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Quantitative Metabolomics and Its Application in Metabolic Engineering of Microbial Cell Factories Exemplified by the Baker's Yeast

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1. Introduction

The baker's yeast *Saccharomyces cerevisiae* and its beneficial properties have been recognized very early by human beings. It has been used in the making of alcoholic beverages, bread and cake long before the term biotechnology has been coined. In addition to its great importance in food industry *S. cerevisiae* strains are nowadays applied in many other fields for example in the production of bio-fuels from corn or sugar containing crops, in the bio-sorption of heavy-metals from sewage, in pharmaceuticals or in the production of precursor compounds for the synthesis of pharmaceuticals or fine chemicals. As a consequence *S. cerevisiae* developed to one of the most important and best investigated microbial cell factories for the industrial (white) biotechnology. Furthermore *S. cerevisiae* is an important model organism used to elucidate the underlying molecular mechanistic principles that are involved in complex diseases (cancer or diabetes) and metabolic disorders (Castrillo and Oliver 2005; Castrillo and Oliver 2006; Nielsen and Jewett 2008). Other important features of *S. cerevisiae* that led to its multifaceted applicability in industry and R&D constitute its GRAS (generally recognized as safe) status and that cells are very easy to cultivate and are readily available.

The physiology of *S. cerevisiae* under various environmental conditions has been investigated intensively in the last 140 years (Racker 1974). The baker's yeast exhibits some very interesting physiological features that render it unique among all other microorganisms. It grows nearly equally fast under aerobic and anaerobic conditions with glucose as the sole carbon source (Nissen et al. 2000a; Visser et al. 1990). Under aerobic conditions and at glucose concentrations above 100 mg/L biomass formation is accompanied by the production of ethanol as a consequence of an overflow metabolism at the pyruvate node (Crabtree-effect, (Crabtree 1928)). After depletion of glucose the ethanol initially formed by the overflow metabolism is further converted into biomass under aerobic conditions (Diauxie). Under anaerobic conditions about 90% of glucose carbon is converted into ethanol and CO_2. The rate of glucose utilization and the specific ethanol yield is higher under anaerobic conditions as compared to the sugar conversion rate and ethanol yield under aerobic conditions (Pasteur-effect, (Racker 1974)). It can reduce a number of keto-compounds to the corresponding chiral alcohols that represent

interesting precursors for pharmaceuticals (Csuk 1991). It can grow as a diploid as well as a haploid which highly facilitates genetic manipulation and permits high-throughput genetic engineering.

Considering the enormous early interest in studying and understanding the physiology of *S. cerevisiae* long before modern omics techniques have been developed, it is not very surprising that it was the baker's yeasts genome that was the first within the domain of eukaryotes that was completely sequenced. Genomic and biological information about *S. cerevisiae* molecular biology is comprehensively collected at the *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org/). Driven by the knowledge of the complete genomic sequence and by the steadily increasing availability of tools developed for genetic engineering, *S. cerevisiae* became a key work horse and the representative eukaryotic model organism in every modern discipline within the biosciences such as molecular and cell biology, functional genomics, systems biology or metabolic and synthetic engineering. Today's genetic work with *S. cerevisiae* cells is highly alleviated by the presence of a wide spectrum of established yeast molecular biology tool kits and availability of many wild-type and mutant strain (e.g. knock-out strains) collections as well as plasmid collections containing *S. cerevisiae* ORFs, gene deletion markers or promoter sets and many more, offered by commercial sources such as EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html), Open biosystems (http://www.openbiosystems.com/Products/) or Addgene (http://www. addgene. org/).

The commercial establishment of genetic manipulation techniques paved the way for *S. cerevisiae* to be exploited in the field of metabolic engineering. Various novel recombinant designer strains capable of either selective formation of one desired product or of producing heterologous compounds or endogenous products from new resources (waste or renewable materials) emerged in the last decades. Metabolic engineering efforts based on *S. cerevisiae* are comprehensively summarized elsewhere and the interested reader is referred to (Bettiga et al. 2010; Nevoigt 2008). A collection of engineered substrate utilization and heterologous or homologous product formation pathways is given in Table 1.

The corresponding underlying engineering principles can be basically broken down into 4 strategies as depicted in Figure 1 panel A-D. Elucidation of the appropriate engineering approach represents the most important step in designing novel cellular properties and targets on the identification of reaction(s) or even entire pathways that are suited for the anticipated metabolic engineering objective. Relevant reaction(s) and associated gene(s) can be extracted by thorough screenings of literature data (US National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed), SciFinder (https://scifinder.cas.org/) or Web of Knowledge (http://wokinfo.com/)) and online databases (KEGG the Kyoto Encyclopedia of Genes and Genomes (http://www. genome.jp/kegg/), the enzyme database BRENDA (http://www.brenda-enzymes.org/), or the SIB bioinformatics Resource Portal ExPASy (http://www.expasy.ch/).

To increase the probability of engineering success identified targets can be subjected to in silico modeling by employing mathematical models like restricted flux balance analysis (FBA) based on a genome-scale stoichiometric network to verify their compatibility with the underlying metabolic network (Cvijovic et al. 2010; Selvarajoo et al. 2010).

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Substrate targets	Applications	References
Xylose	bio-ethanol	(Jeffries and Jin 2004; Petschacher and Nidetzky 2008; van Maris et al. 2007)
L-Arabinose	bio-ethanol	(Wisselink et al. 2007)
Lactose / whey	bio-ethanol	(Domingues et al. 2010)
Galactose	bio-ethanol	(Lee et al. 2010)
Product targets		
Insulin and insulin precursors	pharmaceuticals	(Kjeldsen 2000)
Hepatitis B antigen	pharmaceuticals	(Kuroda et al. 1993)
Cyanophycin	biopolymer	(Steinle et al. 2009)
Organic acids	chemical building blocks	(Abbott et al. 2009; Ishida et al. 2006; Raab et al. 2010)
n-Butanol	bio-fuel	(Steen et al. 2008)
Sesquiterpenes	pharmaceuticals and food	(Asadollahi et al. 2010; Jackson et al. 2003)
Carotenoids	pharmaceuticals and food	(Ukibe et al. 2009)
Diterpenoids	pharmaceutical industry	(Dejong et al. 2006)
Triterpenes	pharmaceutical industry	(Madsen et al. 2011)
Polyketides	pharmaceutical industry	(Mutka et al. 2006)
Five-carbon sugars / alcohols	food ingredients	(Toivari et al. 2007)
Ethylene	synthetic polymers	(Pirkov et al. 2008)
Flavonoids, stilbenoids	pharmaceuticals and food	(Trantas et al. 2009)
SO_2	beer flavor stability	(Yoshida et al. 2008)
Ethanol	bio-fuel	(Nissen et al. 2000b)

Table 1. Collection of metabolic engineering targets in *S. cerevisiae*

To unravel reaction(s) or pathways instead that would compromise substrate conversion and/or production of a desired product in silico modeling by for example FBA (Bro et al. 2006) or MOMA (minimization of metabolic adjustment) (Asadollahi et al. 2009) have been applied and produced potential candidate reactions which otherwise would have been often overlooked (Cvijovic et al. 2010). The open-source software platform OptFlux developed especially for in silico driven metabolic engineering is available at http://www.optflux.org/ (Rocha et al.).

