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PROBLEMS, PROSPECTS

Metabolic Flux Analysis using ^{13}C Isotopes: III. Significance for Systems Biology and Metabolic Engineering[1]

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Abstract—At present, ^{13}C-MFA is a primary method for quantitatively characterizing intracellular carbon fluxes in cells *in vivo* under steady-state conditions. The method has been successfully used to investigate both the fundamental characteristics of prokaryotic and eukaryotic cell metabolism and to improve producer strains for more than twenty years. This publication is the last in a set of reviews that describe various aspects of the method. Here, the authors highlight recent achievements that involved using ^{13}C-MFA to elucidate bacterial metabolism. Analyses of well-characterized bacterial model strains revealed that central metabolism robustness is provided by a set of alternative metabolic pathways; these analyses also helped develop a better understanding of the physiological significance of these pathways and identified previously unknown functions of well-studied metabolic pathways. Several examples of ^{13}C-MFA-based fundamental investigations of poorly characterized bacteria are also analyzed. In applied investigations, flux analysis of strains that produce amino acids, vitamins and antibiotics indicated targets for modifications, suggested unconventional metabolic engineering approaches, and, most importantly, confirmed their utility. In the last section of this article, ^{13}C-MFA prospects, including the monitoring of the dynamics of metabolic flux distribution during culture growth, are discussed.

Keywords: flux coupling, futile cycles, metabolic pathway activity, NADPH balance
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This part is the third of a review of a modern method in systems biology—fluxomics—and one of its most developed approaches, metabolic flux analysis using ^{13}C isotopes (^{13}C-MFA). In the first two parts, the basic principles of the ^{13}C-MFA technique, including the generation of simplified metabolic models and the experimental and mathematical aspects of flux calculations and statistics, have been discussed [1, 2]. This final part briefly describes the main achievements in fundamental and applied investigations that used ^{13}C-MFA. In accordance with the professional interests of the authors, this review focuses on biotechnologically important bacteria. However, ^{13}C-MFA is intensively used on yeast [3], plant [4], mammalian [5] and other cellular systems [6].

^{13}C-MFA CONTRIBUTIONS TO FUNDAMENTAL INVESTIGATIONS

During the past decade, much attention has been paid to ^{13}C-MFA of the metabolic changes that result from the precise inactivation of genes that encode central metabolism enzymes and regulatory proteins. The main aim of such studies was to reveal the roles of

[1] The article was translated by the authors.

Abbreviations: CM—central metabolism; AC—acetate; ACALD—acetaldehyde; ACCOA–acetyl-coenzyme A; AKG–2-oxoglutarate; CoA—coenzyme A; C2/C3—two-carbon and tri-carbon fragments involved in the enzymatic reactions catalyzed by transaldolase and transketolase of the non-oxidizing branch of the PP pathway according to the ping-pong mechanism; CIT—citrate; 13C-MFA (13C-based metabolic flux analysis)—metabolic flux analysis in experiments using substrates containing 13C isotopes of carbon; E4P—erythrose 4-phosphate; ED pathway—Entner—Doudoroff pathway; EMP pathway—Embden–Meyerhof–Parnas pathway; DHAP—dihydroxyacetone phosphate; ICIT—isocitate; F6P—fructose 6-phosphate; FDP—fructose 1,6-diphosphate; FUM—fumarate; G6P—glucose 6-phosphate; G3P—glyceraldehyde 3-phosphate; GC–MS—Gas chromatography–mass spectrometry; GLC—glucose; GLX—glyoxylate; KDPG—2-keto-3-deoxy-phosphogluconate; LC—liquid chromatography; LL-DAP—L,L-diaminopimelic acid; MAL—malate; MS/MS—tandem mass spectrometry; MTHF—methyltetrahydrofolate; NAD(H)—oxidized (reduced) nicotinamide dinucleotide; m so-DAP—meso-diaminopimelic acid; OAA—oxaloacetate; PP pathway—pentose phosphate pathway; PRPP—5-phosphoribosyl-1-pyrophosphate; PTS—phosphoenolpyruvate-dependent phosphotransferase system; Pyr—pyruvate; P5P—phosphorylated pentasaccharide pool (R5P, Ru5P, and Xu5P); PEP—phosphoenolpyruvate; 3PG—3-phosphoglycerate; R5P—ribose 5-phosphate; Ru5P—ribulose 5-phosphate; S7P—sedoheptulose 7-phosphate; SAKP—N-succinyl- [alpha]amino-oxopimelate; SDAP—succinyl-diaminopimelic acid; SUC—succinate; SUCCOA—succinyl-coenzyme A; TCA—Tricarboxylic acid cycle; THDP—tetrahydrodipicolinate; Xu5P—xylulose 5-phosphate; 6PG—gluconate 6-phosphate; 13DPG—1,3-diphosphoglycerate. Amino acids are designated according to the three-letter code.

