports on the growth and production characteristics of wild-type *Aspergillus niger* and a glucoamylase overproducing *A. niger* transformant, grown in continuous culture and in the recycling fermenter with 100% biomass feedback.

Methods

Strains and growth conditions. Wild-type *Aspergillus niger* N402 (*cspA1* derivative of ATCC 9029; Bos *et al.*, 1988) and a glucoamylase overproducing transformant, *A. niger* N402[pAB6-10]B1 (Verdoes *et al.*, 1993), supplied by and constructed at TNO Medical Biological Laboratory, Rijswijk, The Netherlands, were used. Transformation was based on the use of the *amdS* selection marker and a cosmid vector, containing four copies of the glucoamylase gene (*glaA*) (Verdoes *et al.*, 1993). Both organisms were maintained on potato-dextrose agar slants.

The medium composition was as described by Anderson & Smith (1971) with slight modifications. It contained, per litre of distilled water: $(\text{NH}_4)_2\text{SO}_4$, 1.98 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.234 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 6.3 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1 mg; and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3.5 mg. The medium was sterilized in the absence of carbon source by autoclaving. The carbon source was sterilized for 30 min at 100 °C and added aseptically.

In batch and continuous cultures carbon and energy sources were either 10 mM-glucose or 1.64 g maltodextrin l^{-1} (dextrin 10, Fluka). The composition of dextrin 10 is not known exactly, but it contains about 10% of reducing matter, which means that the mean chain

length of this maltodextrin is 10 glucose units. Therefore the mean M_r is 163.8. Thus 1.64 g l^{-1} corresponds to 10 mM of C_6 units. In recycling experiments carbon- and energy-sources were either 20 mM-glucose or 24 mM-xylose.

Initial characterization was carried out in 500 ml Erlenmeyer flasks containing 100 ml of medium. They were grown in a New Brunswick Scientific shaker incubator at 250 r.p.m. at 30 °C. The cultures were harvested 48 h after inoculation and the dry weight and glucoamylase activity were determined.

Aerobic carbon-limited chemostat experiments were carried out in a fermenter apparatus designed and manufactured by the electronics and mechanics workshops of the Faculty of Biology, Vrije Universiteit, Amsterdam, The Netherlands (Hanegraaf *et al.*, 1991). A 2 litre fermenter vessel with a working volume of 1.5 litre was used. The culture volume was controlled by means of a stainless steel standpipe (overflow) inserted through the baseplate; the exhaust gas was led through this pipe also, to avoid accumulation of the fungus in the overflow system. Agitation was obtained by flat blade, propeller-type impellers operating at about 700 r.p.m. When necessary wall growth was removed daily with a Teflon covered ring bar magnet, which could be moved over the wall with an external horseshoe magnet. Culture pH was controlled at 4.5 ± 0.1 by addition of 1 M-KOH, and the temperature was controlled at 30 °C. Steady states were assumed at the moment the rates of CO_2 production and O_2 consumption remained constant. Samples were taken for measurements after at least six volume changes had taken place. For every steady state a new culture was started by inoculation with spore suspension, thus eliminating the possibility of carry-over of material from previous steady states.

Aerobic carbon limited recycling experiments were carried out in a 2 litre fermenter vessel with a working volume of 1 litre. The culture volume was controlled by means of a liquid-level indicator and a recycling unit, both inserted through the top plate of the fermenter. Fig. 1 is a photograph of the internal recycling unit. The Teflon filters used had a pore-size of 0.22 μm (type GV, Millipore). The filters were sealed and placed over a socket of sintered steel. The liquid-level indicator regulated the speed of a peristaltic pump that kept the volume constant by withdrawing filtrate from the culture through the internal recycling unit, thus letting the biomass remain behind in the culture vessel. The whole recycling system has been designed and built by the electronics and mechanics workshops of the Faculty of Biology, Vrije Universiteit, Amsterdam, The Netherlands. Fig. 2 is a diagram of the recycling system. Agitation was obtained by flat blade, propeller-type impellers operating at about 700 r.p.m. Culture pH was controlled at 4.5 ± 0.1 by addition of 1 M-KOH, and the temperature was controlled at 30 °C.

