



Metabolomics tools for the synthetic biology of natural products

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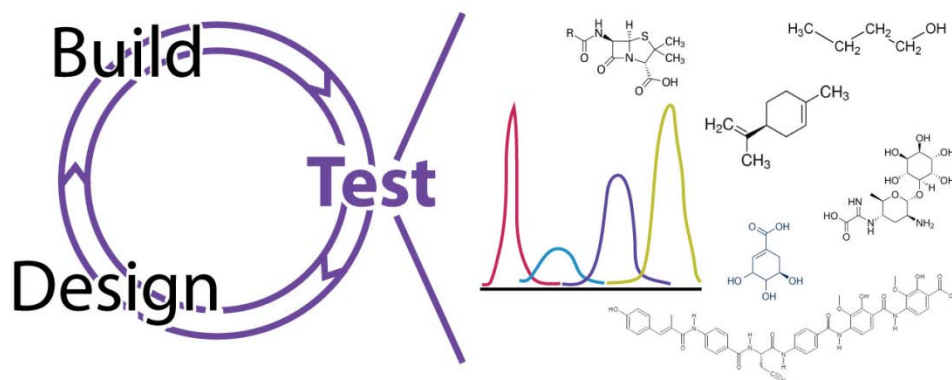
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Abstract

Metabolomics plays an increasingly central role in within the Design – Build – Test cycle of synthetic biology, in particular in applications targeting the discovery, diversification and optimized production of a wide range of natural products. For example, improved methods for the online monitoring of chemical reactions accelerate data generation to be compatible with the rapid iterations and increasing library sizes of automated synthetic biology pipelines. Combinations of label-free metabolic profiling and ¹³C-based flux analysis lead to increased resolution in the identification of metabolic bottlenecks affecting product yield in engineered microbes. And molecular networking strategies drastically increase our ability to identify and characterize novel chemically complex biomolecules of interest in a diverse range of samples.

Graphical abstract



Synthetic biology of natural products

Synthetic biology facilitates the biosynthesis of pharmaceutical ingredients and other high-value chemicals by employing the Design – Build – Test cycle of engineering to guide the systematic enhancement of microbial factories [1-4]. Exemplary successful applications of synthetic biology to natural product production include a one-pot method for menthol biosynthesis in *Escherichia coli* [5], the modular extension of a styrene biosynthesis pathway to produce 2-phenylethanol [6], cannabinoid biosynthesis in yeasts [7], and the heterologous production of antibiotics using extensively refactored biosynthetic gene clusters: myxobacterial α -pyrone antibiotics in *Myxococcus xanthus* [8] and kasugamycin (an aminoglycoside antibiotic isolated from *Streptomyces kasugaensis*) in actinomycetes [9]].

A recent review by Smanski and colleagues [10] provides details of recent advances in the technologies underpinning the Build aspects of the synthetic biology cycle, including pathway construction and pathway screening, while a complementary review by Chen *et al.* [11] focuses

43 on the modelling approaches for the construction and optimisation of cell factories for bio-
44 production, which cover a large part of the Design activities. In the present review, we will
45 turn focus on the Test component of synthetic biology, focusing in particular on advances in
46 metabolomics as a discovery and debugging tool for metabolically enhanced microbial systems.

47
48

49 **Test Analytics – Appropriate Technologies**

50

51 Mass spectrometry (MS) coupled to chromatography remains the domineering technology used
52 for the quantification of natural product targets and is also the most widely used platform for
53 the global profiling of the impact of an engineered biosynthetic pathway on the microbial
54 metabolome. The challenge for the analytical technologies is to achieve the acquisition speed
55 and sensitivity required to meet the high-throughput needs of a synthetic biology-based
56 pipeline. Traditionally, products are measured directly from an aliquot of cell culture medium
57 or – in the case of volatile products – they are captured in solvent overlays and transferred to
58 vials or multi-well plates for analysis. These approaches are often slow (tens of minutes per
59 sample plus preparation time) and provide only a snap shot of what is occurring at a given time.

