Appl Microbiol Biotechnol (1994) 41:658–663

ORIGINAL PAPER

N.C. van de Merbel · G.J.G. Ruijter · H. Lingeman
U.A.Th. Brinkman · J. Visser

An automated monitoring system using on-line ultrafiltration and column liquid chromatography for *Aspergillus niger* fermentations

Received: 11 October 1993 / Received revision: 28 January 1994 / Accepted: 31 January 1994

Abstract An automated system for the monitoring of fermentations of filamentous fungi is described. The system is based on the on-line combination of ultrafiltration, for the removal of cellular and macromolecular material from the fermentation broth, and column liquid chromatography for analysis of the filtrate. The performance of one hollow-fibre and two planar ultrafiltration modules is evaluated. The maximum sampling frequency as well as prevention from clogging by mycelium is strongly dependent on the construction of the module, best results being obtained with a planar membrane and a relatively high flow rate (150 ml/min) of the broth through a single, wide-bore (3 mm) flow channel in the module. The method is used for the study of metabolic and regulatory processes of two different *Aspergillus niger* strains on multiple carbon sources. The selected system can be applied for at least 70 h without any negative effect on either the fermentation or the analytical system. Through an analysis frequency of once per hour detailed information regarding consumption and production of nine different compounds could be obtained.

Introduction

Aspergillus species provide interesting possibilities for studying carbon metabolism for two reasons. Firstly, they are able to metabolize a wide variety of simple and complex carbon sources for growth, including polymers, oligo- and disaccharides, hexoses, pentoses, organic acids, alcohols, polyols and aromatic compounds (McCullough et al. 1986). This requires optimal adaptation to prevailing conditions, which is reflected by the induction or repression of specific catabolic enzymes. A second interesting aspect of *Aspergillus* carbon metabolism is overproduction of primary metabolites. Two groups of compounds are overproduced under certain conditions, namely organic acids, gluconic acid being a well-known example, and polyols such as glycerol and mannitol (Röhr et al. 1992). The molecular mechanisms leading to this overflow metabolism are often not clear.

In order to understand the regulatory mechanisms governing consumption of nutrients and the underlying mechanisms leading to overproduction of primary metabolites, detailed information regarding the kinetics of consumption or production of the various compounds is of utmost importance. As a consequence, the availability of accurate methods for the determination of these key compounds in biological samples is an important requirement. When studying fermentation processes these analyses are generally performed by manual sampling and work-up of a part of the fermentation broth and subsequent analysis by one of a number of chromatographic, spectroscopic and enzymatic techniques. To increase the analysis frequency and, thus, be provided with more detailed information, the application of automated methods is an attractive option, also because they allow unattended operation and give more reliable data than the traditional manual assays. The efficient on-line coupling of an analytical system to a bioreactor is of great importance in achieving this goal; this step is, however, still considered a major bottleneck in the automated monitoring of bioprocesses (Langer 1991).

On-line monitoring usually requires the use of a filtration interface, which removes interfering broth components such as cellular and macromolecular material and permits direct introduction of the filtrate into an analytical system. Several interfaces have been described in the recent literature, for use either outside the fermentor in an external sampling loop (Monseur

N.C. van de Merbel (✉) · H. Lingeman · U.A.Th. Brinkman
Department of Analytical Chemistry, Free University,
De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands,
FAX 31-20-64 61 479

G.J.G. Ruijter · J. Visser
Department of Genetics, Agricultural University,
Dreyenlaan 2, 6703 HA Wageningen, The Netherlands

and Motte 1988; Garn et al. 1989; van de Merbel et al. 1992, 1993) or placed directly in the fermentation medium (Möller et al. 1986; Holzhauer-Rieger et al. 1990; Pique and Corrieu 1992; Marko-Varga et al. 1993). Neither of these configurations is ideal, for *in-situ* filters tend to clog easily in the case of insufficient tangential flow across the membrane and cannot be replaced, whereas in an external loop the physicochemical conditions may differ from those in the bioreactor. Still, it is generally felt that in the near future these systems will be more attractive for automated fermentation monitoring than the use of in-line biosensors, as these still show severe reliability problems (Mattiasson and Håkanson 1993). The choice of the analytical system depends, among others, on the required speed of analysis and the number of compounds to be monitored. The main advantage of column liquid chromatography (LC) over non-chromatographic techniques frequently used for fermentation monitoring, such as flow injection analysis, is its potential for determining several compounds simultaneously. Its advantage over gas chromatography is its compatibility with non-volatile and thermally labile compounds.

