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Application of isotope labeling experiments and ^{13}C flux analysis to enable rational pathway engineering

Allison G McAtee[1], Lara J Jazmin[1] and Jamey D Young[1,2]

Isotope labeling experiments (ILEs) and ^{13}C flux analysis provide actionable information for metabolic engineers to identify knockout, overexpression, and/or media optimization targets. ILEs have been used in both academic and industrial labs to increase product formation, discover novel metabolic functions in previously uncharacterized organisms, and enhance the metabolic efficiency of host cell factories. This review highlights specific examples of how ILEs have been used in conjunction with enzyme or metabolic engineering to elucidate host cell metabolism and improve product titer, rate, or yield in a directed manner. We discuss recent progress and future opportunities involving the use of ILEs and ^{13}C flux analysis to characterize non-model host organisms and to identify and subsequently eliminate wasteful byproduct pathways or metabolic bottlenecks.

Addresses
[1] Department of Chemical and Biomolecular Engineering, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA
[2] Department of Molecular Physiology and Biophysics, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

Corresponding author: Young, Jamey D (j.d.young@vanderbilt.edu)

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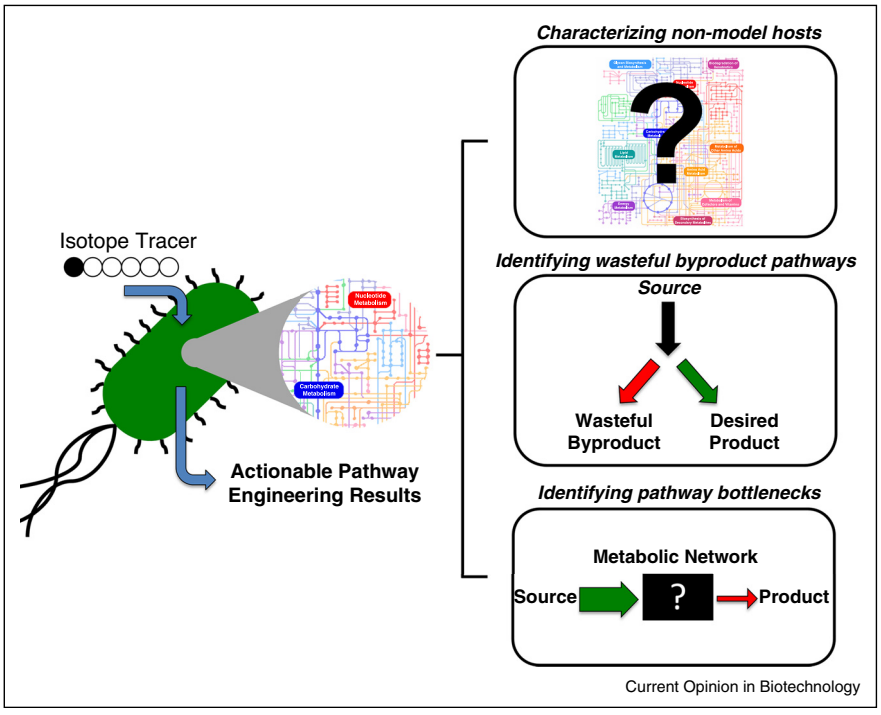
Introduction

Mammalian, plant, yeast, and bacterial cells are used as industrial hosts for the production of commodity chemicals, specialty chemicals, small-molecule drugs, therapeutic proteins, and other biomolecules of commercial interest [1••]. Because these processes rely on living cells as biocatalysts, they are often hindered by toxic byproduct formation, low product yield, and slow production rates. Genome-scale modeling, cell-wide 'omics' platforms, and high-throughput screening approaches have been developed to overcome these challenges by identifying genes that can be engineered to improve host cell performance. However, isotope labeling experiments (ILEs) and metabolic flux analysis (MFA) [2–4] have received limited attention in the biotech and biopharma industries to date, despite the fact that these approaches can provide direct readouts on *in vivo* metabolic pathway activities. This may be partly due to the fact that many companies lack the combined experimental and computational expertise needed to effectively analyze ILEs, but perhaps even more important is the perception that these studies are intrinsically difficult and there have not been enough success stories to justify the requisite effort. Therefore, the purpose of this review is to present some recent examples where ILEs and MFA have been successfully applied to close the 'design-build-test-learn' metabolic engineering cycle.

