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## **Microbial metabolomics: past, present and future methodologies** — [Source link](#)

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1 Running Title: Microbial metabolomics: Past, Present and  
2 Future Methodologies

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24 Spectrometry, Rapid sampling, Quenching, Metabolite Extraction

1 **Abstract**

2

3 Microbial metabolomics has received much attention in recent years mainly because it  
4 supports and complements a wide range of microbial research areas from new drug  
5 discovery efforts to metabolic engineering. Broadly, the term metabolomics refers to the  
6 comprehensive (qualitative and quantitative) analysis of the complete set of all low  
7 molecular weight metabolites present in and around growing cells at a given time during  
8 their growth or production cycle. This mini review focuses on the past, current and future  
9 development of various experimental protocols in the rapid developing area of  
10 metabolomics in the ongoing quest to reliably quantify microbial metabolites formed  
11 under defined physiological conditions. These developments range from rapid sample  
12 collection, instant quenching of microbial activity, extraction of the relevant intracellular  
13 metabolites as well as quantification of these metabolites using enzyme based and or  
14 modern high tech hyphenated analytical protocols, mainly chromatographic techniques  
15 coupled to mass spectrometry (LC-MS<sup>n</sup>, GC-MS<sup>n</sup>, CE-MS<sup>n</sup>), where n indicates the  
16 number of tandem mass spectrometry, and nuclear magnetic resonance spectroscopy  
17 (NMR).

18

19

20

## 1 **Introduction**

2 Quantitative understanding of microbial metabolism and its *in vivo* regulation requires  
3 knowledge of both extracellular and intracellular metabolites. Traditionally, this  
4 knowledge is acquired through fast sampling, instant arrest of metabolic activity and  
5 deactivation of endogenous enzymatic activity, metabolite extraction and subsequent  
6 quantification of intracellular reactants (metabolites). Extracellular metabolites are  
7 quantified in the cell free supernatant obtained either by filtration or centrifugation at low  
8 temperatures. The ongoing quest towards understanding *in vivo* regulation of microbial  
9 metabolic networks has been the primary fuel for the recent rapid developments in  
10 metabolomics, i.e. quantification of the total complement of metabolites inside  
11 (endometabolome) and outside (exometabolome) a cell under different growth  
12 conditions. Although, the term metabolome has only surfaced in the late 90's (Oliver *et*  
13 *al.*, 1998; Tweeddale *et al.*, 1998), metabolomics research has been in existence since  
14 the late 1960 and early 1970's (Harrison & Maitra, 1969; Gancedo & Gancedo, 1973). In  
15 recent years, various extensions of metabolite analysis terminology have also surfaced  
16 so as to differentiate qualitative analysis of both exometabolome and endometabolome  
17 referred to as metabolite footprinting and metabolite fingerprinting, respectively. On the  
18 contrary, quantitative analysis of known pre-defined metabolites is referred to as target  
19 analysis (Jewett *et al.*, 2006). Metabolomics research has become so relevant that it has  
20 recently culminated in the formation of a Metabolomics Society as well as a dedicated  
21 journal called Metabolomics, launched in 2005.

22 However, routine detection and quantification of intracellular metabolites *in vivo* remains  
23 a challenge, therefore most metabolomics research relies on the isolation of metabolites  
24 from biological sample (i.e. *in vitro* analysis). Therefore, successful application of the

1 craft of metabolomics dictates development and integration of robust and reliable  
2 protocols ranging from microbial cultivation techniques (defined biomass), biomass  
3 sampling procedures, isolation/extraction of relevant metabolites of interest as well as  
4 quantitative analysis of these metabolites.

5 Weibel *et al.* (1974) reported a rapid sampling technique for yeast cells with a very short  
6 time interval between harvesting and simultaneous inactivation and intracellular  
7 metabolites extraction from the cells. This method laid the foundation for quantitative  
8 analysis of the microbial metabolome as reported later by Saez & Lagunas, (1976). The  
9 method was further refined and automated by de Koning & van Dam (1992), Gonzalez  
10 *et al.* (1997), Schaefer *et al.* (1999), Theobald *et al.* (1993 and 1997), and Visser *et al.*  
11 (2002).

12 It is desirable that effective instant quenching methods for metabolic activity fulfil some  
13 basic requirements such as: no cell leakage should occur during the process or if  
14 leakage does occur, the leaked metabolites should be quantifiable. Many researchers  
15 have embarked on systematic investigations of various quenching methods and the  
16 outcome has been that most prokaryotic microorganisms (bacteria) behave differently  
17 when exposed to the almost universal cold methanol protocol than eukaryotic  
18 microorganisms (e.g. yeasts and filamentous fungi). Recently, some authors have  
19 evaluated cold methanol protocol as an extraction protocol for extracting intracellular  
20 metabolites in *E. coli* and *Corynebacterium glutamicum* (Maharjan & Ferenci, 2003;  
21 Wittman *et al.*, 2004).

22 Microbial metabolomic strategies generally aim at quantifying microbial substrates and  
23 products at two levels, i.e. outside the cells (extracellular) and inside the cells  
24 (intracellular). It is therefore important to distinguish the origin of the substrates and

1 products from the culture as extracellular or intracellular. The first step in distinguishing  
2 the origin of these metabolites is to separate the cells (biomass) from the culture  
3 supernatant. This challenge is widely achieved by rapid sampling techniques from  
4 bioreactors, followed by rapid quenching (arrest) of metabolic activity. Figure 1 depicts  
5 all common steps involved in metabolomics studies ranging from sample collection to  
6 metabolite analysis. Rapid quenching of microbial metabolic activity is traditionally  
7 achieved by instant change of sample temperature to either low (e.g.  $<-40^{\circ}\text{C}$ ) or high  
8 temperatures (e.g.  $>+80^{\circ}\text{C}$ ), or by applying extreme sample pH to either high (alkali, e.g.  
9 KOH or NaOH) or low (acid, e.g. perchloric acid, HCl or trichloroacetic acid). Following  
10 rapid quenching, the cells are separated from the medium by centrifugation at low  
11 temperatures or filtration, however, the former tend to be the preferred choice. The  
12 biomass is then permeabilized to extract intracellular metabolites usually with organic  
13 solvents, i.e. ethanol or chloroform at high or low temperatures respectively. The organic  
14 solvents are then removed usually by evaporation under vacuum. The remaining residue  
15 is resuspended in small volume of ultra-pure water, centrifuged and the supernatant  
16 stored at low temperatures until analysis with appropriate analytical method (Figure 1).  
17 Metabolite analysis methods vary from enzymatic based methods (Bergmeyer *et al.*  
18 1985) to modern hyphenated techniques such as gas chromatography coupled to mass  
19 spectrometry (GC-MS) or liquid chromatography mass spectrometry (LC-MS/MS) and  
20 most recently capillary electrophoresis mass spectrometry (CE-MS). It is important at  
21 this stage to mention that an ideal quenching and extraction protocol should meet  
22 certain minimum prerequisites, amongst which are:

- 23 1. Quenching procedures should ideally instantly arrest (freeze) cellular metabolic  
24 activity.

- 1        2. No significant cell membrane damage should occur during the quenching  
2            procedure as this might lead to the loss of intracellular metabolites from cells due  
3            to leakage.
- 4        3. The extraction procedure should extract as wide a range of metabolites as  
5            possible.
- 6        4. The procedure should not modify the intracellular metabolites, neither physically  
7            nor chemically, so as to make them unidentifiable.
- 8        5. The resulting sample matrix should be compatible or amenable to the analytical  
9            method of choice.

