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

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Key words: Bacteria, Chromatography, Metabolomics, Filamentous fungi, Yeast, Mass Spectrometry, Rapid sampling, Quenching, Metabolite Extraction

Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

Biomass source

Metabolomics studies requires biomass source which is achieved by growing microorganisms under controlled environment in bioreactors. In a bioreactor, temperature, pH, medium components as well as dissolved gas concentrations such as oxygen (O_2) and carbon dioxide (CO_2) are easily controlled so that the microenvironment is well defined. Well defined growth conditions are necessary in order to establish standard and reproducible reference culture conditions. In addition, bioreactors can be operated in a batch, fed-batch or continuous mode (Figure 2). Recently, the majority of the researchers in metabolomics tend to prefer continuous culture mode of bioreactor operation for several reasons:

- Specific growth rate (μ) = dilution rate (D) can be well defined and fixed.
- One specific growth limiting medium component such as carbon source can be imposed.

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

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

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

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

Rapid Sampling techniques

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

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

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

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

Exometabolome

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

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

Endometabolome

Quenching methods for microbial cells metabolic activity (Table I)

Bacteria

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

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

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

Filamentous fungi

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

Yeast

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

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

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

Extraction methods for intracellular metabolites

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

with minimal degradation. The inherent dilution effects of some of the procedures should be kept as minimal as possible. In the forefront of the extraction agents (Table II) are boiling 75% ethanol (v/v) (Gonzalez *et al*., 1997; Hajjaj *et al*., 1998; Visser *et al*., 2002; Maharjan & Ferenci, 2003; Mashego *et al*., 2004; Villas-Bôas *et al*. 2005a; Nasution *et al*., 2006); 50-100% methanol (Tweeddale *et al*., 1998; Maharjan & Ferenci, 2003; Wittman *et al*., 2004; Villas-Bôas *et al*., 2005a); trichloroacetic acid, acetic acid, hydrochloric acid, perchloric acid,Tris-H_2SO_4/EDTA, ethyl acetate or KOH (Bagnara & Finch, 1972; Lilius *et al*., 1979; de Koning & van Dam, 1992; Theobald *et al*., 1993; Tweeddale *et al*., 1998; Schaefer *et al*., 1999; Chassagnole *et al*., 2002; Oldiges *et al.*, 2004; Kammerer *et al*., 2004; Villas-Bôas *et al*., 2005a; Kayser *et al*., 2005; Weber *et al*., 2005); chloroform or toluene (de Koning & van Dam, 1992; Tweeddale *et al*., 1998; Jensen *et al*., 1999; Maharjan & Ferenci, 2003; Villas-Bôas *et al*., 2005a); as well as hot water (Bhatthacharya *et al*., 1995. Recently, an extraction method of intracellular metabolites in *Mycobacterium bovis* combining deep freezing in liquid nitrogen and mechanical grinding of cells has been reported (Jaki *et. al.*, 2006). Typical metabolites extracted with these methods include intermediates from glycolysis, tricarboxylic acid cycle, pentose phosphate pathway as well as purine and pyridmidine nucleotides, amino acids and other low molecular weight compounds.

Analytic platforms

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

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

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

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

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

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

Conclusion and Future Outlook

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

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

References

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

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

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

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

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

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

Buziol S, Bashir I, Baumeister A, Claaßen W, Noisommit-Rizzi N, Mailinger W, Reuss M (2002) New bioreactor-coupled rapid stopped-flow sampling technique for measurements of metabolite dynamics on a subsecond time scale. Biotechnol Bioeng. 80(6): 632-636.

Castrillo JI, Hayes A, Mohammed S, Gaskell SJ, Oliver SG (2003) An optimized protocol for metabolome analysis in yeast using direct infusion electrospray mass spectrometry. Phytochemistry. 62(6): 929-937.

Cech NB, Enke CG (2001) Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom Rev. 20: 362-387.

Chassagnole C, Noisommit-Rizzi N, Schmid JW, Mauch K, Reuss M (2002) Dynamic modeling of the central carbon metabolism of *Escherichia coli*. Biotechnol Bioeng. 79(1): 53-73.

De Koning W, van Dam K (1992) A method for the determinations of changes of glycolytic metabolites in yeast on a sub second time scale using extraction at neutral pH. Anal Biochem. 204: 118-123.

