At-line Determination of Glucose, Ammonium and Acetate in High Cell Density Cultivations of *Escherichia coli*

By *Thorsten Peuker*, *Michael Riedel*, *Christian Kaiser*, *Andree Ellert*, *Kai Lenz*, *Olaf Elsholz*, and *Reiner Luttmann**

A sophisticated measurement system for at-line determination of the main C-source glucose, the by-product acetate, and the N-source ammonium for high cell density cultivations (HCDC) of *Escherichia coli* K12 TG1 is presented. One flow diffusion technique (FDA) system is used for glucose measurement in the range of 0.5 up to 40 gL^{-1} in the cultivation broth. Another FDA system detects the amount of the undesired by-product acetate. The ammonium concentration in the range of 0.2 to 2.5 gL^{-1} is determined on-line by a flow injection analysis (FIA) system. For verification purposes, an HPLC system which is also connected to the bioreactor for at-line measurements is utilized. Several HCDC with cell densities of more than 100 gL^{-1} have been carried out. The courses of growth-determining substrates have been detected at-line. All used systems have shown an excellent compliance with off-line measurements.

1 Introduction

A very common microorganism in biotechnological applications is *Escherichia coli*, well known for efficient production of recombinant proteins like hormones and enzymes. Since *E. coli* is widely used in the biotechnological world, it is well investigated in molecular biology and biochemical engineering.

Besides the desired products *E. coli* also tends to form metabolic by-products that might cause a decrease in growth and productivity performance [1]. Therefore, it is useful to know more about the presence or, even better, more about the tolerance limit of those metabolic by-products. This information can be obtained from on-line measurement performed during the running cultivation.

In the context of on-line measurement, the focus is on the used carbon source glucose, the formed by-product acetate, as well as the required nitrogen source ammonium.

Circumstances promoting the formation of acetate during cultivation of *E. coli* are well investigated. These conditions are the concentration of the carbon source [2] leading to metabolic overflow if a certain concentration is exceeded [3], the oxygen concentration in case of oxygen limitation [4], and the cell-specific growth rate in case of reaching the critical value μ_{crit} (0.2–0.35 h^{-1}) [5][1)].

Combination of knowledge about the metabolic pathway and the quantity and availability of suitable substrates within the bioreactor allows the directed manipulation of growth conditions.

Based on excellent process engineering conditions and appropriate process strategies, high cell density concentrations can be achieved.

2 Materials and Methods

2.1 Bioreactor

Cultivations are performed in a 15 L BIOSTAT C plant (B. Braun Biotech International, Melsungen, Germany) schematically shown in Fig. 1.

Ambitious analysis technique, connected to the fully equipped fermentor, allows detailed knowledge about the proceeding within the cultivation vessel. Besides classical in-line devices such as pH and pO_2 probes as well as standard off-gas analysis, additional at-line measurement devices, namely, two different flow diffusion analysis systems (FDA) and a flow injection analysis system (FIA), are connected to a cell-free analysis flow F_A. This flow is provided via an Eppendorf sterilizable in-line probe (ESIP).

Furthermore, the reservoirs providing the glucose feeding media (R1) as well as the pH-adjusting agents (T1/T2) and antifoam (AF) are shown. All these reservoirs are placed on balances in order to calculate the mass balances for the whole reaction system.

2.2 Applied at-line Analytics

In total, three different at-line analysis systems are applied during this work. Fig. 2 points out how these different systems are arranged and connected to the cultivation vessel.

A peristaltic pump conveys cell-free media samples via an ESIP from the fermenter to the different measuring devices. These are a ProcessTrace (TRACE Analytics GmbH, Braunschweig, Germany) measuring the acetate concentration (c_{P3M}), a TAS 2000 FIA system (Jüke GmbH, Altenberge, Germany) measuring the ammonium concentration (c_{AltotM}), and, finally, a MERCK HPLC system (VWR Inter-

[*] T. Peuker, M. Riedel, C. Kaiser, A. Ellert, K. Lenz, O. Elsholz, R. Luttmann (author to whom correspondence should be addressed, Reiner.Luttmann@rzbd.haw-hamburg.de), Hamburg University of Applied Sciences, Research Center of Bioprocess Engineering, Lohbrügger Kirchstraße 65, D-21033 Hamburg, Germany.