#### 11        **Biomass source**

12        Metabolomics studies requires biomass source which is achieved by growing  
13        microorganisms under controlled environment in bioreactors. In a bioreactor,  
14        temperature, pH, medium components as well as dissolved gas concentrations such  
15        as oxygen (O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) are easily controlled so that the  
16        microenvironment is well defined. Well defined growth conditions are necessary in  
17        order to establish standard and reproducible reference culture conditions. In addition,  
18        bioreactors can be operated in a batch, fed-batch or continuous mode (Figure 2).  
19        Recently, the majority of the researchers in metabolomics tend to prefer continuous  
20        culture mode of bioreactor operation for several reasons:

- 21            • Specific growth rate ( $\mu$ ) = dilution rate (D) can be well defined and fixed.
- 22            • One specific growth limiting medium component such as carbon source  
23            can be imposed.

- 1 • By fixing the specific growth rate, all other fluxes such as specific substrate  
2 uptake rate ( $q_s$ ); oxygen uptake rate (OUR) as well as carbon dioxide  
3 evolution rate (CER) are fixed.
- 4 • Physiological steady state condition can easily be achieved and  
5 reproduced.

6 However, during growth in a continuous culture mode, the residual substrate  
7 concentration (e.g. glucose) is usually very low, in the range of approximately 12 mg/l  
8 and 20 mg/l for *E. coli* K12 W3110 and for *S. cerevisiae* CEN.PK 113-7D grown at a  
9 dilution rate of  $0.1 \text{ h}^{-1}$  and  $0.05 \text{ h}^{-1}$ , respectively (Chassagnole *et al.*, 2002; Mashego *et*  
10 *al.*, 2003). It is therefore, critical to rapidly collect the sample from the bioreactor and  
11 arrest cellular metabolism instantaneously upon the transfer of broth from the bioreactor,  
12 failure of which would result in the disturbance of the physiological steady state of the  
13 culture due to substrate deprivation. In contrast to the continuous culture mode, rapid  
14 sampling is not of critical importance in batch cultures, mainly because substrate  
15 concentration may be high enough not to lead to a significant change of the  
16 physiological state of the cells.

17 One disadvantage of the chemostat culture conditions is that the culture medium  
18 composition is designed in such a way that only one growth limiting medium component  
19 such as carbon source is limiting whereas other components, e.g. phosphate and  
20 sulphate are in excess. For example, in glucose limited *E. coli* culture medium, typical  
21 residual phosphate and sulphate concentration is in the order of 4 mM and 6 mM  
22 respectively. Therefore, separation of the biomass from the rest of the high residual salt  
23 containing supernatant following the quenching step is critical. After the separation of  
24 the biomass, the high salt content that remains attached to the biomass is removed by



1 washing the cell pellet with similar quenching solution at low temperature (<-40°C). This  
2 washing step is necessary; especially when liquid chromatography coupled to  
3 electrospray ionisation mass spectrometry based analysis method is used, since LC-  
4 ESI-MS/MS is prone to ion suppression (e.g. phosphate and sulphate).

5

## 6 **Rapid Sampling techniques**

7 Rapid sampling techniques have been developed so that the resulting samples  
8 represent *in vivo* conditions. These sampling techniques are necessitated by the high  
9 turnover rates of intracellular metabolites such as glucose-6-phosphate and ATP, which  
10 are usually in the order of 1-2 seconds (Weibel *et al.*, 1974; de Koning & van Dam,  
11 1992). Therefore, successful capture of the *in vivo* snapshot of the metabolic state of the  
12 cells and metabolite pool levels requires that the time between sample collection and  
13 quenching should ideally be shorter than the turnover rates for such metabolites.  
14 Furthermore, during pulse response experiments, in which a growth limiting medium  
15 component such as glucose is instantly increased in a chemostat, simultaneous rapid  
16 sampling and quenching of metabolic activity is indispensable for studying the rapid  
17 dynamics of cellular metabolism, see Figures 3 & 4. Rapid sampling protocols have  
18 been used successfully for yeast, bacteria and filamentous fungi (Theobald *et al.*, 1993;  
19 Weuster-Botz, 1997; Schaefer *et al.*, 1999; Lange *et al.*, 2001; Buziol *et al.*, 2002; Visser  
20 *et al.*, 2002; Ruiters & Visser, 1996).

21 Schaefer *et al.* (1999) reported an automated sampling device capable of a sampling  
22 frequency of 0.22 seconds per sample. The sample flasks are fixed in a transport  
23 magazine moving horizontally by a step engine. Useful application of this sampling  
24 device was demonstrated through measurements of the intracellular metabolites of *E.*

1 *coli* K-12 grown to steady state in a continuous culture and then perturbed by the  
2 instantaneous increase in residual glucose concentration. Furthermore, Buchholz *et al.*  
3 (2002) used the same device to follow over 30 intracellular metabolites response in *E.*  
4 *coli* after glucose or glycerol perturbations. In the quest to capture fast reaction  
5 dynamics in *E. coli* K12 after a glucose pulse, Buziol *et al.* (2002) developed the  
6 stopped-flow sampling technique capable of achieving sampling times as fast as 100  
7 milliseconds between glucose stimulus point and the first sample collection point. This  
8 sampling frequency was facilitated by the applied high bioreactor overpressure of 0.4-  
9 0.5 bar. The stopped-flow sampling technique was successfully used by Chassagnole *et*  
10 *al.* (2002) for following intracellular concentrations of metabolites and coenzymes in *E.*  
11 *coli* at transient conditions.

12 Visser *et al.* (2002) developed a rapid sampling and perturbation device (BioScope)  
13 which is a mini plug flow reactor that can be coupled to the steady state bioreactor that  
14 serves as a source for biomass. The steady state biomass is directed into the BioScope  
15 where it is perturbed with various agents such as ethanol, glucose and most recently  
16 acetaldehyde (Mashego *et al.*, 2006a). This device has become relevant in microbial  
17 research since it has been used with *Penicillium chrysogenum*, *Saccharomyces*  
18 *cerevisiae* and *E. coli* (Nasution *et al.*, 2006; Mashego *et al.*, 2006b) and tends to  
19 generate extensive and rich data sets from a single chemostat as can be seen in  
20 Figures 3 and 4.

21

## 22 **Exometabolome**

23 Measurement of excreted extracellular metabolite levels (exometabolome) as well as  
24 substrate concentrations is indispensable in metabolomics studies. In the literature,

1 many different methods have been described to rapidly arrest metabolic activity for  
2 measurement of the residual substrate concentration in glucose limited chemostat  
3 cultures as well as during dynamic perturbation experiments (Theobald *et al.*, 1993;  
4 Postma *et al.*, 1989; van Hoek *et al.*, 1999). Theobald *et al.* (1993) used pre-cooled 15  
5 ml glass tubes containing 10-15 glass beads (diameter 4 mm) at  $-10^{\circ}\text{C}$  and later pre-  
6 cooled tubes filled with 10-15 stainless steel spheres (4mm diameter) at  $-10^{\circ}\text{C}$  for  
7 rapidly cooling the broth for subsequent analysis of extracellular metabolites. Postma *et*  
8 *al.* (1989), Verduyn *et al.* (1992), van Hoek *et al.*, (1999) and Diderich *et al.* (1999) used  
9 fast sampling of the broth directly into liquid nitrogen, followed by thawing in ice with  
10 gentle shaking to keep the cell suspension at  $0^{\circ}\text{C}$ . The suspension was later  
11 centrifuged to separate biomass from the supernatant. Most recently, Mashego *et al.*  
12 (2003) critically evaluated sampling protocols for reliable determination of residual  
13 glucose concentration in glucose limited chemostat cultures of yeast. These authors  
14 concluded that use of liquid nitrogen as a quenching method to rapidly arrest cellular  
15 metabolism for quantitative analysis of extracellular glucose is not a very reliable  
16 method, whereas the filter syringe steel beads protocol work very well (Figure 3). This  
17 method has been demonstrated to work satisfactory for extracellular metabolites such  
18 as pyruvate, acetate and ethanol in *S. cerevisiae*, *Penicillium chrysogenum* and *E. coli*  
19 (Mashego *et al.*, 2006b; Nasution *et al.*, 2006; unpublished data).