In previous studies it has been shown that, by optimizing the configuration of a hollow-fibre ultrafiltration (UF) interface in an external sampling loop, a wide range of compounds in a variety of complex fermentation broths can be automatedly monitored by LC during various yeast and bacterial fermentations under both aerobic and anaerobic conditions (van de Merbel et al. 1992, 1993). This system could be applied for at least 250 h without any adverse effect on either the fermentation or the analytical system, or a major decrease in filtration performance, and allowed a sampling frequency of, typically, once per 5–10 min. In the present paper, the extension of this method to fermentations of the filamentous fungus *A. niger* is reported and its general applicability to fundamental studies of carbon metabolism is illustrated. Since the morphology of filamentous fungi is likely to cause specific problems with regard to the sampling system, the performance of three different filtration modules is compared. Some illustrative results obtained by applying the system to cultures of two different *A. niger* strains growing on a mixture of glucose and fructose are shown.

Materials and methods

Materials

Products from the following suppliers were used: trypticase peptone and casamino acids (Merk, Darmstadt, Germany), yeast extract (Oxoid, London, UK), ribonucleic acids (BDH Chemicals, Poole, UK), agar (Life Technologies, Breda, Netherlands) and Tween-80 (Ferak, Berlin, Germany). The components of the vitamin solution were obtained from Sigma (St. Louis, Mo., USA). All other chemicals were purchased from J.T. Baker (Deventer, Netherlands). LC-grade water was prepared using a Milli-Q (Millipore, Bedford, Mass., USA) system.

Strains, media and fermentation conditions

The fermentations were carried out with mutants derived from the *A. niger* wild-type strain N400. These mutants were N597 (*csp*A1 *pab*A1), which produces short conidiophores and requires *p*-aminobenzoic acid, and NW101 (*csp*A1 *pab*A1 *gox*C17), which, in addition, is glucose-oxidase negative (Witteveen et al. 1990).

The medium used in agar plates contained per litre: 6.0 g sodium nitrate, 1.5 g potassium dihydrogen phosphate, 0.5 g potassium chloride, 0.5 g magnesium sulphate 7-hydrate, 0.2 ml of the trace metal solution described by Vishniac and Santer (1957), 2.0 g trypticase peptone, 1.0 g casamino acids, 1.0 g yeast extract, 0.5 g yeast ribonucleic acids, 10 g glucose, 1.5 g agar and 1 ml of a vitamin solution. This solution contained per litre: 100 mg thiamine.HCl, 1 g riboflavin, 100 mg *p*-aminobenzoic acid, 1 g nicotinamide, 500 mg pyridoxine.HCl, 100 mg pantothenic acid and 20 mg biotin. Fermentation media consisted of 1.2 g sodium nitrate, 0.5 g potassium dihydrogen phosphate, 0.2 g magnesium phosphate 7-hydrate, 0.5 g yeast extract and 8 μl of the above trace metal solution per litre and were supplemented with 1.37 mg·l^{-1} of *p*-aminobenzoic acid. As a carbon source 10 g glucose and 10 g fructose were added per litre.

A 0.2 l preculture was inoculated with approx. $1 \cdot 10^7$ conidiospores·ml^{-1}, which were harvested from the agar medium with a 10 ml aqueous 0.8% (w/v) sodium chloride, 0.05% (v/v) Tween-80 solution. After 7.0–7.5 h of incubation at 30° C in a rotary shaker at 200 rpm, the preculture was added to 2.0 l medium in the fermentor. The temperature was maintained at 30 ± 0.3° C and the dissolved oxygen tension at 30 ± 5% relative air saturation by addition of pure oxygen and nitrogen and stirring at a rate of 750 rpm. The pH was held constant at 6.0 ± 0.2 by automated addition of 5 M sodium hydroxide.

