Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Biotechnology

Fast sampling for quantitative microbial metabolomics

Walter M van Gulik

Targeted metabolomics, aimed at the study of metabolic reaction networks and their regulation *in vivo*, is a rapidly emerging field in systems biology. Obtaining proper quantitative snapshots of the microbial metabolome requires fast sampling, immediate quenching of enzymatic activity, separation of exometabolome and endometabolome, complete metabolite extraction from the cells, and reliable high-throughput analysis methods. This review addresses the current state of the art of rapid sampling and quenching for microbial metabolomics. Several robust and reliable rapid sampling devices have been developed. Various quenching and separation procedures have been proposed and applied, but with respect to the reliability of the different methods the literature is contradictory. To date a reliable universal method applicable to different microbial species is still lacking.

Address
Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628BC Delft, The Netherlands

Corresponding author: van Gulik, Walter M (w.m.vangulik@tudelft.nl)

Current Opinion in Biotechnology 2010, **21**:27–34

This review comes from a themed issue on
Analytical biotechnology
Edited by Peter Neubauer and Andreas Schmid

Available online 10th February 2010

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2010.01.008

Introduction

To obtain a quantitative systems biology understanding of cellular metabolism and its regulation, measurements on all different hierarchical levels, for example genome, transcriptome, proteome, fluxome, and metabolome are required [1]. At present whole genome sequencing and measurement of genome-wide expression levels have become common practice. Also methods for the quantification of intracellular fluxes, either through metabolite balancing or based on stable isotope (e.g. ^{13}C) labeling, have been well established [2]. Although significant progress has been made in proteomics [3] and metabolomics [4], especially because of the advancement of MS-based analytical procedures, these are still far from being established routine techniques. A prerequisite for obtaining meaningful metabolome data is the application of proper sampling and sample processing procedures. Many metabolites, especially the intermediates of the central metabolic pathways and connected cofactors like ATP and NADH, have turnover times in the order of seconds, as can be calculated from their *in vivo* pool sizes and conversion rates. It should be realized that to obtain a proper snapshot of the intracellular metabolite levels, sampling, and subsequent arrest of metabolic activity should be fast, that is, significantly faster than the turnover time of the metabolite pools. In this review the current state of the art of rapid sampling and quenching for quantitative microbial metabolomics is addressed whereby the reliability of the published procedures is critically investigated.

Fast sampling from bioreactors

Sampling devices

In the past several manually operated devices for rapid sampling from bioreactors have been developed [5,6,7•]. Main disadvantages of these systems were that the operation is relatively laborious, and that the variation in sample volume highly depends on the skills of the operator. This led to the construction of sampling systems with electrically operated valves controlled by a timer [8,9]. Disadvantage of these systems is that they do not allow increasing the sampling frequency much above one sample per five seconds, because of the many manual handlings that have to be performed. Therefore Schaefer *et al.* [10] developed a completely automated sampling device whereby the sampling tubes are fixed in transport racks, moved by a step engine underneath a continuous jet of sample from a bioreactor, allowing a sampling rate of approx. 4.5 samples per second. A completely different approach to increase the sampling frequency, and to minimize the number of handlings required, was developed by Weuster-Botz [11]. The basic idea was to perform sampling, inactivation of metabolic activity, and extraction of intracellular metabolites in a continuous way in a long tube connected to the bioreactor. In this way the highly dynamic metabolite patterns, resulting from a sudden disturbance of the culture in the reactor, were fixed at a certain position in the sampling tube. After sampling the tube was disconnected and immediately frozen at −80°C. To obtain single samples at different reaction times the frozen tube was cut into parts. It was demonstrated that the system could be successfully applied to capture the short time dynamics, on a subsecond scale, of some glycolytic intermediates of chemostat cultivated *Zymomonas mobilis* as a response to a glucose pulse.

A different approach for integrated sampling and extraction from a bioreactor culture has been published by Schaub *et al.* [12]. Hereby short time heating of the

sample was used as the procedure to quench all metabolic activity and at the same time extracting the metabolites from the cells. This was achieved by using a helical coil heat exchanger, which allowed continuous withdrawal of sample from a bioreactor, whereby the broth was rapidly heated to 95°C. The helical geometry was chosen to enhance radial mixing. After extraction the cells were removed by filtration. This sampling device allowed withdrawing five samples per second. The method has been applied for the analysis of the growth rate dependent *in vivo* dynamics of glycolysis in *Escherichia coli* [13].