1) List of symbols at the end of the paper.

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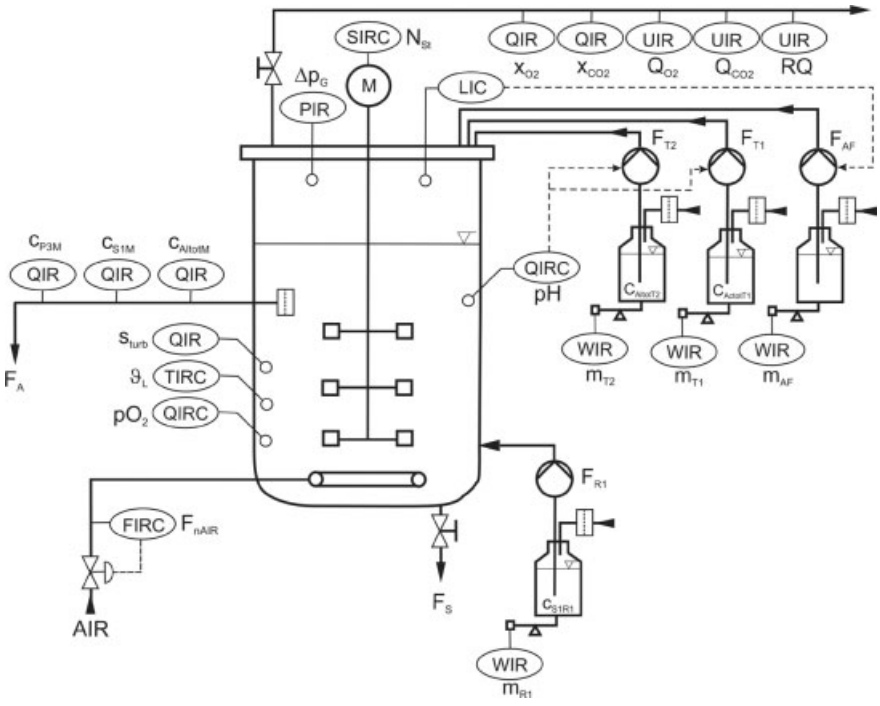

Figure 1. Measurement and control technology of the bioreactor.

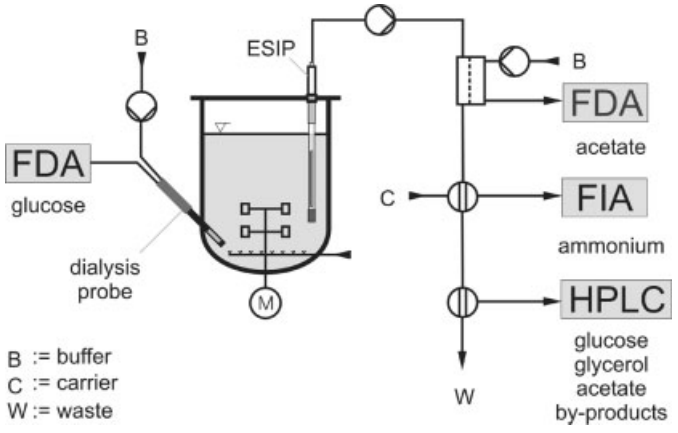

Figure 2. Sampling technique and connection of the at-line measurement systems.

national, Darmstadt, Germany) is in charge of validation of the above-named agents. These devices are connected in series in the cell-free sample flow F_A (see Fig. 1).

In parallel, another ProcessTrace system is connected to the fermenter via a dialysis probe measuring the glucose concentration (c_{S1M}) within the media phase.