Instrumentation

A schematic drawing of the monitoring system is shown in Fig. 1. Fermentations were run in a Multiferm (Quintus, Gouda, Netherlands) 5-l fermentor equipped with temperature, pH, dissolved oxygen tension and stirring control. Filtration was performed with a Bio 2000 TC cross-flow ultrafiltration system (Bio-Flo, Glasgow, UK) equipped with a peristaltic pump and one out of three different ultrafiltration modules, details of which are presented in Table 1. The hollow-fibre module A has been described elsewhere (van de Merbel et al. 1993). Two planar membrane units were used: a Millipore (Etten-Leur, Netherlands) Minitan system (module B) consisting of two stainless-steel half-blocks provided with two membranes, which were separated by 3-mm-thick homemade spacers and an adapted Amicon (Beverly, Mass., USA) CEC1 filtration block (module C, depicted in Fig. 2), containing a 3-mm-deep spiral groove. In all cases the total volume of the external sampling loop, consisting of tubings, pump and filter, was about 30 ml. The filtration system was sterilized by flushing with 0.5 l of 70% ethanol followed by rinsing with 2 l sterile water. Filtration was performed by continuously pumping the broth through the UF module (150 ml/min) and applying a pressure of approx. 0.3 bar. The retentate was pumped back to the fermentor, the filtrate was transferred on-line to a 3.0-μl injection loop mounted on a six-port switching valve (No. 2), which was connected to the LC system. The filtrate was returned to the fermentor, but to prevent eventual contamination it was passed through a sterile barrier, a 0.2 μm mixed cellulose ester microfilter, type culture guard (Microgon, Laguna Hills, Calif., USA). The sampling frequency was set at once per hour; filtration was performed for 20 min and during the remaining 40 min the filtrate outlet was closed by means of an additional valve (No. 1) and the filtration was stopped. Between two fermentation runs the UF module was cleaned by flushing with an aqueous 2.5% Decon Neutracon (Decon Laboratories, Hove, UK) solution, followed by thorough rinsing with water. When not in use, the module was stored in a 0.05% sodium azide solution.

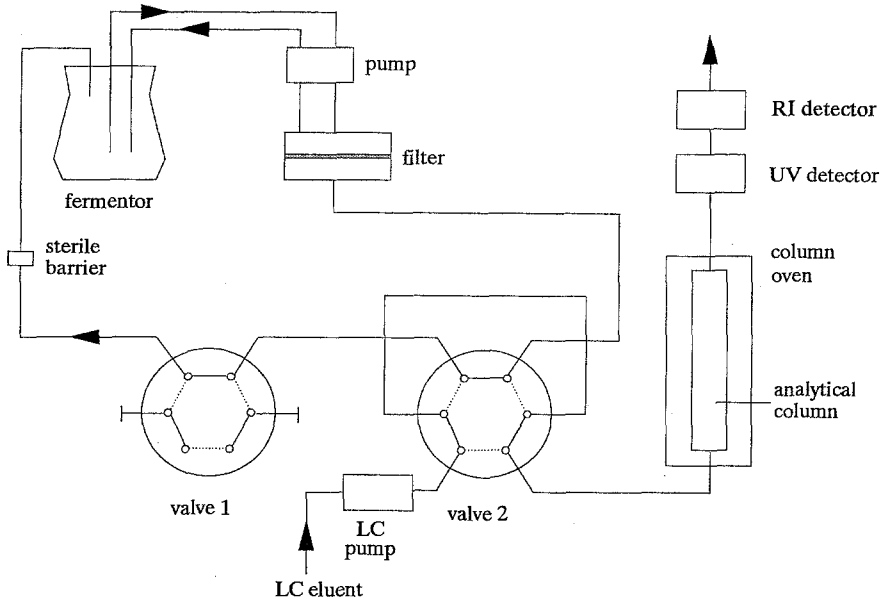

Fig. 1 Schematic set-up of the on-line monitoring system (*RI* refractive index, *UV* ultraviolet, *LC* liquid chromatography)