Metabolic integration of a novel pathway is selected when the utilization of a new substrate (Fig. 1 A) or synthesis of a new product is intended (Fig. 1 B). New in this context means that the original, not engineered, cells are genetically not programmed to perform these reactions. Formation of an undesired side-product (Fig. 1 C) can be attributed to a split in carbon flux at the related node (C) into a productive (C→P) and undesired flux (C→D). Reasons for such flux partition compromising efficient production of the product (P) can be often traced back to the presence of an enzyme or differently regulated isoenzyme competing for the same substrate S or a promiscuous enzyme that in addition to the desired substrate is also active with other substrates (Fig. 1 D).
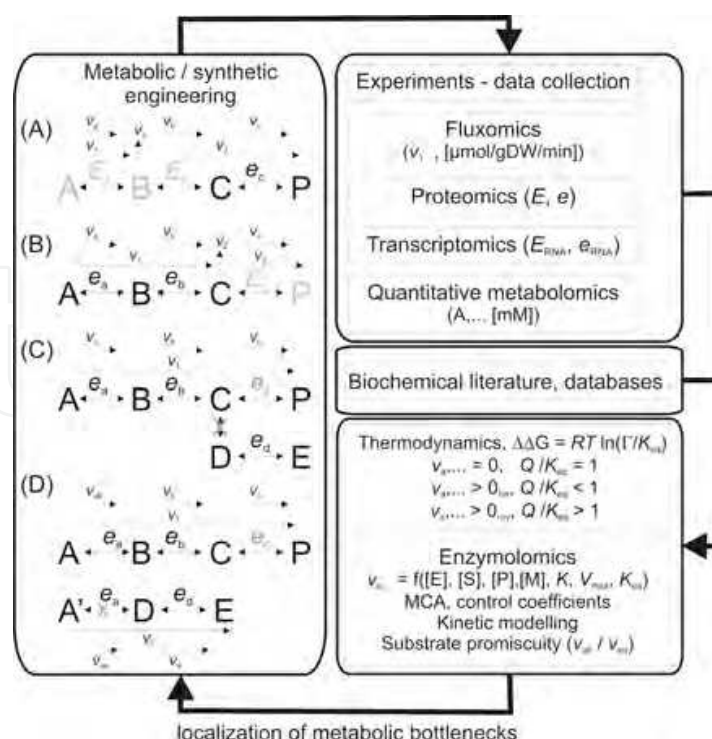
Fig. 1. Typical metabolic engineering principles based on rational design (panels A – D) are linked a suggested experimental work-flow to unravel limiting metabolic sites. Panels A-D refer to enabling of substrate utilization (A) or product formation (B), preventing side product formation by deletion and/or overexpression of an endogenous enzyme (C), increasing selectivity of a substrate promiscuous enzyme (D); Substrate A, intermediate B, product P and enzymes *E* new to the network are indicated in grey. Overexpression of an endogenous enzyme is depicted by a grey e. Knock outs are indicated by grey x's. Subscripts of rate constants *v* given as numbers and small letters refer to fluxes based on stoichiometry (solid arrows) and individual reaction rates of enzymes (dotted arrows), respectively.

Directing the carbon flux towards P can be afforded by deletion of respective gene(s), overexpression of enzymes participating in the productive branch, or replacing the corresponding activity by a less regulated or more selective one. Furthermore unbalanced carbon usage between reaction partners participating in this pathway and/or in the recycling of, for example cofactors, can result in accumulation and release of a pathway intermediate (Krahulec et al. 2009; Krahulec et al. 2010). In this case fine-tuning of all activities involved based on for example a metabolic control analysis (MCA) or kinetic modeling analysis is required to minimize or even completely prevent by-product accumulation (Parachin et al. 2011).

Aside from rational design stochastic methods based on inverse metabolic engineering have been developed for *S. cerevisiae* to identify key target reactions and associated gene sequences enabling the desired new cellular property (Bailey et al. 2002; Bengtsson et al. 2008; Bro et al. 2005; Hong et al. 2010; Jin et al. 2005; Lee et al. 2010). Differently, methods targeting on the induction of a cellular property, such as growth, increase of substrate conversion rate or enhancing resistance to environmental stress, that is hardly to capture by in silico design because of its highly intricate metabolic relations that have to be satisfied, rely on the cellular adaptability to a certain environmental stress by evolution (Cakar et al.

2009; Cakar et al. 2005; Garcia Sanchez et al. 2010; Kuyper et al. 2005; Sonderegger and Sauer 2003; Wisselink et al. 2009).

In the course of establishing systems biology various high-throughput omics techniques such as transcriptomics, proteomics, fluxomics and others have been developed with the objective to comprehensively analyze cellular physiology at all molecular levels (DNA, RNA, protein, flux, and metabolite). Data-driven analysis is often exploited to unravel novel interrelations at the various molecular levels or to obtain a more insightful (quantitative) understanding of cellular processes. It is obvious that metabolic engineering can greatly benefit from the integration of omics techniques in the design of improved microbial cell factories (Nielsen and Jewett 2008). The various omics tools have helped to increase understanding about how cells regulate, communicate and adapt to different environmental conditions.

Depending on the metabolic engineering objective the appropriate omics tool or a combination should be selected after due consideration. For example transcriptome analysis provides a holistic image of mRNA molecule pattern and levels but do not tell us anything about metabolic fluxes. Optimization of the flux towards a specific metabolite however represents one of the major goals in metabolic engineering. Metabolic flux analyses based on stoichiometric models or ^{13}C-isotopomer analysis (provided that cells can grow under the environmental conditions used) are useful tools in this respect (Nielsen and Jewett 2008). To understand the underlying mechanistic relationships between the flux through a particular pathway and the enzymes forming the pathway, providing the relevant information for strain design, detailed knowledge about enzyme-metabolite interactions are required. Consequently quantitative information about metabolites involved together with detailed knowledge of kinetic properties of participating enzymes is mandatory. Within the omics family metabolomics represents the youngest member. This is basically due to the facts that metabolites vary greatly in their physico-chemical properties (polarity, acidity, reactivity, and stability) and are present in a large dynamic concentration range which make it almost impossible to record the entire metabolome on a single analytical platform. Another challenge represents the generation of reliable and representative metabolite data from biological samples. Cell-wall leakage, instabilities and losses of metabolites throughout the sample work-up, or strong matrix effects in the MS analysis are a few of the many causes impairing metabolite data and as a consequence adulterate molecular mechanistic interpretation. Nevertheless in the last years much progress has been made due to enormous efforts of the yeast research community to overcome these obstacles. Protocols of unbiased sample-work-up and different analytical platforms are available today that can cover more than 100 compounds quantitatively.

This review presents current accepted protocols and techniques that enable acquisition of absolute quantitative metabolite data from *S. cerevisiae* cells. The second part focuses on how quantitative metabolite data can help in the development of improved microbial cell factories.

However, before going into the details some definitions of terms used in metabolome analysis should be reminded (Nielsen 2007). Metabolite profiling targets on the qualitative or semi-quantitative analysis of specified metabolites or groups of metabolites. In contrast in metabolite target analysis selected metabolites are quantified. If the entire metabolome or a fraction of it is addressed (or as many metabolites as possible) qualitatively or quantitatively we speak of metabolomics or quantitative metabolomics.

