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Current Opinion in
Biotechnology

Metabolomics-assisted synthetic biology

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As the world progresses from a fossil-fuel based economy to a more sustainable one, synthetic biology will become increasingly important for the production of high-value fine chemicals as well as low-value commodities in bulk. The integration of metabolomics and fluxomics within synthetic biology projects will be vital at all levels, including the initial design of the pathways to be generated, through to the optimisation of those pathways so that more efficient conversion of low-cost starting materials into highly desirable products can be achieved. This review highlights these areas and details the most important and exciting advances being made in this area.

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Current Opinion in Biotechnology 2011, 23:1–7

This review comes from a themed issue on
Analytical biotechnology
Edited by Wei E. Huang and Jizhong Zhou

0958-1669/\$ – see front matter
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DOI 10.1016/j.copbio.2011.10.014

Metabolite and metabolic engineering

One of the main goals of synthetic biology is to generate the desired material with a good conversion from substrate(s) to product whilst reducing unwanted (side-) products. This is not a new goal for biology and has its routes from metabolite/metabolic engineering, whereby genetic and regulatory processes and pathways within cells are optimised with some preconceived idea of which pathways are important in order to increase the production of a whole range of valuable substances. At the same time efforts are also made to reduce the energy costs to the cells, reduce the production of any undesirable substances (both with respect to yield of the end product and also metabolite pathway inhibition), and to reduce overall production costs (Figure 1 illustrates the metabolic engineering process). These can all be highly important aspects, especially when one considers that these may be industrial-scale applications and for

low-valuable products an increase of only 1% production yield could be highly rewarding in financial terms (e.g. carbon conversion into biofuel production).

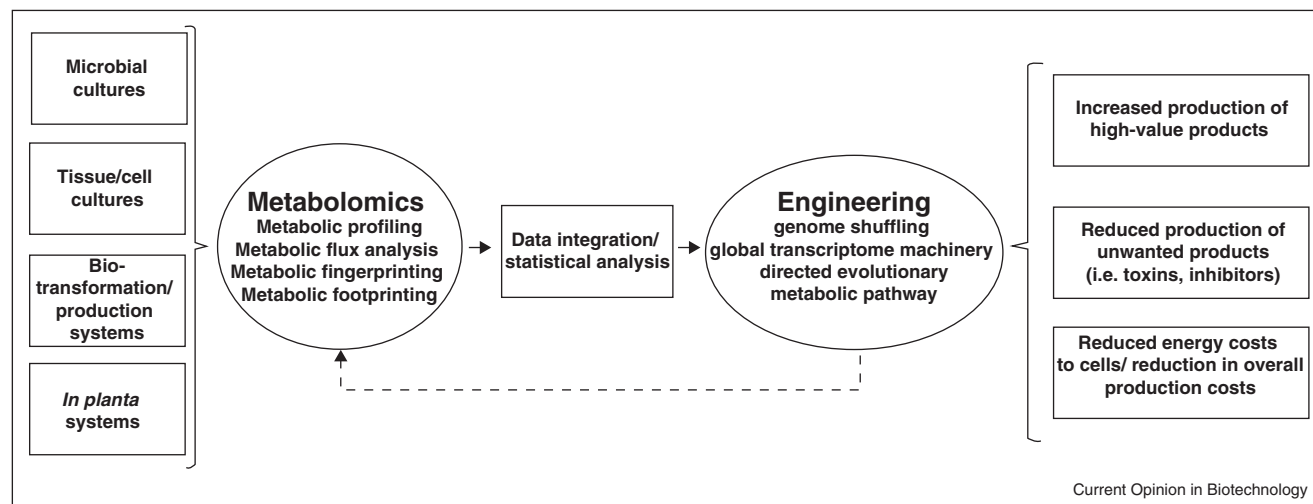
A recent and highly topical study of metabolic pathway engineering based on metabolomics [1] involved the development of novel bio-ethanol producing yeast strains with increased tolerance to inhibitors in lignocellulosic hydrolysates. *Saccharomyces cerevisiae* is used to produce bio-ethanol from lignocellulosic materials from agriculture and industrial wastes, as it is a fast sugar consumer, gives a high ethanol yield from glucose, and is said to have a higher resistance to ethanol and other compounds present in lignocellulosic hydrolysates than bacteria [2]. These toxic compounds, which negatively affect microbial growth, metabolism, and of course ethanol yield, include weak organic acids (such as acetic and formic acids), furan derivatives and phenolics. This study examined the effect of acetic acid on xylose fermentation using metabolite profiling to identify and target genes for improving organic acid tolerance. Results revealed that several metabolites involved in the non-oxidative pentose phosphate pathway (PPP) were significantly accumulated by the addition of acetate, and the authors suggested the possibility that acetic acid slows down this pathway. Therefore, metabolic engineering was focused on the non-PPP. A gene encoding a PPP-related enzyme (transaldolase or transketolase) was overexpressed in the fermenting yeast, which successfully conferred ethanol productivity in the presence of both acetic and formic acid [2].