Applications of isotope labeling experiments in metabolic engineering

ILEs allow strain and process engineers to peek inside the black box of host cell metabolism by tracing the progression and rearrangement of isotopically labeled substrate atoms as they are metabolized through intermediary biochemical pathways [5–7]. For example, ^{13}C-labeled glucose tracers are commonly used to study central carbon metabolism in heterotrophic microbes, while ^{13}C-labeled amino acids and organic acids have been used extensively to assess citric acid cycle flux in mammalian cells. While ILEs can provide useful information about metabolic rates even in the absence of quantitative flux analysis [8•], several flux modeling approaches have been developed to extract additional information from the raw isotope labeling data: MFA [9,10,11•,12], isotopically nonstationary MFA (INST-MFA) [13–15], metabolic flux ratio (METAFoR) analysis [16], and kinetic flux profiling (KFP) [17]. Both KFP and INST-MFA compute fluxes based on transient (rather than steady-state) isotope labeling measurements, which reduces the duration of ILEs and provides increased flux resolution in most cases. METAFoR analysis and KFP rely on targeted isotope labeling measurements to assess the local flux distribution at specific network nodes. In contrast, MFA and INST-MFA are able to integrate multiple independent measurements into a comprehensive flux map while also identifying inconsistent data that would not be readily detected through a localized analysis. The quantitative flux estimates produced by these modeling approaches have direct biological meaning and can be compared across independent experiments. Flux information obtained from ILEs has been effectively applied to (1) characterize new host organisms, (2) identify wasteful pathways that limit product yield, and (3) identify metabolic bottlenecks that restrict production rate (Figure 1). By quantifying fluxes at each major node of

Figure 1



Examples of how ILEs can lead to actionable results for metabolic pathway engineering. ILEs can elucidate genetic targets for improving cell metabolism by enabling researchers to **(1)** characterize the metabolism of non-model organisms, **(2)** identify wasteful processes that lead to yield losses, and **(3)** identify kinetic bottlenecks in metabolic networks that limit production rate.

the metabolic network and determining how these fluxes become re-routed in response to targeted genetic or environmental perturbations, fundamental insights about network regulation can be obtained to guide further rounds of metabolic engineering.

Characterizing non-model host organisms

Model host organisms such as *Escherichia coli*, yeast, and CHO cells have been thoroughly studied and are commonly used as industrial hosts [18,19]. However, non-model host organisms often possess unique metabolic capabilities that make them better suited for production of specific compounds, especially biofuels and specialty chemicals, although the genetic tools required for metabolic engineering may still be underdeveloped. Another potential limitation is that the biochemical pathways of these non-model hosts are often incompletely understood. In this case, ILEs can be used to obtain missing biochemical pathway information by tracing how labeled substrates are broken down and rearranged as they traverse the metabolic network. For example, non-targeted tracer fate detection (NTFD) [20] can locate metabolite peaks within a chromatographic trace that exhibit a mass shift when cells are grown in the presence of an isotopic tracer. These peaks represent downstream intermediates or end-products derived from the tracer substrate, which can be later identified by comparison to standard libraries or by NMR spectroscopy. By collecting a time series of samples following tracer administration, it is possible to obtain progressive snapshots of isotope labeling over time, effectively mapping the progression of the tracer atoms through the unknown metabolic network. In this way, the intermediary pathways are elucidated, which may offer novel targets for improving host metabolism.

The scientific literature provides several illustrative examples where non-model hosts have been successfully profiled by ILEs. For example, Swarup *et al.* [21] used ^{13}C MFA to reconstruct the metabolic network of *Thermus thermophilus* HB8, a potential host for biotech applications due to its ability to thrive at high temperatures, and to characterize its growth phenotype. Recently, ^{13}C MFA was used to explore the largely unknown metabolic phenotypes of 25 marine microbes that use glucose from seawater as their main carbon source [22•]. Results from the ILEs showed that the majority of the marine bacteria utilized a completely different glucose catabolic pathway than their terrestrial counterparts, which was shown to provide resistance against oxidative stress typically found in the marine environment. There has also been increased interest in the use of photosynthetic or methanotrophic organisms as production hosts because of their advantageous ability to use CO_2 or natural gas, respectively, as their sole carbon source. Cyanobacteria and

algae have been thoroughly probed and characterized through the application of ILEs under heterotrophic, mixotrophic, and autotrophic conditions [23–29]. The metabolic networks of methanol-consuming *Corynebacterium glutamicum* [30] and *Pichia pastoris* [31–34] have also been established through the utilization of ILEs. Comprehensively profiling the metabolic capabilities of these hosts has paved the way for rational strain engineering in the future.