20

21

## 1 **Endometabolome**

### 2 **Quenching methods for microbial cells metabolic activity (Table I)**

#### 3 **Bacteria**

4 Jensen *et al.* (1999) used 60% v/v methanol at -35 °C to bring about fast and complete  
5 stop of metabolic activity in *Lactobacillus lactis*. These authors noticed cell leakage of  
6 intracellular metabolites into the quenching solution, although the extent of the leakage  
7 was not quantified. Buchholz *et al.* (2001), Kaderbhai *et al.* (2003), Al Zaid Siddiquee *et*  
8 *al.* (2004); Oldiges *et al.*, (2004) and Hoque *et al.* (2005) quenched *E. coli* cells with 60%  
9 methanol solution buffered with 70mM HEPES at -50°C, -40°C, and -80°C, respectively,  
10 but again these authors neither mentioned nor tested cell leakage during the quenching  
11 procedure. Liquid nitrogen (-196 °C) has been used by Chassagnole *et al.* (2002) for  
12 rapid quenching of *E. coli* metabolic activity. This method requires thawing of the frozen  
13 sample followed by separation of the cells from the medium by centrifugation. It is highly  
14 likely that during the freezing of the biomass, ice crystals may damage the cell  
15 membranes, thus leading to metabolite leakage and hence inaccurate quantification of  
16 the metabolites.

17 Wittmann *et al.* (2004) reported a systematic investigation of the effect of cold shock on  
18 the intracellular quantification of amino acids in *Corynebacterium glutamicum*. They  
19 investigated and compared quenching the cells with 60% methanol/water at -58°C;  
20 10mM HEPES buffered 60% v/v methanol at -58°C, cold 0.9% NaCl at -0.5°C, and quick  
21 filtration. They concluded that all quenching methods tested led to the cell leakage in *C.*  
22 *glutamicum* and hence these methods were found not to be suitable for quantification of  
23 intracellular metabolites in this organism and that quick filtration without quenching was

1 most suitable for quantification of intracellular metabolites exhibiting time constants  
2 significantly larger than the sampling time.

3

#### 4 **Filamentous fungi**

5 Ruijter & Visser (1996) employed 60% v/v methanol buffered with ethanolamine (pH 7.3)  
6 at -45°C to quench metabolic activity of *Aspergillus niger*. Glycolytic intermediates,  
7 pyridine and adenine nucleotides were quantified enzymatically. These authors did not  
8 observe any leakage of metabolites during quenching. Hajjaj *et al.* (1998) compared two  
9 rapid quenching techniques (i.e. liquid nitrogen and cold 10mM HEPES buffered  
10 methanol, 60 % v/v at -40°C) in the filamentous fungus *Monascus ruber*. They found that  
11 arrest of metabolism was equally effective using both methods; however, no data on cell  
12 leakage during the quenching procedure was reported. The cold methanol (60% v/v)  
13 quenching protocol has been recently used in our laboratory for quantification of  
14 intracellular metabolites of glycolysis, TCA cycle, and adenine nucleotides in *Penicillium*  
15 *chrysogenum* (Nasution *et al.* 2006). In this work, the data of ATP analysis used as an  
16 indicator metabolite for leakage suggested that no significant leakage occurred since  
17 ATP was not detected in the quenching nor washing fluid.

18

#### 19 **Yeast**

20 Cold methanol (60%v/v) has been widely used to quench metabolic activity in  
21 *Saccharomyces cerevisiae* (de Koning & van Dam 1992, Gonzalez *et al.* 1997, Visser *et*  
22 *al.* 2002, Mashego *et al.* 2004). This method is popular mainly because it allows instant  
23 quenching of metabolic activity followed by separation of biomass from the growth  
24 medium, so that biomass can be extracted with minimal contamination from medium

1 salts, usually present at high levels, as well as metabolites that are present both  
2 intracellularly and extracellularly such as pyruvate. Castrillo *et al.* (2003) reported an  
3 optimized protocol for metabolome analysis in yeast using direct infusion electrospray  
4 mass spectrometry. These authors tested cold methanol based quenching fluid buffered  
5 with HEPES, PIPES and Tricine and concluded that the latter was more efficient as it is  
6 a non-salt buffer compatible with electrospray mass spectrometry. However, these  
7 authors did not report on cell leakage during the quenching procedure. Furthermore, the  
8 metabolite analysis was not quantitative but rather were qualitative based on the peak  
9 sharpness.

10 Most recently, Villas-Bôas *et al.* (2005a) have reviewed and evaluated the whole sample  
11 preparation procedures for the analysis of intracellular metabolites in a batch grown *S.*  
12 *cerevisiae* CEN.PK 113-7D. These authors observed leakage of intracellular metabolites  
13 (TCA cycle, organic acids and amino acids) in yeast during the methanol/water  
14 quenching procedure. Furthermore, they noticed varying efficacy of six different  
15 extraction procedures as well as losses of metabolites during sample concentration by  
16 lyophilization and solvent evaporation. However, sugar phosphates were not detected in  
17 the quenching fluid, suggesting that leakage of metabolites during the quenching  
18 procedure is not universal but rather metabolite specific.

19

## 20 **Extraction methods for intracellular metabolites**

21 Intracellular metabolites should be exposed to various analytical procedures, usually by  
22 exposing cells to cell membrane permeabilizing agents (Table II). These agents should  
23 neither physically nor chemically modify the metabolites targeted for analysis.  
24 Furthermore, the extraction procedure should extract as many metabolites as possible

1 with minimal degradation. The inherent dilution effects of some of the procedures should  
2 be kept as minimal as possible. In the forefront of the extraction agents (Table II) are  
3 boiling 75% ethanol (v/v) (Gonzalez *et al.*, 1997; Hajjaj *et al.*, 1998; Visser *et al.*, 2002;  
4 Maharjan & Ferenci, 2003; Mashego *et al.*, 2004; Villas-Bôas *et al.* 2005a; Nasution *et*  
5 *al.*, 2006); 50-100% methanol (Tweeddale *et al.*, 1998; Maharjan & Ferenci, 2003;  
6 Wittman *et al.*, 2004; Villas-Bôas *et al.*, 2005a); trichloroacetic acid, acetic acid,  
7 hydrochloric acid, perchloric acid, Tris-H<sub>2</sub>SO<sub>4</sub>/EDTA, ethyl acetate or KOH (Bagnara &  
8 Finch, 1972; Lilius *et al.*, 1979; de Koning & van Dam, 1992; Theobald *et al.*, 1993;  
9 Tweeddale *et al.*, 1998; Schaefer *et al.*, 1999; Chassagnole *et al.*, 2002; Oldiges *et al.*,  
10 2004; Kammerer *et al.*, 2004; Villas-Bôas *et al.*, 2005a; Kayser *et al.*, 2005; Weber *et al.*,  
11 2005); chloroform or toluene (de Koning & van Dam, 1992; Tweeddale *et al.*, 1998;  
12 Jensen *et al.*, 1999; Maharjan & Ferenci, 2003; Villas-Bôas *et al.*, 2005a); as well as hot  
13 water (Bhatthacharya *et al.*, 1995. Recently, an extraction method of intracellular  
14 metabolites in *Mycobacterium bovis* combining deep freezing in liquid nitrogen and  
15 mechanical grinding of cells has been reported (Jaki *et al.*, 2006). Typical metabolites  
16 extracted with these methods include intermediates from glycolysis, tricarboxylic acid  
17 cycle, pentose phosphate pathway as well as purine and pyrimidine nucleotides, amino  
18 acids and other low molecular weight compounds.