Lignocellulosic biomass hydrolysates are said to be increasingly used as feedstock for industrial fermentations [3]. A recent article studied the performance of six industrial relevant microorganisms (two bacteria, two yeasts, and two fungi) in terms of their ability to utilise monosaccharides in the biomass, resistance against inhibitors, and their ability to grow on different types of feedstock hydrolysates. The authors concluded that a substrate-orientated approach, rather than the more commonly used product-oriented approach towards the selection of a microbial production host, would avoid the requirement for extensive metabolic engineering [3].

Whilst many of the studies into biofuels involve approaches utilising terrestrial crops, a recent study involved metabolomic analysis of aquatic microbes, specifically, oxygenic photoautotrophs (AMOPs). Some AMOPs, such as cyanobacteria, are said to offer several advantages over terrestrial crop biofuel production methods, a major one being the direct excretion of

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Figure 1



Flow diagram of the metabolic engineering process highlighting the role metabolomics has to play.

alternative fuel precursors, such as hydrogen [4[•]]. This study used metabolic and genetic engineering to construct a mutant of the cyanobacterium *Synechococcus* sp. which lacked the enzyme for the NADH-dependent reduction of pyruvate to D-lactate. Subsequent metabolomic analysis by nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) showed that autofermentation by this mutant strain resulted in no D-lactate production and higher concentrations of excreted acetate, alanine, succinate, and up to a 5-fold increase in hydrogen production compared to the wild type [4[•]]. Importantly, this genetic elimination of metabolic pathways which improved H₂ levels could be applied to other AMOPs [4[•]], illustrating that even small changes in a low product forming organism with rational metabolic engineering can result in significant product yields.

Other metabolic engineering areas of interest include those involved in the engineering of complex metabolic pathways in microbes. One recent study involved targeted metabolic profiling of what these authors termed 'deleterious interactions between pathway intermediates and host cell metabolism' [5]. This was performed in order to identify modes of toxicity from the accumulation of unwanted metabolites. In addition to identifying the pathways affected (including inhibition of fatty acid biosynthesis) and any toxins involved (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA)), these authors also identified ways to counteract this through the addition of palmitic acid to the growth medium, demonstrating the ability to optimise synthetic biological systems with their approach [5]. This also demonstrates the ability of metabolic engineering to go beyond genetic optimisation of the organism and to consider the

environment in which the organism grows. In the future, the optimisation of the genetic makeup may necessitate careful tailoring of the growth medium so that the host organisms undergo less perturbation.

