Fast filtration for metabolome sampling of suspended animal cells

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Section: Animal Cell Technology

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Abstract A new method for sampling suspended animal cells by fast filtration is presented that allows rapid quenching of cellular metabolism and efficient separation of the cells from culture medium. Compared to sampling with a microstructure heat exchanger or centrifugation without prior quenching, the adenylate energy charge and the measured concentrations especially of metabolites with a high turnover rate or of metabolites early in metabolic pathways were substantially higher. No leakage of ATP from the cells was observed when using iso-osmotic NaCl solution in the washing step. The combination of fast filtration and cold methanol extraction is therefore suitable for intracellular metabolomic studies of suspended animal cell cultures and superior to other methods currently applied.

Keywords Animal cells · Fast filtration · Metabolome · Microstructure heat exchanger · Quenching · Rapid sampling

Introduction

Recent years have seen an increase in systems biological approaches for the investigation of cellular processes. In this respect the ”omics” technologies have not only been developed to investigate the processes within cells but also to use the obtained knowledge to improve cultivation processes. From the classical functional genomics/systems biology tools, metabolomics is the most recent technique. Unlike for bacteria and yeast (Bolten and Wittmann 2008; Faijes et al. 2007; Schaub et al. 2006; Villas-Bôas et al. 2005), there are no standard protocols for sampling and extraction of suspended animal cells. A number of different rapid sampling methods are currently used (Sellick et al. 2009; Wiendahl et al. 2007; Dietmair et al. 2010), but have not been compared to other methods in depth.

It is most important for metabolome sampling to stop the organism’s metabolic activity quantitatively and rapidly. This is to avoid loss of metabolites caused by residual enzyme activity (de Koning and van Dam 1992) or quenching conditions. A standard quenching method for bacteria and yeast is cold methanol, though other protocols using a microstructure heat exchanger

or fast filtration are also applied (Wellerdiek et al. 2009; Bolten and Wittmann 2008). Dietmair et al. (2010) showed that using methanol, as proposed by Sellick et al. (2009), causes increased cell permeabilization and leakage of intracellular components. Thus, alternative methods for the quenching of suspended animal cells are required.

Furthermore, it is of utmost importance to separate the cells from the supernatant before metabolite extraction, as residual extracellular components will interfere with the measurement of low concentrated intracellular metabolites, making filtration or pelleting by centrifugation the most suitable techniques. Both methods require a washing step to remove residual medium from the cells. Phosphate buffered saline (PBS) is widely used as washing solution (Teng et al. 2009), but the high phosphate concentration in the sample after the washing step is problematic. Phosphate is a target for metabolome analysis and in large quantities interferes with analytical methods for metabolite quantification, e.g. GC-MS. Dietmair et al. (2010) use an iso-osmotic saline solution, which is also applied in this work.

Another important step of the sample preparation process is the extraction of metabolites from cells. A number of different extraction methods are currently employed for bacteria and yeast as well as for animal cells using boiling methanol, chloroform/methanol and acidic extractions with acidic acetonitrile/methanol or acetic acid. For suspended animal cells methanol is most commonly used as extraction agent (Ritter et al. 2008; Bennett et al. 2008; Sellick et al. 2009; Dietmair et al. 2010).

Fast filtration as a sampling method for suspended animal cells has already been described by Dietmair et al. (2010) but, due to problems with the metabolite recovery they did not consider it a suitable technique. In this study we describe new method for sampling suspended animal cells using fast filtration and subsequent extraction of metabolites. Furthermore, this method was used for taking metabolite samples during a cultivation of CHO cells. Samples were compared to those taken by a microstructure heat exchanger method derived from Wiendahl et al. (2007) as well as to sampling without quenching.

Material and methods

Cultivation of cells

CHO DP12 cells (ATCC, CRL-12445) were cultivated using the chemically defined medium TC-42 (TeutoCell AG, Germany).

Sampling using fast filtration

The fast filtration set-up contained a custom build two part PTFE filtration device. The lower part accommodated a 50 mm stainless steel filter support frit (Sartorius Stedim Biotechnology, Germany) and a connection for the vacuum pump and controller (CVC3000, Vacuubrand, Germany). The upper part contained a 15 cm polystyrene pipe of 8 mm diam. for application of the sample and washing solution.

For sampling with the fast filtration device a volume of cell suspension correlating to 5×10^7 cells total was drawn from the bioreactor using a luer-lock coupled syringe. The cell suspension was applied to the custom build PTFE filtration device and drawn through a 47 mm depth filter disk of 3 µm nominal pore size (Type A/D, Pall Corporation, USA) using a vacuum of 40 mbar. Subsequently, 30 ml of a 0 °C cold iso-osmotic NaCl solution (290 mOsm kg^{-1}) was drawn through the filter to remove residual culture medium. The filter was taken from the filtration device and placed inside a 50 ml tube pre-cooled to 0 °C. Tube and filter were frozen in liquid N_2, immediately.

Sampling using a microstructure heat exchanger

A microstructure heat exchanger (Forschungszentrum Karlsruhe, Germany) was connected to a cooling unit (Julabo, Germany) maintained at −4 °C. Cell suspension from the bioreactor was drawn through the heat-exchanger with a vacuum of 180 mbar. The cooled cell suspension was collected in a glass bottle on ice and a volume correlating to 5×10^7 cells was transferred to pre-

cooled 50 ml tubes. The tubes were centrifuged for 1 min at −2 °C and 3000 *g* in a fixed-angle centrifuge. The supernatant was discarded and the cell pellets were washed with 30 ml ice-cold NaCl solution. The washed cell pellets were frozen in liquid N_2 and stored at −80 °C until extraction of metabolites.

Reference sampling

The cell suspension was directly drawn into 50 ml tubes. Further treatment was according to the microstructure heat exchanger sampling protocol.