Table 1 Properties of different ultrafiltration modules (*MWCO* molecular weight cut-off value, *n. d.* not determined)

Parameter	Ultrafiltration modules		
	A	B	C
Configuration	Hollow fibres	Two planar membranes	One planar membrane
Membrane material	Polysulphone	Polysulphone	Polysulphone
MWCO (kDa)	54	30	30
Membrane area (cm^2)	90	60	40
Filtrate dead volume (ml)	0.8	2	0.9
Sampling dead time (min) at 0.3 bar	5	8	13
Pure water flux (ml/min per bar)			
Original	5.5	24	3.0
After one (70-h) run	n.d.	18	2.5
Clogging			
After one (70-h) run	Completely	Partly	Negligible

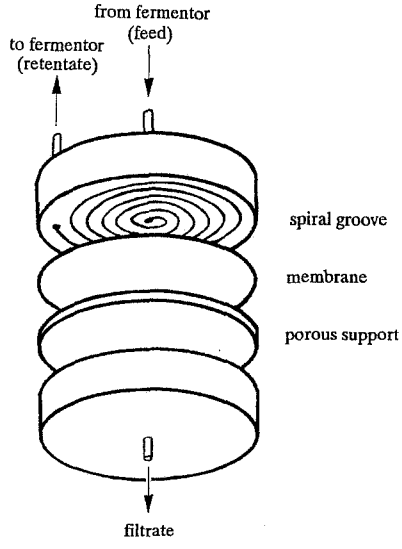

Fig. 2 Schematic drawing of ultrafiltration module C, consisting of a planar membrane, clamped between two perspex blocks

The LC system consisted of a Gilson (Villiers-le-Bel, France) Model 302 high-pressure piston pump, an Interaction Chemicals (Mountain View, Calif., USA) ION-300 column (300 mm × 7.8 mm i.d.) thermostatted at 75° C in a Pharmacia LKB (Uppsala, Sweden) Model 2155 column oven, with 0.005 M sulphuric acid as the eluent (0.5 ml/min). For detection a Pharmacia LKB Model 2142 refractometer and a Model 2141 ultraviolet-visible light (UV-VIS) photometer set at 215 nm were coupled in series. Signals were recorded using a Model 2210 recorder (Pharmacia LKB) and a Hewlett-Packard (Waldbronn, Germany) Model 3396A integrator. Valve switching was controlled by a Prospekt 1.0 (Spark Holland, Emmen, Netherlands).

Results

Filtration

As the efficiency of a sampling system is very much dependent on the geometrical and material properties of the ultrafiltration device, three different modules were compared. Special attention was devoted to the maximum sampling frequency and the amount of fouling of the membrane. The results are summarized in Table 1.

On-line monitoring

In order to be able to distinguish between glucose and gluconate, which could not be separated chromatographically in the present system, a double detection system was used, consisting of a refractive index (RI) detector and a variable-wavelength UV-VIS detector set at 215 nm, coupled in series. Glucose does not show UV absorbance at 215 nm, so gluconate can be selectively determined by the latter detector. Since the RI detector monitors both compounds with equal sensitivity, by combining the responses the concentration of glucose can be calculated. As an illustration, Fig. 3 shows a typical LC-RI chromatogram (recorded during a fermentation of the NW101 strain). It convincingly demonstrates the potential of LC for the synchronous determination of a variety of substrates and metabolites and its general usefulness for on-line monitoring of bioprocesses.

During the runs, the media were regularly inspected microscopically, but no contamination with external micro-organisms was ever observed. In addition, the injection of over 400 filtrate samples in the course of this study did not lead to a decrease in separation efficiency of the LC column. This indicates that the filtration interface provides a proper protection of both the analytical system and the bioprocess.