60

61 To overcome this limitation, much effort has been invested into the development of improved
62 methods for the online monitoring of chemical reactions; which would provide greater control
63 of sampling and provide dynamic results with regards to product turnover. **Definitions of the**
64 **analytical terminology described herein are summarised in Table 1.** In recent work by Yan *et al.*,
65 desorption electrospray ionization (DESI) coupled to ion-mobility mass spectrometry was used
66 for the high-throughput screening of biocatalysis directly from bacterial colonies on agar plates
67 [12], which can in principle be applied to a broad range of substrates and products, including
68 free amines, carboxylic acids, alkaloids and phenols; multiple analytes can be detected in a
69 single analysis thus allowing for the screening of diverse strain libraries with complex product
70 profiles. DESI-MS was also applied to the rapid analysis of enzyme kinetics by Cheng and co-
71 workers [13], who measured product formation in a buffered aqueous medium, explored the
72 possibility of adjusting the pH and solvent composition of the DESI spray to quench the
73 enzymatic reaction and thus improved the accuracy of the kinetic measurements by preventing
74 post-ionization reactions.

75

76 As an alternative to DESI, matrix-assisted laser desorption ionisation mass spectrometry
77 imaging (MALDI-MSI) has readily been applied towards the large-scale phenotyping of bacteria
78 [14, 15]. A related optically-guided MALDI-MS strategy has recently been implemented for the
79 profiling of microbial colonies for rapid screening of natural product analogue libraries [16].
80 This impressive development used optical imaging of microbial colonies to direct the laser
81 coordinates for an automated MALDI-MS screening of approximately 1000 colonies directly
82 from an imprinted glass slide with an MS sampling rate of about one colony per second.
83 Reaction products were screened *in situ* and results overlaid with the optical images;
84 integration of results allowed for subsequent colony picking and recovery of the desired mutant
85 strains. The majority of commercially available MALDI-MS instrumentation permit a spatial
86 resolution of > 100 μm . However, the group of Bernhard Spengler has recently dramatically
87 pushed this boundary towards much better lateral resolutions down to 1.4 μm [17], thus further
88 advancing the technique towards single cell resolution and even higher throughput [18].

89

90 The coupling of microreactors or continuous flow chemical reactors directly to the mass
91 spectrometer provides an enhanced ability to characterise unstable reaction products and
92 reduces the sample volume required (albeit with sufficient mass spectrometer sensitivity). **Link**
93 **et al. [19] provided a comprehensive example of such an application. They**
94 **demonstrated the ability to undertake real-time metabolome profiling by direct**

95 injection of living bacteria, yeast and mammalian cells into a high-resolution mass
96 spectrometer through coupling a peristaltic pump and two six-port valves and
97 automatically sampling from a liquid culture. This approach permitted the automated
98 monitoring of around 300 compounds in 15–30 s cycles over several hours. They investigated
99 the metabolite dynamics in real-time during 2 h starvation and 30 min of growth resumption.
100 The approach suggested that the accumulation of energetically costly metabolites in starved *E.*
101 *coli* reflects the control strategy to favour cheap metabolic pathways for growth resumption.
102 From an analytical perspective the method permitted real-time metabolome profiling that
103 followed the dynamics of metabolic processes in different organisms over extended periods.
104 The method alleviates retrospective manual sampling, sample preparation and sample
105 manipulation associated with traditional off-line methods.

106
107 Progress has also been made on the mass spectrometry techniques available to the synthetic
108 biology community: proton transfer reaction mass spectrometry (PTR-MS) and selected ion
109 flow tube mass spectrometry (SIFT-MS). These techniques are direct injection approaches that
110 utilise chemical ionisation for real time analysis of volatile organic compounds. PTR-MS has
111 been shown to achieve near-to-real-time monoterpene separation and identification, when
112 coupled to a fast gas chromatography, with sensitivity in the range of 1.2 ppbv from plant
113 material [20]. PTR-MS has also been applied to the real time monitoring of the yeast volatilome
114 [21], detecting more than 300 metabolite features, 70 of which were tentatively identified, in
115 the headspace of *Saccharomyces cerevisiae* cultures over 11 days at 4-h time points. Additional
116 development and application of this technique has been demonstrated by Materic *et al.* [22],
117 who used Selective Reagent Ion PTR-MS to investigate the separation of monoterpene mixtures,
118 which are a particularly common target in recent synthetic biology projects *i.e.* geraniol [23],
119 linalool [24] and limonene [25].

122 **Global analysis – Metabolomics**

123 Synthetic biology requires not only the rapid and accurate quantitation of the desired end
124 products; even more important for a systematic engineering of the microbial factories is a
125 thorough understanding of metabolic flux and the regulation of central carbon metabolism to
126 ensure the desired production of target compounds is compatible with maintaining cellular
127 homeostasis and energy balance. Metabolomics, the comprehensive profiling of small molecules
128 in a biological sample, is the obvious method of choice for collecting the necessary data for this
129 kind of analysis, and synthetic biology can build on a continuously refined repertoire of
130 metabolomics approaches [26, 27].