Extraction of metabolites

Pelleted cells and cells on filters were extracted using a similar protocol: 4 ml 85 % (v/v) aqueous methanol at −20 °C was added to the washed filter and cell pellets. As the filter still contains a certain volume of washing solution, 1 ml ice-cold water was added to the pellets. Internal standards for metabolite analysis were added and incubated for 24 h at −20 °C. The filter was removed from the extraction solution by drawing the solution through the porous frit of an empty PD10 column (GE-Healthcare, Sweden). Subsequently, 400 µl chloroform (20 °C) were added and the mixture was vortexed thoroughly for 20 s and spun down for 10 min at 2000 rcf and 20 °C. The supernatant was transferred to a new 15 ml tube and evaporated at 10 °C. Extracts were resuspended in 500 µl water and 500 µl chloroform was added. The suspension was vortexed thoroughly for 20 s and re-centrifuged for 15 min at 2000 *g* and 20 °C. Three 100 µl aliquots of the aqueous phase were transfered to new 1.5 ml tubes. The extracts were stored at −80 °C until analysis.

Analytical methods

A lactate dehydrogenase (LDH) test kit (Roche Diagnostics, Germany) was used to measure cell disruption given as percentage of total cellular LDH activity.

Adenosine triphosphate (ATP) was quantified using a commercial luminescence assay (Biaffin, Germany) or as described below for metabolites from the central energy metabolism.

Amino acids and pyruvate were measured using standard protocols on by HPLC (Büntemeyer 2010).

The concentrations of intracellular metabolites from the central energy metabolism were determined by HILIC-mass spectrometry (Hydrophilic Interaction Liquid Chromatography) on a MonoChrom diol column (Varian, Germany) using acetonitrile with 0.1 % formic acid (A) and 5 mM ammonium acetate with 0.1 % formic acid (pH 3.5, adjusted with ammonia) (B), at 0.2 ml min^{-1}.

For GC-MS analysis, the keto- and aldehyde-functions of the metabolites were converted into their oxime derivatives using methoxyamine hydrochloride. All other relevant functions like amines, carboxylic acids or hydroxyls were masked with trimethylsilyl groups resulting in volatile derivatives. Statistical errors were reduced by using ribitol as internal standard, while systematic bias were avoided by randomization. The derivatives were separated in a GC 3800 chromatograph and detected by a MS4000 mass spectrometer (both Varian, Germany). A FactorFour VF-5ms column (30 m × 0.25 µm × 0.25 µm) consisting of 95 % methyl- and 5 % phenyl-groups was used (Varian, Germany).

Results and discussion

Validation of fast filtration as means of metabolome sampling

A microstructure heat exchanger method proposed by Wiendahl et al. (2007) and the use of methanol/ammonium bicarbonate as proposed by Sellick et al. (2009) have been used for quenching animal cell suspensions to 0 °C in the sub-second scale, but residual metabolic activity still occurs below 0 °C (Sauter 2003). Therefore it is key to authentic metabolome sampling of

animal cells to not only cool the cells down as fast as possible but also to keep overall sampling time to a minimum. Sampling using centrifugation has certain drawbacks in this respect. We found that a centrifugation time of 1 min at 3000 *g* is advisable for reliable results. This results in a total sampling time of about 3 min after quenching when using a washing step. Fast filtration allows sampling within 25 s.

The shear forces caused by drawing cell suspension and washing solution through the filter are a major concern, as they can result in high rates of cell disruption during sampling. Data on LDH release during sampling with different vacuums are shown in Fig. 1.

The release of LDH from the cells is only slightly higher during filtration compared to sampling using centrifugation. Sampling at 40 mbar vacuum results in 5.43 % ± 0.57 % LDH measured in filtrate and wash compared to 2.56 % ± 0.66 % LDH measured in supernatant and wash of reference centrifugation experiments.

An increase in vacuum results in higher amounts of LDH in the filtrate and wash due to higher shear forces caused by the increased transmembrane pressure. Lower vacuums increase the sampling time, as illustrated in Fig. 2, making 40 mbar an optimal vacuum allowing short sampling time with minimal cell damage. Higher vacuums account for increased cell disruption which leads to the loss of intracellular metabolites from the sample.

To reduce residual medium components in the extrac,t a rapid washing step is implemented in the sampling procedure. Fig. 3 shows the cellular content of four exemplary amino acids extracted from filters loaded with 10^7 cells to 6×10^7 cells. For comparison data of reference experiments with centrifugation of 5×10^7 cells are shown.

Cellular amino acid concentrations were independent from the number of cells on the filter, indicating that residual extracellular components are negligible. High amounts of residual amino acids would lead to significantly higher amino acid concentrations at lower cell counts. Data of other amino acids support these findings further. The average amount of residual amino acid in the filter was calculated to be 0.19 % ± 0.01 % of the total amino acid amount in the cell suspension used with a maximum for glycine of 0.22 % ± 0.04 %. These values are in accordance to those

presented by Sellick et al. (2009) for direct quenching in methanol with an additional washing step.

Additionally, the slightly increased cell disruption measured when using fast filtration for sampling has no significant effect on the metabolite content. Both, sampling using fast filtration and centrifugation yielded the same results on intracellular amino acid amounts. Potential differences caused by cell disruption seem to be smaller than the error from sampling and measurement.

Fig. 4 shows the results of experiments on leakage of ATP from the cells during sampling. ATP was chosen as a model for larger polar metabolites and to compare results to data from Sellick et al. (2009), who use solutions of methanol and substances like ammonium bicarbonate for quenching and washing. No detectable leakage was observed when using an iso-osmotic solution of sodium chloride in water and only minor leakage when using pure water as washing solution. In contrast, Sellick et al. (2009) describe ATP leakage of more than 50 % in a washing step. The main reason for this is the disintegration of the cell membrane due to the methanol in the quenching solution, as assessed by Dietmair et al. (2010).