19

## 20 **Analytic platforms**

21 Traditionally, quantitative analysis of exometabolome and endometabolome has been  
22 carried out using enzyme-based assays (Bergmeyer *et al.*, 1985; Hajjaj *et al.*, 1998;  
23 Ruijter & Visser, 1996; Theobald *et al.*, 1993 and 1997). However, the available small  
24 sample volumes and the relatively large volumes needed in those assays limit the

1 analysis to single or a few metabolites per sample. Additionally, the reliable  
2 quantification of intracellular metabolite concentrations is hindered by the low  
3 concentrations of these compounds in cells and is exacerbated by the dilution of the  
4 already low metabolite concentrations during the quenching/extraction steps.  
5 Furthermore, the complex cellular matrix might interfere with the analytical procedures  
6 applied.

7 The advent of high sensitivity liquid chromatography-mass spectrometry (LC-ESI/MS<sup>n</sup>),  
8 gas chromatography-mass spectrometry (GC-MS<sup>n</sup>) and most recently capillary  
9 electrophoresis-mass spectrometry (CE-MS<sup>n</sup>) has broadened the range of techniques  
10 available for the quantification of intracellular and extracellular metabolites (Cech &  
11 Enke, 2001; Tomer, 2001; Buchholz *et al.*, 2001 and 2002; van Dam *et al.*, 2002;  
12 Castrillo *et al.*, 2003; Farre *et al.*, 2002; Wu *et al.*, 2005; Edwards & Thomas-Oates,  
13 2005; Villas-Bôas *et al.*, 2006; Ramautar *et al.*, 2006). These methods combine  
14 chromatographic techniques for separation of metabolites based on their physical and  
15 chemical properties coupled to mass detection with mass spectrometry (Dunn & Ellis,  
16 2005; Dunn *et al.*, 2005). The advantages which have led to the increasing use of these  
17 analytical techniques are the high sensitivity, the simultaneous quantification of many  
18 different metabolites (glycolysis, tricarboxylic acid cycle and pentose phosphate  
19 pathway) and the small sample volumes (10µL) required for analysis with a detection  
20 limit in the picomole range (van Dam *et al.*, 2002; Villas-Bôas *et al.*, 2005b). Although  
21 successful, there are still some challenges with the LC-ESI-MS/MS analytical technique,  
22 amongst which are the high salt content of typical microbial complex media samples that  
23 interferes with the operation of electrospray ion sources by clogging the skimmer and  
24 obscuring or suppressing the ionisation efficiency of the ESI (Shi, 2002; Fernie *et al.*,



1 2004). Additionally, carbon limited growth conditions are advocated for cultivating  
2 reproducibly biomass, in which the medium composition is designed in such a way that  
3 all required nutrients except for the one under investigation such as a carbon source  
4 should typically be in excess, i.e. 20-50% more than minimally required to support a pre-  
5 defined biomass concentration. This excessive extra mineral salts levels invariably leads  
6 to an even higher salt load of the samples and hence ion suppression to the ESI.  
7 However, most recently, ion suppression problem in the ESI has been alleviated by the  
8 introduction of the Mass Isotopomer Ratio Analysis of U-<sup>13</sup>C-Labeled Extracts  
9 (MIRACLE; Mashego *et al.*, 2004) and Isotope Dilution Mass Spectrometry (IDMS; Wu  
10 *et al.*, 2005), which uses fully <sup>13</sup>C-labeled metabolites as internal standard (Figure 4). In  
11 addition, IDMS analytical method eliminates the traditionally required spiking and  
12 standard additions needed for metabolite recovery studies during the extraction  
13 procedures as well as during analysis. Furthermore, successful measurement of <sup>13</sup>C-  
14 label distributions of free intracellular metabolites from steady state grown *S. cerevisiae*  
15 by liquid chromatography–mass spectrometry has been reported (van Winden *et al.*  
16 2005). This method is crucial in metabolic flux analysis studies as well as identification  
17 and validation of metabolic network structure.

18 GC-MS<sup>n</sup> analysis method measures volatile compounds and non volatiles that can be  
19 volatilized through derivatization protocols, thus adding more steps to the analytical  
20 protocols (Koek *et al.*, 2006). Therefore, efficient and reproducible derivatization  
21 methods which are central to the success of GC-MS metabolome analysis methods  
22 need to be developed and fine tuned so as to minimize likely errors propagated by these  
23 additional steps in the quantitative analysis of the metabolome.

1 CE-MS<sup>n</sup> metabolome analysis protocols are still in their infancy, although promising as  
2 has recently been demonstrated (Edwards *et al.*, 2006; Ramautar *et al.*, 2006).

3

#### 4 **Conclusion and Future Outlook**

5 Great progress in microbial metabolomics has been achieved in the last 37 years.  
6 However, it is clear that there appears to be no universal methodology in microbial  
7 metabolomics for instantaneous quenching of microbial metabolic activity, extraction of  
8 all low molecular weight metabolites and analysis of these metabolites of interest. This  
9 challenge is exacerbated by the high degree of chemical diversity such as polar and  
10 non-polar characteristics inherent to low molecular weight metabolites. Obviously, the  
11 current procedures appear to be strongly organism dependent/specific as well, hence  
12 procedures developed for metabolome quantification in prokaryotes (e.g. bacteria)  
13 cannot be directly transferred to eukaryotes (e.g. yeast or filamentous fungi) without  
14 optimization. The main problem that remains to be resolved is leakage of intracellular  
15 metabolites into the surrounding medium during the quenching step especially in  
16 prokaryotes). Therefore, a leakage test is essential - albeit often neglected - when a  
17 quenching protocol is developed for precise quantification of the metabolites. Similar  
18 arguments hold for the extraction protocols, in that the losses of metabolites need to be  
19 established and corrected for during the extraction step or labeled internal standards  
20 have to be used to correct for the possible metabolite losses. The latter procedure, i.e.  
21 labelled internal standards has successfully been used before in *Saccharomyces*  
22 *cerevisiae* (Figure 4) and *Penicillium chrysogenum* (Wu *et al.*, 2005; Nasution *et al.*,  
23 2006). Furthermore, designing a single method that could separate all metabolites  
24 appears to be unthinkable given the wide diversity in chemical and physical properties

1 inherent to the metabolites constituting the microbial metabolome. Therefore, it appears  
2 more practical to develop techniques dedicated to and targeting classes of metabolites,  
3 i.e. sugar intermediates, organic acids, amino acids, and cofactors. As suggested before  
4 by Nielsen & Oliver, (2005) and Griffin, (2006), the development of a metabolomics  
5 database containing accurately measured metabolite concentrations under given sets of  
6 standard culture conditions would serve as a reference guide and could position  
7 metabolomics as an essential part of microbial research and technology.

8

## 1 **References**

2 Al Zaid Siddiquee K, Arauzo-Bravo MJ, and Shimizu K (2004) Metabolic flux analysis of  
3 pykF gene knockout *Escherichia coli* based on <sup>13</sup>C-labeling experiments together with  
4 measurements of enzyme activities and intracellular metabolite concentrations. Appl  
5 Microbiol Biotechnol. 63(4): 407-417.