131
132 Of the many technological advances in recent years, we only highlight the increasing importance
133 of parallel reaction monitoring (PRM) in metabolomics; the quantitation of intermediates of
134 central carbon metabolism, amino acids and shikimate pathway-related metabolites in
135 engineered strains of *E. coli* [28] is just one important example of its application in synthetic
136 biology. PRM permits the quantitative analysis of multiple targets (237 in this example) [29]
137 with excellent linearity of quantitation, as well as high precision and accuracy. In a related
138 approach, all ion fragmentation acquisition has recently been demonstrated to achieve
139 increased accuracy in metabolite identification for a large number of pre-selected compounds,
140 while at the same time acquiring full scan information to allow the identification of additional
141 metabolites that were initially not targeted [30].

142
143 A metabolomics-driven approach was applied to identify non-obvious target genes to further
144 improve the production of 1-butanol [31, 32]. The authors performed quantitative targeted
145 analysis of acyl-CoAs in the CoA-dependent 1-butanol biosynthetic pathway in *Synechococcus*
146 *elongatus* strains *via* ¹³C-labelling of cell extracts as an internal standard and HPLC-MS analysis.
147 The results indicated several targets for potential improvements of 1-butanol production in

148 cyanobacteria, such as possible rate-limiting steps (reductive reaction of butanoyl-CoA to
149 butanal) or effective regeneration of free-CoA from butanoyl-CoA to enhance the conversion of
150 pyruvate to acetyl-CoA. In a parallel study addressing 1-butanol production in *E. coli*, the
151 authors examined the metabolomic impact of the deletion of phosphate acetyltransferase, which
152 was performed in an attempt to reduce the amount of acetate produced and simultaneously
153 increase the acetyl-CoA pool. Metabolomics analysis using a targeted ion pair LC-MS/MS
154 method detected a total of 78 metabolites and pointed to several metabolic perturbations
155 caused by the deletion that seemed to be the consequence of a CoA imbalance or insufficient
156 CoA recycling, which caused the undesirable accumulation of side products. Further
157 metabolomics analysis identified the underlying enzymatic bottleneck, alcohol dehydrogenase,
158 and fine-tuning of this activity resolved the CoA imbalance and led to substantially improved 1-
159 butanol titres [31].

160
161 A metabolomics approach was also implemented to investigate central metabolism of a fructose
162 repressor (*fruR*) knockout in a recombinant L-tryptophan producing strain of *E. coli* (*E. coli* FB-
163 04) [33]. The authors report more than 80 intracellular metabolites that were altered as a result
164 of the knockout, 23 of which were related to tryptophan biosynthesis. The levels of glycolysis,
165 pentose phosphate and TCA cycle intermediates were consistently increased, and levels of
166 shikimate derivatives (direct tryptophan precursors) and L-glutamine were decreased in the
167 knockout strain, which also showed a substantially increased tryptophan production. The
168 interpretation of these results illustrates very clearly the pitfalls of using steady-state
169 metabolome profile information as a proxy for metabolic fluxes, which are of central interest for
170 synthetic biology: based on increased levels of glycolytic and pentose phosphate pathway
171 intermediates, the authors conclude that the *fruR* knockout enhanced metabolic flow through
172 these two pathways which provide the substrates for L-tryptophan biosynthesis. However, the
173 TCA cycle, which directly competes with tryptophan biosynthesis shows an equally increased
174 level of its intermediates, and the only pathway for which direct flux measurements are
175 available, tryptophan biosynthesis itself, shows a consistent decrease in its key intermediates,
176 despite an increase in flux by 62.5% (from 0.024 to 0.039 g/L/h).

177
178 A subsequent study combining metabolomics and ¹³C fluxomics provided more detailed insights
179 into the metabolic flux redistribution in an *E. coli* strain overproducing shikimic acid with high
180 titres and yields: Rodriguez *et al.* [34] used an engineered AR36 *E. coli* strain constitutively
181 expressing six proteins encoded in a synthetic operon promoting high-yield production of
182 shikimic acid from glucose. Comparative metabolomics of a production strain and parental
183 strains (carrying either no plasmid or “empty plasmid”) was used to track the levels of seven
184 exometabolites and 25 endometabolites over time. It revealed a global remodelling of carbon
185 and energy metabolism in the high producer. This resulted in reduced carbon available for
186 oxidative and fermentative pathways and increased levels of endometabolites involved in
187 energy pathways, preventing the depletion of essential intermediates, such as PEP and ATP.
188 Both glycolytic flux and TCA cycle activity were substantially reduced in this overproduction
189 scenario (43 g/L of shikimate in 30 h on complex medium).

