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Biological insights into non-model microbial hosts through stable-isotope metabolic flux analysis

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Stable-isotope metabolic flux analysis is an important approach to unravel the metabolic network and its regulation in organisms. It has become a key analytical technology for biotechnological applications. During recent years non-model microorganisms have received increasing attention because they possess unique metabolic capabilities and can serve as a host for production of biofuels and biochemicals. Stable-isotope metabolic flux analysis has been widely used in these microorganisms for exploring novel pathways, elucidating the operation of central metabolic networks, and revealing the metabolic changes that result from genetic manipulations. Here, we review recent applications of stable-isotope metabolic flux analysis in characterizing non-model microbial hosts, guiding the development of rational engineering strategies for enhancement of biochemical production and extension of substrate range, and understanding of industrial production processes.

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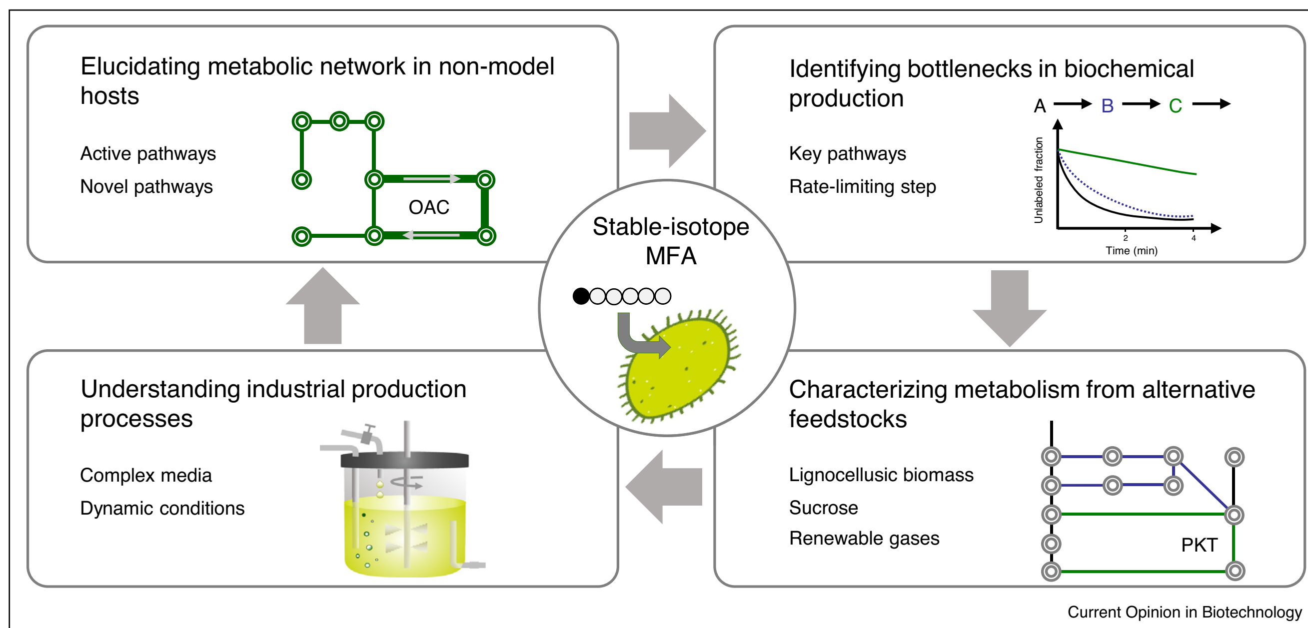
Introduction

Knowledge of metabolic fluxes is crucial to unravel the operation and regulation of metabolic networks and derive rational engineering strategies for biotechnological applications. Metabolic flux analysis (MFA) with isotope tracers provides a rigorous approach to quantify intracellular fluxes through various pathways in complex metabolic networks. Basically, this technique makes use of isotopic tracers (e.g. ^{13}C, ^{15}N, or ^{2}H) combined with isotopic labelling measurements through mass spectrometry (MS) or nuclear magnetic resonance (NMR) techniques as well as mathematical modelling based on isotopomer balancing and metabolite balancing. In addition to model microbes such as *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae*, stable-isotope MFA has been applied to non-model microorganisms, which can serve as a host for production of biofuels and biochemicals. Here, we review recent applications of stable-isotope MFA in non-model microorganisms, including elucidation of metabolic network, identification of bottlenecks in biochemical production, characterization of metabolism from alternative feedstocks, and understanding of industrial production processes (Figure 1).