Stopped flow sampling systems

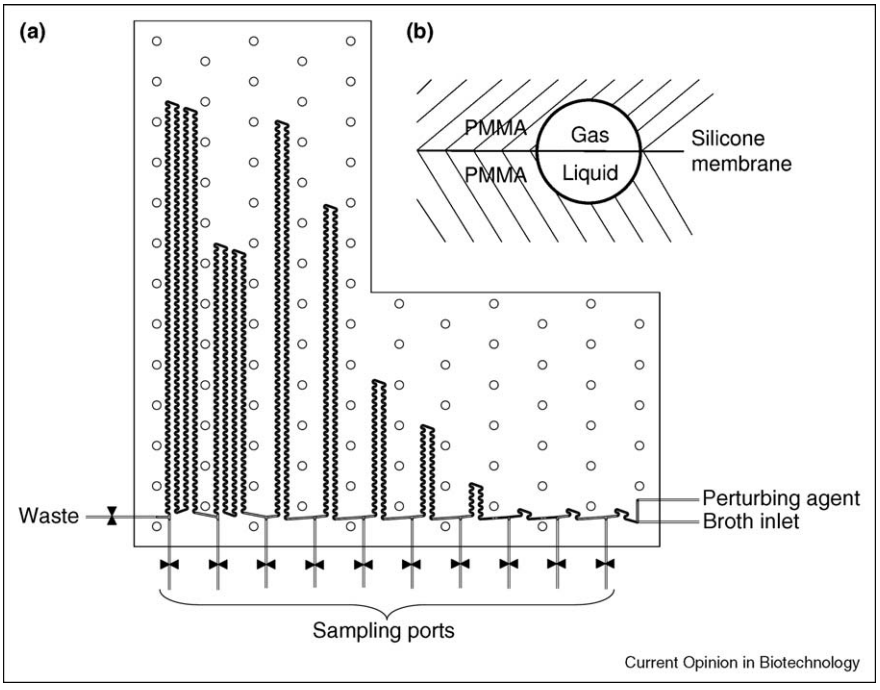
Several authors have applied rapid sampling methods in studies aimed at the elucidation of the *in vivo* kinetic properties of enzymes of metabolic pathways of microorganisms. Typically this can be achieved with so-called stimulus response experiments, whereby a culture growing under well-defined steady-state conditions is suddenly disturbed. This can be achieved for example by injecting a concentrated glucose solution into a glucose limited chemostat, whereafter the dynamic changes in the intracellular metabolite levels are measured within a timeframe of a few hundreds of seconds [10,14,15]. However, with respect to capturing the changes which occur during the first milliseconds to seconds after the disturbance, the pulse response technique has an inherent limitation. After injection into the bioreactor a certain amount of time is required for the compound to be completely mixed with the culture and to come in contact with all cells. This mixing time depends on the reactor type and volume, the stirring speed and the viscosity of the culture, and may easily be several seconds. Another disadvantage of disturbing the complete bioreactor culture is that the steady-state condition is lost after each experiment. This can be circumvented by applying the perturbation outside the bioreactor, for example by using a stopped flow technique. The first authors who applied this technique to microbial cultures, with the aim to capture metabolite dynamics in the milliseconds range, were De Koning and Van Dam [16]. They performed incubations of starved yeast cells in a freeze quench apparatus with a four jet tangential mixer to measure the changes of glycolytic intermediates after adding a pulse of glucose within a time frame of 15–5000 ms. A similar stopped flow apparatus was developed by Buziol *et al.* [17]. The system consisted of a sampling port with a mixing chamber, which was mounted in the wall of the bioreactor. By means of applying an overpressure of 0.4–0.5 bar in the headspace the culture broth was driven out of the reactor through a capillary into the mixing chamber, where it was instantaneously mixed with a concentrated glucose solution. Subsequently the broth/glucose mixture flowed toward a cascade of five three way valves, whereby each valve corresponded with a certain distance from the mixing chamber and thus with a certain contact time with the glucose solution. Samples taken from the valves were quickly frozen at −196°C in liquid nitrogen. With this system a minimum time span between glucose stimulus and quenching of less than 100 ms could be obtained. It should be realized, however, that for aerobic conditions the maximum reaction time with the glucose is determined by the time it takes until the available dissolved oxygen has decreased to a limiting value. This is highly dependent on the biomass concentration in the reactor, as well as the maximum oxygen uptake rate of the microorganism studied, and may range from seconds to a few tenths of seconds.

The solution is to apply a stopped flow system, which allows gas exchange with the broth in the device. This was accomplished by Visser *et al.* [18], who developed a mini plug flow reactor, named the BioScope, wherein the flow channel consisted of an oxygen and carbon dioxide permeable silicon tubing. For practical reasons, and to assure sufficient plug flow characteristics, the tubing was configured in a serpentine geometry. The system was equipped with 11 sample ports, including 1 sample port before the mixing chamber where the perturbing agent was mixed with the culture broth. The oxygen and carbon dioxide transfer characteristics of the BioScope were measured and were found to be sufficient for glucose pulse experiments under the reported conditions. From multiple glucose pulse experiments with *Saccharomyces cerevisiae*, carried out in the BioScope, the authors observed that the obtained dynamic patterns of the glycolytic intermediates were very reproducible and appeared to be highly similar to the patterns obtained from a comparable glucose pulse experiment, carried out by injecting the glucose directly into the chemostat.