6  
7 Bagnara AS, Finch LR (1972) Quantitative extraction and estimation of intracellular  
8 nucleoside triphosphates of *Escherichia coli*. Anal Biochem. 45: 24-34.

9  
10 Bhattacharya M, Fuhrman L, Ingram A, Nickerson KW, Conway T (1995) Single-Run  
11 Separation and Detection of Multiple Metabolic Intermediates by Anion-Exchange High-  
12 Performance Liquid Chromatography and Application to Cell Pool Extracts Prepared  
13 from *Escherichia coli*. Anal Biochem. 232: 98-106.

14  
15 Bergmeyer HC, Bergmeyer J, Grass M (1985) Methods in Enzymatic Analysis, 3rd Ed.  
16 Verlag-Chemie, Weinheim.

17  
18 Buchholz A, Takors R, Wandrey C (2001) Quantification of intracellular metabolites in  
19 *Escherichia coli* K12 using liquid chromatographic-electrospray ionization tandem mass  
20 spectrometric techniques. Anal Biochem. 295(2):129-137.

21  
22 Buchholz A, Hurlbaeus J, Wandrey C, Takors R (2002) Metabolomics: Quantification of  
23 intracellular metabolite dynamics. Biomol Eng. 19: 5-15.

24

1 Buziol S, Bashir I, Baumeister A, Claaßen W, Noisommit-Rizzi N, Mailinger W, Reuss M  
2 (2002) New bioreactor-coupled rapid stopped-flow sampling technique for  
3 measurements of metabolite dynamics on a subsecond time scale. *Biotechnol Bioeng.*  
4 80(6): 632-636.  
5  
6 Castrillo JI, Hayes A, Mohammed S, Gaskell SJ, Oliver SG (2003) An optimized protocol  
7 for metabolome analysis in yeast using direct infusion electrospray mass spectrometry.  
8 *Phytochemistry.* 62(6): 929-937.  
9  
10 Cech NB, Enke CG (2001) Practical implications of some recent studies in electrospray  
11 ionization fundamentals. *Mass Spectrom Rev.* 20: 362-387.  
12  
13 Chassagnole C, Noisommit-Rizzi N, Schmid JW, Mauch K, Reuss M (2002) Dynamic  
14 modeling of the central carbon metabolism of *Escherichia coli*. *Biotechnol Bioeng.* 79(1):  
15 53-73.  
16  
17 De Koning W, van Dam K (1992) A method for the determinations of changes of  
18 glycolytic metabolites in yeast on a sub second time scale using extraction at neutral pH.  
19 *Anal Biochem.* 204: 118-123.  
20  
21 Diderich JA, Schepper M, van Hoek P, Luttk MAH, van Dijken JP, Pronk JT, Klaasen P,  
22 Boelens MJ, Teixeira de Mattos MJ, van Dam K, Kruckeberg AL (1999) Glucose uptake  
23 kinetics and transcription of HXT genes in chemostat cultures of *Saccharomyces*  
24 *cerevisiae*. *J Biol Chem.* 274: 15350-15359.

1 Dunn WB, Ellis DI (2005) Metabolomics: Current analytical platforms and  
2 methodologies. *Trends Analyt Chem.* 24(4): 285-294.  
3  
4 Dunn WB, Bailey NJC, Johnson HE (2005) Measuring the metabolome: current  
5 analytical technologies. *Analyst.* 130: 606-625.  
6  
7 Edwards E, Thomas-Oates J (2005) Hyphenating liquid phase separation techniques  
8 with mass spectrometry: on-line or off-line. *Analyst.* 130: 13-17.  
9  
10 Edwards JL, Chisolm CN, Shackman JG, Kennedy RT (2006) Negative mode  
11 sheathless capillary electrophoresis electrospray ionization-mass spectrometry for  
12 metabolite analysis of prokaryotes. *J Chromatogr A* 1106(1-2): 80-88.  
13  
14 Farre EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001)  
15 Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars,  
16 organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous  
17 fractionation method. *Plant Physiol.* 127(2): 685-700.  
18  
19 Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L (2004) Metabolite profiling: from  
20 diagnostics to systems biology. *Nat Rev Mol Cell Biol.* 5(9): 763-769.  
21  
22 Gancedo JM, Gancedo C (1973) Concentrations of intermediary metabolites in yeast.  
23 *Biochimie.* 55(2): 205-211.  
24

1 Gonzalez B, Francois J, Renaud M (1997) A Rapid and reliable method for metabolite  
2 extraction in yeast using boiling buffered ethanol. *Yeast*. 13: 1347-1356.  
3

4 Griffin JL (2006) The Cinderella story of metabolic profiling: does metabolomics get to  
5 go to the functional genomics ball? *Philos Trans R Soc Lond B Biol Sci*. 361(1465):147-  
6 161.  
7

8 Hajjaj H, Blanc PJ, Goma G, Francois J (1998) Sampling techniques and comparative  
9 extraction procedures for quantitative determination of intra- and extracellular  
10 metabolites in filamentous fungi. *FEMS Microbiol Lett*. 164: 195-200.  
11

12 Harrison DE, Maitra PK (1969) Control of respiration and metabolism in growing  
13 *Klebsiella aerogenes*. The role of adenine nucleotides. *Biochem J*. 112(5): 647-656.  
14

15 Hoque MA, Ushiyama H, Tomita M, Shimizu K (2005) Dynamic responses of the  
16 intracellular metabolite concentrations of the wild type and *pykA* mutant *Escherichia coli*  
17 against pulse addition of glucose or NH<sub>3</sub> under those limiting continuous cultures.  
18 *Biochem. Eng. J*. 26: 38-49.  
19

20 Jaki BU, Franzblau SG, Cho SH, Pauli GF (2006) Development of an extraction method  
21 for mycobacterial metabolome analysis. *J Pharm Biomed Anal*. 41(1): 196-200.  
22

1 Jensen NB, Jokumsen KV, Villadsen J (1999) Determination of the phosphorylated  
2 sugars of the Embden-Meyerhoff-Parnas pathway in *Lactococcus lactis* using a fast  
3 sampling technique and solid phase extraction. *Biotechnol Bioeng.* 63(3): 356-362.  
4

5 Jewett MC, Hofmann G, Nielsen J (2006) Fungal metabolite analysis in genomics and  
6 phenomics. *Curr Opin Biotechnol.* 17(2): 191-197.  
7

8 Kaderbhai NN, Broadhurst DI, Ellis DI, Goodacre R, Kell DB (2003) Functional genomics  
9 via metabolic footprinting: monitoring metabolite secretion by *Escherichia coli* tryptophan  
10 metabolism mutants using FT-IR and direct injection electrospray mass spectrometry.  
11 *Comp Funct Genom.* 4: 376-391.  
12

13 Kammerer B, Kahlich R, Laufer S, Li SM, Heide L, Gleiter CH (2004) Mass  
14 spectrometric pathway monitoring of secondary metabolites: systematic analysis of  
15 culture extracts of *Streptomyces* species. *Anal Biochem.* 335(1):17-29.  
16

17 Kayser A, Weber J, Hecht V, Rinas U (2005) Metabolic flux analysis of *Escherichia coli*  
18 in glucose-limited continuous culture. I. Growth-rate-dependent metabolic efficiency at  
19 steady state. *Microbiology.* 151: 693-706.  
20

21 Koek MM, Muilwijk B, van der Werf MJ, Hankemeier T (2006) Microbial metabolomics  
22 with gas chromatography/Mass spectrometry. *Anal Chem.* 78: 1272-1281.  
23