Diderich JA, Schepper M, van Hoek P, Luttik MAH, van Dijken JP, Pronk JT, Klaasen P, Boelens MJ, Teixeria de Mattos MJ, van Dam K, Kruckeburg AL (1999) Glucose uptake kinetics and transcription of HXT genes in chemostat cultures of *Saccharomyces cerevisiae*. J Biol Chem. 274: 15350-15359.

Dunn WB, Ellis DI (2005) Metabolomics: Current analytical platforms and methodologies. Trends Analyt Chem. 24(4): 285-294.

Dunn WB, Bailey NJC, Johnson HE (2005) Measuring the metabolome: current analytical technologies. Analyst. 130: 606-625.

Edwards E, Thomas-Oates J (2005) Hyphenating liquid phase separation techniques with mass spectrometry: on-line or off-line. Analyst. 130: 13-17.

Edwards JL, Chisolm CN, Shackman JG, Kennedy RT (2006) Negative mode sheathless capillary electrophoresis electrospray ionization-mass spectrometry for metabolite analysis of prokaryotes. J Chromatogr A 1106(1-2): 80-88.

Farre EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L (2001) Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiol. 127(2): 685-700.

Fernie AR, Trethewey RN, Krotzky AJ, Willmitzer L (2004) Metabolite profiling: from diagnostics to systems biology. Nat Rev Mol Cell Biol. 5(9): 763-769.

Gancedo JM, Gancedo C (1973) Concentrations of intermediary metabolites in yeast. Biochimie. 55(2): 205-211.

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

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

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

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

Hoque MA, Ushiyama H, Tomita M, Shimizu K (2005) Dynamic responses of the intracellular metabolite concentrations of the wild type and *pyk*A mutant *Escherichia coli* against pulse addition of glucose or NH_3 under those limiting continuous cultures. Biochem. Eng. J. 26: 38-49.

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

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

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

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

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

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

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

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

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

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

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

Mashego MR, Wu L, Van Dam JC, Ras C, Vinke JL, Van Winden WA, Van Gulik WM, Heijnen JJ (2004) MIRACLE: mass isotopomer ratio analysis of U-^{13}C-labeled extracts. A new method for accurate quantification of changes in concentrations of intracellular metabolites. Biotechnol Bioeng. 85: 620-628.

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

Mashego MR, van Gulik WM, Heijnen JJ (2006b) Metabolome dynamic responses of *Saccharomyces cerevisiae* on simultaneous rapid perturbations in external electron acceptor and electron donor. FEMS Yeast Res. In Press.

Nasution U, van Gulik WM, Kleijn RJ, van Winden WA, Proell A, Heijnen JJ (2006) Measurement of intracellular metabolites of primary metabolism and adenine nucleotides in chemostat cultivated *Penicillium chrysogenum*. Biotechnol Bioeng. 94 (1): 159-166.

Nielsen J, Oliver S (2005) The next wave in metabolome analysis. Trends Biotechnol. 23 (11): 544-546.

Oldiges M, Kunze M, Degenring D, Sprenger GA, Takors R (2004) Stimulation, monitoring, and analysis of pathway dynamics by metabolic profiling in the aromatic amino acid pathway, Biotechnol progr. 20(6): 1623-1633.

Oliver SG, Winson MK, Kell DB, Baganz F (1998) Systematic functional analysis of the yeast genome. Trends Biotechnol. 16(9): 373-378.

Postma E, Scheffers WA, van Dijken JP (1989) Kinetics of growth and glucose transport in glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CBS 8066. Yeast. 5: 159-165.

Ramautar R, Demirci A, de Jong GJ (2006) Capillary electrophoresis in metabolomics. Trends Analyt Chem. 25(5): 455-466.

Ruijter GJG, Visser J (1996) Determination of intermediary metabolites in *Aspergillus niger*. J Microbiol Methods. 25 (8): 295-302.

Sáez MJ, Lagunas R (1976) Determination of intermediary metabolites in yeast. Critical examination of the effect of sampling conditions and recommendations for obtaining true levels. Mol Cell Biochem.13 (2): 73 – 78.