In order to get good filamentous growth, for both chemostat and recycling experiments, the fermenter vessel was supplemented with CaCl_2 , added aseptically after sterilization of the medium to avoid precipitation (final concentration 46.7 mg l^{-1}), inoculated with a spore suspension (10^5 spores ml^{-1} , final concentration), and a batch phase started in which the pH was initially kept at 2.0, air was led over with a flow of 10 l h^{-1} and the agitation speed was kept at 250 r.p.m. to get oxygen limitation. The pH was shifted to a value of 4.5, the airflow to 30 litres h^{-1} , led in through the bottom of the vessel and the agitation speed to 700 r.p.m. after batch growth was nearly completed, and growth in continuous or recycling culture started.

Analytical procedures. Dry weight was determined by filtration over Millipore prefilters of constant weight (type AP 40), washing twice with equal volumes of water and drying at 105 °C. Oxygen uptake and carbon dioxide production were measured with a mass spectrometer (MM8-80F, VG Gas Analysis Systems).

Extracellular products were measured with a Total Organic Carbon Analyzer (type 915A, Beckman). The glucoamylase activity was measured as described by Yamasaki *et al.* (1977) with slight modifications as follows. A reaction mixture containing 0.2 ml 1%

Fig. 1

Fig. 1. Photograph of the internal recycling unit. In the middle, the socket of sintered steel is shown. It is covered by a sealed filter with a pore size of 0.22 µm, shown in the lower part of the picture. The upper part of the picture shows the assembled recycling unit, ready to be placed in the fermenter vessel through the top plate.

Fig. 2

Fig. 2. Schematic diagram of the recycling system. Nutrient is added at a constant speed to the culture vessel via pump A. The volume of the culture fluid in the culture vessel is kept constant by the liquid-level sensor B, which regulates the speed of pump D via level control unit C. Pump D removes the culture fluid through the internal recycling unit E, which is covered by a 0.22 µm Teflon filter, resulting in 100% biomass retention in the culture vessel.

(w/v) maltose, 0.5 ml 1 M-acetate buffer (pH 5.3) and 0.3 ml enzyme solution (culture supernatant sample), was incubated at 40 °C for 10 min. After incubation the reaction was stopped by heating in a boiling water bath. The glucose formed was measured by the UV method (Boehringer Mannheim kit). One unit of glucoamylase is defined as the amount of enzyme which forms 1 µmol glucose min⁻¹ under the conditions described.

Results

Glucoamylase production in shake flask cultures

Aspergillus niger N402 wild-type and transformant B1 (*A. niger* N402[pAB6-10]B1) were initially characterized in Erlenmeyer shake flasks. From Table 1 it can be seen that transformant B1 (containing 20 copies of the *glaA*

gene; see Verdoes *et al.*, 1993) produced 4.6 times more units glucoamylase per gram dry wt than the wild-type strain on both glucose and maltodextrin as sole carbon sources. On maltodextrin both strains produced about twice as much glucoamylase as on glucose.

To investigate the dependency of glucoamylase production on the specific growth rate (μ) of wild-type *A. niger* and overproducing transformant B1, both strains were grown in continuous culture. In this way it is also possible to determine the maximum growth yield (Y_{xsm}) and the maintenance coefficient (m_s), which are important parameters to investigate the energetic costs of glucoamylase production. When steady states are reached, it is possible to observe differences in culture characteristics and mycelium morphology at different specific growth rates.