Intracellular ATP was measured in this study to be about 1.5 fmol $cell^{-1}$. While Dietmair et al. (2010) determined the intracellular ATP amount of a recombinant CHO cell line (clone C2.8 SPF) to be 4.6 fmol $cell^{-1}$. Differences to this study might be caused by a multitude of reasons including differences in the cell line or culture conditions. A comparison with results to data for CHO cells from Sellick et al. (2009) is difficult as they measured intracellular concentrations instead of the cellular amount. Assuming perfectly circular cells of 12 µm diam. the intracellular concentration of 6 mM would translate into an amount of approx. 5.4 fmol $cell^{-1}$. All these intracellular ATP amounts are well within the range Cordell et al. (2008) states as normal for CHO cells.

Pyruvate leakage from cells during fast filtration is shown in Fig. 5. Pyruvate was chosen as model for smaller polar metabolites, especially monocarboxylates, which are prone to 'bleed' during sampling. In experiments with water as washing solution' nearly all pyruvate bled from the cells. When using iso-osmotic NaCl solution for washing about 600 nmol or 50 % of the total pyruvate bled from the cells. The efflux of monocarboxylates, especially lactate, from cells is caused by

concentration driven transport of proton-linked monocarboxylate transporters (MCT) rather than by diffusion across the membrane (Sharpe and Milligan 2003). Therefore, it can be assumed that the much higher release of pyruvate from the cells compared to LDH release during sampling is caused by transporters and, possibly, ion-channels rather than cell disruption. This is supported by the negligible ATP leakage as there are no ATP transporters in the cell membrane.

Application of fast filtration for metabolome sampling

To evaluate the applicability of metabolome sampling of animal cells using fast filtration samples were drawn from a bioreactor cultivation of CHO DP12 cells (Fig. 6) using different sampling protocols. Samples for metabolome analysis were taken on days three, four, and five using fast filtration and microstructure heat exchanger sampling as well as centrifugation as reference.

Fig. 7 illustrates the adenylate energy charge (AEC) measured for each sampling protocol at each sampling point. The AEC was calculated as described by Ataullakhanov and Vitvitsky (2002).

The AEC for the samples taken using the fast filtration method is higher for all sampling points. This indicates a higher efficiency of stopping the metabolic activity, as the turnover rates of ATP to ADP and AMP are rather high (Ataullakhanov and Vitvitsky 2002).

The intracellular glucose concentration for the three sampling points is shown in Fig. 8. The glucose concentration from the samples of day five of the cultivation was below the limit of detection. A higher amount of intracellular glucose is observed for the samples taken by fast filtration. This can be due to a higher quenching efficiency of the fast filtration method or to residual extracellular glucose on the filter. The latter can be ruled out by the fact, that fast filtration samples do not exhibit higher amino acid concentrations than samples taken with the other methods (data not shown).

Large differences were also found for ribose/ribulose 5-phosphate concentrations (Fig. 9). On cultivation day 5 concentrations of up to 0.243 fmol cell^{-1} were measured in fast filtration extracts

and concentrations below 0.038 fmol $cell^{-1}$ and 0.037 fmol $cell^{-1}$ were measured in extracts from samples taken using the microstructure heat exchanger and centrifugation, respectively.

This strongly supports the higher quenching efficiency of the fast filtration method. By using fast filtration the metabolism of the cells was more efficiently stopped and concentrations of metabolites with high turnover rates were maintained at higher concentrations, whereas the metabolism of cells sampled with the microstructure heat exchanger or just centrifugation was stopped less efficiently.

Dietmair et al. (2010) describe considerably lower amounts of extracted metabolites from cells on filters compared to extracts from cell pellets. This was not observed in this study (see also Fig.10).

Conclusions

Fast filtration is a valuable and reliable tool for metabolome sampling of suspended animal cells. Even metabolites with high turnover rates were detected in higher concentrations compared to other sampling methods. Furthermore, leakage of metabolites from the cells during sampling is low, as is cell damage.

There is no obvious advantage using the microstructure heat exchanger method in comparison to centrifugation at −2 °C. This is most likely due to the extended period of time in which the cells are handled at about 0 °C, which is not sufficient to completely stop the metabolic activity.

Depending on the nature and the turnover rates of the target metabolites, a simple sampling method like centrifugation might be sufficient to yield some results. But to generate a snapshot of the metabolic state of a cell at any given moment, the fast filtration method presented in this study is a reliable tool superior to other sampling techniques like microstructure heat exchanger or rapid centrifugation.

The cold methanol extraction described in this work is more capable of releasing metabolites from cells on filters than the method used by Dietmair et al. (2010). This may be due to the longer exposure of the cells to the extraction reagent (i.e. 24 h as opposed to 10 min).

Furthermore, inert borosilicate glass-fiber filter material was used in this study to prevent interactions of metabolites with the filter material. The composite PFTE and PE filters used by Dietmair et al. (2010) might cause adherence of metabolites to the filter material.
Thus, the presented combination of fast filtration and cold methanol extraction can be considered as well applicable for sampling of intracellular metabolites from suspended animal cells.

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Fig. 1 Dependence of LDH release and vacuum applied to the filtration device. Reference experiments with centrifugation of cells at 3000 rcf for 1 min exhibited a release of LDH during sampling of 2.56 % ± 0.66 % compared to the total LDH amount in the sample cell solution. For each cell count three individual experiments were done. LDH measurements were done in triplicates

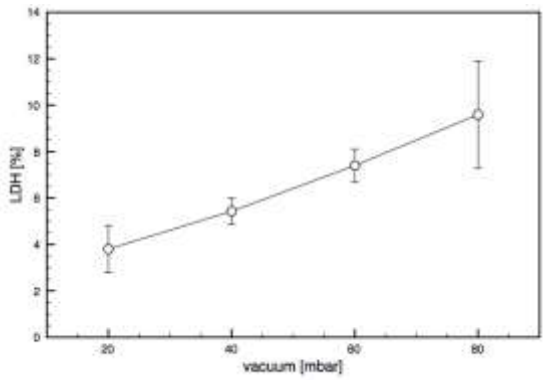

LDH [%]
vacuum [mbar]
0
2
4
6
8
10
12
14
20
40
60
80

Fig. 2 Correlation of vacuum and filtration time for cell suspension and sodium chloride washing solution. Error bars indicate the standard deviation of four individual experiments