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

Shi G (2002) Application of co-eluting structural analog internal standards for expanded linear dynamic range in liquid chromatography/electrospray mass spectrometry. Rapid Commun Mass Spectrom. 17: 202-206.

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

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

Tomer KB (2001) Separations combined with Mass Spectrometry. Chem Rev. 101: 297-328.

Tweeddale H, Notley-McRobb L, Ferenci T (1998) Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("metabolome") analysis. J Bacteriol. 180(19): 5109-5116.

van Dam JC, Eman MR, Frank J, Lange HC, van Dedem GWK, Heijnen JJ (2002) Analysis of glycolytic metabolites in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionisation with tandem mass spectrometric detection. Anal Chim Acta. 460: 209-218.

Van Hoek WPM, Diderich JA, Schepper M, Luttik MAH, van Dijken JP, Pronk JT, Klaasen P, Boelens MJ, Teixeria de Mattos MJ, van Dam K, Kruckeburg AL (1999) Glucose uptake kinetics and transcription of HXT Genes in chemostat cultures of *Saccharomyces cerevisiae*. J Biol Chem. 64: 4226-4233.

van Winden WA, van Dam JC, Ras C, Kleijn RJ, Vinke JL, van Gulik WM, Heijnen JJ (2005) Metabolic-flux analysis of *Saccharomyces cerevisiae* CEN.PK113-7D based on mass isotopomer measurements of $^{(13)}$C-labeled primary metabolites. FEMS Yeast Res. (6-7):559-568.

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

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

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

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

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

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

Weibel KE, Mor JR, Fiechter A (1974) Rapid sampling of yeast cells and automated assays of adenylate, citrate, pyruvate and glucose-6-phosphate pools. Anal Biochem. 58: 208-216.

Weuster-Botz D (1997) Sampling tube device for monitoring intracellular metabolite dynamics. Anal Biochem. 246(2): 225-233.

Wittmann C, Krömer JO., Kiefer P, Binz T, Heinzle E (2004) Impact of the cold shock phenomenon on quantification of intracellular metabolites in bacteria. Anal Biochem. 327(1): 135-139.

Wu L, Mashego MR, van Dam JC, Proell AM, Vinke JL, Ras C, van Winden W A, van Gulik WM, Heijnen JJ (2005) Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly ^{13}C-labeled cell extracts as internal 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_2O	-	-40°C	*L. lactis*	Jensen *et al*., (1999)
60% (v/v) MeOH/H_2O		-40°C	*S. cerevisiae*	Mashego *et al*., (2004)
60% (v/v) MeOH/H_2O		-40°C	*S. cerevisiae*	Villas-Bôas *et al*., (2005a)
60% (v/v) MeOH/H_2O	HEPES	-40°C	*Monascus ruber*	Hajjaj *et al*., (1998)
60% (v/v) MeOH/H_2O	HEPES	-40°C/-50^{o}C	*E. coli*	Buchholz *et al*., (2001); Oldiges *et al.,* 2004
60% (v/v) MeOH/H_2O	HEPES	-40°C/-50^{o}C	*C. glutamicum*	Wittmann *et al*., (2004)
60% (v/v) MeOH/H_2O	HEPES	-40°C/-50^{o}C	*E. coli*	Al Zaid Siddiquee *et al*., (2004)
60% (v/v) MeOH/H_2O	Ethanolamine	-45°C	*A. niger*	Ruijter & Visser, (1996)
60% (v/v) MeOH/H_2O	Tricine	-40°C	*S. cerevisiae*	Castrillo *et al*., (2003)
Liquid nitrogen	-	-150°C	*Monascus ruber*	Hajjaj *et al*., (1998)
Liquid nitrogen	-	-150°C	*E. coli*	Buziol *et al*., (2002); Chassagnole *et al*., (2002)