In an excellent review *Corynebacterium glutamicum*, a species of the class *Actinobacteria* used for the industrial scale production of various amino acids of this species. Nesvera and Patek [6] summarized the latest developments in the field of genetic engineering in *C. glutamicum*, as well as the use of 'omics-based approaches, including transcriptomics, proteomics, metabolomics, and fluxomics [7]. They showed that large-scale datasets from these functional analyses could be combined to produce predictive models, and through integration of the information obtained this could result in a complex description of all cell interactions, one of the aims of systems biology [6]. The review also includes some of the challenges which need to be surmounted in order to implement these tools fully, enabling the construction of superior production strains of this important bacterium. These include the need for co-ordinated changes in metabolism which tune higher flux through the engineered pathway, and avoiding depletion or overloading levels of particular metabolites, which can lead to a more balanced and stable production strain. This is in addition to understanding and describing the control mechanisms within regulatory networks, the ability to control cell division and optimisation of the cell properties and downstream processes [6]. Although it is clear to us that this is incredibly complex to model at this stage, this report demonstrates the potential power of coupling systems biology to metabolic engineering, whereby system-level knowledge about the bacterial host could lead to the identification of potential bottlenecks within the

host, which would then be the targets for improved strain design.

Other reviews include those concerning industrial scale metabolic engineering [8], highlighting the ‘older’ and ‘newer’ tools of metabolic engineering and synthetic biology respectively. In combination with more recent whole cell engineering approaches such as genome shuffling, global transcriptome machinery engineering and directed evolutionary engineering. For a further excellent review covering metabolic engineering and synthetic biology for the optimisation of both medicinal and aromatic plants the reader is directed to Hendrawati *et al.* [9].

Many synthetic biology studies have thus far involved microbes, and given the tractability of the system this is perhaps not surprising. Microbial metabolomics is very popular [10^{*}] and this is not surprising when one considers how much this field supports and complements a wide range of microbial research areas, ranging from metabolic engineering to drug discovery [11]. Winder *et al.* used the metabolic fingerprinting (Table 1) approach of Fourier transform infrared (FT-IR) spectroscopy to monitor whole-cell dioxygenase-catalysed biotransformation of toluene to toluene *cis*-glycol in fed-batch cultures of *E. coli*. PLSR could be used to correlate metabolic fingerprints with the level of product concentration, and their results demonstrated the potential of this high-throughput approach to assess temporal biochemical dynamics in complex bioprocesses, and also provided rapid information on product yields and quality without the requirement for time-consuming chromatographic product analysis [12]. The same group studied global metabolic profiling of *E. coli* by GC-MS for the evaluation of

quenching and extraction processes for the accurate quantification of intracellular bacterial metabolites [13]. The convoluted relationship between intracellular metabolism and metabolic footprinting in microbiology, and how this method can assist in the interpretation of cell communication mechanisms, metabolic engineering and industrial biotechnological processes is also the subject of a review by Mapelli *et al.* [14].

Tissue and cell culture optimisation

Whilst microbes and yeast are very tractable hosts in terms of genetics and bioreactors can be easily scaled up, they are relatively ‘simple’ beasts and the production of recombinant proteins that require correct glycosylation for activity need to be produced in more complex eukaryotic systems. Thus another important area for synthetic biology is product production in tissue and cell culture systems and these too need careful optimisation; metabolomics can also play a valuable role here. One recent study involved two models which were used to evaluate the cellular metabolome and to optimise the growth media [15]. The first of these was NMR-based metabolic profiling for the quantification of metabolites in Chinese hamster ovary (CHO) cell lines engineered to express a recombinant protein, followed by the second model of metabolomic analysis of superfusion media used for drug metabolism and toxicology studies in *in vitro* liver slices. Results from the first model highlighted which culture parameters could be manipulated to optimise growth and protein production. Whilst results from the second model showed that two of the medium components were depleted at a faster rate than any other nutrients, and augmentation of the starting medium with these two components (choline and histidine) improved long-term liver slice viability [15]. A related study involved