selected metabolic and regulatory pathways in response to specific genome rearrangements and changing environmental conditions and to find compensatory reactions that were used during adaptive evolution. In the case of *E. coli*, many studies have been performed in strains from the Keio collection [7]. The first targets for ^{13}C-MFA were mutants in which genes of key central metabolism enzymes or of enzymes that catalyzed reactions at main metabolic branch points were inactivated. Analysis of the PTS-mutants, i.e., mutants that were unable to transport and phosphorylate glucose via the phosphoenolpyruvic acid (PEP)-dependent phosphotransferase system, revealed that they activated alternative pathways of carbohydrate utilization [8, 9]. The *pgi* and *zwf* genes code for enzymes that catalyze the first reactions of the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PP) pathways, respectively. It is clear that inactivation of the one of these genes will lead to redirecting the total carbon flux to an alternative pathway at the EMP/PP pathways branch point. However, flux redistribution in other parts of the central metabolism was not as easily predicted [10, 11]. In a *pgi*-mutant, after glucose transport and phosphorylation, all carbon flux was directed to the PP pathway, and almost no flux went to the Entner-Doudoroff (ED) pathway. Simultaneously, a decrease in carbon flux through the isocitrate dehydrogenase pathway accompanied an increase in glyoxylate shunt flux. The authors discussed that such a flux redistribution compensates for redundant NADPH synthesis, which is toxic for cells. It was also difficult to predict that inactivation of the PEP kinase-catalyzed pyruvate (PYR) synthesis, followed by synthesis of Ac-CoA and fermentation products, could lead to increased PP pathway flux [12, 13]. These examples of ^{13}C-MFA of mutants with knocked-out central metabolism genes are only a few of those that resulted in interesting findings.

Additionally, a set of studies using ^{13}C-MFA of strains with mutations in global regulation genes [see 14, 15 for example] are published. Among them are investigations into flux redistribution resulted from altered expression levels in two-component systems. In particular, an analysis of *arc*A-*arc*B system inactivation under different aeration conditions (anaerobic/microaerobic [16] and aerobic [17]) gave interesting results. Many ^{13}C-MFA studies confirm the plasticity of bacterial metabolism when perturbed. Bacterial adaptation to changing environmental conditions and gene expression results from the presence of different "shunts" and alternative metabolic pathways in branched metabolic networks [18, 19]. In response to inactivation of a key gene, the cell first activates known shunts (ED pathway, glyoxylate shunt, etc.) that are inactive when the cells are normally cultivated on glucose. However, continued adaptive evolution can lead to simultaneous repression of these latent pathways and activation of alternative pathways that improve the effectiveness of the metabolism [18].

^{13}C-MFA finds new significance in well-known central metabolism reactions. For example, in some bacteria, the PP pathway not only synthesizes precursors and regenerates NADPH but can function as the main pathway of glucose catabolism [20]. Two isoforms of *E. coli* transhydrogenases (membrane-bound PntAB and cytoplasmic UdhA) that catalyze electron transfer from NADH to $NADP^+$ and vice versa have different physiological roles. ^{13}C-MFA of specially constructed mutants showed that the H^+-dependent PntAB tranhydrogenase restores $NADP^+$ in *E. coli*. In contrast, energy-dependent NADPH oxidation by UdhA helps cells to avoid NADPH toxicity when it is in excess [10]. Inactivation of *pnt*AB in *E. coli* cells increased the PP pathway flux to compensate for a decrease in NADPH supply. In such a case, redundant carbon is returned to the EMP pathway (at the stages of G6P and G3P synthesis) via reactions in the non-oxidative PP pathway (Fig. 1, [19]).