Transient ILEs in combination with INST-MFA or KFP have been recently used to characterize flux phenotypes in more complex photosynthetic systems, such as the plant *Arabidopsis thaliana*. These experiments have been performed under a variety of experimental conditions, providing new understanding of plant leaf metabolism that could not have been assessed using steady-state ILEs [35•,36,37•]. ILEs have also been applied to transgenic *Nicotiana tabacum* (tobacco) hairy roots as a means to identify targets for increasing geraniol production [38]. These studies represent an important step toward improving product yield and/or biomass productivity in plant hosts, which are not amenable to high-throughput engineering approaches.

Enhancing product yield by identifying wasteful byproduct pathways

A first logical step in many host engineering workflows is to increase product yield by eliminating unwanted byproduct pathways that siphon carbon away from productive pathways. This is because substrate cost is a major driver of bioprocess economics, and commercial viability oftentimes cannot be achieved until product yield approaches the theoretical limit. Furthermore, byproducts may become toxic to the cell culture, thereby inhibiting cell growth and productivity. For example, 'overflow' pathways such as ethanol in yeast [39,40], acetate in *E. coli* [41,42], and lactate in mammalian cells [43–45] can often restrict high-yield production in these hosts, and significant efforts have been devoted to reducing flux into these pathways by either metabolic engineering or bioprocess optimization. ILEs provide a definitive approach to trace the flow of carbon from substrates into unwanted byproducts and to detect when byproduct pathways become activated. For example, ^{13}C MFA recently identified increased acetate production and other flux shifts caused by accumulation of octanoic acid (C8) in the medium of *E. coli* cultures [46]. Based on these results, metabolic engineering strategies to divert carbon flux away from acetate production were proposed as a way to improve product yield and titer.

Maximizing yield is critically important in the production of commodity chemicals and biofuels, where profit margins are razor thin. Ranganathan *et al.* [47] used ^{13}C MFA and genome-scale modeling to identify chain-length-specific genetic manipulations to increase production of various fatty acid species in *E. coli*. They successfully engineered *E. coli* strains that produced ~39% of the theoretical maximum yield of C_{14-16} fatty acids, as opposed to the wild-type species that produced only ~11% of the theoretical yield. Similarly, when the newly discovered bacterial host *Basfia succiniciproducens* [48] was determined to have a natural affinity for bio-succinate production, Becker *et al.* [49] explored its intracellular metabolism using system-wide ^{13}C MFA. The analysis elucidated undesired fluxes occurring through both the pyruvate-formate lyase (PflD) and lactate dehydrogenase (LdhA) pathways, which led to a double knockout strain that drastically reduced lactate production, completely eliminated formate production, and improved succinate yield by 45% compared to the wild-type strain [49]. These studies provide excellent examples of how ILEs can be applied to 'close the loop' in the design-build-test-learn metabolic engineering cycle.

Metabolic engineering can also be used to improve the production of large biomolecules. Nocon *et al.* [50••] utilized ILEs and MFA to identify overexpression and knockout targets in *Pichia pastoris* to overcome the metabolic burden of recombinant protein expression. Specifically, the ILEs pinpointed fermentation pathways as knockout targets, and the engineered strains exhibited significant increases in product yield after these pathways were removed [50••]. Similarly, Zhou *et al.* [51] reduced lactate production in CHO cells by 90% by knocking down lactate dehydrogenase and pyruvate dehydrogenase kinases to ultimately increase specific productivity of a monoclonal antibody (mAb) by 75% and 68%, respectively, when compared to the parental line.

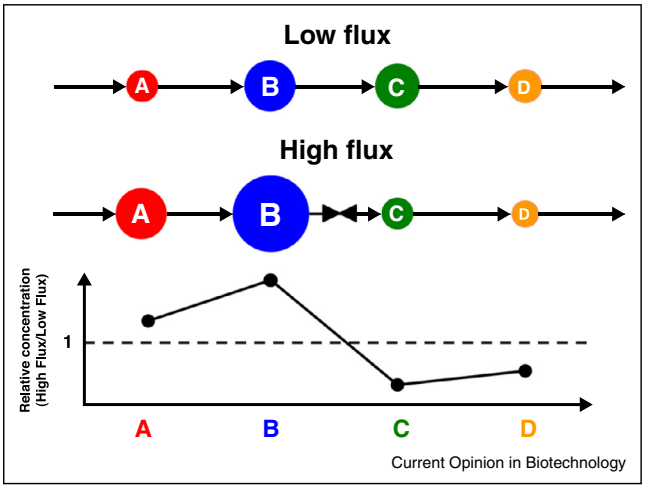
Enhancing production rate by identifying pathway bottlenecks