Table 1

Glossary of some of the terminology used within metabolomics

Metabolome	All low-molecular weight metabolites (i.e. metabolic intermediates, hormones and other signalling molecules, and secondary metabolites, >1000 kDa) to be found in a biological sample/system, such as a single organism, which are the end products of gene expression.
Metabolomics	The non-biased and non-targeted identification and quantification of all metabolites in a biological system.
Fluxomics	Specific labelled (¹³ C or ¹⁵ N) substrates are fed to bacterial, yeast or tissue cultures in order to follow the destination of carbon or nitrogen within metabolic pathways, using MS- or NMR-based mass isotopomer analyses.
Metabolic profiling	Identification and quantification of a selective number of pre-defined metabolites, which are generally related to a specific metabolic pathway.
Metabolic fingerprinting	Global, high-throughput, rapid analysis to provide sample classification. Can be used as a rapid high-throughput screening tool to discriminate between samples from different biological status, origin, and processes. Enables rapid biochemical information regarding production yields at set points within and/or the end of production lines for example.
Metabolic footprinting	Analysis of the metabolites secreted/excreted by an organism, also known as the exometabolome; if the organism is growing in media/culture this will include its environmental and growth substances. Some of which can be valuable products whilst others may be unwanted.
Metabolic/metabolite engineering	Optimisation of genomic and regulatory processes within cells and tissues to increase production of valuable substances, and/or reduce production of unwanted substances (i.e. toxins). Can also lead to more energy efficient biochemical processes and reduce large-scale production costs.
Metabolite target analysis	Qualitative and quantitative analysis of one, or several, metabolites related to a specific metabolic reaction.

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a metabolomics-based approach for the improvement of CHO cell growth. This utilised LC–MS to identify extracellular metabolites (the exometabolome, also known as metabolic footprinting [16,17]) in the medium of fed-batch CHO reactor cultures, with the aim to improve cell numbers through metabolic engineering. This was achieved after the exometabolome showed that amongst the metabolites identified malate was the most significant, and metabolic engineering to overexpress malate dehydrogenase resulted in a 1.9 fold increase in integral viable cell numbers [18]. This is a very nice example of where a data-driven analytical process (metabolomics) can lead to ‘hypotheses’ or suggestions for *highly focused* rationale metabolic engineering which resulted in improved synthesis.

Other studies involving nutrient feed, CHO and murine myeloma non-secreting (NS0) cell lines include work by Ma *et al.* [19]. Here, a chemically defined nutrient feed (CDF) coupled with basal medium preloading was developed to replace a hydrolysate-containing feed for a fed-batch NS0 process. Results showed that the CDF enabled a completely chemically defined feed with an increased monoclonal antibody titre of 115%. Tests of the CDF in a CHO process were also indicative of CDF being able to replace hydrolysate-containing feed with a subsequent 80% increase in product titre [19]. Sellick *et al.* have undertaken a body of work in this area including rapid monitoring of recombinant antibody production in CHO and NS0 cell lines. This was achieved using the metabolic fingerprinting [20,21] technique of FT-IR spectroscopy combined with multivariate statistical analyses (e.g. partial least squares regression (PLSR)) and was employed to predict anti-body levels. It was clearly demonstrated that a spectroscopic approach could be an appropriate starting point for the potential on-line monitoring and measurement of antibody production in industrial-scale bioreactors [22]. Other studies by this group include the optimisation of technologies for the global metabolomic profile of CHO cell lines, including the evaluation of extraction processes [23] and effective quenching processes [24]. In combination, this has led to enhanced recombinant antibody production (over double) by specific tailoring of the nutrient feed to CHO cells [25]. We expect this approach to be widely used to enhance protein yields, as feeding specific substrates through growth can elongate production times and thus increase product formation.

Stable isotope tracers for engineering

Metabolomics techniques are known to provide very large datasets and allow for the high-throughput quantification of metabolites, yet the resultant data-flood [26] from these methods has been said to provide minimal information on the rates and connectivity of metabolic pathways [27••]. After all, as Henry Nix once said in his 1990 Keynote address to AURISA (Australasian Urban and Regional Information Systems Association Inc.):

“Data does not equal information; information does not equal knowledge; and, most importantly of all, knowledge does not equal wisdom. We have oceans of data, rivers of information, small puddles of knowledge, and the odd drop of wisdom.”