One of the meaningful ^{13}C-MFA contributions to fundamental cell metabolism studies is the identification of "new" reactions that were not earlier suggested in organisms with well-characterized metabolisms. Here, the term "new" is used with a special meaning. ^{13}C-MFA uses a user-generated metabolic model that includes all biochemical reactions that are part of the metabolism that is being studied. Flux calculations numerically characterize carbon fluxes in reactions. If a calculated flux is zero (within confidence intervals), the corresponding reaction, which was introduced in the model, is inactive under the investigated conditions (see Fig. 2; [21]).

Thus, ^{13}C-MFA cannot predict "new" biochemical reactions that are not included in the metabolic model. The method can find combinations of flux parameter values that best fit the simulated data points to those of the experiments. The contribution of ^{13}C-MFA to fundamental investigations should be evaluated based on this specific meaning of the word "new".

^{13}C-MFA confirmed that "forward" and "reverse" reactions simultaneously function even then catalyzed by different enzymes. For example, EMP pathway reactions have been observed to be concurrently active with gluconeogenesis; coupling between terminal parts of the EMP pathway with the TCA cycle forms the so-called PEP-PYR-oxaloacetate (OAA) node. Such interconnections between different parts of metabolism provide cell plasticity under changing environmental conditions at the expense of ATP hydrolysis [23]. Under glucose-limited conditions, oxidation and anaplerosis occur by the PEP-glyoxylate shunt rather than the PEP-carboxylase reaction that is normal under glucose-rich conditions (Fig. 3; [24]). Accurate co-factor (ATP, HADH, and NADPH) balancing that is based on calculated carbon flux distri-