Growth and glucoamylase production in continuous culture

The growth parameters of continuous culture experiments in which *A. niger* N402 wild-type and transformant B1 were grown on glucose as limiting carbon and energy source are given in Table 2. The maximum growth yields, Y_{xsm} and Y_{xom} , and the maintenance coefficients, m_s and m_{O_2} were calculated using the linear equation of substrate utilization (Pirt, 1965) as shown in equation 1

$$q_{s(O_2)} = \frac{\mu}{Y_{xsm(xom)}} + m_{s(O_2)} \quad (1)$$

Table 1. Copy numbers of *glaA* and glucoamylase production of *A. niger* N402 wild-type and glucoamylase overproducing transformant B1 after 48 h incubation in shake flasks with glucose and maltodextrin as sole carbon sources

Copy numbers are as determined by Verdoes *et al.* (1993).

Strain	<i>glaA</i> copy number	Glucoamylase activity [units (g dry wt) ⁻¹] on:	
		Glucose	Maltodextrin
N402 (wild-type)	1	127.7	268.7
N402[pAB6-10]B1	20	579	1236

Table 2. Growth parameters of *A. niger* N402 and transformant B1 grown aerobically in glucose limited continuous culture

q_s , q_{O_2} and q_{CO_2} are, respectively, specific rates of glucose and oxygen consumption and CO_2 production [$mmols (g \text{ dry wt})^{-1} h^{-1}$]. Y_{xsm} and Y_{xom} are, respectively, maximum growth yields on glucose and oxygen ($g \text{ dry wt mol}^{-1}$). m_s , m_{O_2} and m_{CO_2} are, respectively, maintenance utilization of substrate and oxygen and production of CO_2 [$mmols (g \text{ dry wt})^{-1} h^{-1}$]. The values of maintenance requirements and maximum yield were calculated by means of linear regression considering all data obtained from dilution rates within the range of $0.025 h^{-1} \leq D \leq 0.225 h^{-1}$; 95% confidence intervals are given in parentheses.

Parameter	<i>A. niger</i> N402 (wild-type)	<i>A. niger</i> N402[pAB6-10]B1
q_s	$0.061 \pm 10.00 \mu$	$0.029 \pm 10.385 \mu$
q_{CO_2}	$0.245 \pm 19.488 \mu$	$0.371 \pm 19.336 \mu$
q_{O_2}	$0.369 \pm 18.287 \mu$	$0.426 \pm 18.47 \mu$
Y_{xsm}	100 (88.14–115.6)	96.29 (85.94–109.5)
Y_{xom}	54.68 (46.08–67.25)	54.14 (42.59–74.28)
m_s	$0.061 (-0.136-0.258)$	$0.029 (-0.108-0.110)$
m_{CO_2}	$0.245 (-0.290-0.779)$	$0.371 (-0.350-1.093)$
m_{O_2}	$0.369 (-0.130-0.868)$	$0.426 (-0.175-1.027)$
Carbon balance	94.4%	97.1%
Energy balance	105.4%	104.9%

Fig. 3. Specific glucoamylase production (q_{GLA}) versus the specific growth rate (μ) of glucose- (a) and maltodextrin- (b) limited chemostat cultures of wild type *A. niger* N402 (●) and transformant *A. niger* N402[pAB6-10]B1 (○).

in which q is the specific rate of substrate consumption, Y is the molar growth yield and m is the maintenance coefficient. As can be seen from Table 2, there was no significant difference between the values of the measured

Table 3. Specific rate of glucoamylase production (q_{GLA}) [units ($g \text{ dry wt})^{-1} h^{-1}$] of *A. niger* N402 wild-type and transformant B1, grown aerobically in glucose- and maltodextrin-limited continuous culture

Data were fitted within the range of $0.025 h^{-1} \leq \mu \leq 0.15 h^{-1}$ in the case of maltodextrin-limitation and within the range of $0.025 h^{-1} \leq \mu \leq 0.225 h^{-1}$ in the case of glucose-limitation.