Elucidation of metabolic network in non-model hosts

The stable-isotope MFA has been used to study the glucose metabolism in various non-model microorganisms, which shed significant insight into *in vivo* operation of their metabolic networks. A prominent study has investigated the glucose metabolism in 25 marine microbes that represent various classes of the Bacteriodetes phylum [1]. The flux results showed that 90% of the marine bacteria studied use the Entner-Doudoroff (ED) pathway for glucose catabolism [1]. The ED pathway was also identified as the main route of glucose catabolism for other bacteria including Zymomonas mobilis that is used for ethanol fermentation, *Pseudomonas putida* that is harnessed to synthesize fine chemicals, and Rhodococcus opacus that is a promising host for lignocellulosic biomass conversion [2••,3,4•,5]. These studies indicate that while model microbes such as *E. coli* use the Embden-Meyerhof-Parnas (EMP) pathway for glucose catabolism, many non-model bacteria rely on the ED pathway. Despite a low ATP yield, the ED pathway has no carbon loss and requires fewer enzymes than the EMP pathway. Moreover, the ED pathway could provide abundant NADPH for protection against oxidative stress in bacteria [1]. The ED pathway operated in a cyclic mode along with several reactions from the pentose phosphate pathway (PPP) and EMP pathway, which forms so-called EDEMP cycle, was reported for *P. putida* [3,4•]. Interestingly, *Gluconobacter oxydans*, which is industrially applied for production of vitamin C and other chemicals, was found to use the PPP as the predominant route of glucose catabolism [6]. Moreover, the ^{13}C MFA showed the cyclic carbon flux through the oxidative part of the PPP in *G. oxydans* [6].

Figure 1



Applications of stable-isotope MFA in non-model microbes.

The ^{13}C MFA has also been used to study the metabolism in thermophilic or fast-growing microorganisms. For example, Cordova *et al.* used the ^{13}C MFA to quantify the fluxes in three extremely thermophilic bacteria *Geobacillus* sp. LC300, *Thermus thermophilus* HB8, and *Rhodothermus marinus* DSM 4252 [7•]. The results showed that although all three thermophilic strains rely on the EMP pathway for glucose catabolism, their redox metabolism is significantly different [7•]. The ^{13}C MFA revealed that the central carbon metabolism in the fast-growing bacterium *Vibrio natriegens* is similar to that in *E. coli* [8•]. The ^{13}C MFA was also used to study the central metabolism in a model cellulolytic bacterium *Clostridium thermocellum* [9,10•]. The EMP pathway was found as the predominant route for glucose catabolism [9]; however, pyruvate kinase is absent in this bacterium. Both the pyruvate phosphate dikinase and the malate shunt, which is catalyzed by phosphoenolpyruvate (PEP) carboxykinase, malate dehydrogenase and malic enzyme, contribute to conversion of PEP to pyruvate in *C. thermocellum* [10•].

There has been increased interest in the metabolism of photosynthetic microorganisms, since the MFA was first applied to the model cyanobacterium *Synechocystis* sp. PCC 6803 [11]. The isotopically nonstationary ^{13}C MFA has been used to elucidate the photoautotrophic metabolism [12], and the steady-state ^{13}C MFA has been used to examine the heterotrophic and photomixotrophic metabolism in cyanobacteria [13•]. The results showed that the fluxes through the CO_2-fixing Calvin-Benson-Bassham (CBB) cycle, oxidative PPP, and EMP pathway in *Synechocystis* are flexibly altered under different growth conditions, whereas the flux through the tricarboxylic acid (TCA) cycle is maintained at very low levels [13•]. The CBB cycle has the highest activity under photoautotrophic condition with sufficient light. The ^{13}C MFA was also applied to a fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 [14]. This strain exhibits a substantial flux through the CBB cycle and small fluxes towards oxidative PPP and sugar storage pathway [14]. Tao *et al.* used the ^{13}C MFA to elucidate the anaerobic photoheterotrophic metabolism in a nonsulfur purple bacterium *Rhodobacter sphaeroides*. The ED pathway was identified as the major glucose catabolic pathway, while the CBB cycle also contributes to glucose catabolism in *R. sphaeroides*, leading to an increased yield of pyruvate from glucose [15].