2. Data acquisition for quantitative metabolomics in *Saccharomyces cerevisiae*

Determination of unbiased intracellular concentrations of metabolites is without doubt a prerequisite for serious interpretation of cellular properties at the molecular – kinetic level. Unbiased refers here to the physiological state, the sample work-up and preparation for metabolite analysis and metabolite quantification and calculation of intracellular molar concentration. Consequently four experimental tasks that have to be fulfilled can be formulated as follows:

- Harvesting and quenching of cells at a predefined physiological state (representative) and separation from extracellular compounds (exometabolome) without leakage of intracellular metabolites (quantitative)
- Destruction of metabolic activity and complete extraction of metabolites by maintaining the metabolite composition quantitatively unchanged, concentration and if required chemical preparation for metabolite analysis
- High-throughput quantitative analysis by employing the appropriate instrument
- Relation of molar concentrations obtained from quantitative analysis to cell specific parameters such as cell dry weight (μmol / gCDW) or cell number (μmol / cell) or if absolute molar intracellular concentrations are required to the cell (compartment) volume.

However, before we go into the details and hurdles of each task it should be emphasized here that altering the cellular network by metabolic engineering will always result in a quasi new strain with completely new properties and behaviors. In the worst case the complete sample work-up protocol established for the wild-type strain might not be applicable for the mutant strains. It is therefore strongly recommended that protocols already established for the absolute quantification of metabolites have to be re-assessed and verified for the created strains.

2.1 Representative harvesting and quantitative quenching

The concentration of a metabolite in the cell is not directly linked to genes but is determined by its formation and utilization rates. Conversion rates in turn depend on the enzymes associated catalytically with this metabolite and their kinetic parameters with respect to this metabolite as well as effectors (inhibitors, activators). In particular metabolites from catabolic reactions and reactions involved in energy metabolism display high turnover rates. For example for the frequently used metabolite ATP (~16% of all reactions present in a genome-scale stoichiometric model of *S. cerevisiae* involve ATP (Förster et al. 2003)) turnover rates of ~1.5 mM/s were reported (Rizzi et al. 1997). Hence, quenching of metabolic activity within a very short time window is required without altering the metabolomic state of the sample. Another highly desirable feature is that the extracellular environment containing substrates, products, salts and the exometabolome, that can affect subsequent metabolite analysis, is separable from the cells without losing any intracellular metabolites due to disruption of or leakage through the cell wall. Different to for example *E. coli* the cell wall of *S. cerevisiae* is less leaky and both requirements can be achieved for *S. cerevisiae* cells by spraying a defined volume of cell suspension into an appropriate quenching solution at sufficiently low temperatures. It was

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found that the volume ratio of sample to quenching solution affects the quenching quality and a ratio of at least 1 to 5 was suggested (Canelas et al. 2008a).

Today two methods cold methanol and cold glycerol-saline have achieved wide acceptance within the metabolomics community (Canelas et al. 2008a; Villas-Bôas and Bruheim 2007). See Fig. 2 for details. In addition to the quenching temperature and time or the volume ratio between sample and quenching solution, the time between quenching and separation by centrifugation and the centrifugation time can influence metabolic activity and metabolite leakage significantly. Harvesting by rapid sampling is often coupled to quenching. Rapid sampling is especially important in continuous steady-state cultivations and for pulse-experiments in which changes of intracellular metabolite pools induced by a certain environmental impulse are analyzed at the sub-second time scale. Ingenious devices have been developed in the last years that enable rapid sampling and quenching simultaneously at the millisecond scale. The various manual, semiautomatic and fully automatic rapid sampling techniques and their pros and cons have been comprehensively discussed and the reader is referred to (Reuss et al. 2007; van Gulik 2010; Villas-Bôas 2007a). A disadvantage in this context is that most of these devices are not available on the market and therefore not accessible to the scientific community. Commercial accessibility however would be of great importance in the context of comparability, reproducibility and standardization in quantitative metabolomics studies. In batch cultivations manual transfer of the cell suspension to the quenching solution by using a pipette or a syringe is widely accepted as environmental conditions might not change significantly during the sample transfer (3 – 6 s) (Villas-Bôas 2007b). These assumptions might hold for anaerobic or microaerobic conditions but should be reconsidered in case of aerobic cultivations for which for example the O_2/CO_2 ratio may vary and induces changes (oxygen limitation) in cell metabolism during sampling.

2.2 Quantitative metabolite extraction

The next challenge represents the quantitative extraction of all metabolites or at least of those to be of interest without reactivating any metabolic activity, enabling chemical reactions and minimizing metabolite disruption. Metabolic activity and chemical reactions can be controlled by the temperature. The addition of a denaturing (inactivating) agent bears some risks as it might be also reactive with metabolites or provides the environment (pH) for chemical reactions (Villas-Bôas 2007b). Control of metabolite degeneration or reactivity during extraction and in the following process steps represents a complex and difficult task. Furthermore metabolite degeneration is a highly specific process and strongly depends on the metabolite species, extraction solution and extraction parameters (pH, T). For example the redox cofactors NAD^+ and $NADP^+$ are stable at acid to neutral pH even at high temperatures (80-90°C) for 3 minutes (Klimacek et al. 2010). On the contrary their reduced counterparts are highly unstable under acidic conditions with NADPH generally less stable than NADH (Chaykin 1967; Lowry et al. 1961). At high pH where NAD(P)H are stable NAD(P) in turn destruct rapidly (Lowry et al. 1961). Decomposition can also be catalyzed by phosphate a prominent compound in metabolite extracts (van Eunen et al. 2010) by forming an adduct with NADH across the pyridine group (Alivisatos et al. 1964; Chaykin 1967).
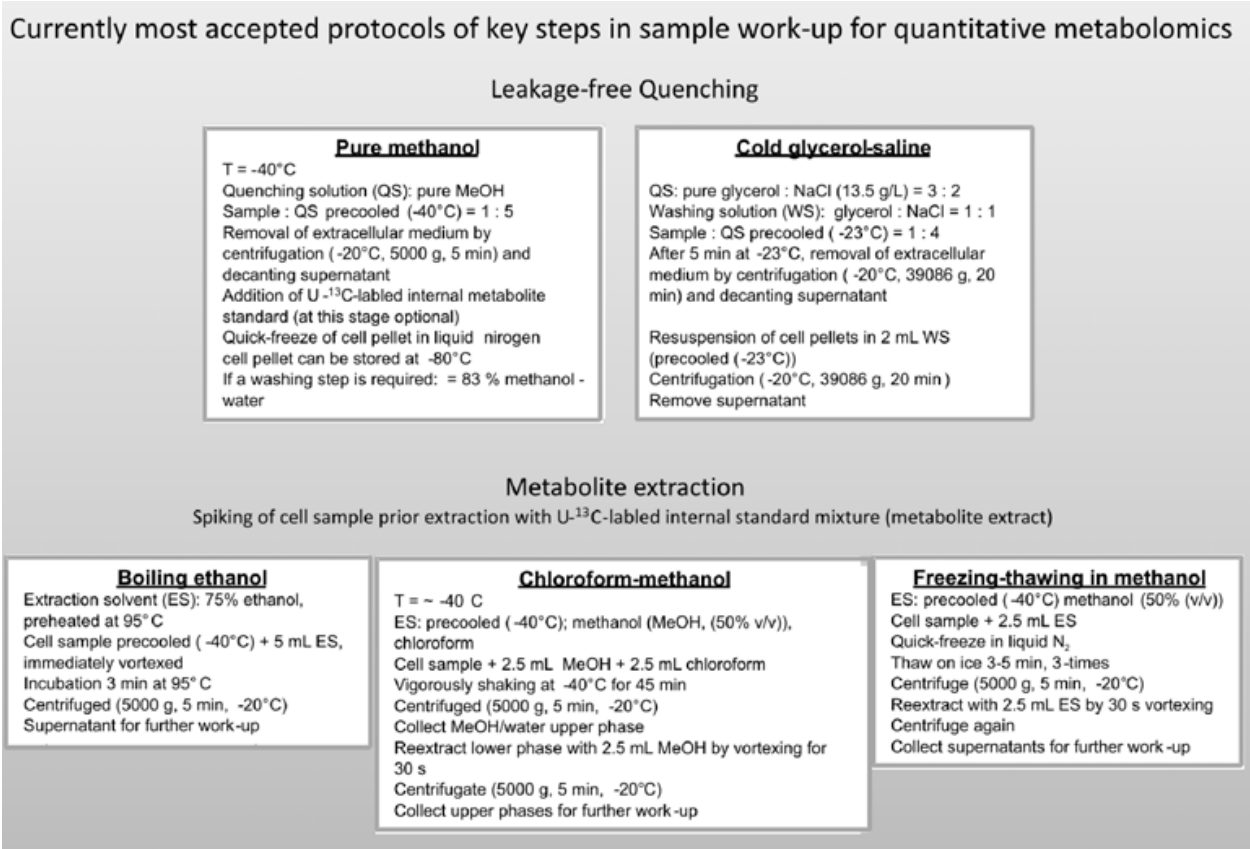
Currently most accepted protocols of key steps in sample work-up for quantitative metabolomics