Carbon source	<i>A. niger</i> N402 (wild-type)	<i>A. niger</i> N402[pAB6-10]B1
Glucose	$11.6 \pm 271.6 \mu$	$30.46 \pm 663.75 \mu$
Maltodextrin	$6.8 \pm 948.21 \mu$	$13.94 \pm 1106.9 \mu$

parameters of the wild-type and transformant B1. Carbon recovery was somewhat lower than 100%, because the carbon balances were calculated without considering product formation. The energy balance was calculated as described by Metwally *et al.* (1991), using the balance method described by Roels (1980).

Fig. 3(a) shows the specific rate of glucoamylase production, q_{GLA} (units per gram dry wt per h) versus the specific growth rate of wild-type *A. niger* N402 and transformant B1, grown in glucose-limited continuous culture. In Table 3 the calculated relation between the specific rate of glucoamylase production and the specific growth rate is given for both strains, grown on glucose. The specific rate of glucoamylase production of transformant B1 was about twice that of the wild-type strain.

The growth parameters of continuous culture experiments in which *A. niger* N402 wild-type and transformant B1 were grown on maltodextrin as limiting carbon and energy source are given in Table 4. The same

Table 4. Growth parameters (on a C_6 base) of *A. niger* N402 and transformant B1 grown aerobically in maltodextrin-limited continuous culture

See Table 2 legend for parameter definitions.

Parameter	<i>A. niger</i> N402 (wild-type)	<i>A. niger</i> N402[pAB6-10]B1
q_s	$0.061 + 11.14 \mu$	$0.0003 + 11.357 \mu$
q_{CO_2}	$0.298 + 19.646 \mu$	$0.293 + 18.157 \mu$
q_{O_2}	$0.292 + 19.082 \mu$	$0.253 + 18.39 \mu$
Y_{xsm}	89.60 (79.47–103.12)	88.05 (80.80–96.72)
Y_{xom}	52.41 (44.90–62.93)	54.37 (48.07–62.58)
m_s	$0.057 (-0.146-0.259)$	$0.0003 (-0.149-0.150)$
m_{CO_2}	$0.298 (-0.133-0.729)$	$0.293 (-0.013-0.599)$
m_{O_2}	$0.292 (-0.155-0.739)$	$0.253 (-0.101-0.608)$
Carbon balance	85.5 %	87.11 %
Energy balance	106 %	106.7 %

Fig. 4. Mycelium of *A. niger* growing in submerged culture. (a) Mycelium growing at a dilution rate of about 0.05 h^{-1} ; (b) mycelium growing at a dilution rate of about 0.22 h^{-1} . Bars, $20 \mu\text{m}$.

calculations have been carried out as with the glucose-limited cultures. For a good comparison between the glucose and maltodextrin data, the specific rate of maltodextrin consumption, the maximum growth yield on maltodextrin and the maintenance requirements for maltodextrin are expressed on a C_6 basis. Here again the determined parameters of transformant B1 were not significantly different from those of the wild-type.

Fig. 3(b) shows the specific rate of glucoamylase production, q_{GLA} (units per gram dry wt per h) versus the specific growth rate of wild-type *A. niger* N402 and transformant B1, grown in maltodextrin-limited continuous culture. In Table 3 the calculated relation between the specific rate of glucoamylase production and the specific growth rate in the first linear part of Fig. 3(b) is given for both strains, grown on maltodextrin. At specific growth rates higher than 0.15 h^{-1} , the specific rate of glucoamylase production by the transformant did not increase with increasing μ . Therefore the relation between q_{GLA} and μ in the case of maltodextrin-limitation as mentioned in Table 3 only applies to specific growth rates lower than 0.15. There was no apparent difference

in specific rate of glucoamylase production between the wild-type strain and the transformant. Moreover, there was no difference in specific rate of glucoamylase production between transformant B1, grown on glucose and the two strains, grown on maltodextrin in the range $\mu = 0 \text{ h}^{-1}$ to 0.15 h^{-1} , to which linear regression was applied.