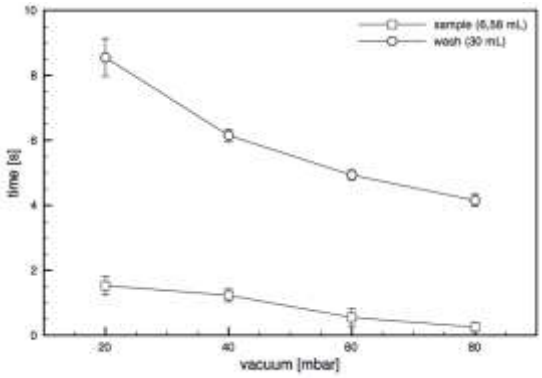

sample (6,56 mL)
wash (30 mL)
time [s]
vacuum [mbar]
0
2
4
6
8
10
20
40
60
80

Fig. 3 Correlation of cell number on filter and extracted metabolite content compared to a reference sample taken by centrifugation. Error bars indicate standard deviation of three individual experiments

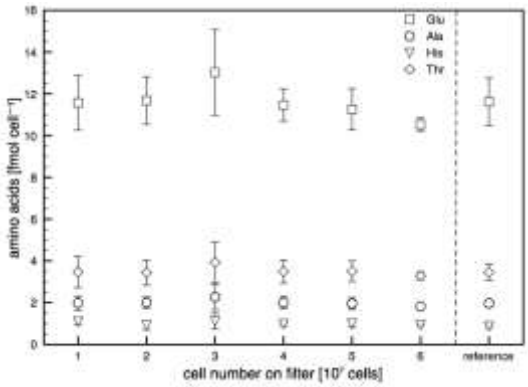

Glu
Ala
His
Thr
amino acids [fmol cell⁻¹]
cell number on filter [10⁷ cells]
reference

Fig. 4 Leakage of ATP after sampling using fast filtration. Leakage was assessed by comparison of the ATP amount measured in extracts from 5×10^7 cells on filter and measured in the washing solution. Error bars indicate the standard deviation of three individual experiments

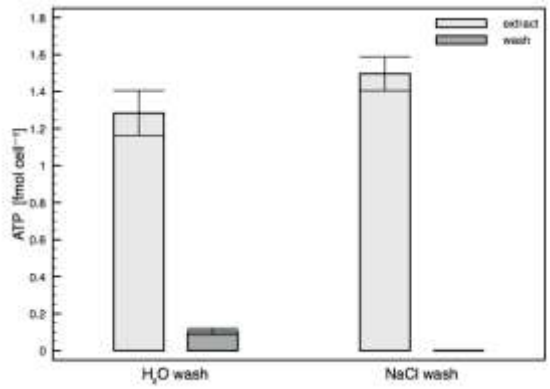

extract
wash
ATP [fmol cell⁻¹]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H2O wash
NaCl wash

Fig. 5 Leakage of pyruvate after sampling with fast filtration. Leakage was assessed by comparison of the pyruvate amount measured in extracts from 5×10^7 cells on filter and measured in the washing solution. Error bars indicate the standard deviation of three individual experiments

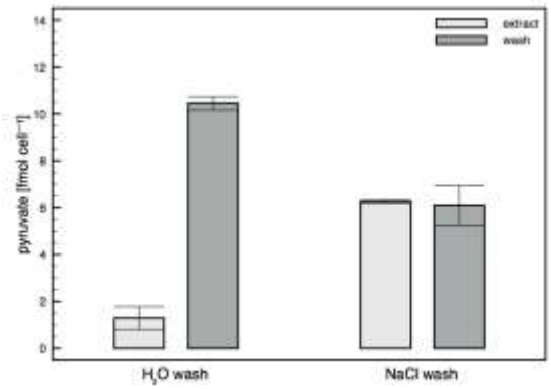

extract
wash
pyruvate [fmol cell⁻¹]
0
2
4
6
8
10
12
14
H2O wash
NaCl wash

Fig. 6 Cultivation of CHO DP12 cells in a 2 l bioreactor. Arrows indicate sampling points for metabolome analysis. Initial cell density was 5×10^5 cells ml^{-1}. The temperature was controlled at 37 °C. The stirring speed with two Rushton turbines was 150 rpm. PO and pH were controlled at 40 % DOT and pH 7.2, respectively

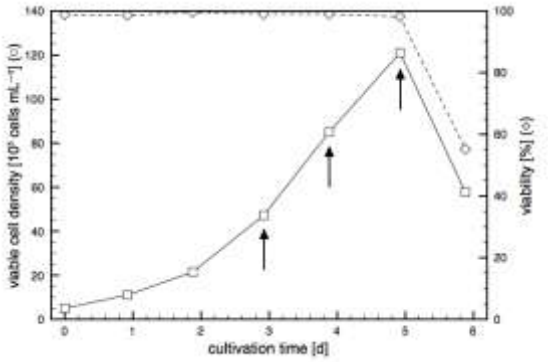

viable cell density [10⁵ cells mL⁻¹] (□)
viability [%] (◇)
cultivation time [d]
0
20
40
60
80
100
120
140
0
1
2
3
4
5
6

Fig. 7 Adenylate energy charge at different time points in CHO bioreactor cultivation using different sampling methods. Error bars indicate the standard deviation of four individual measurements