The monitoring system was used to examine the consumption of nutrients and formation of products by two different *A. niger* strains, isogenic except for the presence of glucose oxidase. When cultured on a medium containing a mixture of equal amounts of glucose and fructose, both strains consumed glucose preferentially to fructose (Fig. 4). In the case of strain N597 the available glucose was very rapidly converted into gluconate (Fig. 4A). When all glucose was consumed, the concentration of gluconate was 9 g/l, which corresponds to a carbon yield of 83%. Strain NW101 did not produce gluconate (Fig. 4B). Following its production, gluconate was slowly consumed in the wild-type strain N597. When gluconate was added to a culture of the glucose oxidase negative mutant NW101 gluconate consumption was also observed; it was, however, essentially negligible before complete exhaustion of the glucose (Fig. 4C). As regards fructose consumption, we observed a much slower rate in N597 than in NW101. Upon addition of gluconate to a culture of NW101, fructose consumption was also slowed down.

A large amount of oxalate was produced by strain NW101 (13 g/l after 70 h). A much lower rate of oxalate

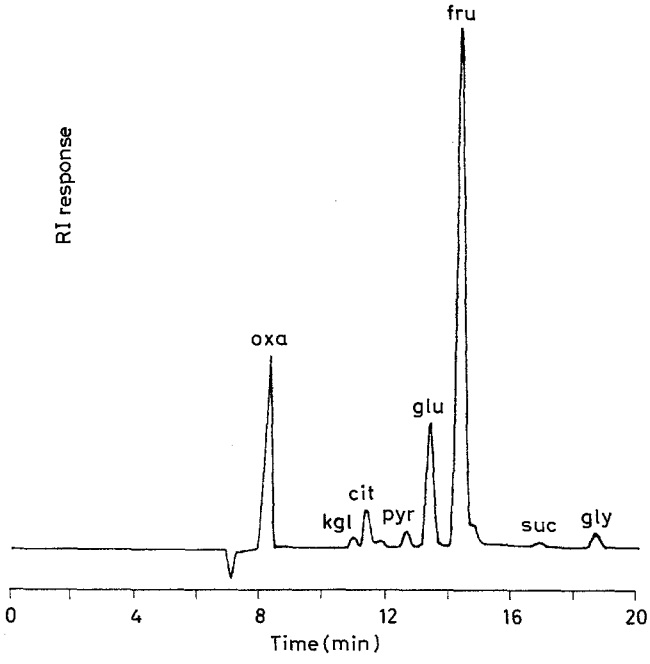

Fig. 3 LC-RI chromatogram of an *Aspergillus niger* NW101 fermentation medium sample at 30h fermentation time: for LC details, see text. (*oxa* oxalate, *kgl* 2-ketoglutarate, *cit* citrate, *pyr* pyruvate, *glu* glucose, *fru* fructose, *suc* succinate, *gly* glycerol