Later on an improved and more robust version of the BioScope device was developed by Mashego *et al.* [19•]. In this design the serpentine channels were milled into PMMA. Two mirror images of a PMMA block, with semicircular two-dimensional serpentine channels milled in the surface of each block, were screwed together, separated by a silicone membrane (Figure 1). In this way a two channel system was obtained, whereby one of the channels was used for the flow through of cell culture broth and the opposite channel for gas transfer, for example for oxygen supply and carbon dioxide removal in case of aerobic conditions, or for carbon dioxide and oxygen removal by nitrogen flushing in case of anaerobic conditions. A clear advantage, compared to the first BioScope design, is that in this system the gas transfer, that is oxygen, carbon dioxide, or other gaseous compounds, can be controlled by variation of the gas composition in the gas channel. The observation window was between 1 and 30 s for the highest and 5 and 180 s for the lowest flow rate applied.

The above shows that a variety of sampling systems, both manually operated and semiautomatic, for obtaining fast

Figure 1



(a) 2D serpentine channel geometry of the BioScope and **(b)** cross-section of the BioScope channel.

representative samples from bioreactor cultures of microbes has been developed and successfully applied.

Quenching methods

Quenching procedures for metabolome measurements in microorganisms can be divided in two main groups, namely procedures which allow separation of cells and supernatant, and procedures which do not. The last category also contains methods wherein quenching and metabolite extraction are combined. It should be realized that methods which do not allow separation of the cells after quenching, are only suitable to measure compounds of which the total amounts present in the supernatant are negligible compared to the total intracellular amounts. Although extracellular metabolite concentrations may often be several orders of magnitude lower than intracellular concentrations, the contribution to the measured amount in total broth samples may still be significant, because the volume of the extracellular medium in laboratory cultivations is often two orders of magnitude larger than the total cell volume.

In the early days of fast sampling from microbial cultures separation of cells from the supernatant, for example by filtration or centrifugation, was often applied before quenching of the metabolism. The main reason for this approach was to obtain a concentrated cell sample, such that the metabolite levels were above the detection limits of the analysis method applied. The disadvantage of this approach was that metabolism continued for several minutes under undefined conditions with respect to temperature and the availability of substrate, oxygen, and other nutrients, and thus that measured metabolite levels are not representative for the applied cultivation conditions. For this reason several researchers used immediate quenching by direct sampling into cold perchloric acid, followed by a series of freezing–thawing cycles, thus achieving quenching, release of intracellular metabolites, and inactivation of enzymes [20].

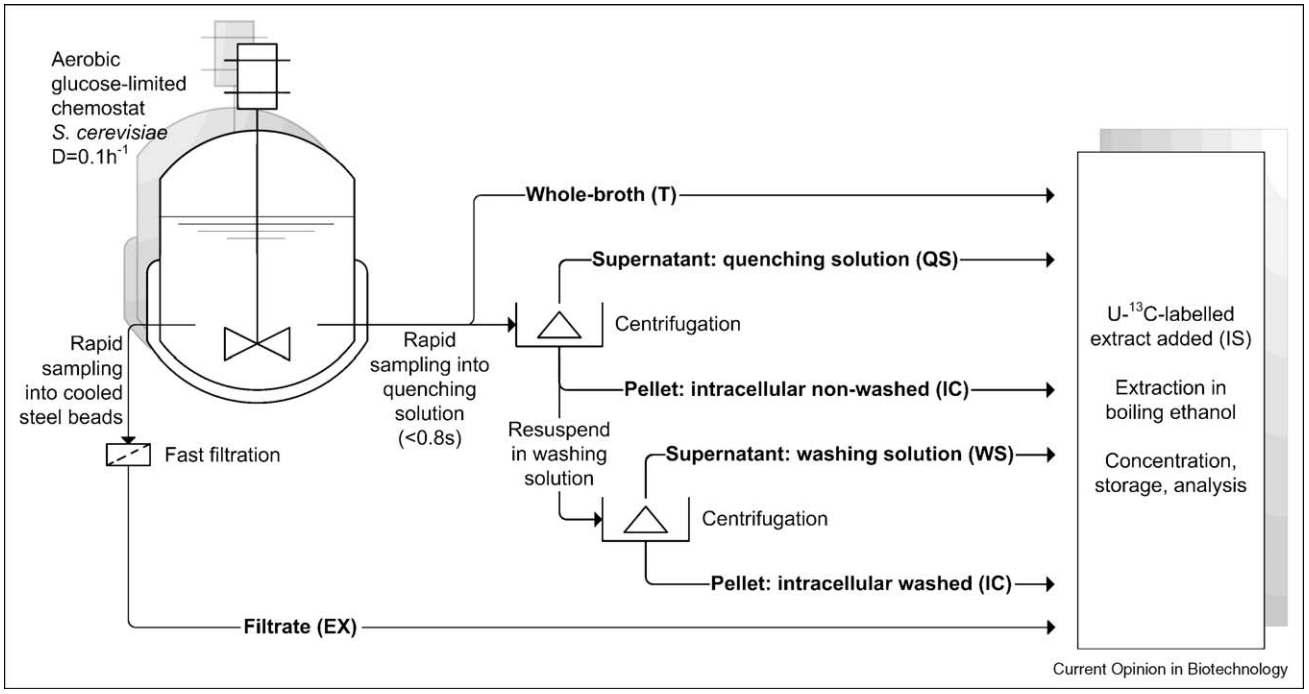
Most popular quenching method: cold methanol/water