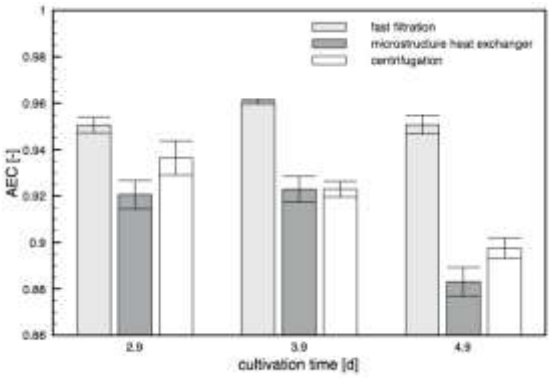

fast filtration
microstructure heat exchanger
centrifugation
AEC [-]
1
0.98
0.96
0.94
0.92
0.9
0.88
0.86
2.9
3.9
4.9
cultivation time [d]

Fig. 8 Intracellular glucose concentration measured by GC-MS for the different sampling methods during the cultivation. The ratio of glucose peak to ribitol peak (internal standard) is given. Error bars indicate the standard deviation of six individual measurements

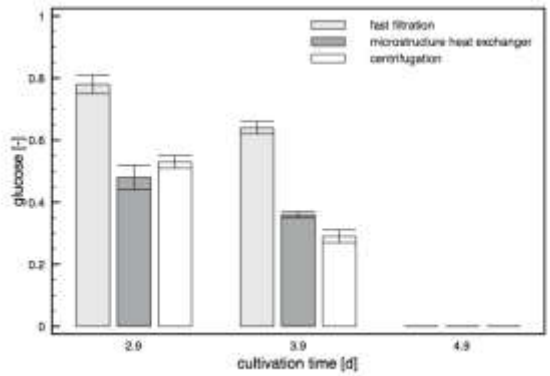

fast filtration
microstructure heat exchanger
centrifugation
glucose [-]
0
0.2
0.4
0.6
0.8
1
2.0
3.9
4.9
cultivation time [d]

Fig. 9 Ribose/ribulose 5-phosphate concentration in the extracts measured by LC-MS for the different sampling methods during the cultivation. Due to the similar chemical structure a distinction between ribose 5-phosphate and ribulose 5-phosphate was not possible. Error bars indicate the standard deviation of four individual measurements