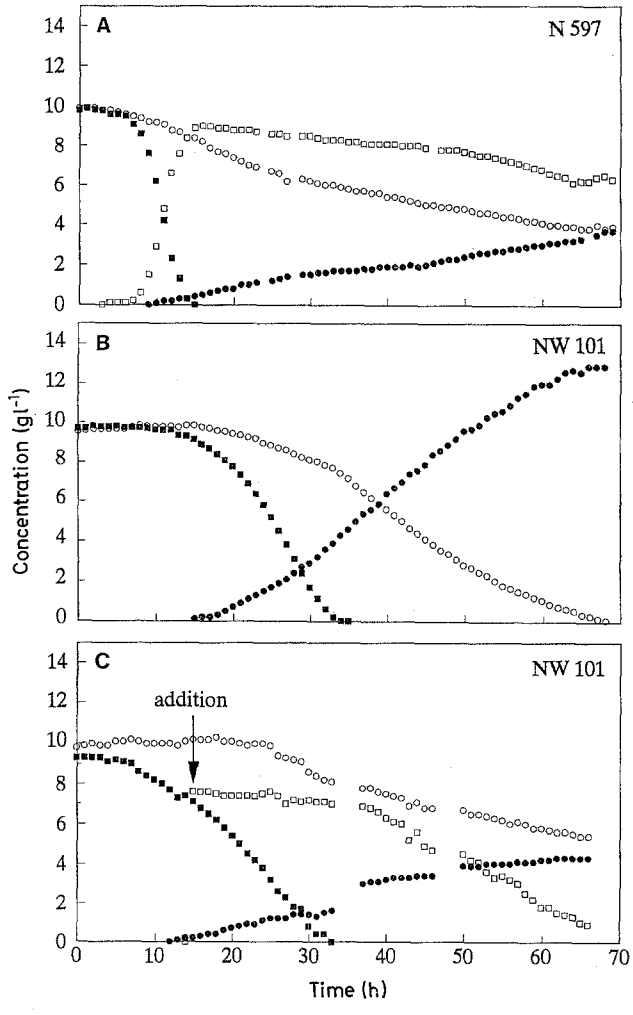

Fig. 4 Concentration profiles of the nutrients glucose (■) and fructose (○) and the major products gluconate (□) and oxalate (●) during a fermentation of (**A**) *A. niger* N597, (**B**) *A. niger* NW101 and (**C**) *A. niger* NW101 with external addition (*arrow*) of gluconate at $t = 15$ h. Due to technical problems, the data from 34–36 h and from 47–49 h have not been recorded in fermentation **C**. For fermentation details, see text

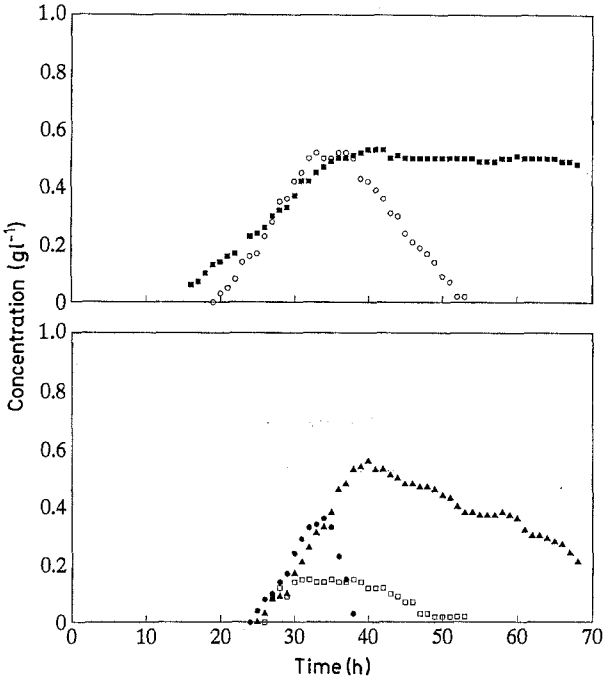

Fig. 5 Concentration profiles of the minor metabolites citrate (■) glycerol (○) pyruvate (●) 2-ketoglutarate (▲) and succinate (□) during a fermentation of *A. niger* NW101. For fermentation details, see text

formation was observed in the other experiments (approx. 4 g/l), both in the case of N597 and of NW101 with externally added gluconate. In addition to gluconate and oxalate, a number of minor metabolites were produced during the various fermentations. For example, NW101 produced citrate, glycerol, pyruvate, 2-ketoglutarate and succinate (Fig. 5), whereas N597 only produced citrate and glycerol (data not shown). In the case of NW101, glycerol, pyruvate, succinate and part of the 2-ketoglutarate were consumed again.

Discussion

Filtration

Two important features of an efficient sampling system are (i) the ability to provide representative samples with a minimal time delay, so that rapidly changing processes can be monitored accurately, and (ii) sufficient stability to allow reproducible sampling during a complete fermentation run without any intervention. The time required to obtain a representative sample, i.e. with 100% analyte recovery, is called the sampling dead time. It depends primarily on the dead volume of the filtration module and the filtration rate (or flux) through the membrane which, in its turn, is influenced by such membrane parameters as pore size and surface area (van de Merbel et al. 1993). The construction of the filtration module therefore exerts a major influence on the sampling frequency. A hollow-fibre filtration unit is in principle most advantageous for obtaining a high sampling frequency since it combines a large membrane area with a low dead volume.