The basic measurement proceeding is very similar in both applications. An aqueous solution containing the substance that needs to be measured (donor) on one side and a carrying buffer solution (acceptor) on the other side of a membrane allows diffusion of this specific substance of interest across the membrane (see Fig. 3).

Enriched with this particular substance, the acceptor solution passes an enzymatic reaction which causes H_2O_2 formation that is evaluated by an ammeter [6].

Using ProcessTrace with the prototype acetate application requires adaptation of the cultivation media. Certain inhibitors such as zinc ions or the complex binder EDTA greatly influence the enzymatic acetate detection.

The FIA system TAS 2000 allows at-line determination of ammonium within the sample broth during cultivation of *E. coli* (see Fig. 4).

A defined sample segment is injected into a H_2O carrier (C). This particular flow is admixed with a reagent flow (R1). Later on, two more reagents (R2 and R3) are added to the liquid stream that passes a tempered water bath. Finally, a photometer detects a change in absorption at a wavelength of 605 nm. Depending on whether the ammonium detection is running at-line during cultivation or off-line in the course of validation the sampling proceeding varies.

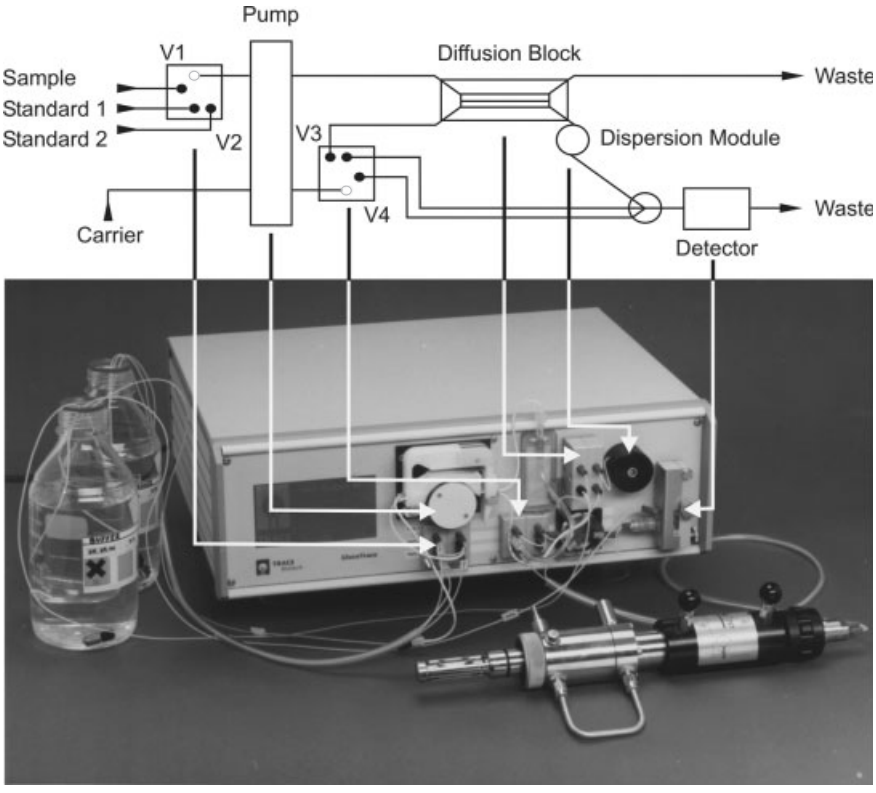

Figure 3. Principle of flow diffusion technique by the analysis system ProcessTrace.

