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REVIEW

Microbial metabolomics: past, present and future methodologies

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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 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 metabolic 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 ($LC\text{-}MS^n$, $GC\text{-}MS^n$, $CE\text{-}MS^n$), where n indicates the number of tandem mass spectrometry, and nuclear magnetic resonance spectroscopy (NMR).

Keywords Bacteria · Chromatography · Filamentous fungi · Mass spectrometry · Metabolomics · Metabolite extraction · Quenching · Rapid sampling · Yeast

Glossary

Metabolomics	Quantification of the total metabolites complement inside and outside a cell under defined growth conditions
Exometabolome	Total metabolites excreted outside the cell (culture supernatant)
Endometabolome	Total metabolites located inside the cell (Intracellularly)
Footprinting	Qualitative analysis of exometabolome
Fingerprinting	Qualitative analysis of endometabolome

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Target analysis	Quantitative analysis of known pre-defined metabolites concentrations
Quenching	Instantaneous arrest of endogenous metabolic activity
GC-MS	Gas chromatography coupled to mass spectrometry
LC-ESI-MS	Liquid chromatography coupled to electrospray ionisation mass spectrometry
Q-TOF	uattro Time of light
FT-ICR	Fourier transform-ion cyclotron resonance
CE-MS	Capillary electrophoresis coupled to mass spectrometry
Metabolite turnover rate	The inverse of the metabolite pool size to metabolite flux ratio

Introduction

Biotechnologically based industrial production of high value added bio-products, such as vitamins, amino acids, enzymes, bulk chemicals, antibiotics and bio-pharmaceuticals, has been accomplished in the past with the use of micro-organisms. These microbial mutants with improved product specific production rates compared with the wild type parents were mainly created by classical strain development programmes based on random strain mutagenesis and selection procedures. These procedures were successful, especially in the biotechnological production of amino acids such as glutamic acid and lysine (Aida et al. 1986), as well as antibiotics such as penicillin. However, the effected mutations were largely undefined; the selection programmes were labour intensive and based on a trial and error.

The advent of recombinant DNA technology in the late 1970s has led to specific direct introduction of specific product pathway of interest such as in the production of insulin by *E. coli* and *S. cerevisiae* respectively or the enhancement of the flux to the products naturally produced by the organisms (Nielsen 2001). Most of the biotechnologically derived products from micro-organisms are secondary products of metabolism, meaning that they are not directly linked to the primary metabolism or specific growth rate directly. However, their production rates and concentrations are indirectly linked to the primary metabolism as primary central carbon metabolism provide precursors, cofactors in the form of NAD(H) and NADP(H) as well as ATP (primary energy currency of the cells). It is therefore logical that successful, engineering of superior microbial cells (metabolic engineering) for the enhancement of microbial products production using laboratory strains to date requires firm understanding of primary cellular metabolism and its regulation in vivo (Bailey 1991).

Knowledge of metabolite levels has led to the identification of bottlenecks in the metabolic reaction network. For example, reactions far from equilibrium, such as phosphofructokinase (PFK) and pyruvate kinase (PK), have been confirmed as key regulatory points of glycolytic flux through metabolomics (Theobald et al. 1993). Furthermore, metabolomics constitute reactants and products of in vivo reactions, which are directly connected to cellular metabolism (phenotypes)—other than transcriptome (mRNA) and proteome (proteins) (Oliver et al. 1998). Metabolite concentrations plays an important direct regulatory role through which rapid response to metabolic flux changes via allosteric or feedback inhibition mechanism of enzymes as a mode of pathway flux control is effected. Metabolomics thus complement genomics, transcriptomics, proteomics as well as fluxomics data and facilitate metabolic engineering and system biology efforts towards designing superior biocatalysts and cell factories.

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 in different environments, growth conditions or genetic perturbations. Although the term metabolome has only surfaced in the late 1990s (Oliver et al. 1998; Tweeddale et al. 1998), metabolomics research has been in existence since the late 1960s and early 1970s (Harrison and Maitra 1969; Gancedo and 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 (Kell et al. 2005). On the contrary, quantitative analysis of known predefined metabolites is referred to as target analysis (Jewett et al. 2006). Especially in a systems biology context, 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 (Goodacre 2005).