In addition to the CBB cycle, the Wood-Ljungdahl (WL) pathway is another carbon fixation pathway that is energetically more efficient and found exclusively in anaerobic microorganisms. *C. thermocellum* lacks the formate dehydrogenase, which catalyzes the reduction of CO_2 to formate in the WL pathway. Using the ^{13}C tracer approach, Xiong *et al.* demonstrated that *C. thermocellum* converts CO_2 into formate via a rPFOR-PFL shunt, which is catalyzed by pyruvate: ferredoxin oxidoreductase (PFOR) in the reversed direction and pyruvate-formate lyase (PFL) [16••]. In another study, ^{13}C tracer experiments were used to investigate the metabolic function of an incomplete WL pathway in an organohalide-respiring

bacterium *Dehalococcoides mccartyi* [17]. The incomplete WL pathway cleaves acetyl-CoA to generate the precursor for methionine biosynthesis, accompanying with carbon monoxide accumulation. This unique one-carbon metabolism is important for bacterial growth and interactions in anaerobic communities [17].

Recently several stable-isotope MFA studies have identified novel amino acid metabolic pathways. For example, using ^{15}N and ^{13}C MFA approaches, Romagnoll *et al.* elucidated an alternative pathway for arginine metabolism in the yeast *Kluveromyces lactis*, which involves guanidinobutyrase as a key enzyme [18]. In a more recent study, Zhang *et al.* combined dynamic ^{15}N and ^{13}C tracer experiments, metabolomics analysis, and mathematical modelling to determine the metabolic responses of cyanobacteria to sudden nitrogen availability [19••]. Cycling between ornithine and arginine in response to the perturbation was identified. The results led to the discovery of an ornithine-ammonia cycle (OAC) that involves an arginine dihydrolase and the identification of the function of OAC as nitrogen storage-and-remobilization machinery [19••].

Identification of bottlenecks in biochemical production

The ^{13}C MFA has been used to compare the metabolic fluxes between wild-type and production strains or between different engineered strains. This enables to identify the key pathways and pinpoint the knockout or overexpression targets for improvement of biochemical production in non-model microorganisms. For example, undesired fluxes through PFL and lactate dehydrogenase were identified in a succinate-producing bacterium *Basfia succiniciproducens*. This led to generation of a double knockout strain, which shows drastically reduced formation of by-products (formate and lactate) and enhanced succinate production [20].

To reveal the bottlenecks in fatty acid production, ^{13}C MFA has been performed in the oleaginous yeast *Yarrowia lipolytica*. The results showed that the flux through oxidative PPP is dramatically increased to supply NADPH for fatty acid production in engineered *Y. lipolytica* [21,22]. ^{13}C MFA has also been applied to recombinant expression microbial hosts. For recombinant protein production in the yeast Pichia pastoris, the TCA cycle flux is increased, resulting in enhanced ATP production that may satisfy the increased energy demand for synthesis of amino acids and recombinant protein [23]. While several studies found a downregulation of TCA cycle in the fission yeast *Schizosaccharomyces prombe* and in *P. pastoris* producing a different recombinant protein, an increase in protein production was observed when the TCA cycle flow was enhanced by supplementing acetate or glutamate to cultures [24,25]. Thus, these ^{13}C MFA studies identified the TCA cycle as a potential target for improvement of heterologous protein production in microbial hosts. The increased TCA cycle flux has also been found to be important for chemical production in photosynthetic microorganisms, such as hydrogen production in *R. sphaeroides* and ethylene production in *Synechocystis* [15,26].

Although ^{13}C MFA can be used to identify the pathways that should be targeted to improve biochemical production, it remains unclear which particular step within a given linear pathway is flux-controlling. Integrating flux analysis with targeted metabolomics and dynamic labelling experiments are opening novel possibilities for in-depth investigation of specific pathways. For example, isopentenyl pyrophosphate (IPP) isomerase was identified as a bottleneck enzyme in the methylerythritol phosphate (MEP) pathway for isoprene production in cyanobacterium *S. elongatus* based on metabolomics analysis [27••]. The labelling kinetics of pathway intermediates from dynamic labelling experiments demonstrated that the reaction catalyzed by 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (IspG) is another flux-controlling step in the MEP pathway [27••]. Jazmin *et al.* performed isotopically nonstationary ^{13}C MFA on isobutyraldehyde-producing *S. elongatus*, which revealed a metabolic bottleneck at the pyruvate kinase (PK) reaction [28••]. Overexpression of PK or enhancement of the PK bypass pathway catalyzed by PEP carboxylase, malate dehydrogenase and malic enzyme resulted in increased isobutyraldehyde productivity [28••].