Mycelium morphology at different specific growth rates

At specific growth rates lower than 0.12 h^{-1} we observed a change in mycelium morphology: the hyphae were more branched and conidiation took place. At specific growth rates higher than 0.12 h^{-1} the hyphae were less branched and no conidiophores were formed. These morphological changes at different specific growth rates were most obvious in chemostat cultures with the wild-type strain. Photographs of culture samples taken at a low growth rate (μ about 0.05 h^{-1}) and a high growth rate (μ about 0.22 h^{-1}) are shown in Fig. 4.

Because of these observations we analysed the chemostat experiments in two domains, although, when linear

Fig. 5

Fig. 6

Fig. 5. Specific rate of glucose consumption (q_{glucose}) (a) and oxygen consumption (q_{O_2}) (b) versus the specific growth rate (μ) in glucose-limited continuous culture of wild-type *A. niger* N402. Linear regression has been carried out in two domains I and II. The regression lines for domain II are shown in the figure. Domain I: $Y_{\text{xsm}} = 101.55 \text{ g (mol glucose)}^{-1}$; $m_s = 0.0998 \text{ mmols glucose g}^{-1} \text{ h}^{-1}$; $Y_{\text{xom}} = 112.3 \text{ g (mol oxygen)}^{-1}$; $m_{\text{O}_2} = 1.045 \text{ mmols oxygen g}^{-1} \text{ h}^{-1}$. Domain II: $Y_{\text{xsm}} = 78.25 \text{ g (mol glucose)}^{-1}$; $m_s = -0.447 \text{ mmols glucose g}^{-1} \text{ h}^{-1}$; $Y_{\text{xom}} = 40.26 \text{ g (mol oxygen)}^{-1}$; $m_{\text{O}_2} = -0.79 \text{ mmols oxygen g}^{-1} \text{ h}^{-1}$.

Fig. 6. Specific rate of glucose consumption (q_{glucose}) (a) and oxygen consumption (q_{O_2}) (b) versus the specific growth rate (μ) in glucose-limited continuous culture of wild-type *A. niger* N402. Non-linear regression has been carried out using all data points. Linear regression lines, supposing growth rate independent maintenance requirements to be zero are shown as dotted lines. No maintenance requirements are supposed at $\mu = 0.12 \text{ h}^{-1}$.

regression considering all data was carried out, good fits for linearity were found (see the confidence limits in Tables 2 and 4). The values of maintenance requirements and maximum yield shown in Tables 2 and 4 were calculated by means of linear regression considering all data points.

The results for the glucose-limited continuous culture with wild-type *A. niger* N402 are shown in Fig. 5. In domain I ($\mu < 0.12 \text{ h}^{-1}$) the Y_{xsm} and m_s values were not significantly different from the values determined considering all data points (compare with Table 2). The Y_{xom} and m_{O_2} values in domain I were both significantly higher, indicating an increased energy need when compared with a fit with all data or domain II ($\mu > 0.12 \text{ h}^{-1}$). It is shown that when linear regression in domain II is used, both maximum yields and maintenance requirements on glucose (a) and oxygen (b) are lowered, compared to results of linear regression when all data points are considered.

These low, even negative, maintenance requirements indicate decreasing costs for maintenance requirements at increasing specific growth rates up to the shift from domain I to II, and increasing costs again at increasing specific growth rates in domain II. Considering maintenance requirements to be growth rate dependent, we applied non-linear regression to the data points of Fig. 5. The results are shown Fig. 6. The dotted lines in Fig. 6 are linear regression lines, supposing growth rate independent maintenance requirements to be zero. For clarity no maintenance requirements are supposed at $\mu = 0.12 \text{ h}^{-1}$, the μ value at which a shift from domain I to domain II occurs. This presentation clearly shows that maintenance requirements decrease with increasing growth rates up to $\mu = 0.12 \text{ h}^{-1}$ and again increase with increasing growth rates at higher μ values.