190
191 Given its importance as a provider of essential precursors for a diverse range of
192 biotechnologically important biochemicals, it is not surprising that the shikimate pathway has
193 been the target of dedicated metabolomics method development: *e.g.*, Lai *et al.* [35] contributed
194 a robust HPLC method for the quantification of aromatic substrates, products and pathway
195 intermediates in order to accelerate strain engineering for industrial production of aromatics as
196 biosynthetic molecules. The achieved limits of detection between 10⁻¹⁰ – 10⁻¹³ mol make the
197 method suitable for endometabolome and exometabolome analysis of engineered strains.

198
199 Another example of a metabolomics-based strategy for strain engineering (this time utilising a
200 GC-MS analytic platform) is the study by Teoh *et al.* [36] investigating phenotypic differences in

201 growth rates and metabolite profiles of nineteen single-deletion *S. cerevisiae* mutant strains
202 cultivated under stress-free and under 1-butanol stress conditions (growth inhibition caused by
203 higher alcohols (*e.g.* 1-butanol) is considered as a bottleneck in their biosynthetic production).
204 Metabolites associated with improved growth rates under stress conditions were identified, and
205 new stress-resistant mutant yeast strains were successfully predicted based on their metabolite
206 profiles. This approach illustrates the potential of metabolomics as a predictive screening tool to
207 inform semi-rational strain engineering approaches.

208
209 Finally, metabolomics has been applied for the monitoring of isoprenoid precursors production,
210 another classic target for synthetic biology [37, 38]. In a study by Kirby *et al.* [39], who report
211 for the first time the functional expression of an extensively engineered functional 1-deoxy-D-
212 xylulose 5-phosphate (DXP) pathway in *S. cerevisiae* which normally utilizes the mevalonate
213 pathway, which has a lower theoretical yield. Metabolite-guided DXP pathway balancing, by LC-
214 MS quantification of intermediates in cultures exhibiting various levels of flux, appeared to be a
215 successful approach for identifying a bottleneck in the pathway. An engineered strain
216 exclusively using the DXP pathway achieved an endpoint biomass 80% of that of the same strain
217 using the mevalonate pathway under low aeration conditions.

218
219

220 **Molecular networking – moving forward**

221 The main challenge of untargeted metabolomics is compound annotation; the persistent
222 difficulties of confidently identifying the detected metabolites currently seriously limits the
223 utility of the MS data acquired. Molecular networking, a visualisation method for tandem MS
224 data, is a powerful complement to traditional de-replication methods [40]. This approach allows
225 for the detection of sets of spectra from related molecules (“spectral networks”), even in the
226 cases when these spectra are not matched to any known compounds. The approach is based on
227 the assumption that similar molecules have similar MS fragmentation patterns so they will tend
228 to cluster closely within a network. Each spectrum (ideally derived from a single compound) is
229 visualised as a network node, and the edges between nodes represent a degree of similarity
230 between spectra. The thicker the line, the more MS/MS fragment ions are shared by the two
231 connected nodes. Nodes can be supplemented by such information as a compounds abundance,
232 biochemical activity, origin *etc.* Molecular networking led to the development of Global Natural
233 Products Social Molecular Networking (GNPS), a metabolomic data-driven platform for the
234 storage, sharing, analysis, and knowledge dissemination of tandem MS spectra where one is
235 able to annotate natural product data *via* continuous de-replication. [41].

236
237 Although improvements are still required to obtain unambiguous analysis of molecular
238 networks such as efficient integration with existing LC-MS detection strategies, enhancement of
239 pre-processing and universal optimal acquisition methods [40, 42, 43], its applications are
240 expanding fast [44-49] and it will soon become an indispensable metabolomics tool in
241 exploratory analyses for the synthetic biology of novel natural products. For example, in a
242 recent study Crüseman and colleagues [46] screened 146 marine *Salinispora* and *Streptomyces*
243 strains using HPLC-MS/MS, molecular networking, and the Global Natural Products Social
244 (GNPS)[41] platform and explored the impact of differing culturing and extraction techniques.
245 The systematic investigation of the effect of these parameters clearly demonstrated how much
246 inherent chemical diversity could be missed when just one culture and extraction protocol is
247 utilised to assess metabolic capacity. This example demonstrated how the application of
248 molecular networking permits the rapid optimisation of experimental parameters that can
249 subsequently be implemented early in the discovery workflow.