One method to address this problem is the use of stable isotope tracers. Whereby an isotopically labelled substrate (e.g. glucose labelled with ^{13}C , or a ^{15}N labelled amino acid or nitrogen substrate) is fed to the (micro-)organisms being studied, the products themselves become labelled and can then be measured using a combination of chromatographic, mass spectrometry, or NMR spectrometry. This form of analysis can then be exploited for metabolic pathway elucidation and metabolic flux determination, which may then provide targets for engineering approaches. One recent review of this area termed this form of mass isotopomer analysis as “time and relative differences in systems (TARDIS)-based analysis”, owing to the fact that it both measures and quantifies the temporal sequential emergence of these labelled products [28•].

A recent and very significant study presented a new method, termed non-targeted tracer fate detection (NTFD) [27••]. This combined the use of stable isotope tracers and gas chromatography-mass spectrometry (GC–MS) with computational analysis, enabling the quantification of all measurable metabolites derived from a specific labelled compound, without any *a priori* knowledge of a reaction network or compound library. Using a mixture of labelled and non-labelled, tracer the NTFD approach can be applied to bacterial cultures, eukaryotic cell cultures, whole animal systems, and even non-biological chemical systems. Further, this novel method provides information about relative flux magnitudes into each metabolic pool through the determination of mass isotopomer distribution (MID) for all labelled compounds, and is said to provide a framework for global analysis of metabolic fluxes. The NTFD approach truly adds new knowledge to this area and owing to its non-targeted approach, adds information about biochemical reactions and metabolites that were previously unknown [27••]. As the authors themselves rightly state, NTFD adds a new dimension to the metabolic toolbox [27••]. Reviews of note in this area include those concerning metabolic flux distributions, genetic information, computational predictions and experimental validation of strain engineering [29], and steady-state metabolic flux analysis (MFA) in plants for the measurement of multiple fluxes in the core network of primary carbon metabolism [30].

Whilst metabolomics gives only snapshots of metabolite levels these TARDIS-based analyses allow the flux of carbon or nitrogen through pathways to be discovered. The caveat is that cells are cultured in a sole carbon or

nitrogen source and for complex eukaryotic systems this is not always possible and so isotope calculations can be more difficult. Though we do believe these flux analyses will be very valuable for ensuring that the flow of starting materials is directed towards the product of interest rather than off to some side branches of the metabolic pathway.

Data integration/analysis processes for synthetic biology

As the above studies using flux analyses elegantly demonstrate (and as is the case with all 'omics' and related disciplines involving large and highly complex datasets), data integration and analytical methods are absolutely vital, the key which can unlock doors to new insights, add to knowledge, and open up completely new dimensions within synthetic biology. A study by Wisselink *et al.* stated that one of the challenges in strain improvement by engineering is the subsequent determination of the molecular basis of the improved properties which were enriched from natural genetic variation during selective conditions [31^{••}]. The authors demonstrated their approach to this challenge through transcriptome analysis, intracellular metabolite measurements and metabolic flux analysis, of glucose-limited and arabinose-limited anaerobic chemostat cultures of metabolic and evolutionary engineered *S. cerevisiae* (IMS0002) and its non-evolved ancestor IMS0001. Results identified key genetic changes contributing to efficient arabinose utilisation by the engineered *S. cerevisiae* strain. Such as confirmation that the galactose transporter is essential for growth on arabinose, and genes which may be involved in flux-controlling reactions in arabinose fermentation could be identified. They tested this by deleting these genes which caused a 21% reduction of the maximum specific growth rate on arabinose [31^{••}]. This is a very nice example of how multi 'omics' data can be integrated to generate new knowledge about carbohydrate utilisation which can be tested and confirmed to be true. Within systems biology, this allows the loop within the inductive approach to knowledge discovery to be completed.