For the sake of brevity, exhaustive specific examples of metabolomics contribution and integration with other—omics platforms to the characterization of microbial phenotypes for biotechnology process improvement are covered in excellent reviews elsewhere (Jewett et al. 2006; Kummel et al. 2006; Wendisch et al. 2006a, b; Lee et al. 2005; van der Werf et al. 2005; Stephanopoulos et al. 2004). In addition, these reviews provides detailed metabolomics success stories about understanding the fundamentals of cellular processes such as the regulation of metabolic flux and redirection of flux distribution in whole cells which is relevant to systems biology and biotechnology (Kitano 2002; Kell 2006).

Although not exhaustive, this review focuses exclusively on the past, present and future methodologies in microbial metabolomics and its current and future challenges. It highlights the current state of affairs in metabolomics in general, emphasizing biomass cultivation techniques, rapid sampling, biomass quenching protocols, extraction of intracellular metabolites and subsequent analysis. The authors acknowledge and recognize the exciting developments in other metabolomics research areas especially in plants and humans, for excellent reviews on these areas; the reader is referred to Fiehn et al. (2000), Fiehn (2006), Glinski and Weckwerth (2006), Sumner et al. (2003), Griffin (2006), Bino et al. (2004).

Current challenges facing metabolomics

Routine detection and quantification of wide ranging intracellular metabolites in vivo remains a formidable challenge, mainly because 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 experimental protocols ranging from microbial cultivation techniques (defined biomass), biomass sampling procedures, isolation/extraction of relevant targeted or non-targeted metabolites of interest as well as quantitative analysis of these metabolites.

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 the systematic investigations of various quenching methods and the outcome has been that in general, most prokaryotic microorganisms (bacteria) behave differently when exposed to the almost universal cold methanol protocol than eukaryotic microorganisms (e.g. yeasts and filamentous fungi). Prokaryotic cells such as *Corynebacterium glutamicum* and *Escherichia coli* tend to leak intracellular metabolites when exposed to cold

methanol protocol, most probably due to their apparent less robust cell wall and membrane structures as well as composition which are known to be different from those of eukaryotic cells. This observation might have prompted the recent evaluation of a cold methanol protocol as a possible extraction protocol for extracting intracellular metabolites from these organisms (Maharjan and Ferenci 2003; Wittman et al. 2004). Eukaryotic organisms such as *Saccharomyces cerevisiae, Penicillium chrysogenum* and *Aspergillus niger* appear to be less leaky when exposed to a cold methanol quenching protocol (Villas-Bôas et al. 2005a; Nasution et al. 2006).

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). Furthermore, this can be achieved via two modes, i.e. sequential or simultaneous sample handling. Table 1 compares and contrasts the advantages and disadvantages of both sample handling procedures.

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°C) or high temperatures (e.g. >+80°C), or by applying extreme sample pH, i.e. either high alkali (e.g. KOH or NaOH) or high 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 (Fig. 1).

Metabolite analysis methods vary widely from enzymatic assay-based methods (Bergmeyer et al. 1985) to modern hyphenated techniques such as gas chromatography coupled to mass spectrometry (GC-MS) or liquid chromatography coupled to mass spectrometry (LC-MS/MS) and most recently, capillary electrophoresis coupled to mass spectrometry (CE-MS). It is important at

Table 1 Comparisons of the advantages and disadvantages between sequential and simultaneous sample processing for metabolomics studies