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

Extraction agent	Temperature	Microorganism	Reference
75% Ethanol	> 80°C	*S. cerevisiae, Monascus ruber*	Gonzalez *et al*., (1997); Hajjaj *et al*., (1998); Castrillo *et al*., (2003)
75% Ethanol	> 80°C	*S. cerevisiae*, *E. coli*, *P. chrysogenum*	Visser *et al*., (2002)
Perchloric acid	-25°C, -80°C	*S. cerevisiae*	Theobald *et al*., (1993 and 1997)
Perchloric acid	-80°C, -25°C	*Monascus ruber*	Hajjaj *et al*., (1998)
Perchloric acid	-80°C, -25°C	*E. coli* K-12 W3110	Chassagnole *et al*., (2002); Oldiges *et al.,* 2004
Tris-H_2SO_4/EDTA	90°C	*E. coli* K-12 W3110	Buziol *et al*., (2002); Chassagnole *et al.,* (2002)
Ethyl acetate	ambient	*Streptomyces spheroides; Streptomyces roseochromogenes*	Kammerer *et al.* (2004)
Water	100°C	*E. coli*	Bhattacharya *et al*. (1995)
KOH	ambient	*S. cerevisiae*	Theobald *et al*., (1993 and 1997)
KOH	ambient	*Monascus ruber*	Hajjaj *et al*., (1998)
KOH	ambient	*E. coli*	Chassagnole *et al*., (2002)
KOH	ambient	*A. niger*	Ruijter & Visser, (1996)
α-aminobutyrate	100°C	*C. glutamicum*	Wittmann *et al*., (2004)
Chloroform	-	*Monascus ruber*	Hajjaj *et al*., (1998)
Chloroform	-	*L. lactis*	Jensen *et al*. (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 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 h^{-1}.