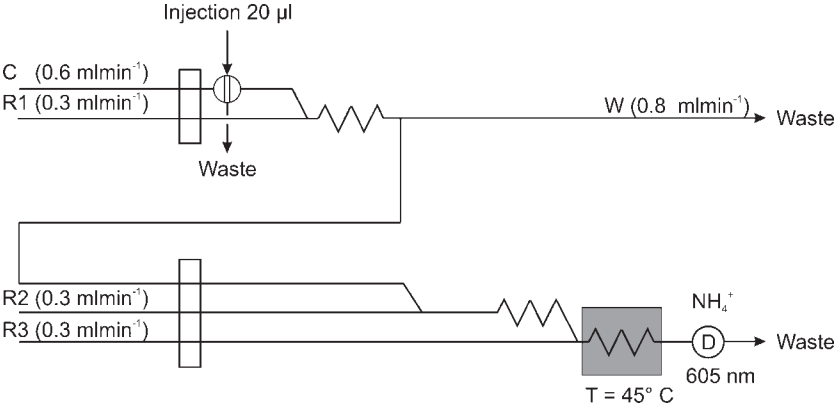

Figure 4. Flow scheme of FIA system TAS 2000.

In the first case, sampling is performed by an automated injector while the off-line measurement occurs after manual injection with a syringe.

2.3 Cultivation Strategy

All cultivations were started with a batch phase followed by a fed-batch phase on glucose. The cell-specific growth rate μ was controlled in the fed-batch phase on a smaller value than μ_{max} via an exponential feeding profile.

3 Results

With respect to the three known and, what is more significant, detectable carbon sources glucose, glycerol, and acetate, the batch phase is divided into three successive sections. In Fig. 5 these sections are headed by roman numbers.

Glucose (c_{S1M}) is the major carbon source in the first labelled section (I), followed by glycerol (c_{S2M}) in the second section (II). In the third section (III) of this batch phase, the former product acetate (c_{P3M}) acts as last remaining substrate. As a matter of fact, the declining flank of the on-line acetate course in the time span from t = 8.5 h to t = 12.2 h does not properly indicate the acetate concentration within

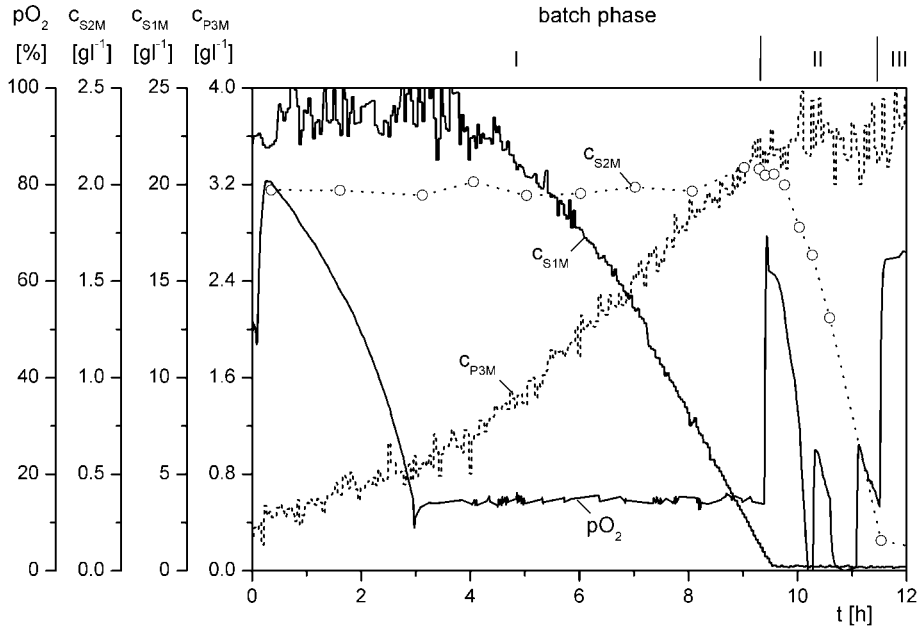

Figure 5. Batch phase of an *E. coli* cultivation: I = glucose uptake, II = glycerol uptake, III = acetate uptake.

the cultivation broth due to a sampling failure (see Fig. 7). The switches in consumption of different carbon sources are indicated by the peaks in the dissolved oxygen tension pO_2.