	Advantage	Disadvantage
Sequential sample processing procedure		
Cold methanol or liquid N_2 quenching	Separation of biomass from supernatant (target specific)	Possible metabolite leakage during quenching
Separate metabolites extraction method	Sample matrix is cleaner (less salts)	Multiple extraction procedures for specific metabolites
	Separate extraction step (target metabolites of choice)	Laborious
Simultaneous sample processing procedure		
Simultaneous quenching and direct extraction with either, alkali, acid, boiling ethanol or water	Procedure is simpler to perform	Difficult to interpret the data
	No separation of biomass from supernatant required	Sample matrix complex
	Total quantification of both (intra and extracellular) metabolites	High salt content in samples
	Non-specific	Metabolites are too dilute leading to poor detection and quantification

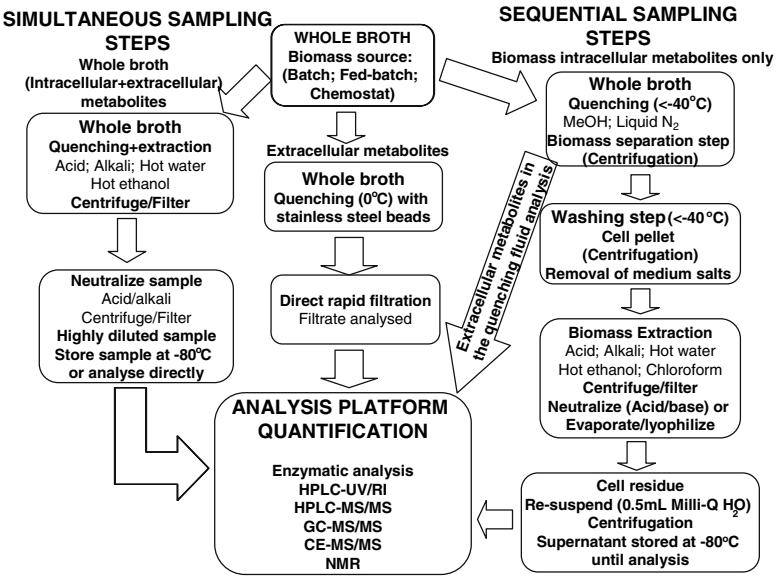

Fig. 1 Flow diagram of sampling procedure, metabolic activity quenching, intracellular metabolite extraction (endometabolome), extracellular metabolite (exometabolome) and analysis procedures for quantification

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 render them unidentifiable or undetectable.
5. The resulting sample matrix should be compatible or amenable to the analytical method of choice.

Biomass source

Metabolomics studies require 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 O_2 and 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 (Fig. 2).

Recently, the majority of the researchers in metabolomics tend to prefer continuous culture

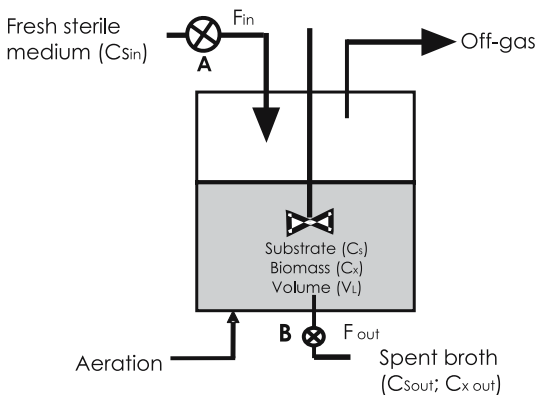

Fig. 2 Typical bioreactor. When valves A and B are closed, the bioreactor is operating in a batch mode. When valve A is open and fresh medium is fed to the bioreactor, the bioreator is operating in a fed-batch mode, and volume does not remain constant, but increases. This mode of bioreactor operation 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

mode of bioreactor operation for several reasons:

- Specific growth rate (μ) = dilution rate (D) can be well defined and fixed.
- By fixing the specific growth rate, all other fluxes such as specific substrate uptake rate (q_s), O_2 uptake rate (OUR) as well as CO_2 evolution rate (CER) are fixed.
- One specific growth limiting medium component such as carbon source can be imposed.
- Physiological reference steady state condition can be easily 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 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 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 reference steady state of the culture due to substrate deprivation. In contrast to the continuous cultivation 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 (metabolome).