Another study also views reverse engineering of high-throughput 'omics' data to infer biological networks as one of the challenges in biology. Here, the authors focused on a systematic analysis of metabolomic network inference from *in silico* metabolome data from *E. coli* and yeast and showed that it may be possible to predict the organism's response and thus underlying metabolic network to different intrinsic and environmental conditions [32]. Another article of interest involved comparative analysis of several mathematical and statistical methods using synthetic datasets produced by simulation of realistic biochemical network models. Using this approach, it was possible to study how inferences were degraded by noise, and this allow the study of the extent to which correlation of metabolomics datasets are capable of recovering features from these biochemical systems. Results

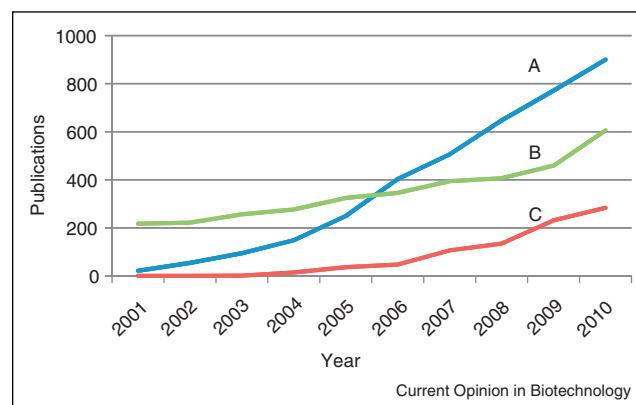
from these analyses identified a number of major metabolic regulatory configurations that result in strong metabolite correlations and demonstrated the utility of biochemical simulation/modelling for the analysis of 'omics' data [33].

Finally, a recent and highly cogent review focuses on a critical assessment of mathematical techniques used to infer metabolic networks from time-resolved metabolomics data [34[•]]. This employed the simulation of data and analysed four representative methods, as well as including an overview of sampling and methods currently used, compared with reverse engineering methods. Most significantly, the authors identified large discrepancies between the requirements of reverse engineering of metabolic networks and current practice (if a full inference of a real-world metabolic network is the goal). Recommendations were also provided for the improvement of time-resolved experimental designs [34[•]].

Conclusion

The marriage of synthetic biology with metabolomics is a growing area (Figure 2) and recent studies of interest in areas not yet mentioned include those involved in plant biotechnology, such as the work involving overproduction of tryptophan in GM rice by Matsuda *et al.* [35], and of course the many excellent overviews in this area, including the use of plant and algae as cell factories to produce numerous high-value compounds such as carotenoids [36], as well as the engineering of carotenoid formation in tomatoes and the application and potential of both systems biology and synthetic biology approaches [37]. In terms of systems biology, there are of course a number of excellent reviews covering areas such as industrial systems biology [38], systems biology of industrial microorganisms [39], and metabolomics, modelling, and machine learning in systems biology [40].

Figure 2



Number of publications per year from Thomas Reuters Web Of Science, illustrating the increasing research activity and publication trends from the three related areas; (A) Metabolomics, (B) Metabolic Engineering, and (C) Synthetic Biology.

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The long-term and mutually beneficial relationship between synthetic biology and metabolomics incorporating fluxomics is clearly important for rational metabolic engineering. Genome-scale metabolic network reconstructions are being generated almost weekly for different species (and strains thereof) and systematic investigations of these will help pinpoint the so-called bottlenecks (or what used to be referred to as the rate-limiting steps <http://pubs.acs.org/doi/pdf/10.1021/ed058p32>) in metabolic pathway constructions in new host organisms. Synthetic biology is here to stay and rational metabolic engineering of bacteria, yeast, fungi and mammalian-based systems will become an important growth area, urgently needed to sustain the planet's needs as the population continues to expand at alarming rates whilst consuming valuable non-renewable resources.

Acknowledgements

The authors would like to thank the UK BBSRC and EPSRC (BB/C008219/1) for financial support of the MCISB (Manchester Centre for Integrative Systems Biology).

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