The long-term stability of a membrane-based sampling system depends on the degree of membrane fouling, which is controlled by the physico-chemical properties of both the membrane and the sample. In addition, when working with unhomogeneous fermentation broths, such as cultures of filamentous fungi, it is essential that no clogging of the sampling system by broth constituents occurs.

The results (Table 1) show that the filtrate flux is influenced by parameters other than just the membrane area and pore size, as can be concluded from the fact that module B, which has less favourable values for these parameters than module A, yielded a fourfold higher flux. This may be due to the use of a thinner and/or more porous (i.e. more pores per unit area) membrane, values of which are not often reported. However, despite the fact that it is far superior in filtrate flux, module B did not yield the shortest sampling dead time because of its relatively unfavourable dead volume. Furthermore, the influence of membrane fouling was found to be comparable for modules B and C. The so-called pure water flux (i.e. the flux of distilled water through the membrane at a pressure of 1 bar), had decreased by about 15% for both modules after one *A. niger* fermentation of 70 h. This indicates that in order to maintain an optimal filtrate flux and, thus, a maximal sampling frequency, membranes should be regularly renewed. In the present investigation, not more than three fermentations were monitored using the same membrane. Finally, as regards the prevention of clogging of the filtration unit by mycelium, here the construction of the module turned out to be of major importance. Module A, consisting of a bundle of approx. 50 hollow fibres sealed into a housing, became clogged completely after just a few hours of operation. With this module, the fermentation broth was passed through the housing outside the fibres where there were several parts in the flow path with a considerably lower flow rate; consequently, the mycelium could easily accumulate there and obstruct the filter. In module B the fermentation broth was divided over nine flow channels and it was found that in the course of 70 h (one complete run) three to four of these were clogged, probably because the flow-rate could not be kept at 150 ml/min in all of the channels. The filtration rate was, however, not affected. In module C the flow-rate could be maintained throughout its single channel and no significant accumulation of mycelium was observed during a fermentation run. Therefore, despite the least favourable sampling dead time (13 min which allows a sampling frequency of about five per hour), module C was used in further experiments.

On-line monitoring

The detailed information obtained with the present system, which turned out to be robust and did not require special maintenance, enabled the observation of a number of interesting phenomena. The data were, however, mainly meant as an illustration of the applicability of the system and more research will be undertaken in future to elucidate the underlying mechanisms.

Firstly, it is not surprising that the available glucose was very rapidly converted into gluconate by strain N597, as the enzymes that catalyse this (two-step) process, glucose oxidase and lactonase, are located extracellularly in *A. niger* (Witteveen et al. 1992). Also, it was to be expected that, due to the absence of a functional glucose oxidase, strain NW101 did not produce any gluconate. Secondly, the inhibition of gluconate consumption by glucose is most probably caused by repressing expression of the genes involved in gluconate catabolism. Repression of the consumption of various carbon sources exerted by glucose and mediated by the wide domain regulatory protein CRE A is well documented in the related fungus *A. nidulans* (Dowzer and Kelly 1991). It is more surprising that fructose consumption was slowed down by the presence of gluconate, as fructose is usually considered to be a readily metabolisable carbon source.

As regards the oxalate formation, evidence has shown that in *A. niger* it involves hydrolytic cleavage of oxaloacetate to oxalate and acetate catalysed by oxaloacetate hydrolase (Müller 1975; Lenz et al. 1976; Kubicek et al. 1988). During culturing of NW101 on a mixture of glucose and fructose, no acetate was found in the medium. Furthermore, after depletion of glucose, all fructose was converted to oxalate. This suggests conversion of the acetate formed to oxalate, involving the glyoxylate cycle. Interestingly, a much lower rate of oxalate formation was observed in the presence of gluconate in the broth. In addition, there was no substantial difference between the oxalate yields of N597 and of NW101 with external addition of gluconate. A possible explanation is that oxalate formation is affected by gluconate, but alternatively the rate of oxalate formation may be related to the rate of fructose metabolism. Finally, consumption of the minor metabolites only started when glucose was exhausted, indicating glucose repression as in the case of gluconate.