1 Lange HC, Eman M, van Zuijlen G, Visser D, van Dam JC, Frank J, de Mattos MJ,  
2 Heijnen JJ (2001) Improved rapid sampling for in vivo kinetics of intracellular metabolites  
3 in *Saccharomyces cerevisiae*. *Biotechnol Bioeng.* 75(4): 406-415.  
4

5 Lilius E, Multanen V and Toivonen V (1979) Quantitative extraction and estimation of  
6 intracellular nicotinamide nucleotides of *Escherichia coli*. *Anal Biochem.* 99: 22-27.  
7

8 Maharjan RP, Ferenci T (2003) Global metabolite analysis: the influence of extraction  
9 methodology on metabolome profiles of *Escherichia coli*. *Anal Biochem.* 313(1):145-154.  
10

11 Mashego MR, van Gulik WM, Vinke JL, Heijnen J J (2003) Critical evaluation of  
12 sampling techniques for residual glucose determination in carbon-limited chemostat  
13 culture of *Saccharomyces cerevisiae*. *Biotechnol Bioeng.* 83 (4): 395-399.  
14

15 Mashego MR, Wu L, Van Dam JC, Ras C, Vinke JL, Van Winden WA, Van Gulik WM,  
16 Heijnen JJ (2004) MIRACLE: mass isotopomer ratio analysis of U-<sup>13</sup>C-labeled extracts.  
17 A new method for accurate quantification of changes in concentrations of intracellular  
18 metabolites. *Biotechnol Bioeng.* 85: 620-628.  
19

20 Mashego MR, van Gulik WM, Vinke JL, Visser D, Heijnen JJ (2006a) In vivo kinetics  
21 with rapid perturbation experiments in *Saccharomyces cerevisiae* using a second-  
22 generation BioScope. *Metab Eng.* 8: 370-383.  
23

1 Mashego MR, van Gulik WM, Heijnen JJ (2006b) Metabolome dynamic responses of  
2 *Saccharomyces cerevisiae* on simultaneous rapid perturbations in external electron  
3 acceptor and electron donor. FEMS Yeast Res. In Press.  
4  
5 Nasution U, van Gulik WM, Kleijn RJ, van Winden WA, Proell A, Heijnen JJ (2006)  
6 Measurement of intracellular metabolites of primary metabolism and adenine  
7 nucleotides in chemostat cultivated *Penicillium chrysogenum*. Biotechnol Bioeng. 94 (1):  
8 159-166.  
9  
10 Nielsen J, Oliver S (2005) The next wave in metabolome analysis. Trends Biotechnol.  
11 23 (11): 544-546.  
12  
13 Oldiges M, Kunze M, Degenring D, Sprenger GA, Takors R (2004) Stimulation,  
14 monitoring, and analysis of pathway dynamics by metabolic profiling in the aromatic  
15 amino acid pathway, Biotechnol progr. 20(6): 1623-1633.  
16  
17 Oliver SG, Winson MK, Kell DB, Baganz F (1998) Systematic functional analysis of the  
18 yeast genome. Trends Biotechnol. 16(9): 373-378.  
19  
20 Postma E, Scheffers WA, van Dijken JP (1989) Kinetics of growth and glucose transport  
21 in glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066. Yeast. 5:  
22 159-165.  
23

1 Ramautar R, Demirci A, de Jong GJ (2006) Capillary electrophoresis in metabolomics.  
2 Trends Analyt Chem. 25(5): 455-466.  
3  
4 Ruijter GJG, Visser J (1996) Determination of intermediary metabolites in *Aspergillus*  
5 *niger*. J Microbiol Methods. 25 (8): 295-302.  
6  
7 Sáez MJ, Lagunas R (1976) Determination of intermediary metabolites in yeast. Critical  
8 examination of the effect of sampling conditions and recommendations for obtaining true  
9 levels. Mol Cell Biochem.13 (2): 73 – 78.  
10  
11 Schaefer U, Boos W, Takors R, Weuster-Botz D (1999) Automated sampling device for  
12 monitoring intracellular metabolite dynamics. Anal Biochem. 270: 88-96.  
13  
14 Shi G (2002) Application of co-eluting structural analog internal standards for expanded  
15 linear dynamic range in liquid chromatography/electrospray mass spectrometry. Rapid  
16 Commun Mass Spectrom. 17: 202-206.  
17  
18 Theobald U, Mailinger W, Reuss M, Rizzi M (1993) In vivo analysis of glucose-induced  
19 fast changes in yeast adenine nucleotide pool applying a rapid sampling technique. Anal  
20 Biochem. 214: 31-37.  
21  
22 Theobald U, Mailinger W, Baltés M, Reuss M, Rizzi M (1997) In vivo analysis of  
23 metabolic dynamics in *Saccharomyces cerevisiae*: I. Experimental observations.  
24 Biotechnol Bioeng. 55: 305-316.

1 Tomer KB (2001) Separations combined with Mass Spectrometry. Chem Rev. 101: 297-  
2 328.  
3  
4 Tweeddale H, Notley-McRobb L, Ferenci T (1998) Effect of slow growth on metabolism  
5 of *Escherichia coli*, as revealed by global metabolite pool ("metabolome") analysis. J  
6 Bacteriol. 180(19): 5109-5116.  
7  
8 van Dam JC, Eman MR, Frank J, Lange HC, van Dedem GWK, Heijnen JJ (2002)  
9 Analysis of glycolytic metabolites in *Saccharomyces cerevisiae* using anion exchange  
10 chromatography and electrospray ionisation with tandem mass spectrometric detection.  
11 Anal Chim Acta. 460: 209-218.  
12  
13 Van Hoek WPM, Diderich JA, Schepper M, Luttik MAH, van Dijken JP, Pronk JT,  
14 Klaasen P, Boelens MJ, Teixeira de Mattos MJ, van Dam K, Kruckeberg AL (1999)  
15 Glucose uptake kinetics and transcription of HXT Genes in chemostat cultures of  
16 *Saccharomyces cerevisiae*. J Biol Chem. 64: 4226-4233.  
17  
18 van Winden WA, van Dam JC, Ras C, Kleijn RJ, Vinke JL, van Gulik WM, Heijnen JJ  
19 (2005) Metabolic-flux analysis of *Saccharomyces cerevisiae* CEN.PK113-7D based on  
20 mass isotopomer measurements of <sup>13</sup>C-labeled primary metabolites. FEMS Yeast Res.  
21 (6-7):559-568.  
22

1 Verduyn C, Postma E, Scheffers WA, Van Dijken JP (1992) Effect of benzoic acid on  
2 metabolic fluxes in yeasts: A continuous culture study on the regulation of respiration  
3 and alcoholic fermentation. *Yeast*. 8: 501-517.  
4

5 Villas-Bôas SG, Hojer-Pedersen J, Akesson M, Smedsgaard J, Nielsen J (2005a) Global  
6 metabolite analysis of yeast: evaluation of sample preparation methods. *Yeast*. 22(14):  
7 1155-1169.  
8

9 Villas-Bôas SG, Mas S, Akesson M, Smedsgaard J, Nielsen J (2005b) Mass  
10 spectrometry in metabolome analysis. *Mass Spectrom Rev*. 24(5): 613-646.  
11

12 Villas-Bôas SG, Noel S, Lane G A, Attwood G, Cookson A (2006) Extracellular  
13 metabolomics: a metabolic footprinting approach to assess fiber degradation in complex  
14 media. *Anal Biochem*. 349(2): 297-305.  
15