Leakage-free Quenching

Pure methanol

T = -40°C
Quenching solution (QS): pure MeOH
Sample : QS precooled (-40°C) = 1 : 5
Removal of extracellular medium by centrifugation (-20°C, 5000 g, 5 min) and decanting supernatant
Addition of U-^{13}C-labled internal metabolite standard (at this stage optional)
Quick-freeze of cell pellet in liquid nirogen cell pellet can be stored at -80°C
If a washing step is required: = 83 % methanol - water

Cold glycerol-saline

QS: pure glycerol : NaCl (13.5 g/L) = 3 : 2
Washing solution (WS): glycerol : NaCl = 1 : 1
Sample : QS precooled (-23°C) = 1 : 4
After 5 min at -23°C, removal of extracellular medium by centrifugation (-20°C, 39086 g, 20 min) and decanting supernatant

Resuspension of cell pellets in 2 mL WS (precooled (-23°C))
Centrifugation (-20°C, 39086 g, 20 min)
Remove supernatant

Metabolite extraction
Spiking of cell sample prior extraction with U-^{13}C-labled internal standard mixture (metabolite extract)

Boiling ethanol

Extraction solvent (ES): 75% ethanol, preheated at 95°C
Cell sample precooled (-40°C) + 5 mL ES, immediately vortexed
Incubation 3 min at 95° C
Centrifuged (5000 g, 5 min, -20°C)
Supernatant for further work-up

Chloroform-methanol

T = ~ -40 C
ES: precooled (-40°C); methanol (MeOH, (50% v/v)), chloroform
Cell sample + 2.5 mL MeOH + 2.5 mL chloroform
Vigorously shaking at -40°C for 45 min
Centrifuged (5000 g, 5 min, -20°C)
Collect MeOH/water upper phase
Reextract lower phase with 2.5 mL MeOH by vortexing for 30 s
Centrifugate (5000 g, 5 min, -20°C)
Collect upper phases for further work-up

Freezing-thawing in methanol

ES: precooled (-40°C) methanol (50% (v/v))
Cell sample + 2.5 mL ES
Quick-freeze in liquid N$_2$
Thaw on ice 3-5 min, 3-times
Centrifuge (5000 g, 5 min, -20°C)
Reextract with 2.5 mL ES by 30 s vortexing
Centrifuge again
Collect supernatants for further work-up

Fig. 2. Collection of state-of-the-art protocols for leakage-free quenching and quantitative extraction. Relevant literature and details can be found in the text.

It is obvious from this example considering just four metabolites that the ideal extraction procedure with which the complete metabolite consortium is extractable without any losses may not exist and calls for a compromise in the selection of conditions used for metabolite extraction. This "inconvenience" however can be circumvented, provided that subsequent metabolite detection is based on mass spectroscopy, by the addition of an aliquot of U-^{13}C-labled internal standard (IS) compounds to the biomass subsequent to quenching or prior to metabolite extraction (Büscher et al. 2009; Canelas et al. 2009; Klimacek et al. 2010; Mashego et al. 2004; Wu et al. 2005). Metabolite losses due to incomplete quantitative work-up of samples can be addressed by application of one selected IS compound. However metabolite specific instabilities, matrix effects, ion suppression, non-linear responses and day-to-day variations can be only identfed and appropriately corrected for by the addition of U-^{13}C-labled IS. A representative mixture of labeled metabolites can be easily prepared from *S. cerevisiae* wild-type and/or mutant cells cultivated in standard mineral medium supplemented with U-^{13}C-labled substrate (glucose, fructose, galactose,…) under the cultivation condition selected and by respective appropriate quenching and extraction procedures. Internal referencing by using an IS displaying a metabolite composition that is representative for the cellular state to be studied should be always taken into account because intracellular metabolite levels can vary considerably in dependence on the cultivation conditions used or on the cellular alterations introduced by pathway engineering (Klimacek et al. 2010).

Various extraction protocols with respect to extraction solvent (acids, bases, ethanol or methanol, organic solvents), buffered or non-buffered solutions, pH, temperature, etc have been tested (Villas-Bôas 2007b), evaluated and verified for *S. cerevisiae* cells in terms of metabolite coverage, efficacy and recovery (Klimacek et al. 2010) in the last years (Canelas et al. 2009; Villas-Bôas et al. 2005b). Today three extraction procedures have achieved some acceptance and are likewise used within the yeast research community (see Fig. 2 for details). That is boiling ethanol (BE; pioneered for *S. cerevisiae* cells by (Gonzalez et al. 1997)), chloroform-methanol (CM; pioneered for *S. cerevisiae* cells by (de Koning and van Dam 1992)) and to some extent freeze-thawing in methanol (FTM; pioneered for *S. cerevisiae* cells by (Villas-Bôas et al. 2005b) for which however controversial results with respect to its applicability are present in the literature. While (Villas-Bôas et al. 2005b) found extraction performance of FTM sufficient, others (Canelas et al. 2009) concluded that FTM cannot effectively prevent metabolite conversion throughout the extraction process and considered FTM therefore as not appropriate for metabolite extraction. Differences in evaluation criteria and growth conditions were used as a basis to explain the different outcomes. It should be however noted that (Canelas et al. 2009) investigated metabolite extraction performances from *S. cerevisiae* cells grown under two different physiological conditions (glucose limitation and glucose saturation; a bioreactor coupled to a rapid sampling device was used), used identical U-^{13}C-labled compounds as IS and three different analytical methods for quantification of a broad range of different compounds. Quality and metabolite recovery of FTM instead was judged by (Villas-Bôas et al. 2005b) by the application of an IS mixture composed of compounds each a representative for a substance class analyzed. The mixture was added to the quenched cells prior to extraction. Cells were cultivated under aerobic conditions in shake flasks and metabolites were quantified by established GC-MS after metabolite derivatization with methyl chloroformate. In Fig. 2 brief descriptions of respective protocols are compiled. A broad spectrum of compounds covering a wide range of different chemical properties such as acidity, polarity, size and responsiveness can be addressed with either of these extraction protocols. Details with respect to extraction method specific component coverage can be extracted from (Buescher et al. 2010; Büscher et al. 2009; Canelas et al. 2008a; Canelas et al. 2009; Klimacek et al. 2010; Villas-Bôas and Bruheim 2007; Villas-Bôas et al. 2005b).