Figure 1

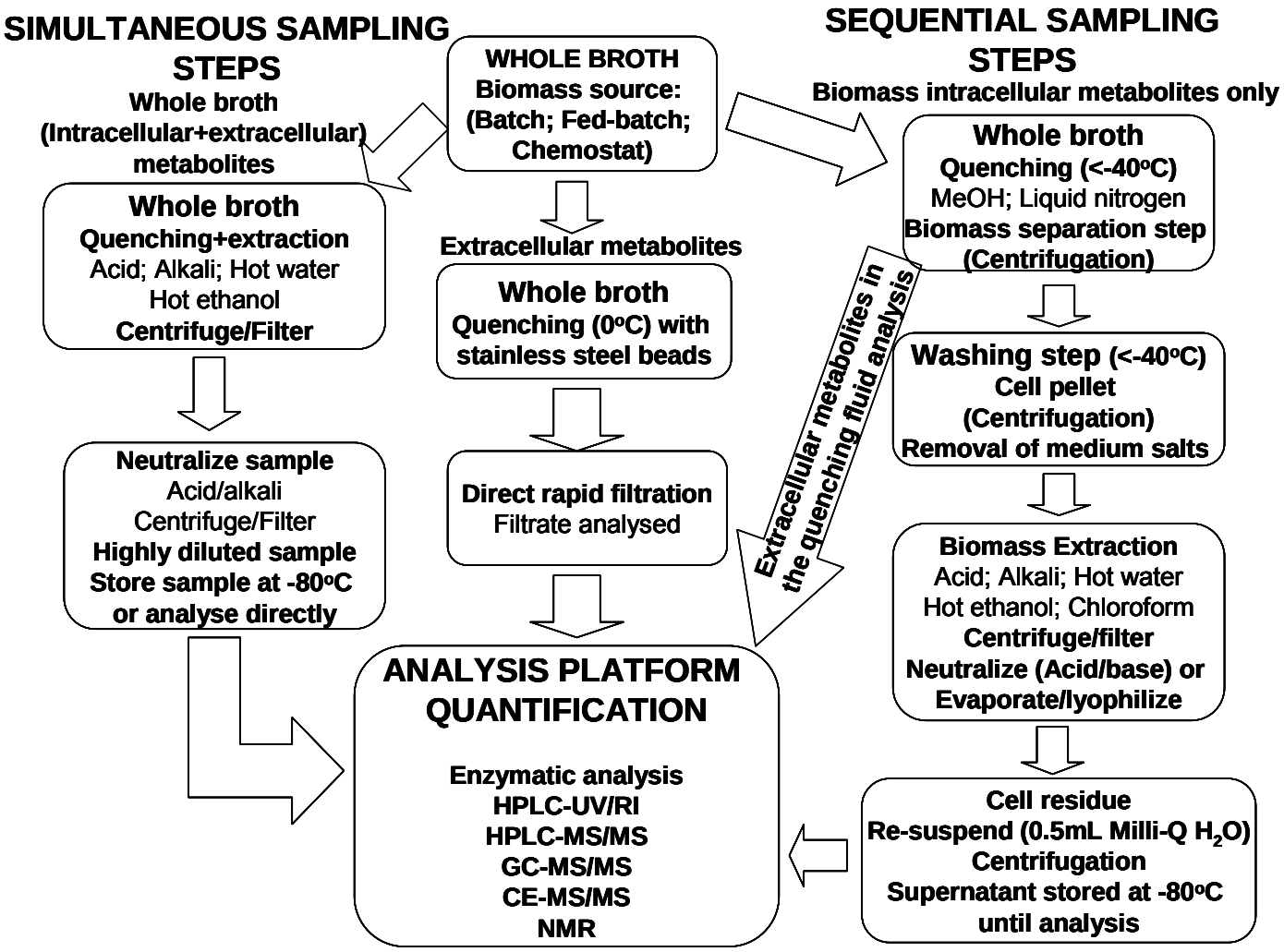

SIMULTANEOUS SAMPLING STEPS
Whole broth (Intracellular+extracellular) metabolites
Whole broth
Quenching+extraction
Acid; Alkali; Hot water
Hot ethanol
Centrifuge/Filter
Neutralize sample
Acid/alkali
Centrifuge/Filter
Highly diluted sample
Store sample at -80°C
or analyse directly
WHOLE BROTH
Biomass source: (Batch; Fed-batch; Chemostat)
Extracellular metabolites
Whole broth
Quenching (0°C) with stainless steel beads
Direct rapid filtration
Filtrate analysed
Extracellular metabolites in the quenching fluid analysis
SEQUENTIAL SAMPLING STEPS
Biomass intracellular metabolites only
Whole broth
Quenching (<-40°C)
MeOH; Liquid nitrogen
Biomass separation step
(Centrifugation)
Washing step (<-40°C)
Cell pellet
(Centrifugation)
Removal of medium salts
Biomass Extraction
Acid; Alkali; Hot water
Hot ethanol; Chloroform
Centrifuge/filter
Neutralize (Acid/base) or
Evaporate/lyophilize
Cell residue
Re-suspend (0.5mL Milli-Q H2O)
Centrifugation
Supernatant stored at -80°C
until analysis
ANALYSIS PLATFORM QUANTIFICATION
Enzymatic analysis
HPLC-UV/RI
HPLC-MS/MS
GC-MS/MS
CE-MS/MS
NMR

Figure 2

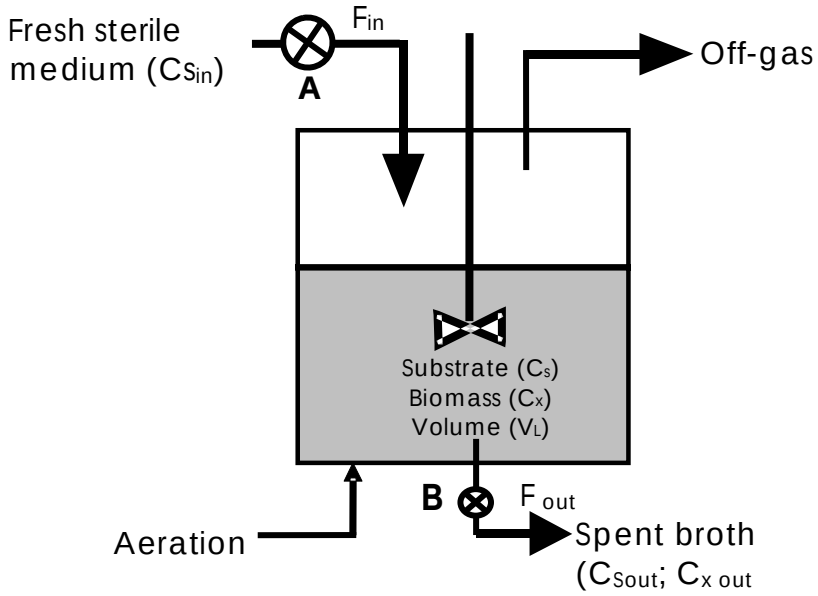

Fresh sterile
medium (C_{Sin})
F_{in}
A
Off-gas
Substrate (C_s)
Biomass (C_x)
Volume (V_L)
B
F_{out}
Aeration
Spent broth
(C_{Sout}; $C_{x\ out}$