In order to point out the quality and reliability of the applied at-line measurement devices, several validation experiments are performed. The results of these experiments are presented separately in the following. Regular glucose at-line measurement during cultivation is performed by ProcessTrace in a high range from c_{S1M} = 0.5 gL^{-1} to 40 gL^{-1}. After cultivation is finished, glucose is measured in samples, which were taken during cultivation using an off-line HPLC system. Since glucose measurement via ProcessTrace is well known and already established, the lower end of the measuring range (0.05 to 5 gL^{-1}) is of more interest.

Verification of the at-line glucose measurement is performed with a second ProcessTrace calibrated on the lower measuring range from c_{S1M} = 0.05 gL^{-1} to c_{S1M} = 5 gL^{-1} during this cultivation. Fig. 6 points out that ProcessTrace with the low measuring range as well as HPLC off-line detection show synchronous results. In comparison, ProcessTrace with the high measuring range that is approved to a low measuring range of c_{S1M} = 0.5 gL^{-1} indicates only a slight offset of approximately 0.2 gL^{-1} (see Fig. 6).

With the purpose to view a decrease of glucose concentration during the batch phase this offset throughout the glucose limited fed-batch phase is tolerable, especially since glucose accumulation, e.g. t = 27.5 h in Fig. 6, is suitably indicated. These particular accumulations of glucose within the cultivation broth result from shifts in the feeding rate F_{R1} due to dynamic response experiments.

Standard acetate at-line measurement during cultivation is performed with ProcessTrace with acetate application in the measuring range from c_{P3M} = 0.5 gL^{-1} up to c_{P3M} = 5 gL^{-1}.

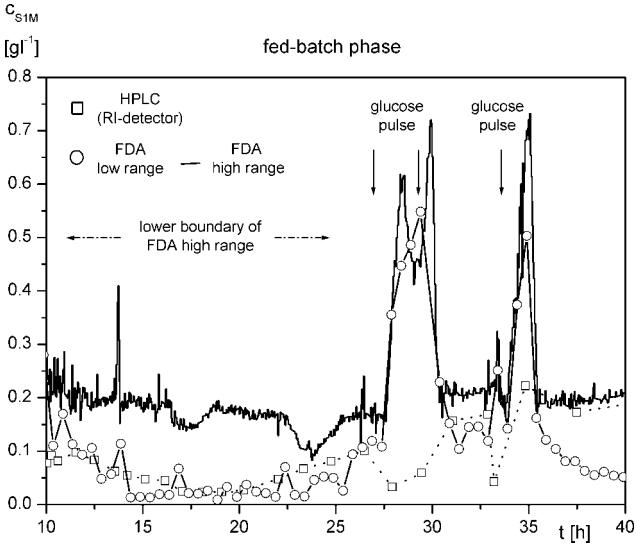

Figure 6. Verification of glucose at-line measurement.

After the cultivation, acetate is measured in samples taken manually during cultivation applying an off-line HPLC system.

Application of an enzymatic test kit (Roche Diagnostics, Penzberg, Germany) verifies these acetate measurements after the cultivation. In order to obtain the highest possible validity, a comparison of measurements is performed only for those spots where at-line ProcessTrace signals differ significantly from off-line determined HPLC results (see Fig. 7).