2.3 Quantification of intracellular metabolites

Approximately 600 metabolites are present in *S. cerevisiae* cells and their concentrations range from sub-μM to mM (Nielsen 2007). Their levels vary considerably in dependence on the environmental conditions applied or mutation introduced. Hence analysis tools suited for the determination of intracellular metabolites should be able to cover quantitatively as many metabolites as possible in wide concentration ranges.

Early studies that have focused on analyzing intracellular metabolites used enzymatic assays (Ciriacy and Breitenbach 1979; de Koning and van Dam 1992; Gonzalez et al. 1997; Grosz and Stephanopoulos 1990; Kötter and Ciriacy 1993; Theobald et al. 1997) or NMR analysis (den Hollander et al. 1981; Navon et al. 1979; Shanks and Bailey 1988) for quantification of a few compounds present in the cell. To increase the coverage towards a holistic quantitative record of the endometabolome enormous efforts were put on the development of new methods and techniques in the last years. In particular mass

spectrometry revealed to be excellently suited in this respect and analytics of metabolite targeting switched therefore from an enzyme assay- or NMR-based to a mass spectrometry-based technique. Today three platforms dominate the analytical part of metabolomics. That is mass spectrometric detection in the form of a single, tandem or triple quadrupole or orbitrap mass spectrometer (MS) coupled via an electrospray ionization source (ESI) to a component separation device such as gas chromatography (GC), reverse-phased ion-pairing or anion exchange liquid chromatography (LC) or capillary electrophoresis (CE). Cross-platform comparison with respect to quantitative metabolomics revealed LC as the best suited separation technique for analysis on a single platform in terms of versatility and robustness. It was suggested by the authors that it is best complemented by the use of the GC platform (Büscher et al. 2009). More than 100 metabolites could be successfully quantified by GC-MS (~100 metabolites, (Villas-Bôas and Bruheim 2007; Villas-Bôas et al. 2003; Villas-Bôas et al. 2005b; Villas-Bôas et al. 2005c)), reverse phase ion pairing LC coupled to a triple quadrupole (138 metabolites, (Buescher et al. 2010)) or an orbitrab mass spec (137 metabolites, (Lu et al. 2010)). Especially the LC-MS platform has been investigated intensively with respect to quantitative metabolite coverage, sensitivity and robustness and revealed to be very suited for the comprehensive analysis of the central carbon metabolism (Buescher et al. 2010; Büscher et al. 2009; Lu et al. 2010). Almost all metabolites involved in glycolysis, pentose phosphate pathway and TCA cycle could be addressed. In addition amino acids and their precursors, redox cofactors, nucleotides, coenzyme A esters and many more can be analyzed in one sample run in approximately half an hour (Buescher et al. 2010; Lu et al. 2010). Current LC-techniques used in metabolomics are however limited to water soluble analytes (Buescher et al. 2010).

As mentioned above absolute quantification of intracellular metabolites is indispensably linked to the use of U-^{13}C-labled internal metabolite standards. Consequently the number of compounds to be analyzed doubles which makes data analysis more demanding. Residual amounts of substrates, products and ionic components in the labeled and unlabeled metabolite extracts can significantly alter the elution profile and ionization characteristics of compounds analyzed (Buescher et al. 2010). These so called matrix effects are hardly to predict and are typically experienced as an increase of the base line signal associated with a high signal-to-noise ratio and as shifts in metabolite-specific retention times. Consequently peak-to-peak resolution and base line separation of peaks can become badly affected. Exacerbate and tedious manual peak integration is in these cases required. Alternatively complex often erroneous peak deconvolution algorithms are applied. To reduce matrix effects to a minimum demands preparation of "clean" - meaning free of disturbing media compounds - metabolite extracts for both biological samples and IS.

The recommended procedure for internal referencing by identical U-^{13}C-labled compounds involves addition of a defined volume of IS to the biological sample (prior to extraction) and to all analytical standard dilutions (minimum 6 dilutions). A standard mixture containing all the compounds at known concentrations to be analyzed is commonly used. As some metabolites are not very stable it is recommended to store a master mixture containing just the stable compounds and add the sensitive components prior to analysis. Metabolites are quantified by comparing the ratio of [12]C- to [13]C- signals with the ^{12}C/^{13}C signals of the representative standard compounds.

2.4 Calculation of molar intracellular metabolite concentrations

The ultimate goal of quantitative metabolomics is the presentation of intracellular metabolite pools in the form of absolute molar concentrations. Only the knowledge of molar concentrations enables reliable integration of metabolome data in thermodynamic analysis, application in MCA based on fundamental enzyme kinetics or kinetic modeling. In the last step of absolute quantification of intracellular metabolite pools, the unbiased molar concentrations of metabolites obtained by the methods described above have to be therefore somehow related to the cell volume. For this reason metabolite concentrations and cell volume are based on the cell dry weight (CDW) producing specific parameters for metabolite concentrations in μmol/gCDW (= [metabolite]*volume of metabolite extract / ([dry cells]*volume of biological sample)) and for the cell volume in mL/gCDW. By dividing these parameters the intracellular metabolite concentration in mM is eventually obtained. Hence accurate determination of the CDW and cell volume is mandatory for calculation of reliable intracellular metabolite data and for molecular mechanistic interpretation relying on this data. Different methods can be found in the literature. Briefly, first the physiological state has to be specified at which the CDW should be analyzed. Second an aliquot of cell suspension is separated from the medium by vacuum filtration or centrifugation. The cell pellet is washed with ice-cold water or physiological NaCl solution to remove residual medium components and subsequently dried at 100-105°C until constancy of mass is verified. One should however keep in mind that application of NaCl can compromise resulting CDW values significantly when low amounts of biomass are addressed. The volume of *S. cerevisiae* cells at a particular physiological state can be determined by applying a Coulter counter analyzer or by microscopic techniques (Lord and Wheals 1981; Tamaki et al. 2005).

The cell volume of the baker's yeast was found to depend on the growth conditions and environmental parameter settings and can vary considerably. For example, the cell volume varies by a factor of 2-3 (16 – 42 μm³) with doubling time (Lorincz and Carter 1979; Tyson et al. 1979) and the type of substrate metabolized (Johnston 1977; Tamaki et al. 2005). The lower the doubling time the larger the cells (Tyson et al. 1979). Cells grown on glucose, the most favored carbon source, are larger than those grown on a nonfermentable carbon source (Johnston et al. 1979; Lorincz and Carter 1979; Porro et al. 2003; Tyson et al. 1979). Cells cultured in the presence of ethanol showed an enlarged size (Kubota et al. 2004). In contrast nitrogen starved cells are abnormally small (Johnston 1977). The level of repression/derepression also contribute significantly to the cell size (Mountain and Sudbery 1990). Interestingly therefore that on the basis of the more relevant parameter for quantification the specific cell volume, *S. cerevisiae* cells do not show significant variation with the growth rate (Brauer et al. 2008) and values in the range of 1.5 – 1.9 mL/gCDW can be found in the literature for the strain CEN.PK 113-7D (Canelas et al. 2011; Cipollina et al. 2008; van Eunen et al. 2009). A slightly higher value of 2.38 mL/gCDW has been reported for another strain CBS 7336 (ATCC 32167) (Ditzelmüller et al. 1983). We can conclude that although the cell volume is highly sensitive to conditions applied the specific cell volume is rather constant. Nevertheless a 1.6-fold higher specific cell volume results in likewise lower metabolite concentrations which in some cases (e.g. [substrate] < K_m) may have an influence on the data interpretation. In the light of metabolic engineering it is hard to predict whether this "constant" likewise translates to recombinant cells. For example *S. cerevisiae* cells

adapted to high ethanol concentrations displayed an altered cell size (Dinh et al. 2008). Or overexpressing mannitol-1-phosphate dehydrogenase (M1PDH) in *S. cerevisiae* to produce mannitol from glucose caused a substantial increasing of the size of cells (Costenoble et al. 2003).