The first authors who combined fast quenching and subsequent separation of cells and supernatant in one procedure were De Koning and Van Dam [16]. They used cold (−40°C) 60% methanol for instant quenching of yeast metabolism by spraying 15 mL of culture broth into 60 mL of the cold solution. After the mixture was cooled for approx. 5 min, the sample/methanol mixture was centrifuged for 5 min at 5000 rpm at −20°C to obtain a cell pellet which was subsequently extracted. This method is currently one of the most frequently applied quenching methods in microbial metabolomics [21,22], and in principle allows determining *in vivo* intracellular metabolite levels without interference of the extracellular amounts.

Metabolite leakage

An important requirement for this method to be applicable is that the metabolites remain in the cells during cold methanol quenching. Several authors have

Figure 2



Sample fractions where metabolite concentrations were measured to investigate the fate of the metabolites during quenching and further sample processing of *S. cerevisiae*. Figure from Canelas *et al.* [23••].

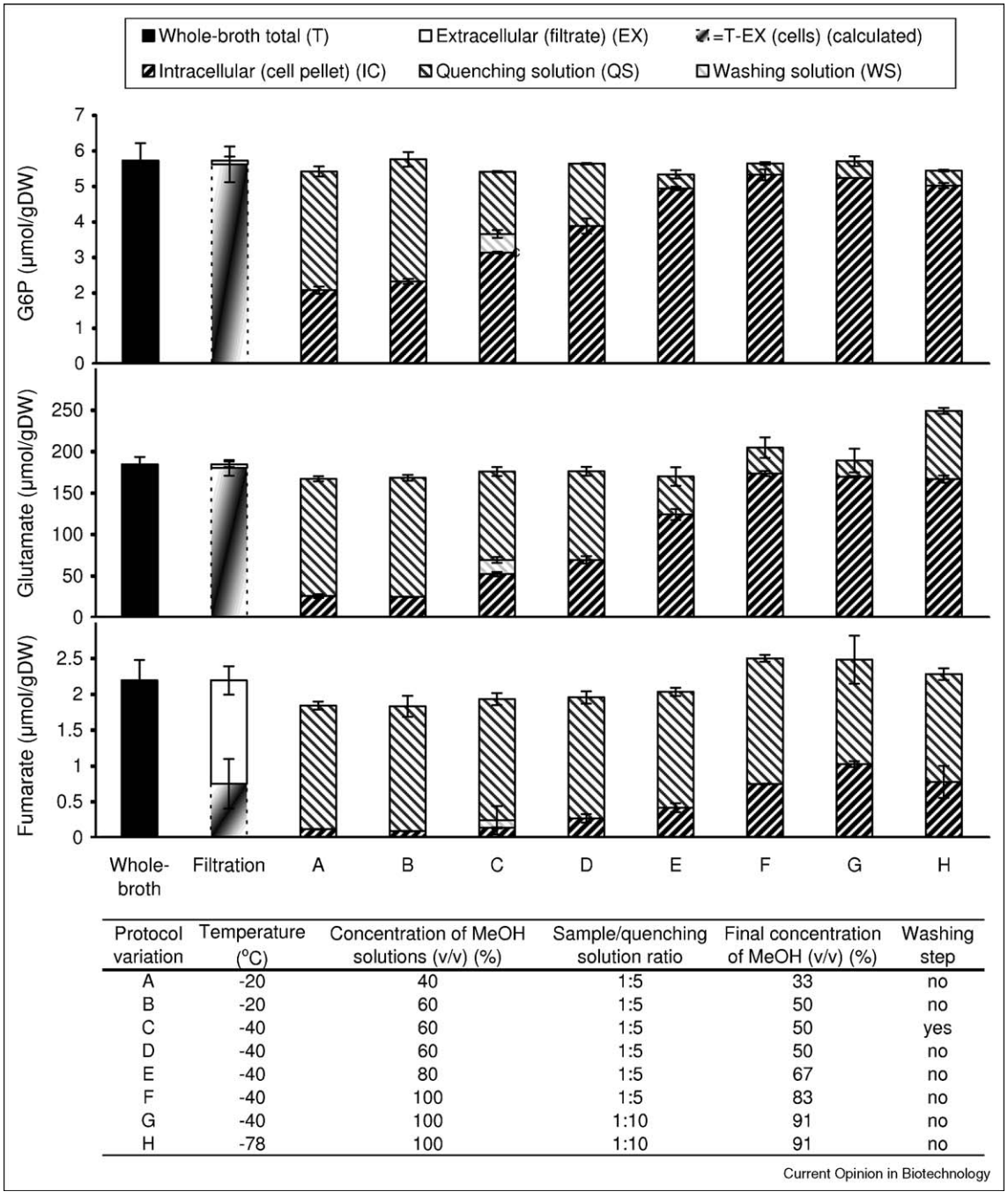
checked this for different microorganisms but the outcomes have been contradictory (see Canelas *et al.* [23••] and references therein) which has mainly been caused by differences in sensitivity of the applied analytical methods. However, more recent research, whereby sensitive MS-based analytical methods have been applied, clearly shows that significant amounts of metabolites diffuse out of the cells into the cold methanol, both for yeast [23••,24] and bacteria [25,26•,27•]. Canelas *et al.* accurately quantified metabolite leakage during cold methanol quenching of the yeast *S. cerevisiae*, by determining the levels of a large range of metabolites in total broth and different sample fractions (see Figure 2) using isotope dilution mass spectrometry (IDMS), whereby 100% U-^{13}C labeled cell extract was used as internal standard [23••]. In this way the authors could set up accurate mass balances and track down the fate of each metabolite during the quenching procedure. They confirmed that leakage of metabolites from yeast cells does occur during conventional cold methanol quenching, and presented evidence that the levels of most metabolites have been previously underestimated by at least twofold. It was shown that metabolite leakage could be entirely prevented by quenching in pure methanol, at a temperature of -40°C or lower (see Figure 3).