Figure 3

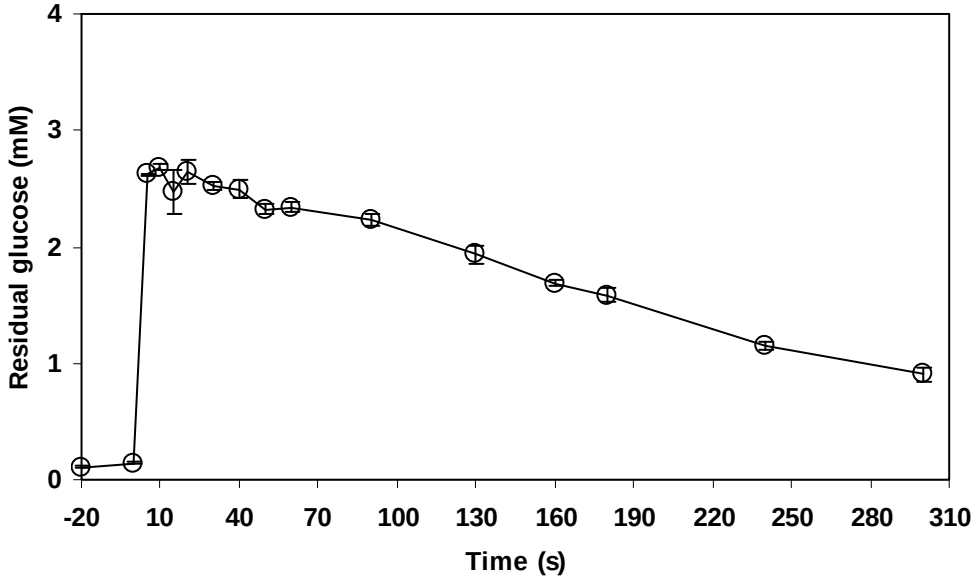
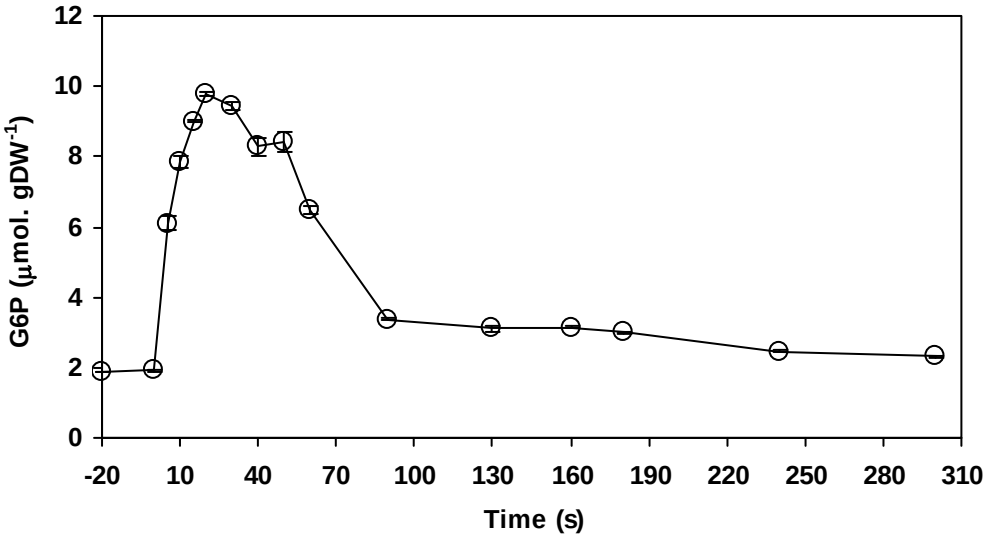

Residual glucose (mM)
4
3
2
1
0
-20
10
40
70
100
130
160
190
220
250
280
310
Time (s)

Figure 4


12
10
8
6
4
2
0
G6P (µmol. gDW-1)
-20
10
40
70
100
130
160
190
220
250
280
310
Time (s)