As for all eukaryotic organisms *S. cerevisiae* metabolism is compartmented (cytosol, mitochondrion, vacuoles) which poses a problem for the accurate determination of concentrations of relevant intracellular metabolites. Current techniques for extracting metabolites and isolating organelles do not allow for absolute separation from the cytosol without altering the respective metabolite composition and pattern. Indirect strategies based on metabolic engineering or on fundamental thermodynamic principles have been developed to address this obstacle and gave first preliminary and semi-quantitative insights into the distribution of metabolites between cytosol and mitochondrion.

Functional expression of M1PDH from *E. coli* in *S. cerevisiae* was used as indicator reaction to determine the cytosolic free NAD to NADH ratio (Canelas et al. 2008b). M1PDH catalyzes the reversible NAD(H)-dependent interconversion of fructose 6-P (F6P) and mannitol 1-P (M1P). This reaction is directly connected to the central carbon metabolism and represents a dead-end reaction in the metabolism of yeast under the conditions applied in this study. Based on the assumption that the M1PDH reaction is at equilibrium the authors were capable of calculating the NAD/NADH ratio from the equilibrium constant and the intracellular concentrations of F6P and M1P. Data were verified by thermodynamic analysis. The cytosolic ratio of NAD/NADH was found to be ~10-fold higher as compared to the same ratio when based on the whole cell. Under anaerobic conditions however mannitol is formed from M1P implying that this approach is not yet universally applicable (Costenoble et al. 2003).

A different approach based on a network-embedded thermodynamic analysis later termed anNET (Zamboni et al. 2008) was used by (Kümmel et al. 2006) to resolve intracompartmental feasible concentration ranges from cell-averaged metabolome data.

Although these first results are promising there is large open space for the development of novel strategies combined with appropriate experimental techniques that enable precise compartment-specific quantification.

3. How can metabolic engineering of *S. cerevisiae* benefit from quantitative metabolomics?

In the typical metabolic engineering approach a bunch of new recombinant strains are designed and created with respect to a particular objective (see Fig. 1) or obtained from evolutionary adaption. Their new phenotypes are tested by fermentation or conversion experiments from which the substrate uptake rate and the product pattern in the form of specific product yields are determined. Results are often applied to FBA for verification. Intracellular enzyme activities of the introduced reactions as well as of those catalyzing reactions relevant for the new phenotype are measured from cell-free extracts. This data set usually provides many valuable details about the production efficiency in terms of conversion rate (how do intracellular activities of target enzymes compare to the conversion rate measured) and product selectivity (identification of side-products and oftentimes the reactions or pathways involved).

So how do metabolomics and more specifically quantitative metabolomics come into play? As described above the composition of intracellular metabolites together with their levels represent a direct signature of the physiological state of the cells investigated. Comparing metabolite profiles of wild-type and mutant strain(s) was often used to identify target reactions limiting the conversion rate (Hasunuma et al. 2011; Kahar et al. 2011; Klimacek et al. 2010; Kötter and Ciriacy 1993; Wisselink et al. 2010; Zaldivar et al. 2002) or extract the metabolite pattern representative for the new phenotype (Canelas et al. 2008b; Devantier et al. 2005; Ding et al. 2010; Hou et al. 2009; Kamei et al. 2011; MacKenzie et al. 2008; Pereira et al. 2011; Raamsdonk et al. 2001; Ralser et al. 2007; Thorsen et al. 2007; Usaite et al. 2009; Villas-Bôas et al. 2005a; Villas-Bôas et al. 2005c; Yoshida et al. 2008). Even apparently silent phenotypes of S. *cerevisiae* single deletion mutants can be uncovered with respect to the underlying mutation based on the developed metabolome (Raamsdonk et al. 2001). The rate however at which a compound's carbon skeleton is channeled through a certain pathway is directly linked to the level of active enzymes present and their affinity to the participating reactants as well as to fundamental thermodynamic laws of the reactions involved. Consequently knowledge about intracellular concentration of metabolites and enzyme activities combined with thermodynamic and enzyme kinetic analysis can provide novel and valuable insights into the kinetic organization of the engineered pathway or even the associated metabolic network which eventually exposes key regulatory or flux limiting sites. Differently to the general holistic approach usually found in systems biology pathway analysis in metabolically engineered cells can be reduced in most cases to the components involved in the new pathway and those connecting this pathway to the central carbon metabolism (Parachin et al. 2011).

3.1 Thermodynamic pathway analysis

If we are interested in analyzing a pathway or network of pathways on the basis of thermodynamic rules with the aim to extract pathway or network relevant mechanistic relationships, knowledge of exact quantitative metabolite data of all reactants involved is mandatory. On the contrary we can also check quantitative metabolome data with respect to its thermodynamic consistency (Kümmel et al. 2006) but most importantly we can get first hits of potential candidate reactions for metabolic engineering within a metabolic network without any knowledge about enzyme activity and kinetic parameters. Consider the following reaction

$$A + B \longleftrightarrow C + D \tag{1}$$

The chemical equilibrium constant (K_{eq}) associated with this reaction can be defined according to the law of mass action as

$$K_{eq} = c_C{}^{eq} c_D{}^{eq} / (c_A{}^{eq}\ c_B{}^{eq}) \tag{2}$$

The superscript ^{eq} relates to the concentrations c of reactants at the chemical equilibrium. The standard Gibbs energy of a chemical reaction ($\Delta_r G^0$, usually given in J/mol) is related to K_{eq} by the fundamental relationship

$$\Delta_r G^0 = \sum_{i=1}^{N_s} v_i \Delta_f G_i^0 = -RT \ln K_{eq} \tag{3}$$

in which v_i and $\Delta_f G_i^0$ correspond to the stoichiometric coefficient of reactant i and to the standard reaction Gibbs energy of formation of species i at a specified T, P and ionic strength, respectively. R and T denote the general gas constant (8.314 J/mol/K) and absolute temperature in Kelvin (K), respectively. The Gibbs energy of formation of a reactant i ($\Delta_f G_i$) is further defined by

$$\Delta_f G_i = \Delta_f G_i^0 + RT \ln(c_i) \tag{4}$$

c_i in Equation (4) refers to the concentration of reactants involved. The Gibbs energy of a reaction eventually is described by

$$\Delta_r G = \sum_{i=1}^{N_s} v_i \Delta_f G_i^0 + RT \sum_{i=1}^{N_s} v_i \ln c_i = \Delta_r G^0 + RT \ln Q \tag{5}$$