Chemostat experiments as described above are convenient for relatively high values of specific growth rate. In chemostat cultures with *Bacillus licheniformis* it

Fig. 7. Glucoamylase production (a, b) and product formation (P_t) (c, d) versus time in maltodextrin- (a, c) and xylose- (b, d) limited recycling cultures of wild-type *A. niger* N402 (●) and transformant *A. niger* N402[pAB6-10]B1 (○).

was found that exocellular protease production was negatively correlated with the specific growth rate (Frankena *et al.*, 1986). To investigate glucoamylase production at low growth rates by *A. niger* wild-type and overproducing transformant B1, both strains were grown in the recycling fermenter with 100% biomass retention. This fermentation technique resembles industrial processes, which are usually carried out in fed-batch type fermentations.

Growth and glucoamylase production in the recycling fermenter

A. niger N402 wild-type and transformant *A. niger* N402[pAB6-10]B1 were grown in xylose- and maltodextrin-limited recycling fermenters with 100% biomass feedback (van Verseveld *et al.*, 1986; Frankena *et al.*, 1988) with a rate of substrate addition corresponding to 1 mmol of C_6 units h^{-1} . Production of biomass, total product (measured as total organic carbon) and glucoamylase were determined.

Fig. 7(a, b) shows the total production of glucoamylase by both strains versus time for the maltodextrin- (a) and xylose- (b) limited recycling cultures. It is clear from Fig. 7(a, b) that much more glucoamylase (about 30 times) was produced on maltodextrin as limiting carbon source than on xylose. This was expected, since

maltodextrin highly induces the expression of the *glaA* gene, while xylose strongly represses it (Fowler *et al.*, 1990). Overproduction of glucoamylase by transformant B1 could be clearly observed when maltodextrin was used as sole carbon source.

During the whole experiment transformant B1 produced about 4.5 times more glucoamylase than the wild-type strain, as was found in shake flask cultures. The total amount of product, measured as total organic carbon, produced by both wild-type and transformant, was about the same during the course of recycling cultivation as shown in Fig. 7(c, d). Glucoamylase production on xylose was negligible.

The model used for the description of growth and product formation in the recycling fermenter has been treated extensively by van Verseveld *et al.* (1986), Stouthamer & van Verseveld (1987) and Ykema *et al.* (1989). It is based on carbon- and energy-balances (Roels, 1980), the linear equation for substrate utilization, $r_s = m_s x_t + \frac{r_s}{Y_{psm}} + \frac{r_p}{Y_{psm}}$ (Pirt, 1965) and a linear equation for product formation, $r_p = ax_t + br_{x_s}$. Equation 2 gives the amount of biomass formed with time

$$x_t = \frac{r_s}{m_s} + \left[x_0 - \frac{r_s}{m_s} \right] e^{-m_s^* Y_{psm}^* t} \quad (2)$$

in which r_s is the rate of substrate consumption (mols h^{-1}), $m_s^* = m_s + \frac{a}{Y_{psm}}$ is the maintenance coefficient

Fig. 8. Total amount of biomass formed versus time in maltodextrin-limited recycling cultures of wild-type *A. niger* N402 (●) and transformant *A. niger* N402[pAB6-10]B1 (○).

Fig. 9. Product formation (P_t) (●) and rate of product formation (r_p) (○) versus time in maltodextrin-limited recycling cultures of wild-type *A. niger* N402 (a) and transformant *A. niger* N402[pAB6-10]B1 (b).

[mols substrate (g biomass)⁻¹ h⁻¹], not corrected for product formation and $Y_{xsm}^* = [\frac{1}{Y_{xsm}} + \frac{b}{Y_{psm}}]^{-1}$ is the maximum yield of biomass on substrate (g mol⁻¹), not corrected for product formation. The full derivation of this equation is given by van Verseveld *et al.* (1986, 1991). According to equation 2 the increase in the amount of biomass, i.e., the growth rate, will decrease with time. Fig. 8 shows that this happened in the maltodextrin-limited recycling cultures. The increase in

biomass was practically zero after about 100 h in the wild-type culture and after about 150 h in the culture with the transformant strain.