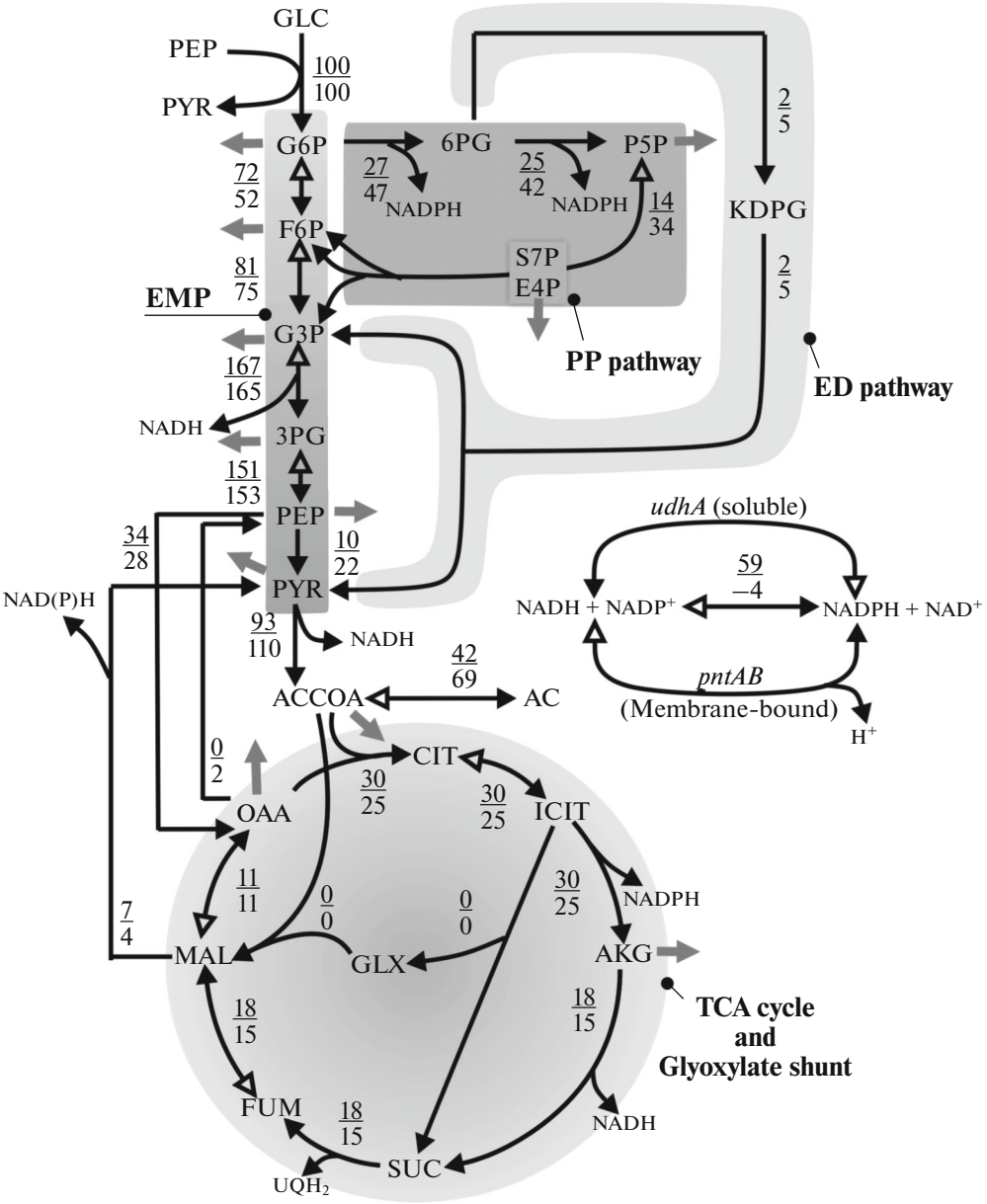
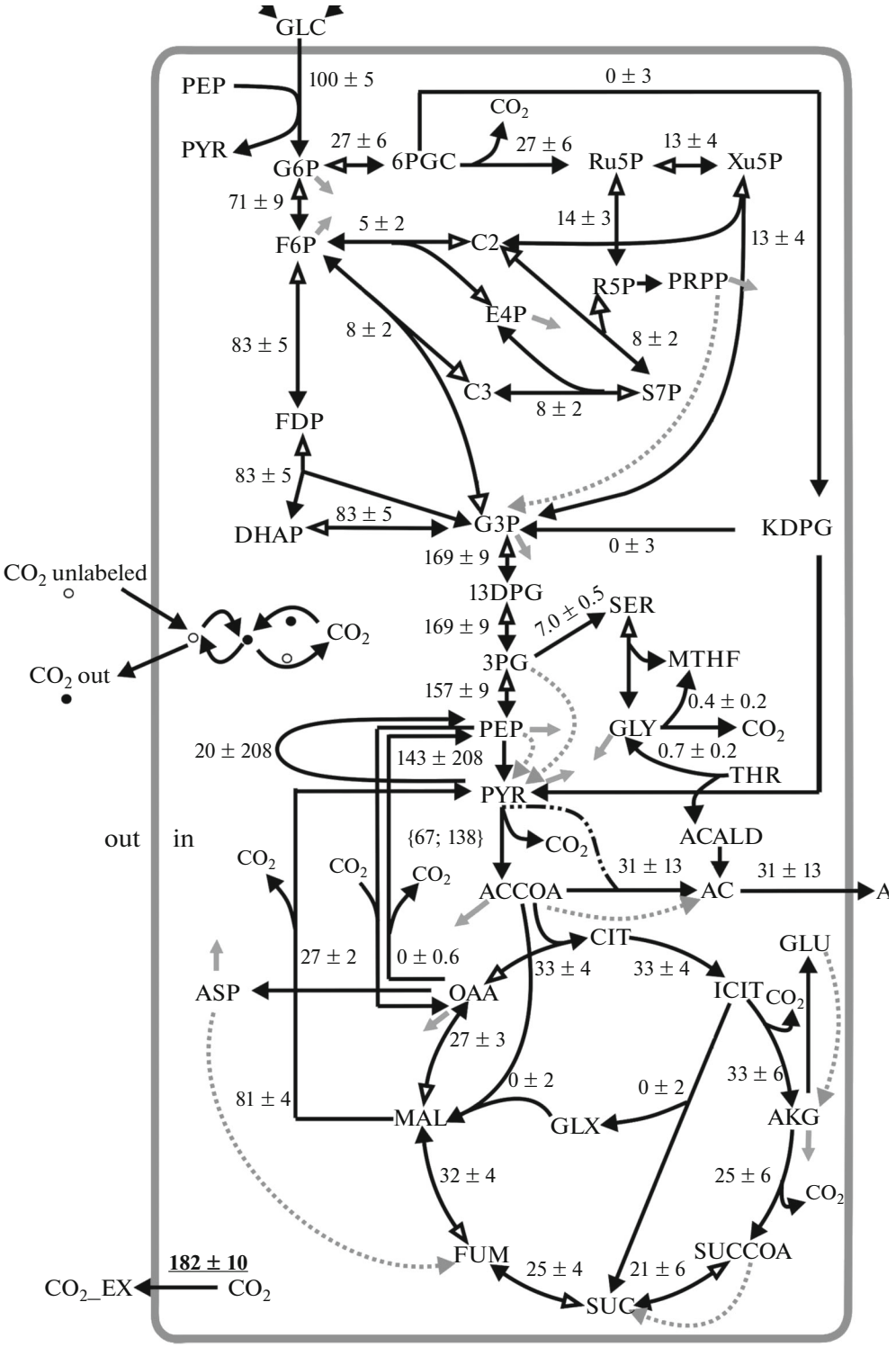