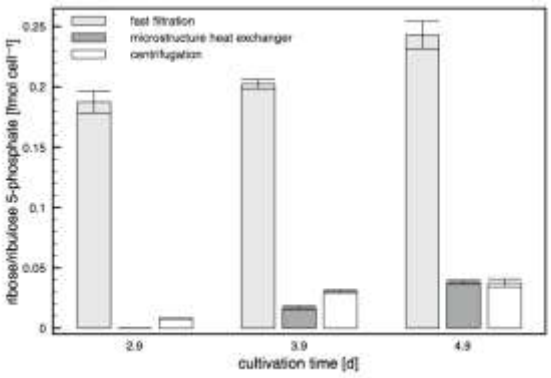
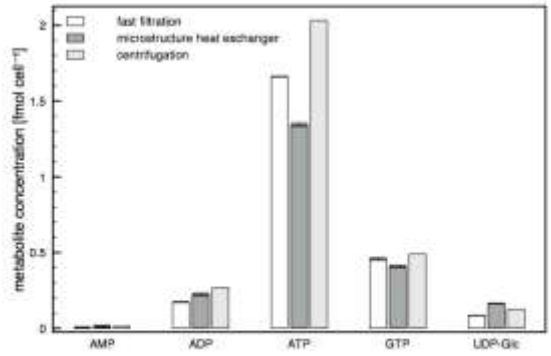
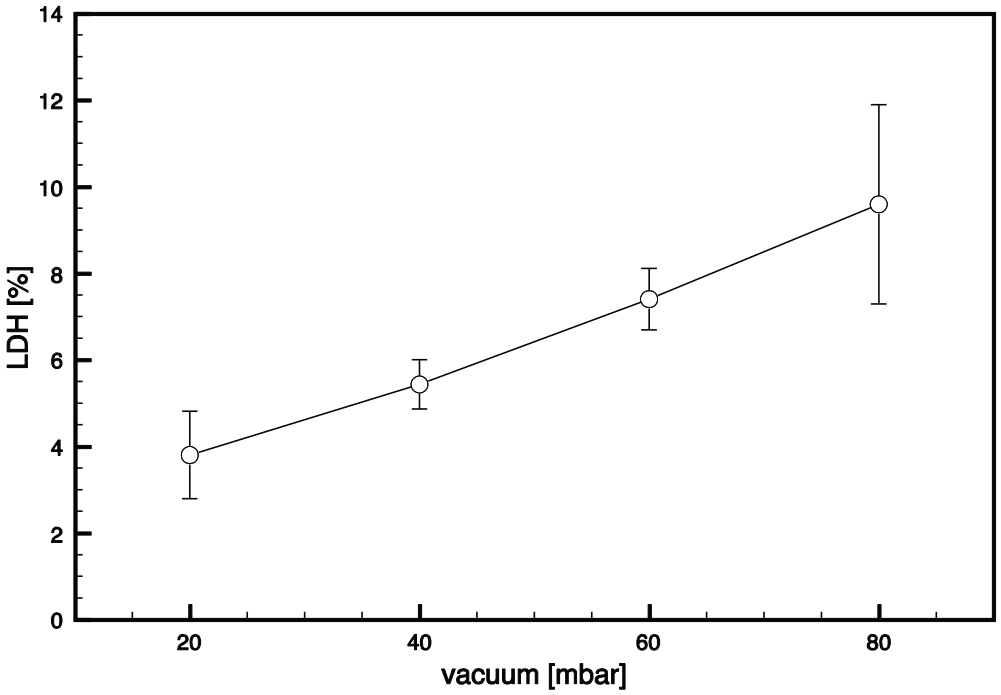
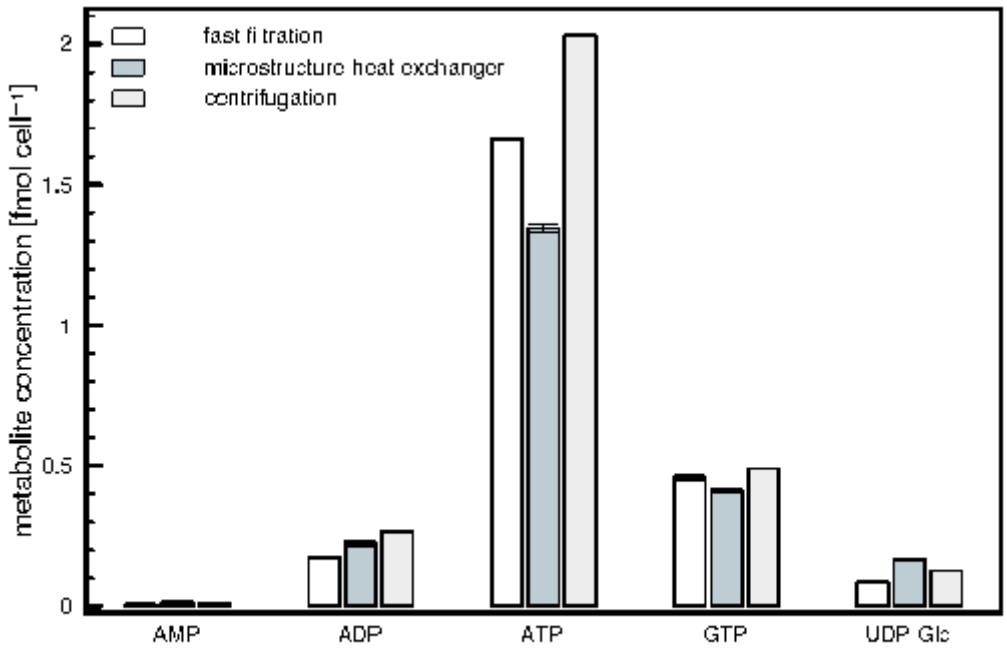
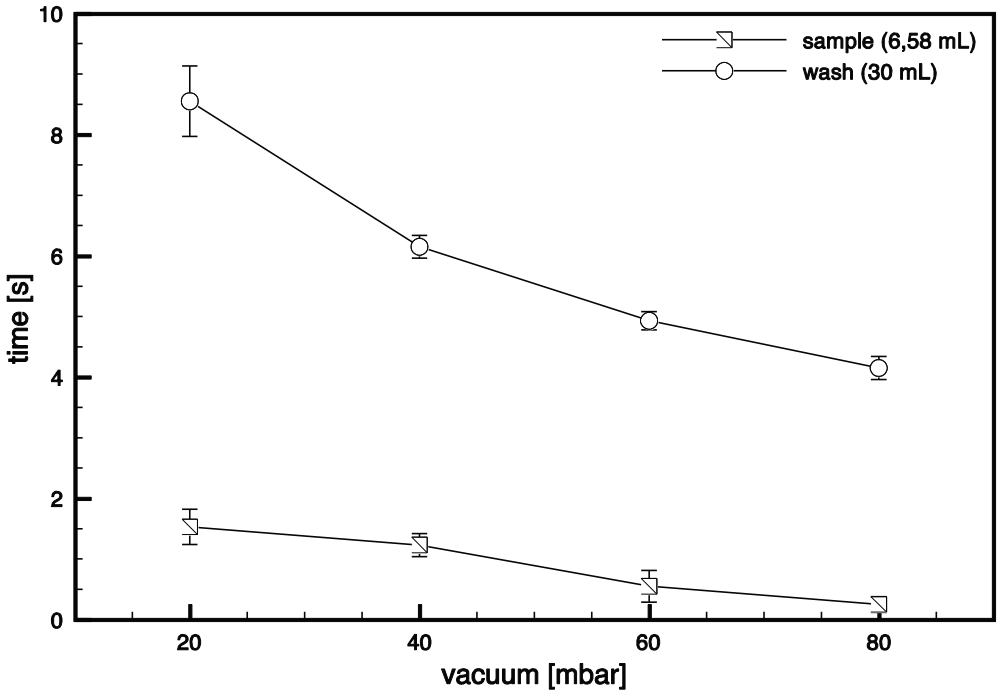

fast filtration
microstructure heat exchanger
centrifugation
ribose/ribulose 5-phosphate [fmol cell⁻¹]
0.25
0.2
0.15
0.1
0.05
0
2.0
3.9
4.9
cultivation time [d]

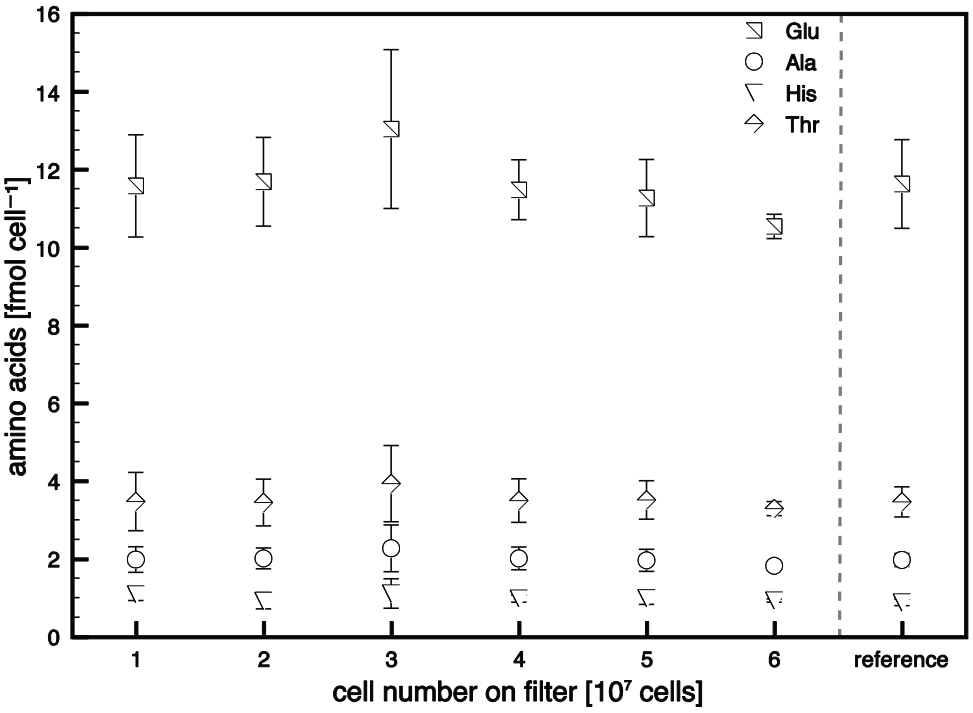
Fig. 10 Metabolite concentrations in the extracts measured by LC-MS for the different sampling methods. Samples are from the first sampling point during the cultivation (day 3). Error bars indicate the standard deviation of four individual measurements