A comparable study was carried out to quantify leakage of amino acids during cold methanol quenching of five phylogenetically different yeast strains [28]. Surprisingly, this study showed that amino acid leakage from *S. cerevisiae* cells was negligible during conventional cold methanol quenching. An explanation for this discrepancy could be that the cultivation methods (glucose-limited chemostats versus shake-flask cultures), as well as the yeast strains used, were different.

Quenching of prokaryotes

Also for bacteria metabolite leakage during quenching has been investigated. Bolten *et al.* [26•] applied the cold methanol method for quenching of different Gram positive and Gram negative bacteria, grown in shake flasks. They observed that the method led to drastic loss (>60%) of all metabolites measured because of leakage. Similar findings were published by Taymaz-Nikerel *et al.* [27•], for cold methanol quenching of *E. coli* cells grown in glucose-limited chemostats. These authors applied the same procedure as previously applied to quantify metabolite leakage from yeast cells [23••], whereby the fate of the different metabolites was tracked down by high accuracy measurements in all sample fractions, using IDMS. Because of the observed massive leakage of metabolites into the cold methanol quenching solution, they proposed, and successfully applied, a differential method whereby the intracellular amounts were obtained from metabolite measurements in total broth and extracellular culture liquid.

Figure 3



Protocol variation	Temperature (°C)	Concentration of MeOH solutions (v/v) (%)	Sample/quenching solution ratio	Final concentration of MeOH (v/v) (%)	Washing step
A	-20	40	1:5	33	no
B	-20	60	1:5	50	no
C	-40	60	1:5	50	yes
D	-40	60	1:5	50	no
E	-40	80	1:5	67	no
F	-40	100	1:5	83	no
G	-40	100	1:10	91	no
H	-78	100	1:10	91	no

Full mass balance for the metabolites measured in the different sample fractions (see Figure 2) under eight different variations in sample treatment protocol (see Table), exemplified for G6P, glutamate, and fumarate. The intracellular metabolite levels were calculated from the difference of the concentrations in whole-broth and extracellular medium (T − EX). Samples were from glucose limited chemostat cultures at $D = 0.1\ h^{-1}$. Data are averages ± standard deviation of at least two replicate samples, each analyzed at least in duplicate. Figure adapted from Canelas *et al.* [23••].

Surprisingly Winder *et al.* [29] concluded that 60% cold (−48°C) methanol solution is the most appropriate method to quench metabolism of *E. coli*. Their conclusion was based on the measured relative recovery of some selected metabolites and the total number of peaks detected, when comparing two variations of the cold methanol quenching method (with and without tricine buffer, 0.5 mM, pH 7.4), and hot ethanol quenching. Leakage of metabolites was observed but not quantified, and appeared more pronounced during hot ethanol

quenching. This is not surprising, because incubation in hot ethanol is an efficient and often applied procedure for the extraction of metabolites from microbial cells [30].