Characterization of metabolism from alternative feedstocks

To increase the economic feasibility of biochemical production, microorganisms are expected to be capable of metabolizing inexpensive and alternative feedstocks including lignocellulosic biomass, sucrose, and renewable gases. ^{13}C MFA has been used to trace the metabolism of these feedstocks in non-model microorganisms. Particularly, the *in vivo* activity of unusual pathways for conversion of these feedstocks was identified.

Lignocellusic biomass is composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose can be hydrolyzed to monosaccharides that include mainly glucose, xylose, and arabinose. The ^{13}C MFA has been used to study the xylose metabolism in non-model microorganisms. The flux results showed highly active PPP, EMP pathway, and TCA cycle in an extremely thermophilic, fast-growing *Geobacillus* strain [29]. Co-metabolism of glucose and xylose by an evolved *T. thermophilus* strain was elucidated by ^{13}C MFA, which showed that the fluxes through EMP pathway and non-oxidative PPP are changed flexibly with varying sugar availability, while the TCA cycle flux remains relatively constant [30]. The ^{13}C MFA has been used to identify the *in vivo* activity of the phosphoketolase (PKT) pathway, which was originally known as an alternative sugar catabolic pathway in certain *Lactobacillus* and *Bifidobacterium* species and filamentous fungi. The existence of a native PKT

pathway in clostridia and cyanobacteria was revealed by ^{13}C MFA [26,31,32,33]. For example, the PKT pathway was found to contribute to up to 40% of the xylose catabolic flux in the solvent-producing anaerobe *Clostridium acetobutylicum* [31].

Lignin is a complex aromatic polymer. Recently Varman *et al.* used the ^{13}C MFA to study the ligninolysis in a soil bacterium *Sphingobium* sp. SYK-6 [34••]. The results demonstrated that the catabolic pathway of vanillin derived from lignin is coupled with the tetrahydrofolate-dependent one-carbon metabolic pathway and serves as the major contributor of NAD(P)H synthesis [34••].

Sucrose is a disaccharide of glucose and fructose. Lange *et al.* investigated the sucrose metabolism in a rumen bacterium *B. succiniciproducens* DD1 by using ^{13}C MFA [35•]. According to the flux results, a previously unidentified fructokinase was overexpressed, resulting in improved succinate production [35•].

Utilization of renewable gases for chemical production has received increasing attention. The ^{13}C tracer experiments have been used to trace the incorporation of CO_2 into microbial metabolism. For example, Jones *et al.* used the ^{13}C tracer experiments to demonstrate the concurrent utilization of hexose and syngas ($CO/CO_2/H_2/N_2$ mixture) in the anaerobe *Clostridium ljungdahlii* [36••]. In addition, methane and methanol that can be obtained from natural gas are also attractive feedstocks. It has long been assumed that the core metabolism must be similar on the two substrates (methane and methanol) for the obligately methylotrophic bacterium Methylomicrobium buryatense 5GB1. However, by using the ^{13}C tracer analysis, Fu *et al.* recently showed an incomplete TCA cycle and increased fluxes through both the ED pathway and the partial serine cycle in methanol-grown cells, compared with the fluxes in methane-grown cells [37].

Understanding of industrial production processes

Industrial production processes typically involve the use of large-scale bioreactors and complex media. The ^{13}C MFA approach has been adapted to investigate the effect of industrial cultivation conditions on microbial metabolism. For example, Adler *et al.* used parallel labelling experiments with different ^{13}C tracers to quantify carbon fluxes in several strains of lactic acid bacteria (LAB) grown in the complex media of cocoa pulp fermentation under laboratory conditions [38]. The LAB strains exhibit significant differences in the central carbon metabolism. Moreover, the metafluxome of consortia of different LAB strains were also analyzed, indicating that a single strain, *Lactobacillus fermentum* NCC 575, plays a dominant role in the microbial consortium [38]. By using the same experimental strategy, the metabolism of acetic acid bacteria (AAB) during simulated cocoa pulp fermentation was also investigated [39]. AAB exhibit a functionally separated metabolism during co-utilization of the two major substrates, ethanol and lactate. Ethanol is used solely for acetate production, while lactate serves for biomass and acetone formation in AAB [39]. Recently, by combining parallel ^{13}C labelling experiments with multiple NMR-based and MS-based techniques, Schwechheimer *et al.* rigorously analyzed the contribution of various medium components to riboflavin (vitamin B_2) biosynthesis in the fungus *Ashbya gossypii*, an important industrial riboflavin producer [40••]. According to the flux results that suggest a one-carbon limitation, formate was added to the culture medium, leading to significantly improved production of riboflavin [40••]. Intracellular fluxes in *A. gossypii* were quantified, which showed a highly active TCA cycle during both the growth phase and the riboflavin production phase [41]. The importance of yeast extract in riboflavin production was revealed, which serves as the main carbon source for bacterial growth and also contributes significantly to the formation of riboflavin [41].