Q in Equation (5) indicates the reaction quotient $c_C\, c_D\, / \,(c_A\, c_B)$, which is also known under the term "mass action ratio" that is frequently abbreviated by Γ. Rearranging Equation (4) yields

$$\Delta_r G = RT\, \{\ln(Q/K_{eq})\} \tag{6}$$

Now the reaction in Equation (2), reading from the left to the right, takes place freely in the forward direction A,B → C,D at $\Delta_r G < 0$ ($K_{eq} > Q$), is at equilibrium and displays no net flux when $\Delta_r G = 0$ ($K_{eq} = Q$) and needs support by an external driving force when $\Delta_r G > 0$ ($K_{eq} < Q$). In other words the reverse reaction is favored under these Q-conditions implying a net flux in the back direction, C+D → A+B. Equation (6) therefore provides a very convenient and important expression that permits immediate assignment of the net flux state of a reaction within a pathway or a large metabolic network provided that K_{eq} and all reactants participating in the particular reaction are known. The value of K_{eq} is dependent on the temperature, the ionic strength and the pressure (Alberty 2003). As a consequence only those K_{eq}s should be applied that were determined under conditions representative for the cell's physiological state investigated. In particular temperature, ionic strength and pH are of considerable interest as pressure can be in most cases assumed as a constant. Thereof only the temperature during the cultivation experiment, that is usually 30°C for *S. cerevisiae*, is known. Unfortunately most K_{eq}s tabulated have been determined at 25°C. For *S. cerevisiae* cellular ionic strength and pH are often assumed to be 0.1 M and 7.0, respectively. This may be sufficient for many thermodynamic network analyses. The validity of these assumptions however should be tested in any event prior pathway or network analysis. Algorithms are available that allow to some extent compensation for ionic strength and temperature.

Equilibrium constants can be determined by three different methods based on either in vitro assay with isolated enzymes, compound-specific standard Gibbs energy of formation (see Equation (3)) or in vivo experiments that combine FBA with quantitative metabolomics.

In case of using an isolated enzyme a reaction assay is designed such that known concentrations of substrate(s) and product(s) are solved in a buffer with defined ionic strength and pH. The reaction is started by the addition of the enzyme and processed at a certain temperature as long as the equilibrium state is reached. Reactant concentrations are then analyzed by appropriate techniques and the apparent K_{eq} (K'_{eq}) at a specified pH, ionic

strength and temperature is calculated with respect to the underlying stoichiometry of the reaction. Values for K'_{eq} are usually applied in the context of network analysis. Importantly all reactants and the enzyme must be of highest purity and must be stable along the time of incubation. The composition of the reaction mixture at the equilibrium must not change in the subsequent component analysis.

When the $\Delta_f G_i^0$ for all reactants participating in the investigated reaction system are known, the respective values for K_{eq} can be calculated (Alberty 1991). Values for standard Gibbs energies of formation $\Delta_f G_i^0$ have been tabulated for a number of compounds (Alberty 2003). Computer programs are available with which one can calculate transformed standard Gibbs energies of formation ($\Delta_f G_i^{'0}$) from $\Delta_f G_i^0$ for a specified pH and ionic strength (Alberty 2003; Zamboni et al. 2008). In addition all possible dissociation forms of a compound are also lumped into a single reactant in $\Delta_f G_i^{'0}$. The respective transformed Gibbs energy of formation for a reactant at a certain concentration ($\Delta_f G_i'$) is described by

$$\Delta_f G_i' = \Delta_f G_i^{'0} + RT \ln(c_i) \tag{7}$$

Applying $\Delta_f G_i'$ and $\Delta_f G_i^{'0}$ instead of $\Delta_f G_i$ and $\Delta_f G_i^0$ in Equations (4) and (5) permit calculation of K'_{eq} of a particular reaction. Large-scale thermodynamic studies for which availability of proper K'_{eq}s are crucial often bemoan large uncertainties with which current thermodynamic data obtained from enzyme assays or based on Gibbs energies are afflicted and therefore not sufficient for data analysis.

A way to circumvent these obstacles was introduced by (Canelas et al. 2011) who developed a new method to derive apparent equilibrium constants under real in vivo conditions. The basic idea of this work was based upon that the rate v of a certain reaction is directly dependent on the maximal turnover number V_{max}, the kinetic properties (β) of the enzyme catalyzing this reaction and on the net flux state of the reaction in relation to the respective equilibrium ($1-Q/K'_{eq}$). That is $v = V_{max} \beta(1-Q/K'_{eq})$. *S. cerevisiae* was cultivated at 30°C in chemostats under aerobic conditions and glucose (7.5 g/L) as the sole carbon source in standard mineral medium at 32 different dilution rates spanning a wide range of specific growth rates (0.03 to 0.29 h^{-1}) and as a consequence substrate conversion rates. Extracellular and intracellular metabolites were quantified at each dilution rate. Specific substrate and product fluxes given in mmol/gCDW/h were applied to a FBA based on a stoichiometric model and stoichiometric fluxes (v) for each reaction of the model network were calculated. Mass ratio coefficients Q of 27 network reactions were calculated from quantitative metabolite data at each v. From plots of Q vs. v the authors were able to extract values for K'_{eq}s. It could be shown that Q of reactions operating close to the equilibrium state display a negative linear dependency of v. Values of K'_{eq}s representing the thermodynamic equilibrium in the cell were determined by linear extrapolation to the y-axis ($v = 0$ and $K'_{eq} = Q$) under the assumption that V_{max} and kinetic properties of enzymes involved are not dependent on v. Intracellular K'_{eq}s obtained differed in part significantly (up to a factor of 9) from their in vitro determined counterparts implying that reaction conditions in vivo (pH and ionic strength) deviate from that specified in in vitro assays or by the in silico approach. The big advantage of this approach is that in vivo K'_{eq}s can be accurately determined without knowing anything about the intracellular pH and ionic strength. However only reactions located at or close to the equilibrium state are addressable.

Combining quantitative metabolomics data with thermodynamic rules on reactions constituting an operating metabolic network enables one to map reactions according to their location with respect to K'_{eq} (Crabtree et al. 1997; Klimacek et al. 2010; Kümmel et al. 2006; Wang et al. 2004). That is close to or far away from equilibrium. A reaction reaches K'_{eq} when the activity of an enzyme catalyzing a reaction downstream is low. On the other hand reactions located far away from K'_{eq} are often catalyzed by enzymes that have regulatory functions in the cell or by flux limiting enzymes representing potential targets for strain improvement (Klimacek et al. 2010). Differently to the relative location of a particular reaction to its equilibrium, the sign (plus or minus) of $\Delta_r G'$ or whether $Q >$ or $< K'_{eq}$ gives important information about the direction of the reaction or of an entire pathway. Information about flux directions can be readily implemented as further restrictions into a stoichiometric network to increase reliability of flux distributions (Hoppe et al. 2007; Kümmel et al. 2006).

3.2 Metabolic control analysis based on quantitative metabolomics in metabolic engineering of *S. cerevisiae*

Identification of those reactions exerting significant control of flux through a particular pathway is crucial to develop strategies for flux improvement. As mentioned above reactions suspected in flux limitation can be elucidated elegantly by thermodynamic analysis. Once identified reactions can be further analyzed with respect to principles of MCA. The concept of MCA was developed and introduced by (Heinrich and Rapoport 1974; Kacser and Burns 1973) and is comprehensively described in (Stephanopoulos et al. 1998). MCA strictly applies only to steady-state conditions. To unravel whether the amount of enzyme or of a reactant participating in the reaction catalyzed by this enzyme contribute to flux limitation through this particular reaction two terms have been defined, namely the flux control coefficient (FCC) and the elasticity coefficient (ε). The FCC defines the relative change in the steady-state flux resulting from an infinitesimal change in the activity of an enzyme of the pathway divided by the relative change of the enzyme activity, represented by the relationship FCC = $E\ dJ$ / ($J\ dE$), in which E and J stand for the concentration or activity of the enzyme and the steady-state flux, respectively. The ε is intrinsically linked to the inherent enzyme kinetic properties and is defined by the ratio of the relative change in the reaction rate resulted by an infinitesimal change in the metabolite concentration and can be described by $\varepsilon = c_j\ \delta v_i$ / ($v_i\ \delta c_j$), where c_j and v_i represent the concentration of reactant j and the reaction rate of enzyme i. FCC and ε are related by the flux-control connectivity theorem (Kacser and Burns 1973).