Because of the fact that these, and other [26•,27•], authors observed metabolite leakage during cold methanol quenching of prokaryotes, this method should not be considered suited for these organisms. Clearly quenching of prokaryotes seems much more problematic in comparison with eukaryotic microbes, which could be a matter of cell membrane composition, cell surface to volume ratio, which is much larger for prokaryotes because they are much smaller than their eukaryotic counterparts, and/or the phenomenon of cold shock.

Cold shock

Leakage of metabolites from bacteria during quenching accomplished by a fast decrease of the temperature, has been attributed to the so-called cold shock phenomenon [25], whereby the decrease in temperature results in an increase of the permeability of the cell membrane. Wellerdiek *et al.* [31•] applied a microstructure heat exchanger to analyze the cold shock effect on a time scale of seconds using *Corynebacterium glutamicum* as a model organism. They observed that fast cooling (within 220 ms) of cell suspension from 30 to 0°C resulted in rapid and significant release (1.5–3-fold increase of the concentration in the supernatant) of central metabolites from the cells within 1.5 s or less. From 10 s after cooling to 0°C, further release was negligible for most metabolites. Release of metabolites after rapid cooling to 0°C has also been observed for *E. coli* [27•] and it was concluded that in this case rapid sampling and fast filtration for measurement of the exometabolome should not include a rapid cooling step, for example by sampling onto ice cold steel beads as proposed by Mashego *et al.* [32].

Alternatives for cold methanol quenching

Recently several attempts have been undertaken to develop new or adapt existing quenching procedures for individual microorganisms, with the aim to minimize metabolite leakage [23••,24,34,35]. Villas-Bôas and Bruheim [24] proposed a cold glycerol-saline solution as most promising quenching fluid for yeast and bacteria. Spura *et al.* [33] attempted to apply the glycerol-saline method for the quenching of yeast and bacteria, but concluded that it did not meet the requirements for an applicable quenching method. They reported that the protocol appeared very laborious, that it was impossible to get rid of the glycerol sticking to the compact cell pellets, and that the obtained chromatograms showed a dominating glycerol peak, masking other metabolites.

In an attempt to develop a quenching procedure applicable to eukaryotes as well as to Gram positive and Gram negative prokaryotes, an alternative quenching solution composed of 40% ethanol and 0.8% (w/v) sodium chloride at −20°C was proposed [33]. The performance was compared with cold methanol quenched and unquenched samples, held at 4°C in 0.9% sodium chloride solution. Unfortunately the relatively high final temperatures of the quenched (above −10°C), and unquenched (4°C), samples do not exclude that enzymatic conversion has taken place, which, depending on the metabolite, can lead to increased or decreased levels. In accordance with this, the authors reported that experiments aimed at detecting cell leakage yielded inconsistent results, that is, a direct relation between reduced levels in the extract, and increased levels in the supernatant, which would be a clear indication of cell leakage, could not be observed.

Faijes *et al.* [34] proposed quenching in cold (−40°C) 60% (v/v) methanol buffered with 0.85% (w/v) ammonium carbonate or with 70 mM HEPES, as most suitable for metabolomics studies in *Lactobacillus plantarum*. However, they based their conclusion solely on measurements of the leakage of ATP from the cells during quenching and subsequent washing of the cell pellet. Published results on accurate measurements of metabolite leakage during quenching of *S. cerevisiae* and *E. coli*, show that the extent of leakage is very different for different compounds [23••,27•], whereby relatively large polar molecules such as ATP show less leakage than smaller and less polar molecules.

Link *et al.* [35] proposed quenching in a cold mixture of methanol and glycerol (3:2 v/v) at −50°C as the preferred method for metabolome measurements in *E. coli*. This method was compared with quenching in cold (−50°C) buffered and unbuffered 60% methanol/water. Metabolite leakage was quantified by measuring the adenylates ATP, ADP, and AMP, in cell extracts and supernatants after cold centrifugation of the quenched sample. The authors reported that leakage of adenylates was significantly reduced, but not completely avoided, when the quenching was performed with cold methanol/glycerol, and that increased concentrations in the cell extract corresponded with decreased concentrations in the quenching fluid.