250
251 Okada *et al.* [50] used molecular networking for the investigation of the influence of
252 trimethoprim (Tmp) antibiotic on the secreted metabolome of *Burkholderia thailandensis* E264.
253 The untargeted comparison of Tmp-induced and uninduced samples (utilising HPLC-QToF-

254 MS/MS) resulted in ~240 metabolites of interest (with >100 compounds observed only for the
255 induced samples). Organising them into 14 sub-networks followed by NMR analysis enabled
256 rapid identification of 40 compounds including analogues of known compounds and a group of
257 new molecules, acybolins, showing that molecular networking aids rapid identification of
258 compounds compared to traditional workflows.

259
260 In related work, von Eckardstein *et al.* [47] used bioactivity-guided untargeted LC-MS/MS
261 analysis and molecular networking in the search of new antibiotic agents from *Xanthomonas*
262 *albilineans*. Over 20,000 MS/MS spectra acquired from crude extracts and bioactive fractions
263 were organised into a molecular network *via* the GNPS portal, which allowed for the
264 identification of potential derivatives in the albicidin sub-network. The group reported eight
265 new natural albicidin derivatives with unambiguous identification.

266
267

268 **Conclusions**

269 There are still some challenges to overcome, but both synthetic biology and metabolomics are
270 very dynamic fields that are forging an ever-closer alliance. An example that illustrates the
271 integral role of metabolomics in synthetic biology pipelines is the recently published multi-
272 omics workflow to characterise strain variation in engineered *E. coli* [51]. It is certain that in
273 coming years we will see a rapid deepening of the technical and conceptual integration of
274 metabolic profiling methods within the Design – Build – Test cycle, and in particular the
275 emergence of additional tools to facilitate the flow of data and insights between the analytical
276 machinery (Test) and its users in the Design and Build stages of strain engineering.

277

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286
287

288 **References and recommended reading**

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290 • of special interest

291

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455 **Table 1**
 456 Glossary of analytical technologies

Technique/Approach	Full Name	Description
Metabolomics	—	The untargeted, non-biased detection and identification of all low-molecular weight compounds (metabolites) present within a biological sample or system.
MS	Mass Spectrometry	Analytical technique based on the ionisation of analytes (e.g., by DESI, MALDI, PTR or SIFT; see below), the subsequent separation of ions according to mass/charge ratio, and their detection and quantification.
MSI	Mass Spectrometry Imaging	Mass spectrometry is conducted in a spatial manner thus permitting the visualisation of the two-dimensional localisation of analytes within a sample, for example across a microbial colony growing on an agar plate.
DESI-MS	Desorption Electrospray Ionization Mass Spectrometry	Ambient ionization technique using a nebulized electrospray. Highly charged microdroplets collect analytes from the surface of the sample prior to secondary droplets carrying the analyte to the MS. This ionization technique is particularly suitable for MSI.
IM-MS	Ion Mobility Mass Spectrometry	A variant of MS, with additional separation of ions according to the time it takes for them to travel through a drift tube with a homogeneous, continuous electric field in the presence of a neutral gas. This leads to separation of ions according to size and shape (collision cross section), complementing the mass/charge information available in traditional MS
MALDI-MS	Matrix Assisted Laser Desorption Ionization Mass Spectrometry	Ionization approach whereby a matrix (an energy-absorbing small organic compound) is applied to/mixed with a sample. A laser applied to the matrix:sample mix excites the matrix molecules and leads to the generation of volatilized ions which subsequently enter the MS. This technique is suitable for MSI.
PTR-MS	Proton Transfer Reaction Mass	A soft ionization technique using an ion beam of protonated water

	Spectrometry	molecules, H_3O^+ , as an ion source to protonate (and thus ionize) volatile analytes. This technique permits for real-time monitoring of organic molecules in the gas phase.
SIFT-MS	Selected-Ion Flow-Tube Mass Spectrometry	Similar to PTR-MS, this soft ionisation technique uses precursor ions in the gas phase to ionize volatile analytes. The precursor ions are generated by a microwave plasma ion source, and a single ion species can be selected (H_3O^+ , NO^+ or O_2^-) to perform as reactant ion. Neutral volatile analyte molecules react with the precursor ions and undergo ionization. This technique permits for real-time monitoring in the gas phase.
Molecular Networking	—	A computational method for MS data analysis that allows for the identification of sets of spectra from chemically related molecules ("spectral networks"), based on similarities in molecular fragmentation patterns, even in the cases when the spectra are not matched to any known compounds.

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