Once economically viable product yields and titers have been achieved, the next step in developing an industrial host often involves increasing the rate of product formation so that the required bioreactor capacity can be minimized. This typically involves identifying specific bottleneck enzymes that can be (1) overexpressed, (2) deregulated, or (3) bypassed to improve productivity. ILEs are extremely useful for comparing the flux phenotypes of producing versus non-producing strains to identify principal nodes where flux is re-routed to accommodate increased product formation. Amplifying the rate-limiting enzymes that control flux at these nodes can be an efficient strategy to 'push' more flux toward product formation. A classic example is the application of ILEs to identify pyruvate carboxylase as an overexpression target to debottleneck lysine production in *C. glutamicum* [52]. A more recent example is the finding that CHO cells exhibit increased citric acid cycle (CAC) and pentose phosphate pathway (PPP) fluxes when engineered to express mAbs at high yield [45,53]. Engineering increased flux into these pathways has been hypothesized as a possible strategy to improve specific mAb productivity [19]. Similarly, Nie *et al.* [54] used ILEs to identify upregulation of CAC as a potential target in

P. pastoris for increased heterologous protein synthesis, while Toya *et al.* [55] and Wasylenko *et al.* [56] identified PPP flux as a target for increased cellulase production in *Bacillus subtilis* and lipid production in *Yarrowia lipolytica*, respectively. Comparison of ILEs in producing versus non-producing strains has also pinpointed increased CAC flux as being important for *S*-adenosyl-L-methionine (SAM) production in *Saccharomyces cerevisiae* [57] and ethylene production in *Synechocystis* 6803 [58]. Finally, ILEs of xylose-consuming *S. cerevisiae* strains have identified a metabolic bottleneck in lower glycolysis that leads to less efficient utilization of xylose relative to glucose [59,60].

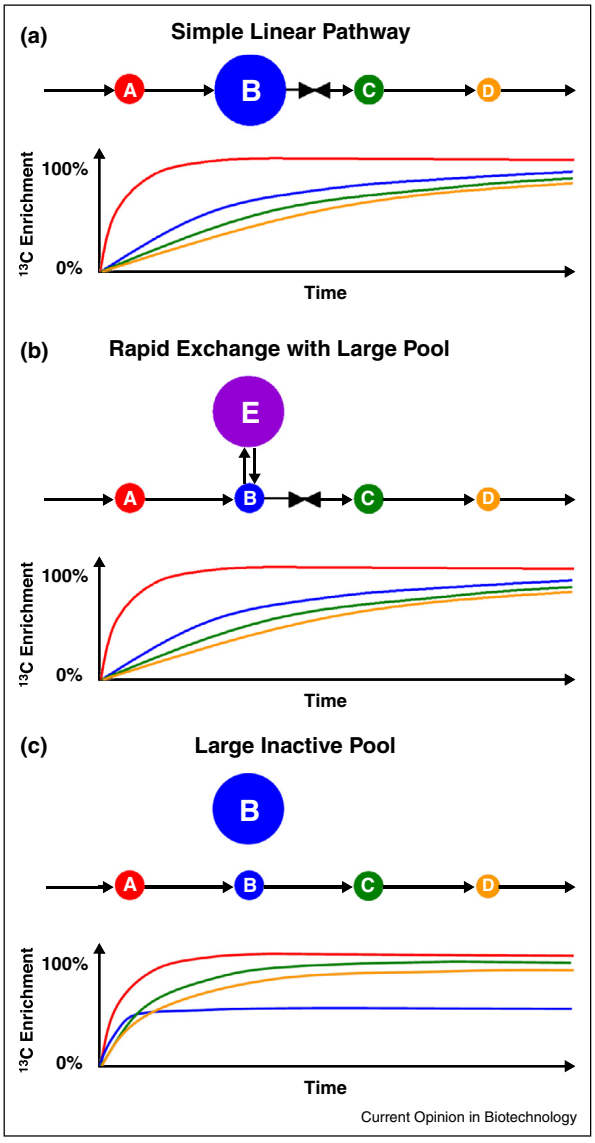
Several recent studies provide full-circle examples of how ILEs can be used to debottleneck metabolic pathways. Klein *et al.* [61••] performed a ^{13}C MFA experiment on maltase-secreting *Schizosaccharomyces pombe*, which identified metabolic bottlenecks at the PPP, CAC, and pyruvate nodes. To overcome these bottlenecks, the authors fed acetate to the cultures as an additional carbon source. With dual feeds of glucose/acetate and glycerol/acetate, maltase production increased 1.5-fold and 2.1-fold higher when compared to glucose-grown cultures, respectively. Lysine production in *C. glutamicum* has also continued to provide illustrative examples of pathway debottlenecking based on ILEs, with recent studies showing how rational engineering of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and succinyl-CoA synthetase genes led to 60% higher lysine yields relative to parental strains [62,63•].