$$\sum_{i=1}^{L} FCC_i^J \varepsilon_{c_j}^i = 0 \tag{8}$$

Equation (8) implies that large elasticities – concentration of reactant exerts large control on the enzyme reaction rate – are associated with small FCCs – the overall flux through the pathway is not very dependent on the enzyme activity – and vice versa. Consequently knowledge of either of these coefficient is only required. FCC can be determined by increasing the amount of active enzyme for example by expressing this particular enzyme at different expression levels and measuring the change in flux through the pathway. The level of enzyme present can be judged by determining its specific activity in cell-free extracts. If

the specific activity (μmol/mg$_{protein}$/min) for the isolated enzyme, the cellular protein content (mg$_{protein}$/gCDW) and the specific cell volume are known then the molar concentration of this enzyme can be calculated. Enzyme levels can be also determined by proteomics techniques and to some extent extrapolated from transcriptome data. Alternatively suitable antibodies that selectively bind to the target enzymes or fusion of an indicator peptide or protein tag to the enzyme to be analyzed permitting quantification either directly in vivo (GFP) or in vitro after separation of the target enzyme by affinity chromatography (e.g. His-tag, strep-tag) can be used for determining intracellular enzyme concentrations. If rate equations and associated kinetic parameters of all enzymes involved and at least their relative activity levels are known mathematical models can be applied to estimate FCCs and ε's. Rate equations based on the steady-state or rapid equilibrium assumption for enzyme catalyzed reactions are comprehensively summarized in (Segel 1993). However kinetic parameters typically used in this approach are determined by in vitro assays and it has been shown that in vitro generated data can significantly differ from those observed in vivo (Aragón and Sánchez 1985; Mauch et al. 2000; Reuss et al. 2007). This discrepancy is most likely due to the incomplete knowledge of the cellular composition and associated enzyme metabolite interactions as well as the difficulty to analyze enzymes under in vivo-like conditions at the lab bench. A promising step forward towards generation of more reliable in vivo-like in vitro enzyme kinetic data was reported recently (van Eunen et al. 2010). The authors suggested to measure enzyme activities in a buffered reaction mixture that simulates the intracellular cellular medium composition of *S. cerevisiae*.

Using the non-linear lin-log formulation developed for metabolic network analysis by (Visser and Heijnen 2003) FCC and ε can be estimated without any prior knowledge of enzyme-specific kinetic parameters (Visser and Heijnen 2002). In this approach the reaction rate is a nonlinear function of metabolite concentrations and is proportional to enzyme levels. lin-log kinetics is suited when large perturbations on the systems are analyzed. Furthermore statistical evaluation of parameter estimates is simplified (Wu et al. 2004). Nevertheless it is an approximation that fits to fundamental enzyme-reactant properties only in a certain range of perturbation (Wu et al. 2004).

3.3 Substrate promiscuous enzymes

Enzymes that have evolved relaxed or broad substrate specificity are substrate promiscuous (Hult and Berglund 2007). The presence of substrate promiscuous enzymes in a metabolic network considerably aggravates accurate network formulation and analysis if the accurate flux partition between the individual substrates is not known. This problem is further enhanced when the utilized substrates are highly connected within the network by other (substrate promiscuous) reactions. This is possibly best exemplified by the coenzyme promiscuous enzyme xylose reductase (XR) from *Candida tenuis* (Petschacher et al. 2005). To enable metabolic integration of xylose by *S. cerevisiae* XR and xylitol dehydrogenase XDH have to be integrated (Petschacher and Nidetzky 2008). Different to XDH which is strictly dependent on NAD(H) (Nidetzky et al. 2003), XR can oxidize both NADH and NADPH. These coenzymes in turn form the redox state of a cell and are important currency metabolites in all catabolic and anabolic pathways of the cell. Hence, keeping the redox state with respect to anabolism and catabolism balanced is crucial for the cell to stay alive. It was found based on stoichiometric considerations that the NADPH usage of XR is in strong

correlation with by-product formation in the form of xylitol, the reaction product of XR and the substrate of XDH in the subsequent reaction (van Maris et al. 2007). Usage of up to (0-52)% NADPH by XR was compatible with a genome-scale metabolic network (Krahulec et al. 2010). Detailed kinetic analysis from in vitro studies showed that the XR almost exclusively utilizes NADPH in terms of catalytic efficiency ($k_{cat}/K_{m,coenzyme}$) and the selectivity parameter R_{sel} ($k_{cat}/(K_{i,coenzyme}K_{m,xylose})$)) which are widely used as marker parameter for coenzyme discrimination. Based on this data even a rough estimation of the coenzyme usage of XR in the cell is not possible. To solve the coenzyme usage riddle of XR we determined intracellular concentrations of NADH and NADPH (Klimacek et al. 2010) and integrated this information together with the relevant kinetic parameters (Petschacher et al. 2005) into the mechanistically appropriate enzyme kinetic rate expression (Banta et al. 2002; Petschacher and Nidetzky 2005). A balanced coenzyme usage perfectly in line with physiology observed was obtained for the XR (Klimacek et al. 2010). Information about the correct flux partition of a particular substrate promiscuous enzyme can be implemented as further restrictions into a stoichiometric network to increase reliability of flux distributions. Furthermore this approach could be successfully applied on a series of wild-type and mutant forms of XR to predict reliably formation of xylitol (Krahulec et al. 2011).

4. Conclusions

Quantitative metabolomics is especially suited to help identifying key sites limiting an engineered metabolic route either within the created pathway but also apart from it. State-of-the-art protocols for sample work-up and LC-MS and GC-MS analysis permit absolute quantification of metabolites from *S. cerevisiae* cells provided that a U-^{13}C-labled IS is applied. Quantitative data in turn are indispensible for reliable pathway and network analysis in the form of a thermodynamic analysis, MCA or kinetic modelling. In combination with other omics techniques it represents a powerful tool to create designer microbial cell factories exposing improved or novel phenotypes.

5. Acknowledgement

6. References

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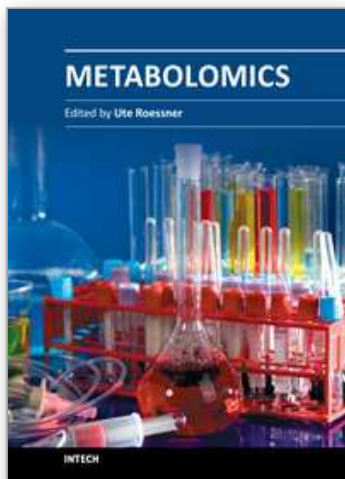
Metabolomics

Edited by Dr Ute Roessner

Metabolomics is a rapidly emerging field in life sciences, which aims to identify and quantify metabolites in a biological system. Analytical chemistry is combined with sophisticated informatics and statistics tools to determine and understand metabolic changes upon genetic or environmental perturbations. Together with other 'omics analyses, such as genomics and proteomics, metabolomics plays an important role in functional genomics and systems biology studies in any biological science. This book will provide the reader with summaries of the state-of-the-art of technologies and methodologies, especially in the data analysis and interpretation approaches, as well as give insights into exciting applications of metabolomics in human health studies, safety assessments, and plant and microbial research.

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