16 Visser D, van Zuylen GA., van Dam JC, Oudshoorn A, Eman MR, Ras C, van Gulik WM,  
17 Frank J, van Dedem GW, Heijnen JJ (2002) Rapid sampling for analysis of in vivo  
18 kinetics using the BioScope: a system for continuous pulse experiments. *Biotechnol*  
19 *Bioeng*. 79 (6): 674-681.  
20

21 Weber J, Kayser A, Rinas U (2005) Metabolic flux analysis of *Escherichia coli* in  
22 glucose-limited continuous culture. II. Dynamic response to famine and feast, activation  
23 of the methylglyoxal pathway and oscillatory behaviour. *Microbiology*. 151:707-716.  
24

1 Weibel KE, Mor JR, Fiechter A (1974) Rapid sampling of yeast cells and automated  
2 assays of adenylate, citrate, pyruvate and glucose-6-phosphate pools. Anal Biochem.  
3 58: 208-216.  
4  
5 Weuster-Botz D (1997) Sampling tube device for monitoring intracellular metabolite  
6 dynamics. Anal Biochem. 246(2): 225-233.  
7  
8 Wittmann C, Krömer JO., Kiefer P, Binz T, Heinzle E (2004) Impact of the cold shock  
9 phenomenon on quantification of intracellular metabolites in bacteria. Anal Biochem.  
10 327(1): 135-139.  
11  
12 Wu L, Mashego MR, van Dam JC, Proell AM, Vinke JL, Ras C, van Winden W A, van  
13 Gulik WM, Heijnen JJ (2005) Quantitative analysis of the microbial metabolome by  
14 isotope dilution mass spectrometry using uniformly <sup>13</sup>C-labeled cell extracts as internal  
15 standards. Anal Biochem. 336: 164-171.

Table I. Comparison of various quenching protocols for microbial metabolic activity

| Quenching agent                 | Buffer       | Temperature | Microorganism         | Reference   |
|---------------------------------|--------------|-------------|-----------------------|---|
| 60% (v/v) MeOH/H <sub>2</sub> O | -            | -40°C       | <i>L. lactis</i>      | Jensen <i>et al.</i> , (1999)                                     |
| 60% (v/v) MeOH/H <sub>2</sub> O |              | -40°C       | <i>S. cerevisiae</i>  | Mashego <i>et al.</i> , (2004)                                    |
| 60% (v/v) MeOH/H <sub>2</sub> O |              | -40°C       | <i>S. cerevisiae</i>  | Villas-Bôas <i>et al.</i> , (2005a)                               |
| 60% (v/v) MeOH/H <sub>2</sub> O | HEPES        | -40°C       | <i>Monascus ruber</i> | Hajjaj <i>et al.</i> , (1998)                                     |
| 60% (v/v) MeOH/H <sub>2</sub> O | HEPES        | -40°C/-50°C | <i>E. coli</i>        | Buchholz <i>et al.</i> , (2001); Oldiges <i>et al.</i> , 2004     |
| 60% (v/v) MeOH/H <sub>2</sub> O | HEPES        | -40°C/-50°C | <i>C. glutamicum</i>  | Wittmann <i>et al.</i> , (2004)                                   |
| 60% (v/v) MeOH/H <sub>2</sub> O | HEPES        | -40°C/-50°C | <i>E. coli</i>        | Al Zaid Siddiquee <i>et al.</i> , (2004)                          |
| 60% (v/v) MeOH/H <sub>2</sub> O | Ethanolamine | -45°C       | <i>A. niger</i>       | Ruijter & Visser, (1996)  |
| 60% (v/v) MeOH/H <sub>2</sub> O | Tricine      | -40°C       | <i>S. cerevisiae</i>  | Castrillo <i>et al.</i> , (2003)                                  |
| Liquid nitrogen                 | -            | -150°C      | <i>Monascus ruber</i> | Hajjaj <i>et al.</i> , (1998)                                     |
| Liquid nitrogen                 | -            | -150°C      | <i>E. coli</i>        | Buziol <i>et al.</i> , (2002); Chassagnole <i>et al.</i> , (2002) |

Table II. Comparison of various microbial biomass extraction protocols for intracellular metabolites

| Extraction agent                          | Temperature  | Microorganism  | Reference  |
|---|--------------|--|--|
| 75% Ethanol                               | > 80°C       | <i>S. cerevisiae</i> , <i>Monascus ruber</i>                             | Gonzalez <i>et al.</i> , (1997); Hajjaj <i>et al.</i> , (1998); Castrillo <i>et al.</i> , (2003) |
| 75% Ethanol                               | > 80°C       | <i>S. cerevisiae</i> , <i>E. coli</i> , <i>P. chrysogenum</i>            | Visser <i>et al.</i> , (2002)  |
| Perchloric acid                           | -25°C, -80°C | <i>S. cerevisiae</i>   | Theobald <i>et al.</i> , (1993 and 1997)   |
| Perchloric acid                           | -80°C, -25°C | <i>Monascus ruber</i>  | Hajjaj <i>et al.</i> , (1998)  |
| Perchloric acid                           | -80°C, -25°C | <i>E. coli</i> K-12 W3110  | Chassagnole <i>et al.</i> , (2002); Oldiges <i>et al.</i> , 2004                                 |
| Tris-H <sub>2</sub> SO <sub>4</sub> /EDTA | 90°C         | <i>E. coli</i> K-12 W3110  | Buziol <i>et al.</i> , (2002); Chassagnole <i>et al.</i> , (2002)                                |
| Ethyl acetate                             | ambient      | <i>Streptomyces spheroides</i> ;<br><i>Streptomyces roseochromogenes</i> | Kammerer <i>et al.</i> (2004)  |
| Water                                     | 100°C        | <i>E. coli</i>   | Bhattacharya <i>et al.</i> (1995)  |
| KOH                                       | ambient      | <i>S. cerevisiae</i>   | Theobald <i>et al.</i> , (1993 and 1997)   |
| KOH                                       | ambient      | <i>Monascus ruber</i>  | Hajjaj <i>et al.</i> , (1998)  |
| KOH                                       | ambient      | <i>E. coli</i>   | Chassagnole <i>et al.</i> , (2002)   |
| KOH                                       | ambient      | <i>A. niger</i>  | Ruijter & Visser, (1996)   |
| α-aminobutyrate                           | 100°C        | <i>C. glutamicum</i>   | Wittmann <i>et al.</i> , (2004)  |
| Chloroform                                | -            | <i>Monascus ruber</i>  | Hajjaj <i>et al.</i> , (1998)  |
| Chloroform                                | -            | <i>L. lactis</i>   | Jensen <i>et al.</i> (1999)  |



## Figure legends

Figure 1. Flow diagram of sampling procedure, metabolic activity quenching, intracellular metabolite extraction (endometabolome), extracellular metabolite (exometabolome) and analysis procedures for quantification

Figure 2. Typical bioreactor. When valves A and B are closed, the bioreactor is operating in a batch mode. When valve A is open and fresh medium is fed to the bioreactor, the bioreactor is operating in a fed-batch mode, and volume does not remain constant, but increases. This mode of bioreactor operation is widely used in industry. When both valve A and B are open and  $F_{in} \approx F_{out}$ , the bioreactor is operated in continuous mode. The bioreactor volume remains constant.

Figure 3. Extracellular glucose concentration profile obtained by rapid sampling after a glucose pulse applied to aerobically, glucose-limited grown *S. cerevisiae* at  $D=0.05 \text{ h}^{-1}$ .

Figure 4. Intracellular glucose-6-phosphate concentration profile obtained by rapid sampling, quenching and extraction of biomass after a glucose pulse applied to aerobically, glucose-limited grown *S. cerevisiae* at  $D=0.05 \text{ h}^{-1}$ .