Acknowledgements The loan of the Minitan system by and useful discussions with Dr. D. Böck (Millipore) are highly appreciated. This work was supported by the Dutch Foundation for Technical Science, Grant No. 349–2119

References

Dowzer CEA, Kelly JM (1991) Analysis of the *cre*A gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol Cell Biol 11:5701–5709

Garn M, Gisin M, Tommen C, Cevey P (1989) A flow injection analysis system for fermentation monitoring and control. Biotechnol Bioeng 34:423–428

Holzhauer-Rieger K, Zhou W, Schügerl K (1990) On-line high-performance liquid chromatography for the determination of cephalosporin C and by-products in complex fermentation broths. J Chromatogr 499:609–615

Kubicek CP, Schreferl-Kunar G, Wöhrer W, Röhr M (1988) Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. Appl Environ Microbiol 54:633–637

Langer OU (1991) Measurement, modelling and control in biotechnology Biotechnol Forum Eur 8:216

Lenz H, Wunderwald P, Eggerer H (1976). Partial purification and some properties of oxalacetase from *Aspergillus niger*. Eur J Biochem 65:225–236

Marko-Varga G, Buttler T, Gorton L, Grönsterwall C (1993) A study of the use of microdialysis probes as a sampling unit in on-line bioprocess monitoring in conjunction with column liquid chromatography. Chromatographia 35:285–289

Mattiasson B, Håkanson H (1993) Sampling and sample handling – crucial steps in process monitoring and control. Trends Biotechnol 11:136–142

McCullough W, Roberts CF, Osmani SA, Scrutton MC (1986) Regulation of carbon metabolism in filamentous fungi. In: Morgan MJ (ed) Carbohydrate metabolism in cultured cells. Plenum, New York, pp 287–355

Merbel NC van de, Kool IM, Lingeman H, Brinkman UATh, Kolhorn A, Rijke LC de (1992) On-line monitoring of fermentation processes by ultrafiltration and column liquid chromatography. Chromatographia 33:525–532

Merbel NC van de, Lingeman H, Brinkman UATh, Kolhorn A, Rijke LC de (1993) Automated monitoring of biotechnological processes using on-line ultrafiltration and column liquid chromatography. Anal Chim Acta 279:39–50

Möller J, Hiddessen R, Niehoff J, Schügerl K (1986) On-line high-performance liquid chromatography for monitoring fermentation processes for penicillin production. Anal Chim Acta 190:195–203

Monseur X, Motte JC (1988) On-line monitoring during fermentation of whey based on ultrafiltration and liquid chromatography. Anal Chim Acta 204:127–134

Müller HM (1975) Oxalate accumulation from citrate by *Aspergillus niger* I. Biosynthesis of oxalate from its ultimate precursor. Arch Microbiol 103:185–189

Pique D, Corrieu G (1992) Performances of aseptic sampling devices for on-line monitoring of fermentation processes. Biotechnol Bioengin 40:919–924

Röhr M, Kubicek CP, Kominek P (1992) Industrial acids and other small molecules. In: Bennett JW, Klich MA (eds) *Aspergillus:* biology and industrial applications. Butterwort-Heinemann, Boston, pp 91–131

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195–213

Witteveen CFB, Vondervoort P van de, Swart K, Visser J (1990) Glucose oxidase overproducing and negative mutants of *Aspergillus niger*. Appl Microbiol Biotechnol 33:683–686

Witteveen CFB, Veenhuis M, Visser J (1992) Localization of glucose oxidase and catalase activities in *Aspergillus niger*. Appl Environ Microbiol 58:1190-1194