Transport limitations and non-ideal mixing in large-scale bioreactors can lead to gradients in availability of substrates and oxygen. To investigate the influence of substrate gradients on penicillin G-producing *Penicillium chrysogenum*, de Jonge *et al.* used dynamic ^{13}C tracer experiments and a hybrid modelling approach to determine the intracellular fluxes during cycles of substrate excess to substrate starvation [42]. This study indicated that the synthesis and degradation of storage compounds are increased in *P. chrysogenum*, which contribute to the decrease in productivity under dynamic conditions [42].

Conclusions and perspectives

Stable-isotope MFA is a powerful technique to elucidate microbial metabolism and to guide the development of appropriate metabolic engineering strategies for improvement of production performance. This technique has been extensively used for exploring novel pathways and revealing the contribution of various pathways to central metabolism in non-model microbes. However, understanding the control of fluxes is crucial for rational modification of metabolic pathways. Thus, integrating flux analysis with dynamic labelling experiments and targeted metabolomics would be extensively utilized to provide an insight into the flux control of metabolic pathways under *in vivo* conditions [27••,28••,43]. This approach can be used to pinpoint the bottleneck steps in important biosynthetic pathways such as polyketide or isoprenoid pathways, and in small-sized metabolic networks. The currently available software tools for nonstationary ^{13}C MFA could greatly aid in design of dynamic labelling experiments and analysis of measurement data of metabolite labelling kinetics [44••]. Moreover, improvement in analytical techniques, in particular, advances in mass spectrometers, allows accurate determination of the pool size and isotope labelling of a large

number of intermediate metabolites involved in various pathways [45••,46]. With the insightful information on the flux control of metabolic pathways, precise metabolic engineering can be performed to eliminate the pathway bottlenecks and enhance the biochemical production by microorganisms.

The study of eukaryotic cell metabolism needs quantification of fluxes in compartments. The same metabolite can exist in different subcellular compartments. However, the current methods of metabolite extraction before MS or NMR analysis allow only measurements of the average labelling pattern and metabolite level within a cell. Many ^{13}C MFA studies of *S. cerevisiae* rely on a compartmentalized model with constrains on localization of individual enzymatic reactions. However, such a metabolic model is rarely available for non-model eukaryotic microorganisms due to the lack of localization information [47]. Genetically encoded fluorescent metabolite reporters provide a means of compartment-specific quantification of several metabolites, such as NADH and NADPH [48,49]. Combination of these metabolite sensors with multiple isotope tracers could reveal the activities of metabolic reactions in different subcellular compartments [50,51]. An alternative approach is rapid organelle isolation based on immunopurification, which has been used in mammalian cells [52]. Nevertheless, there is need for caution about the possibility of metabolite alterations, either due to persistent enzymatic activity or metabolite leakage during the organelle isolation process. With the precise quantification of metabolic fluxes in compartments within eukaryotic cells, stable-isotope MFA could provide strong support for the efforts of harnessing organelles for metabolic engineering [53].

With the remarkable progress in microbiome studies, there is growing interest in metabolic interactions between species in microbial consortia. A current strategy is to isolate each strain and analyze the fluxome individually [38]. However, isolation attempts often fail. The metafluxome analysis using standard ^{13}C MFA generally cannot distinguish contribution from different species in consortium. With the advent of mass spectrometers with high-sensitivity and high multi-dimensional resolution, a peptide-based approach has been proposed, which may allow investigation of metabolic interactions in microbial communities [54,55]. In this approach, the detected peptide sequence can be used to identify the microbial species, from which the peptide originates, and the measured peptide labelling patterns are used to infer intracellular fluxes [54,55]. However, bioinformatics resources and generalized framework for spectra analysis need to be developed for this approach. A wide field of new discoveries can be expected with development in both experimental and computational methods for isotopically tracing metabolic pathways in microbial communities.

Conflict of interest statement

Nothing declared.

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