Figure 1

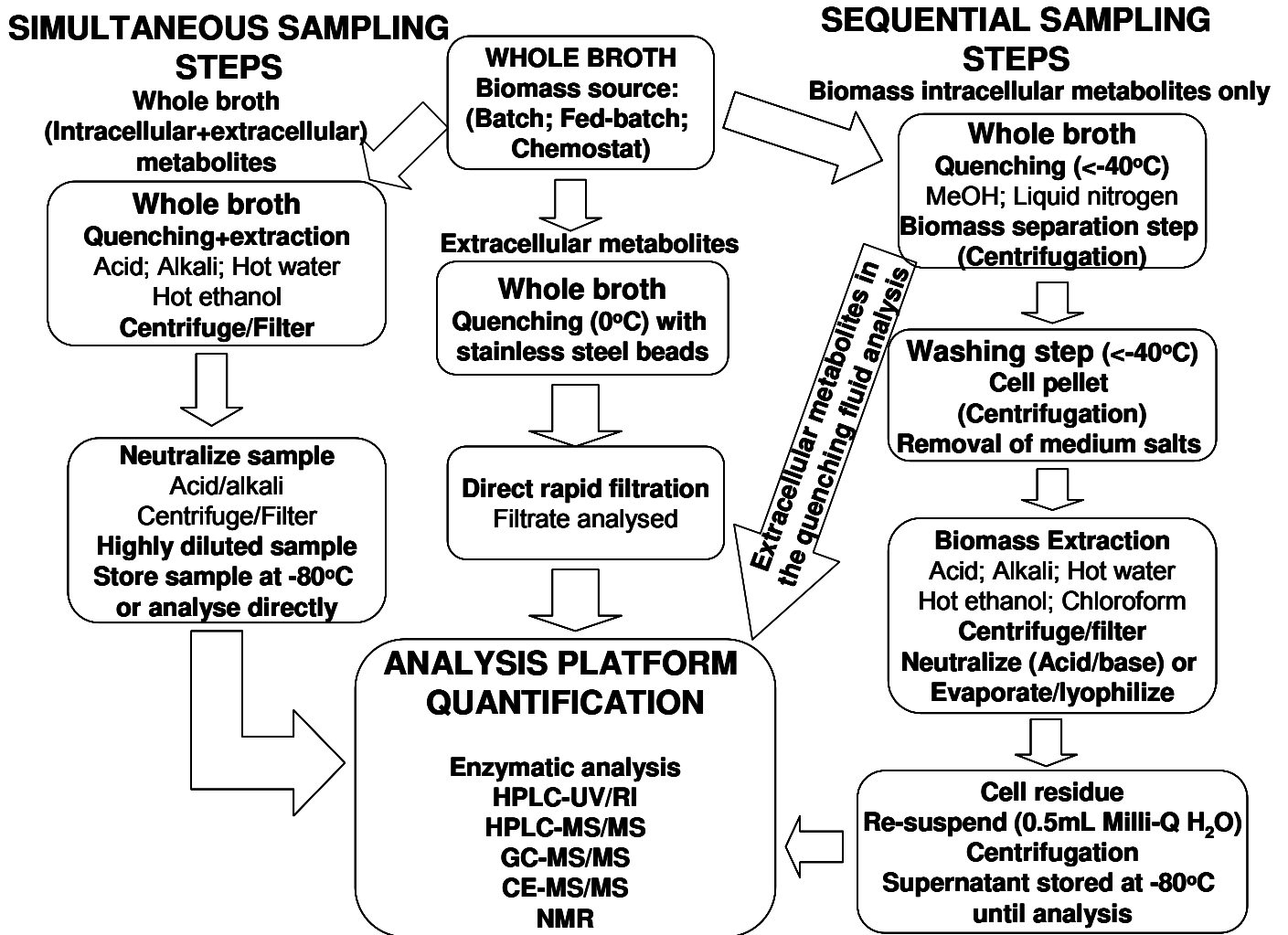


Figure 2

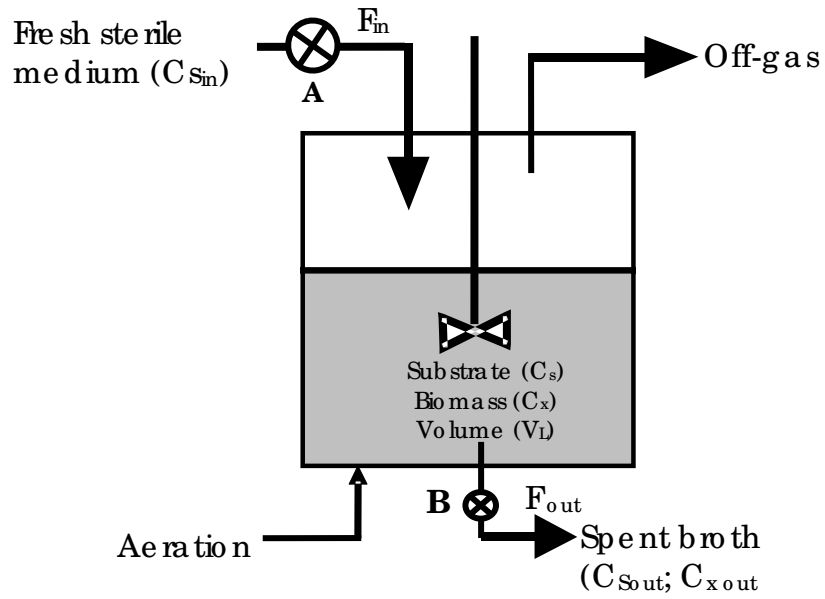


Figure 3

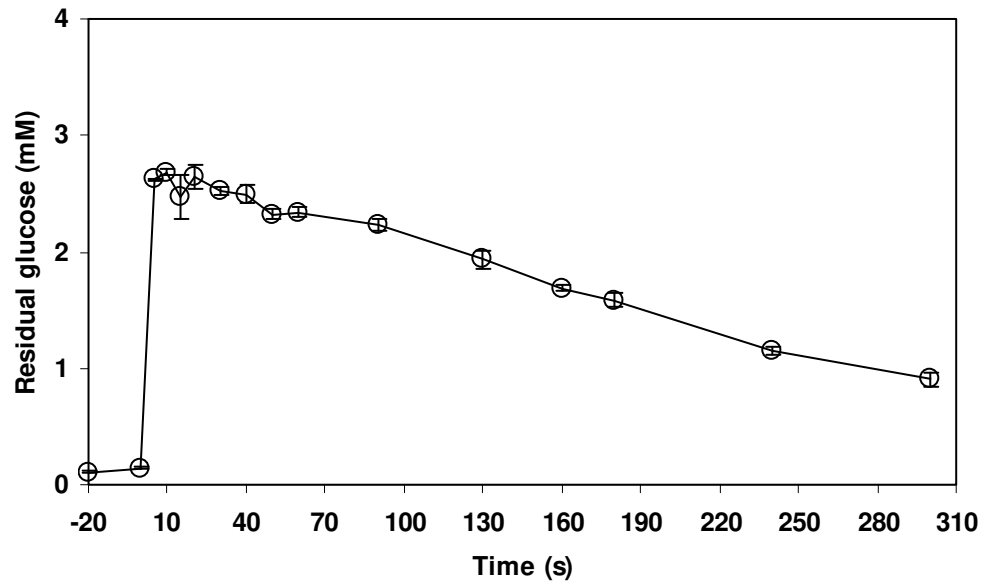


Figure 4