Figure 2



Example of using crossover analysis to identify bottleneck steps in a linear pathway. The steady-state concentrations of pathway intermediates are measured under two different flux conditions: a low-flux steady state and a high-flux steady state. The concentration fold-change is plotted for each intermediate in order of its position along the pathway. Flux-controlling steps are identified by a crossover of the dotted line (from a fold-change >1 to <1). In this example, there is a flux bottleneck in the step from B to C that manifests in the high-flux steady state.

Figure 3



Example of using dynamic ^{13}C labeling experiments to complement metabolomics analysis. A step-change in ^{13}C enrichment is achieved by introducing an isotope tracer (e.g. ^{13}C-labeled glucose) to a culture at steady state. **(A)** In a simple linear pathway, the enrichment of each metabolite intermediate is determined by its turnover rate (flux/concentration) and by the enrichment of its upstream precursor. The labeling dynamics should confirm the metabolomics measurements by exhibiting a time-delay at any step where a large intermediate pool exists (e.g. immediately upstream of a pathway bottleneck). **(B)** Different pathway architectures may give rise to discordance between metabolomics measurements and ^{13}C-labeling trajectories. For example, if the concentration of intermediate B is small but it is in rapid equilibrium with another high-concentration metabolite E, the labeling data may reflect a bottleneck at the B → C step even though the metabolomics data do not. **(C)** The opposite situation can arise if there is a large segregated pool of B that is metabolically inactive. In this case, the metabolomics data will indicate a bottleneck at the B → C step, but the B labeling data will exhibit a fast initial enrichment followed by a plateau at <100% enrichment due to mixing of active and inactive pools upon cell extraction.

Conclusions

There are now many examples of how ILEs and flux analysis can be used to identify specific pathways that can be targeted for up- or down-regulation in order to improve host performance. However, it is not always clear which particular enzymatic step within a given linear pathway is rate-controlling, and typical steady-state ILEs alone cannot provide this information. In some cases, it is necessary to manipulate each intermediate step in a systematic way to arrive at such information [64]. On the other hand, integrating flux analysis with targeted metabolomics and 'crossover analysis' (Figure 2) may become a viable approach to pinpoint specific enzymatic bottlenecks within linear pathways [65]. Combining dynamic ILEs with metabolite pool size measurements may further suggest latent pathway interactions, such as exchange with metabolites in adjacent pathways or the presence of large inactive pools, which can obfuscate the interpretation of metabolomics data sets (Figure 3). Compartment-specific 'sensor metabolites' may also be used to deconvolute metabolite contributions from different organelles [8•].

In order for ILEs to become a more widely used tool for pathway engineering, it is important for practitioners to properly balance the trade-off between information content and experimental difficulty according to the objectives of the study. For example, it is unnecessary to perform a comprehensive flux analysis if only the branch ratio at a single principal node is known to control product formation. In this case, a localized flux analysis such as METAFoR or KFP may be more appropriate for rapid strain optimization. In other cases, a qualitative analysis of the isotope labeling data (e.g. comparison of ^{13}C enrichments or labeling patterns across strains) may be sufficient to guide pathway engineering without the need for quantitative flux modeling, which requires expertise that may not be readily available in many labs. Understanding how to match the scope of the experimental and computational effort to the needs of the study is of paramount importance in order to avoid wasted time and effort.

In this article, we have highlighted several recent examples from the literature that illustrate how ILEs can be used to close the design-build-test-learn metabolic engineering cycle. We expect that the use of ILEs and flux analysis in the biotech and biopharma industries will become increasingly established as the track record of success stories continues to grow. These studies can aid researchers in characterizing the metabolic capabilities of novel host organisms. They can also help to identify wasteful pathways that divert energy and raw materials away from product formation, or bottleneck enzymes that limit production rate. In the future, these studies may also be used to investigate the role of specific metabolic pathways in controlling product quality, such as the glycosylation profile of recombinant proteins [66]. By providing actionable information about cellular metabolic rates that cannot be obtained from alternative methods, ILEs enable researchers to rationally engineer improved cellular hosts for biomanufacturing. This can ultimately lead to reduced time-to-market, increased production capacity, lower capital requirements, and reduced raw material costs.

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