Marks obtained from HPLC analysis correspond to the validation measurement performed at selective sights (see Fig. 7). Especially in the time span between t = 8.1 h and

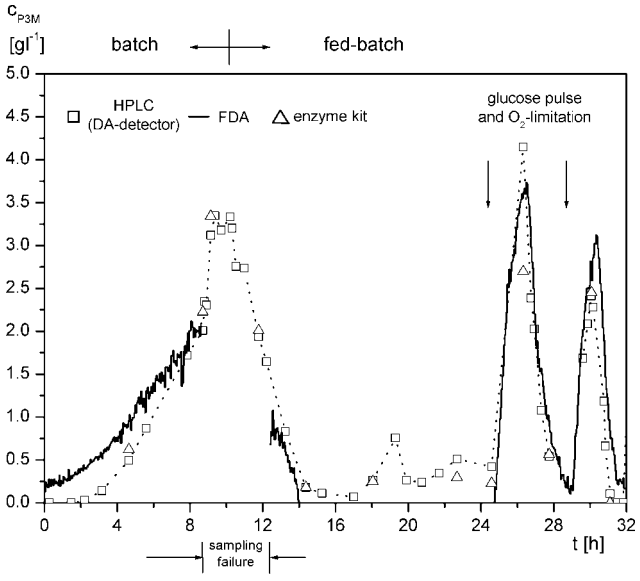

Figure 7. Verification of acetate at-line measurement.

t = 12.5 h the at-line ProcessTrace indicates wrong results, due to temporarily insufficient sampling via ESIP.

Regular ammonium at-line measurements during cultivation are performed by the FIA system TAS 2000 in the measuring range from c_{AltotM} = 0.2 gL^{-1} to c_{AltotM} = 2.5 gL^{-1}. After the cultivation is finished, ammonium is measured again in samples taken manually during cultivation by the same TAS 2000 system with varied sampling procedure.

Validation of the ammonium measurement is performed with a gas-sensitive electrode manufactured by Mettler Toledo, Gießen, Germany.

Up to t = 20 h the ammonium at-line measurement, shown in Fig. 8, is out of range. Comparing the available results from the off-line TAS 2000 measurements with the results obtained from the gas-sensitive electrode a proper fitting is implied (obvious). Being aware of the fact that measurement with the gas-sensitive electrode only allows ammonium detection with respect to orders the fitting is good (see Fig. 8).

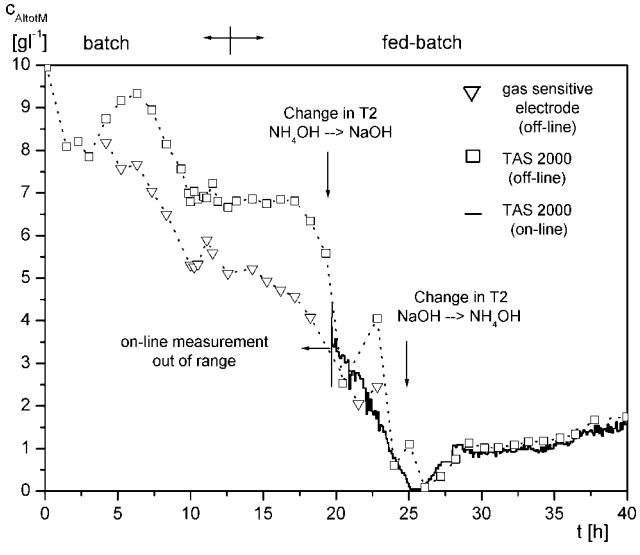

Figure 8. Verification of ammonium at-line measurement.

Since t = 20 h at-line results of the ammonium concentration are available from TAS 2000. These results correspond well to the respective off-line measurements. After t = 23 h no more measurements with the gas-sensitive electrode are available. Nevertheless, suitable fitting of both electrode and TAS 2000 at-line measurement with the TAS 2000 off-line measurement indicates proper ammonium detection during the whole cultivation time.

This is the basis to control the ammonium concentration, and, therefore, the nitrogen supply, during cultivation.

Controlling of certain growth parameters in general offers optimum and stable growth and production conditions.

4 Conclusions

On-line availability of information regarding the parameters of a running microbial cultivation allows better understanding, verification or determination of important cultivation parameters.

This information is very valuable for the design of new cultivations or optimization of already existing processes ending up in a higher yield of the desired agents.