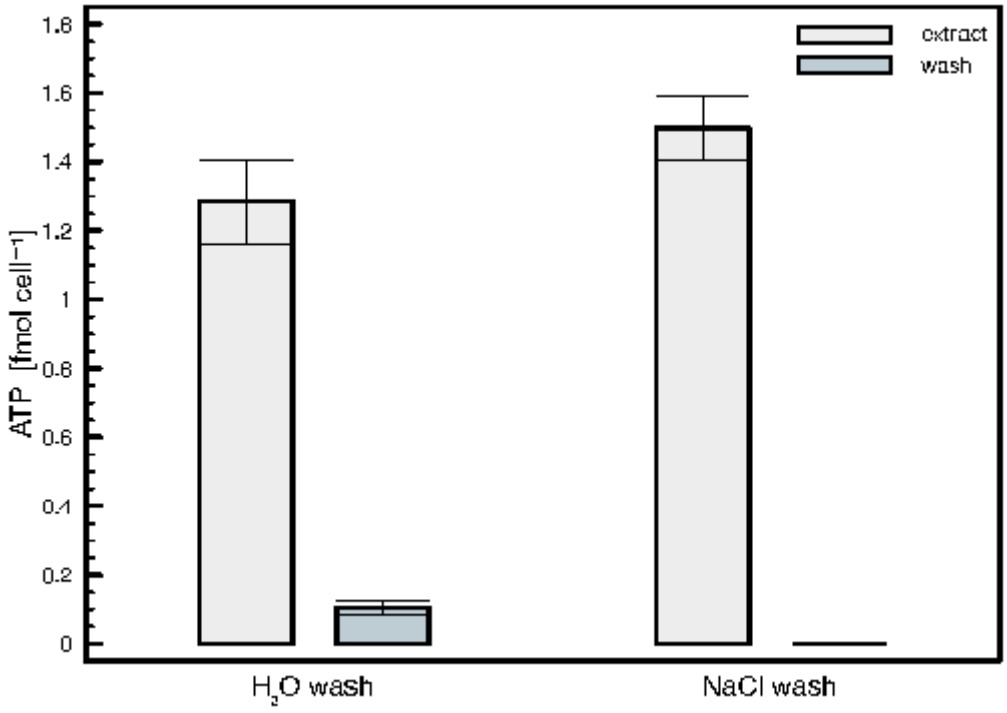

fast filtration
microstructure heat exchanger
centrifugation
metabolite concentration [fmol cell⁻¹]
0
0.5
1
1.5
2
AMP
ADP
ATP
GTP
UDP-Glc

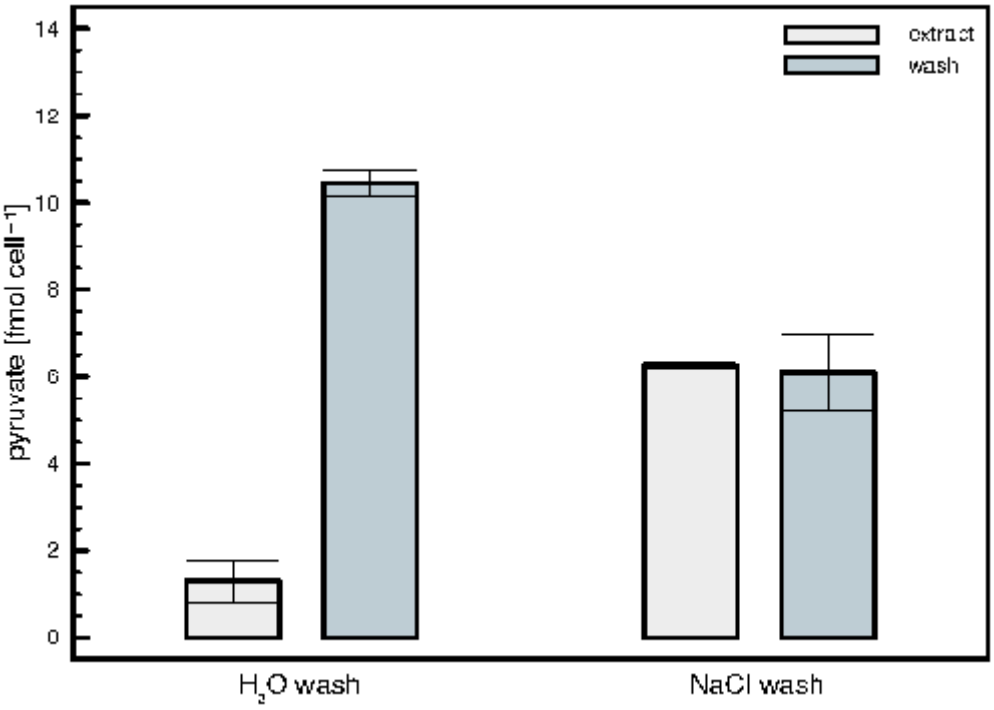

14
12
10
8
6
4
2
0
LDH [%]
20
40
60
80
vacuum [mbar]