Limited metabolomics research has been performed using batch (Villas-Bôas et al. 2005a; Kabir et al. 2005) and fed-batch (Oldiges et al. 2004) cultures despite the fact that these culture modes are widely used in industrial bioprocesses. However, the batch cultures are physiologically less defined and poorly reproducible mainly because substrate as well as dissolved gasses, such as the CO_2 concentration, is continually changing. This leads to a less physiological defined reference biomass, especially when quantitative physiological characterization of phenotypes and comparison with a reference physiological steady state is the objective of the research. Quantitative metabolomics have been studied in fed-batch cultures (Oldiges et al. 2004). However, fed-batch cultivations are experimentally quite challenging especially for routine high-throughput screening purposes for comparative data generations, whereas continuous cultivation mode is experimentally less complex and can be operated in such a way that it mimics closely a fed-batch cultivation mode.

One disadvantage of the chemostat culture condition is that the culture medium composition is designed in such a way that only one growth limiting medium component such as, but not limited to, 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 are in the order of 4 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°C). This washing step is necessary; especially when liquid chromatography coupled to electrospray ionisation mass spectrometry based metabolites 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 collected samples represent in vivo conditions as closely as possible. 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 s (Weibel et al. 1974; de Koning and van Dam 1992). Holms (1996) estimated that isocitrate turns over 2.7 times per second in *E. coli* ML 308 growing on acetate. 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 a carbon source 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 Fig. 3A and B (Mashego et al. 2006b).

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 Sáez and Lagunas (1976). The method was further refined and automated by de Koning and van Dam (1992), Gonzalez et al. (1997), Schaefer et al. (1999), Theobald et al. (1993, 1997) and Visser et al. (2002).

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 and Visser 1996).

Schaefer et al. (1999) reported an automated sampling device capable of a sampling frequency of 0.22 s 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 subsequently 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 ms 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.

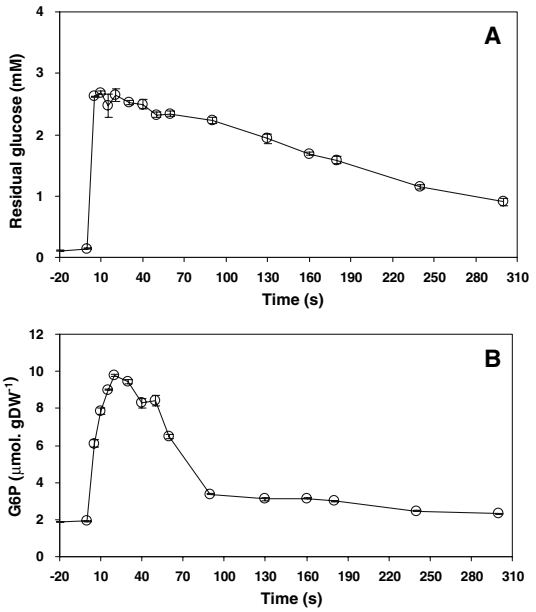

Fig. 3 Extracellular glucose and intracellular glucose 6-phosphate concentration profile obtained by rapid sampling after a glucose pulse applied to aerobically, glucose-limited grown *S. cerevisiae* at $D = 0.05\ h^{-1}$

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 reference 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. 2006b). 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 Fig. 3A and B.

Quenching protocols for microbial cells metabolic activity

Exometabolome

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

have been described to rapidly arrest metabolic activity for measurement of excreted metabolites and 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°C and later pre-cooled tubes filled with 10–15 stainless steel spheres (4 mm diameter) at –10°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 N_2, followed by thawing in ice with gentle shaking to keep the cell suspension at 0°C. The suspension was later centrifuged to separate biomass from the supernatant.

More 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 as can be seen in Fig. 3A. This method has been demonstrated to work satisfactory for excreted metabolites such as pyruvate, acetate and ethanol in *S. cerevisiae*, *Penicillium chrysogenum* and *E. coli* (Mashego et al. 2006b; Nasution et al. 2006, MR Mashego et al., unpublished data).