Fig. 9 shows the total amount of product (measured as organic carbon) and the rate of production (in carbon units per h) versus time in maltodextrin-limited recycling cultures of wild-type *A. niger* N402 and transformant *A. niger* N402[pAB6-10]B1. At the time the amount of mycelial biomass in the fermenter vessel stopped increasing, the carbon production rate strongly decreased and consequently the total amount of organic carbon units produced almost remained stable. This phenomenon was most obvious in the culture with transformant B1, because this culture lasted long enough to reach this stage in the recycling process. The last data point from the recycling culture with the wild-type strain (pointed out with an arrow in Fig. 9a) suggests that this phenomenon was about to happen in this culture as well.

Discussion

In continuous culture we did not observe overproduction of glucoamylase by the transformant strain when maltodextrin was the growth-limiting substrate. Under glucose-limitation there was obvious glucoamylase overproduction as can be seen from Fig. 3 and from Table 3. These results are in agreement with Metwally *et al.* (1991), who found overproduction under glucose-limitation and not under maltose-limitation. As expected, the specific rate of glucoamylase production was higher under maltodextrin-limitation than under glucose-limitation, because glucose either induces the *glaA* gene at a lower level than maltodextrin or acts as a carbon catabolite repressor (Fowler *et al.*, 1990). The difference in production rates, when growing on the two growth-limiting substrates in the case of the transformant, was nevertheless rather small. There may be a maximum limit for glucoamylase production in the chemostat by the fungus due to a shortage of specific regulatory proteins in the presence of a large number of *glaA* gene copies, as suggested by Verdoes *et al.* (1993). This also explains why transformant B1, grown in shake flask culture, only overproduces glucoamylase 4.6 times, while provided with 20 copies of the *glaA* gene.

Brown & Zainudeen (1977) and Metwally *et al.* (1991) found two distinct domains of specific growth rates for the specific rate of substrate and oxygen consumption in continuous cultures of *Trichoderma viride* and *A. niger* respectively. Our data show a comparable deviation of the linear equation of substrate utilization, showing two domains of linearity at specific growth rates lower than 0.12 h⁻¹ and higher than 0.12 h⁻¹. The shifting point 0.12 h⁻¹ is taken somewhat arbitrarily, but it fits best in the graphs for both q_{glucose} and q_{O_2} data. The shift takes

place somewhere between $\mu = 0.11 \text{ h}^{-1}$ and $\mu = 0.14 \text{ h}^{-1}$, because at specific growth rates of 0.11 h^{-1} and lower we observed changes in the mycelium morphology and differentiation (conidia formation) as shown in Fig. 4. The deviation mentioned above was probably caused by these morphological changes, demanding higher maintenance requirements at specific growth rates of 0.11 h^{-1} and lower. Pitt & Bull (1982) observed the same phenomenon in cultures of the filamentous fungus *Trichoderma aureoviride*. Since the respiratory quotient (RQ) equalled 1 at all specific growth rates, this deviation cannot be explained by changes in the pathways of glucose catabolism, as found by Carter & Bull (1969) in chemostat cultures with *Aspergillus nidulans*.