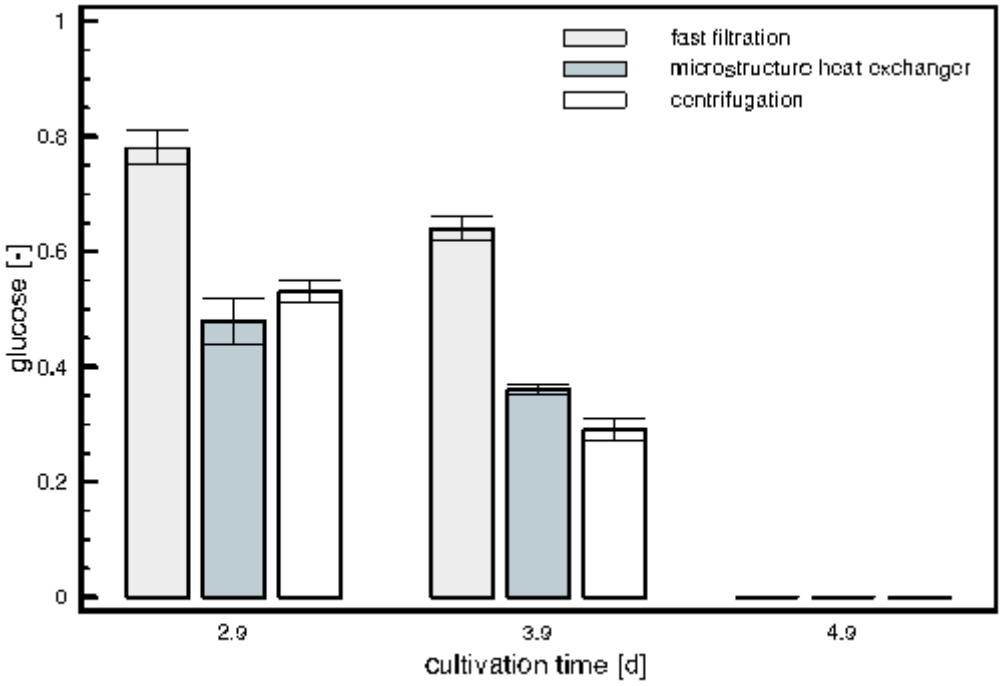

fast filtration
microstructure heat exchanger
centrifugation
metabolite concentration [fmol cell⁻¹]
2
1.5
1
0.5
0
AMP
ADP
ATP
GTP
UDP Glc

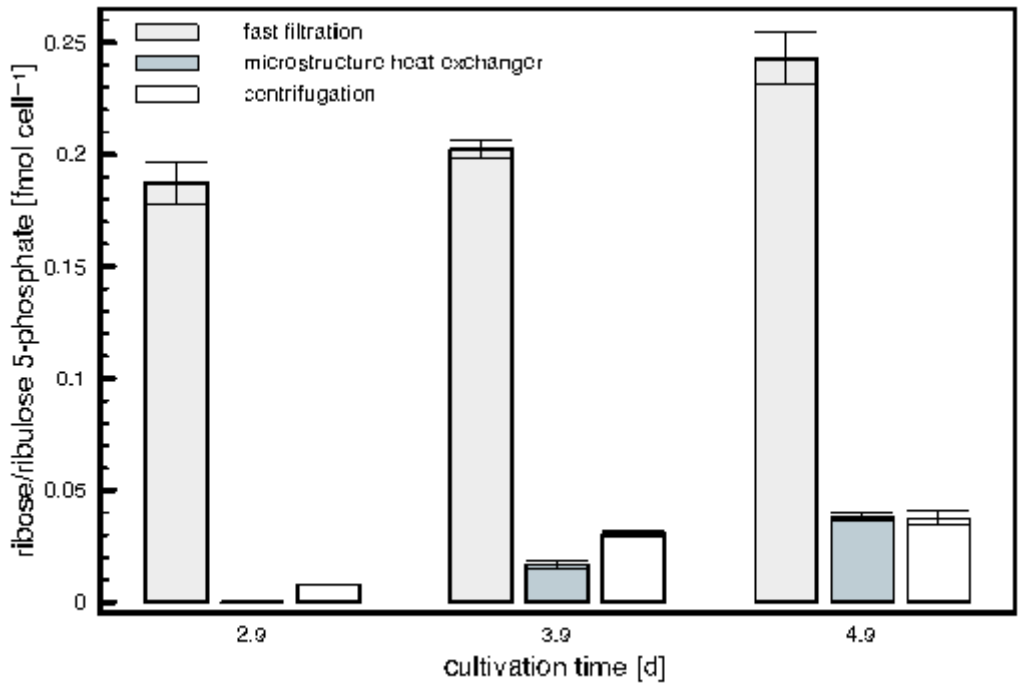

sample (6,58 mL)
wash (30 mL)
time [s]
vacuum [mbar]
0
2
4
6
8
10
20
40
60
80


Glu
Ala
His
Thr
amino acids [fmol cell⁻¹]
0
2
4
6
8
10
12
14
16
1
2
3
4
5
6
reference
cell number on filter [10⁷ cells]


extract
wash
ATP [fmol cell^{-1}]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H_2O wash
NaCl wash


extract
wash
pyruvate [fmol cell^{-1}]
0
2
4
6
8
10
12
14
H_2O wash
NaCl wash


fast filtration
microstructure heat exchanger
centrifugation
glucose [-]
1
0.8
0.6
0.4
0.2
0
2.9
3.9
4.9
cultivation time [d]


fast filtration
microstructure heat exchanger
centrifugation
ribose/ribulose 5-phosphate [fmol cell⁻¹]
0.25
0.2
0.15
0.1
0.05
0
2.9
3.9
4.9
cultivation time [d]