Endometabolome (Table 2)

Bacteria

Jensen et al. (1999) used 60% (v/v) methanol at –35°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 70 mM HEPES at –50, –40, and –80°C, respectively, but again these authors neither mentioned nor tested cell leakage during the quenching procedure. Liquid N_2 (–196°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°C, 10 mM HEPES buffered 60% (v/v) methanol at –58°C, cold 0.9% NaCl at –0.5°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 (amino acids).

Filamentous fungi

Ruijter and Visser (1996) employed 60% (v/v) methanol buffered with ethanolamine (pH 7.3) at –45°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 N_2 and cold 10 mM HEPES buffered methanol, 60% (v/v) at –40°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

Table 2 Comparison of various quenching protocols for microbial metabolic activity

Quenching agent	Buffer	Temperature	Microorganism	References
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°C	*E. coli*	Buchholz et al. (2001), Oldiges et al. (2004)
60% (v/v) MeOH/H_2O	HEPES	–40°C /–50°C	*C. glutamicum*	Wittmann et al. (2004)
60% (v/v) MeOH/H_2O	HEPES	–40°C /–50°C	*E. coli*	Al Zaid Siddiquee et al. (2004)
60% (v/v) MeOH/H_2O	Ethanolamine	–45°C	*A. niger*	Ruijter and Visser, (1996)
60% (v/v) MeOH/H_2O	Tricine	–40°C	*S. cerevisiae*	Castrillo et al. (2003)
Liquid N_2	–	–150°C	*Monascus ruber*	Hajjaj et al. (1998)
Liquid N_2	–	–150°C	*E. coli*	Buziol et al. (2002), Chassagnole et al. (2002)

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 cell leakage suggested that no significant leakage occurred since ATP was neither 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 and 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 was 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 quenching procedure. 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 3).

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 3) are boiling 75% (v/v) ethanol (Gonzalez et al. 1997; Hajjaj et al. 1998; Visser et al. 2002; Maharjan and Ferenci 2003; Mashego et al. 2004; Villas-Bôas et al. 2005a; Nasution et al. 2006), 50–100% methanol (Tweeddale et al. 1998; Maharjan and Ferenci 2003; Wittman et al. 2004; Villas-Bôas et al. 2005a), trichloroacetic acid, acetic acid, hydrochloric acid,

Table 3 Comparison of various microbial biomass extraction protocols for intracellular metabolites

Extraction agent	Temperature	Microorganism	References
75% (v/v) Ethanol	>80°C	*S. cerevisiae, Monascus ruber*	Gonzalez et al. (1997), Hajjaj et al. (1998), Castrillo et al. (2003)
75% (v/v) 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, 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, 1997)
KOH	Ambient	*Monascus ruber*	Hajjaj et al. (1998)
KOH	Ambient	*E. coli*	Chassagnole et al. (2002)
KOH	Ambient	*A. niger*	Ruijter and 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)

perchloric acid,TrisH_2SO_4/EDTA, ethyl acetate or KOH (Bagnara and Finch 1972; Lilius et al. 1979; de Koning and 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 and van Dam 1992; Tweeddale et al. 1998; Jensen et al. 1999; Maharjan and Ferenci 2003; Villas-Bôas et al. 2005a), as well as hot water (Bhatthacharya et al. 1995. Notably, Villas-Bôas et al. (2005a) noticed varying efficacy of six different extraction procedures as well as losses of metabolites during sample concentration by lyophilization and solvent evaporation. 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.

Although, the boiling ethanol extraction protocol is suitable for extracting a wide set of metabolites such as from glycolysis, TCA cycle as well as pentose phosphate pathway, it has been noticed that for extensive coverage of the wide chemically diverse metabolites, extraction protocols have to be classified in order to target classes of like metabolites. This is achieved by classifying the extraction protocols so that polar solvents extract polar metabolites and non-polar solvents extract a class of metabolites that are non-polar. Similarly, acid stable metabolites should be typically extracted with acids whereas alkali stable metabolites should be extracted with alkali. Finally, thermolabile metabolites should be preferably extracted under low temperatures whereas higher temperatures could be used for extracting thermostable metabolites. These forgoing discussions suggests that the ambitious goal of quantitative coverage of the extensive cellular metabolome requires development of specific individualized extraction protocols targeting various classes of metabolites, and not the one-size-fits-all strategy that has been widely adopted thus far.