When non-linear regression is applied to these data, as shown in Fig. 5, it is obvious that maintenance requirements are dependent upon the specific growth rate. At a specific growth rate of 0.12 h^{-1} , the shift from domain I to domain II, maintenance requirements could be virtually zero or at the most very low, as has been found before for other filamentous fungi (Pitt & Bull, 1982). At increasing specific growth rates, when $\mu > 0.12 \text{ h}^{-1}$, maintenance requirements will again increase with increasing specific growth rates. This may be caused by the increasing protein and RNA contents of the fungal biomass, resulting in higher energy demands for protein and RNA turnover at increasing specific growth rates, as found before in chemostat experiments with the yeast *Saccharomyces cerevisiae* (Furukawa *et al.*, 1983; Verduyn *et al.*, 1990) and the filamentous fungus *T. aureoviride* (Pitt & Bull, 1982). These phenomena should always be considered when the linear equation of substrate utilization is applied to cultures of filamentous fungi.

It is difficult to reach a steady state situation when growing a filamentous fungus in continuous culture, mainly because of wall growth and (partial) blocking of the outlet pipe. For this reason one should always take into account that non-homogeneity can influence the culture results. This can lead to pseudo steady states, partial recycling of the culture and inaccurate dry weight determinations. In our experiments we confirmed that a steady state was reached at the moment the rates of CO_2 production and O_2 consumption remained constant. We found no significant effect of glucoamylase production on yield parameters, as shown in Tables 2 and 4, because glucoamylase overproduction was low in glucose-limited continuous culture and absent in maltodextrin-limited continuous culture.

In the recycling fermenter the overproduction of glucoamylase by the transformant strain was obvious and equal to the results found in shake flask cultures, when maltodextrin was the limiting carbon source. In xylose-limited recycling culture the glucoamylase pro-

duction was almost completely repressed as can be seen in Fig. 7. For this reason it can be concluded that the introduced extra copies of the *glaA* gene in the overproducing transformant were subject to the same regulatory mechanisms as the parental *glaA* gene.

In the yeast *S. cerevisiae* secretion of exocellular proteins happens at the bud where vesicles, transporting these proteins from the Golgi apparatus, fuse with the plasma membrane to secrete their contents (Novick *et al.*, 1981). In filamentous fungi the hyphal tip is the chief site of secretion of exocellular enzymes into the medium (Chang & Trevithick, 1974). The apical vesicles, produced by the Golgi apparatus and accumulating in the hyphal tips of a growing fungus, have two essential functions in hyphal tip extension: they carry and secrete enzymes and/or materials for the growth of the plasma-membrane and the cell wall and they extend the plasma-membrane surface (Girbardt, 1969; Bartnicki-Garcia, 1973).

We assume therefore that without growth of the fungus, there will be a strongly decreased secretion of exocellular enzymes. As shown in Figs 7 and 8, the rate of production of glucoamylase was still increasing when the amount of biomass in the culture vessel stayed constant. On the other hand, the total product formation, measured as organic carbon, produced by the transformant strain did not increase after about 150 h. Consequently, from this point of time the total production rate (r_p) decreased rapidly to almost zero (Fig. 9(b)). Most likely this phenomenon also happened in the wild-type culture, indicated by a decreasing r_p after 100 h. As opposed to the glucoamylase production data, no overproduction of total products was found. After about 150 h, dry weight did not increase, while glucoamylase was still produced, suggesting hyphal tip extension was still taking place.

Because of the above observations we assume that after about 150 h, death of the mycelium took place at the same rate as growth at the hyphal tips, resulting in a constant dry weight. Mycelial death could be caused by autolysis due to starvation, and by mechanical damage to the mycelium by the impeller. The contents of the broken mycelial threads would be released into the medium and act as an extra carbon source for the growing parts of mycelium. To confirm these conclusions further investigations (e.g. at a microscopic level) are required. It has been reported that during glucose starvation of the mycelial fungus *Penicillium chrysogenum*, autolysis and cryptic growth on cell contents took place (Trinci & Righelato, 1970). Release of contents of hyphae probably took place before 150 h as well. The increase in the amount of total products, measured as carbon, produced in the first 150 h suggests that in this case there was no carbon-limitation, but only energy-limitation. After this time growth became carbon-

and energy-limited again, when an equilibrium was established between carbon production and consumption.

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