The results presented above point out the necessity of robust and easy to use measuring devices. ProcessTrace as well as TAS 2000 convince by exact and reproducible measurements serving the properties named above in a suitable way.

On the other hand, the applied prototype ProcessTrace acetate lacks the desired exactness at certain stages of cultivation with *E. coli*. Eventually, this is not even a matter of the device itself, but the combination of ProcessTrace acetate with sampling via an ESIP still requires too much adaptation to an existing cultivation process at this stage of development.

However, the potential of this device is obvious. Formation of the by-product acetate during cultivation of *E. coli* still is one of the major problems on the industrial production scale. Optimization with respect to acetate formation could offer the ability of faster microbial growth or higher production yields.

Several cultivations with *E. coli* K12 TG1 are performed, starting in batch mode and followed by a fed-batch phase. At-line and off-line measurements during these cultivations indicate that fermentation by-products like acetate are formed in considerable amounts during both phases.

The use of single measurement applications is discussed in detail in the following.

At-line observation of glucose during microbial cultivation is well known and already established.

ProcessTrace is a suitable measuring device with respect to handling as well as to the results obtained.

For this article the higher measuring range of ProcessTrace from 0.5 L^{-1} to 40 gL^{-1} is applied in order to observe

the glucose concentration during the batch phase and also during the substrate limited fed-batch phase in a rush without any recalibration or changes in the equipment. In this context, there is no difference between the two possible sampling proceedings, dialysis probe and ESIP filtration probe.

Validation has shown that it does not lack exactness and resolution even in the lower end of the measuring range (0.1 gL^{-1}), allowing suitable control of the cell-specific growth rate μ.

Because of the measuring principle evaluating negative peak signals, in combination with the used enzyme that is inhibited besides acetate by several molecules, the acetate measurement is susceptible to overdetermination of acetate. Furthermore, the at-line measurement with ProcessTrace seems to be disturbed during certain periods of cultivation resulting in indication of wrong acetate concentrations (see Fig. 7). Using a different sampling procedure, like the dialysis probe known from the glucose application, might help to minimize this deviation.

For pH control and nitrogen supply purposes, ammonia was fed into the reactor. Ammonium detection with TAS 2000 is possible in a measuring range from 0.2 gL^{-1} to 2.5 gL^{-1} in the cell-free cultivation broth. Hence, the consumption of the nitrogen source as well as the increase in ammonium concentration within the cultivation broth after changing the pH adjusting-agent (NH_4OH to NaOH and vice versa) is observable (see Fig. 8).

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Symbols used

c_{IJ}	[gL^{-1}]	concentration of component *I* in compartment *J*
F_A	[Lh^{-1}]	analysis sampling rate (ESIP)
F_{Tj}	[Lh^{-1}]	titration rate of acid ($j = 1$) and base ($j = 2$)
N_{St}	[rpm]	stirrer agitation speed
p_G	[Pa]	total pressure in the gaseous phase
pO_2	[%]	dissolved oxygen tension
Q_{CO2}	[$gL^{-1}h^{-1}$]	volumetric carbon dioxide discharge rate
Q_{O2}	[$gL^{-1}h^{-1}$]	volumetric oxygen supply rate
RQ	[–]	molar respiratory quotient
S_{turb}	[V]	signal of turbidity probe
t	[h]	cultivation time
x_{IG}	[–]	molar fraction of component I in the gaseous phase
x_I	[%]	off-gas measurement signal of component *I*
μ	[h^{-1}]	cell-specific growth rate
ϑ	[°C]	temperature

Indices

Ac	acid (phosphoric acid)
AF	antifoam
AIR	air
Al	alkali (ammonia)
G	gaseous phase
L	reaction (liquid) phase
M	cell-free media phase
max	maximum value
n	normalized gas conditions
P3	product acetate = substrate 3 (S3)
R1	glucose reservoir
S1	substrate glucose
S2	substrate glycerol
Ti	titration tank i (1 = acid, 2 = alkali)
tot	total
w	set point

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