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 and Visser 1996; Theobald et al. 1993, 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.

Mass spectrometry is a well established analytical instrument for analysis of diverse chemicals as well as bio-molecules on the basis of ion molecular mass-to-charge ratio (m/z). Its popularity in the biosciences research has been made possible by the development in the late 1980s of two soft ionization techniques i.e. electrospray ionization (ESI) and matrix assisted laser desorption ionization electrospray ionization (MALDI) (Domon and Aebersold 2006). Various configurations of mass spectrometers are available today ranging from Quattro LC, Quattro Time of light (Q-TOF) and Fourier transform-ion cyclotron resonance (FT-ICR).

The advent of high sensitivity liquid chromatography-mass spectrometry (LC-ESI/MS^n) through the development of liquid to gas interfaces, 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 and Enke 2001; Tomer 2001; Buchholz et al. 2001, 2002; Fiehn et al. 2000; Fiehn 2006; van Dam et al. 2002; Castrillo et al. 2003; Farre et al. 2001; Wu et al. 2005; Edwards and Thomas-Oates 2005; Brown et al. 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 and 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 (10 μl) required for analysis with a detection limit in the picomole range (Wunschel et al. 1997, 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 ionization 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), analytical methods which uses fully ^{13}C-labeled metabolites as internal standard (Fig. 3B). In addition, the 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 reaction network structure.

The 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 (Soga et al. 2002, Edwards et al. 2006, Ramautar et al. 2006). It is based on the separation of compounds based on their molecular size and charge and subsequently detected and identified by mass spectrometry. The main advantage of CE-MS is its ability to concurrently measure cationic, anionic and nucleotides using as little as 30 nl sample volume (Soga et al. 2003). The main drawback of routine use of CE-MS lies in the difficulty of interfacing CE with mass spectrometry, although good progress is being made in this area.

Conclusions 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 quenching 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 to overcome the leakage of intracellular metabolites into the surrounding medium during the quenching step especially in prokaryotes, such as *E. coli* (Maharjan and Ferenci 2003) due to the inherent differences in cell wall structure and membrane composition in these organisms. Therefore, a leakage test is essential, albeit often neglected, when a quenching protocol is developed for precise quantification of the intracellular metabolites.

Alternatively, the total broth (cells and medium) should be extracted together, thus yielding total metabolites and then followed by the determination of extracellular metabolites normally present in the medium separately, i.e. filtrate or supernatant. Subsequently, these metabolite concentrations should be subtracted from each other as depicted by equation 1 below.

$$IC = T - EX \quad (1)$$

where IC is the intracellular metabolite concentration (μmol/g dry wt), T is the total sum of both intracellular and extracellular metabolites in broth (biomass + supernatant) (μmol/g dry wt), EX is the extracellular metabolite concentration in the filtrate (μmol/g dry wt).

However, this procedure would be applicable with simultaneous quenching and extraction protocol that do not include biomass separation step, provided that the extracellular metabolites are present in very low concentrations or totally absent or undetectable in the cell free supernatant. 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* (Fig. 3B) 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 both anionic and cationic, i.e. sugar intermediates, organic acids, amino acids, and

cofactors. As suggested before by Nielsen and 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.

Ultimately, metabolomics data should be integrated quantitatively with other-omics data i.e. genomics, transcriptomics, proteomics and fluxomics making use of bioinformatics, as demonstrated in *Arabidopsis* (Weckwerth et al. 2004) so as to facilitate meaningful development of strains that are efficient and productive enough to surpass current capacities and thereby justify the enormous investment involved.

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