Conclusions

To aid the measurement of true intracellular metabolite levels of microorganisms, various manual, semiautomatic and fully automated rapid sampling techniques have been constructed and successfully applied. For elucidation of *in vivo* kinetic properties stopped flow systems have been developed to carry out pulse response experiments without disturbing the culture in the bioreactor. In contrast to the development of rapid sampling systems, the development of reliable sample processing methods has been relatively poor. To obtain meaningful metabolome data immediate quenching of enzyme activity is required followed by the separation of the exometabolome and

endometabolome, without leakage of metabolites from the cells to the quenching solution. Preferably the method should be applicable to different species, both eukaryotes and prokaryotes. In the literature various methods have been proposed to be suitable for particular microbial species. In many cases, however, the suitability of these methods is not confirmed by the presented results. Therefore additional effort should be directed toward the improvement of sample processing procedures for quantitative microbial metabolomics.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. De Backer P, De Waele D, Van Speybroeck L: **Ins and outs of systems biology vis-à-vis molecular biology: continuation or clear cut?** *Acta Biotheor* 2009. published online October 24.

2. Tang YJ, Martin HG, Myers S, Rodriguez S, Baidoo EE, Keasling JD: **Advances in analysis of microbial metabolic fluxes via (13)C isotopic labeling**. *Mass Spectrom Rev* 2009, **28**:362-375.

3. Gstaiger M, Aebersold R: **Applying mass spectrometry-based proteomics to genetics, genomics and network biology**. *Nat Rev Genet* 2009, **10**:617-627.

4. Oldiges M, Lütz S, Pflug S, Schroer K, Stein N, Wiendahl C: **Metabolomics: current state and evolving methodologies and tools**. *Appl Microbiol Biotechnol* 2007, **76**:495-511.

5. Lønsmann Iversen JJ: **A rapid sampling valve with minimal dead space for laboratory scale fermenters**. *Biotechnol Bioeng* 1981, **23**:437-440.

6. Theobald U, Mailinger W, Reuss M, Rizzi M: **In-vivo analysis of glucose-induced fast changes in yeast adenine nucleotide pool applying a rapid sampling technique**. *Anal Biochem* 1993, **214**:31-37.

7. • Schädel F, Franco-Lara E: **Rapid sampling devices for metabolic engineering applications**. *Appl Microbiol Biotechnol* 2009, **83**:199-208.
Comprehensive overview of devices for rapid sampling from bioreactors.

8. Larsson G, Törnkvist M: **Rapid sampling, cell inactivation and evaluation of low extracellular glucose concentrations during fed-batch cultivation**. *J Biotechnol* 1996, **49**:69-82.

9. Lange HC, Eman M, van Zuijlen G, Visser D, van Dam JC, Frank J, Teixeira de Mattos MJ, Heijnen JJ: **Improved rapid sampling for in vivo kinetics of intracellular metabolites in *Saccharomyces cerevisiae***. *Biotechnol Bioeng* 2001, **75**:406-415.

10. Schaefer U, Boos W, Takors R, Weuster-Botz D: **Automated sampling device for monitoring intracellular metabolite dynamics**. *Anal Biochem* 1999, **270**:88-96.

11. Weuster-Botz D: **Sampling tube device for monitoring intracellular metabolite dynamics**. *Anal Biochem* 1997, **246**:225-233.

12. Schaub J, Schiesling C, Reuss M, Dauner M: **Integrated sampling procedure for metabolome analysis**. *Biotechnol Prog* 2006, **22**:1434-1442.

13. Schaub J, Reuss M: **In vivo dynamics of glycolysis in *Escherichia coli* shows need for growth rate dependent metabolome analysis**. *Biotechnol Prog* 2008, **24**:1402-1407.

14. Theobald U, Mailinger W, Baltes M, Rizzi M, Reuss M: **In vivo analysis of metabolic dynamics in *Saccharomyces cerevisiae*: I. Experimental observations**. *Biotechnol Bioeng* 1997, **55**:305-316.

15. Rizzi M, Baltes M, Theobald U, Reuss M: **In vivo analysis of metabolic dynamics in *Saccharomyces cerevisiae*. 2. Mathematical model**. *Biotechnol Bioeng* 1997, **55**:592-608.

16. De Koning W, Van Dam K: **A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH**. *Anal Biochem* 1992, **204**:118-123.

17. Buziol S, Bashir I, Baumeister A, Claassen W, Noisommit-Rizzi N, Mailinger W, Reuss M: **New bioreactor-coupled rapid stoppedflow sampling technique for measurements of metabolite dynamics on a subsecond time scale**. *Biotechnol Bioeng* 2002, **80**:632-636.

18. Visser D, van Zuylen GA, van Dam JC, Oudshoorn A, Eman MR, Ras C, van Gulik WM, Frank J, van Dedem GWK, Heijnen JJ: **Rapid sampling for analysis of in vivo kinetics using the BioScope: a system for continuous-pulse experiments**. *Biotechnol Bioeng* 2002, **79**:674-681.

19. • Mashego MR, van Gulik WM, Vinke JL, Visser D, Heijnen JJ: **In vivo kinetics with rapid perturbation experiments in *Saccharomyces cerevisiae* using a second-generation BioScope**. *Metab Eng* 2006, **8**:370-383.
Description and characterization of an improved version of the BioScope plug flow reactor for rapid perturbation experiments outside the bioreactor and subsequent rapid sampling.

20. Weibel EK, Mor J-R, Fiechter A: **Rapid sampling of yeast and automated assays of adenylate, citrate, pyruvate and glucose-6-phosphate pools**. *Anal Biochem* 1974, **58**:208-216.

21. Oldiges M, Takors R: **Applying metabolic profiling techniques for stimulus–response experiments: chances and pitfalls**. *Adv Biochem Eng/Biotechnol* 2005, **92**:173-196.

22. Mashego MR, Rumbold K, De Mey M, Vandamme E, Soetaert W, Heijnen JJ: **Microbial metabolomics: past, present and future methodologies**. *Biotechnol Lett* 2007, **29**:1-16.

23. •• Canelas AB, Ras C, ten Pierick A, van Dam JC, Heijnen JJ, van Gulik WM: **Leakage-free rapid quenching technique for yeast metabolomics**. *Metabolomics* 2008, **4**:226-239.
First authors who carried out highly accurate measurements, using isotope dilution mass spectrometry, of a large number of different metabolites in different sample fractions to verify metabolite leakage during cold methanol quenching and further sample processing in *S. cerevisiae.* On the basis of these results an improved version of the cold methanol quenching method is proposed.

24. Villas-Bôas SG, Bruheim P: **Cold glycerol-saline: the promising quenching solution for accurate intracellular metabolite analysis of microbial cells**. *Anal Biochem* 2007, **370**:87-97.

25. Wittmann C, Kromer JO, Kiefer P, Binz T, Heinzle E: **Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria**. *Anal Biochem* 2004, **327**:135-139.

26. • Bolten CJ, Kiefer P, Letisse F, Portais JC, Wittmann C: **Sampling for metabolome analysis of microorganisms**. *Anal Chem* 2007, **79**:3843-3849.
Different rapid sampling procedures were tested for the quantification of metabolite leakage from different Gram positive and Gram negative bacteria.

27. • Taymaz-Nikerel H, De Mey M, Ras C, ten Pierick A, Seifar RM, Van Dam JC, Heijnen JJ, van Gulik WM: **Development and application of a differential method for reliable metabolome analysis in *Escherichia coli***. *Anal Biochem* 2009, **386**:9-19.
The authors carried out highly accurate measurements, using isotope dilution mass spectrometry, of a large number of different metabolites in different sample fractions to verify metabolite leakage during cold methanol quenching and further sample processing in *E. coli.* On the basis of the observed massive leakage of metabolites they proposed and successfully applied a differential method for metabolome measurement in *E. coli*.

28. Bolten CJ, Wittmann C: **Appropriate sampling for intracellular amino acid analysis in five phylogenetically different yeasts**. *Biotechnol Lett* 2008, **30**:1993-2000.

29. Winder CL, Dunn WB, Schuler S, Broadhurst D, Jarvis R, Stephens GM, Goodacre R: **Global metabolic profiling of *Escherichia coli cultures*: an evaluation of methods for**

quenching and extraction of intracellular metabolites. *Anal Chem* 2008, **80**:2939-2948.

30. Gonzalez B, Francois J, Renaud M: **A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol**. *Yeast (Chichester, England)* 1997, **13**:1347-1355.

31. Wellerdiek M, Winterhoff D, Reule W, Brandner J, Oldiges M:
• **Metabolic quenching of *Corynebacterium glutamicum*: efficiency of methods and impact of cold shock**. *Bioprocess Biosyst Eng* 2009, **32**:581-592.
Interesting application of a microstructure heat exchanger to examine the short-term impact of rapid cooling on the release of metabolites from bacteria.

32. Mashego MR, van Gulik WM, Vinke JL, Heijnen JJ: **Critical evaluation of sampling techniques for residual glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae***. *Biotechnol Bioeng* 2003, **83**: 395-399.

33. Spura J, Reimer LC, Wieloch P, Schreiber K, Buchinger S, Schomburg D: **A method for enzyme quenching in microbial metabolome analysis successfully applied to Gram-positive and Gram-negative bacteria and yeast**. *Anal Biochem* 2009, **394**:192-201.

34. Faijes M, Mars AE, Smid EJ: **Comparison of quenching and extraction methodologies for metabolome analysis of *Lactobacillus plantarum***. *Microb Cell Fact* 2007, **6**:27.

35. Link H, Anselment B, Weuster-Botz D: **Leakage of adenylates during cold methanol/glycerol quenching of *Escherichia coli***. *Metabolomics* 2008, **4**:240-247.