Fig. 1. ^{13}C-MFA results of carbon flux distribution in an *E. coli* wt strain (top values) and its Δ(*udhA-pntAB*) derivative (bottom values) that were grown aerobically on minimal media with glucose as the sole carbon source [19]]. Only net flux values are displayed. Flux values are normalized by the mean values of the measured specific glucose uptake rate (7.5 ± 0.5 (wt) and 8.4 ± 0.2 mmol gDW^{-1} h^{-1} (mutant)), which has been set to equal 100%. Reversible reactions are represented by bidirectional arrows. The flux values of these reactions are equal to the module of the difference between the forward and reverse reaction rates. The direction of the net flux of reversible reactions is indicated by the painted end of the bidirectional arrow. Biomass formation is accounted trough the drain of corresponding precursors.

butions in different bacteria revealed latent pathways of their biosynthesis and equilibration [25].

Many studies have reported carbon flux distributions in well-characterized microorganisms such as *E. coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae*. Special attention has been paid to systematic metabolic engineering of high-performance producer strains [26–28]. Recent progress in ^{13}C-MFA accuracy and precision enabled the use of this method for investigation into poorly characterized organisms [29, 30].

Flux analysis of *Pseudomonas aeruginosa* model strain PAO1 and its 17 clinical isolates revealed that all strains utilize the ED pathway for glucose metabolism and then perform respiratory metabolism without acetate synthesis. This pathway synthesizes enough NADPH to exceed anabolic requirements and could serve to protect cells against the oxidative stress. At the same time, the PP pathway and, in particular, its non-



oxidative branch are exclusively used for biomass precursor synthesis (E4P and P5P). Another feature that was reported was that the flux distribution at the glycolytic part of metabolism was very similar among strains. The flux variation through the pyruvate branch point, TCA cycle and glyoxylate shunt probably resulted from individual adaptation of the isolates during infection [30].

Fig. 2. Carbon flux distribution in MG1655 strain as estimated (by the authors) from labeling experiments with [20%-^{13}U]/[80%-^{12}U]-glucose and using OpenFLUX2 [21] software. Cells were grown aerobically on minimal media with glucose as the sole carbon source. The metabolic map includes the main central metabolic pathways and accounts for the following important features: separate fluxes for each label that was used in experiment; ping-pong kinetic mechanisms of transaldolase and transketolase reactions; carbon exchange with atmospheric carbon dioxide and coupling of biomass synthesis reactions with central metabolism. Biosynthetic pathways of AA and nucleoside synthesis are expressed explicitly. Summarizing the biomass synthesis equation, the consumption of materials by protein, RNA and DNA synthesis is given by drain of the corresponding building blocks. Biosynthetic requirements of other biomass compartments are accounted for by the drain of the corresponding precursors. The flux values that correspond to minimal deviation between experimentally measured and simulated mass isotopomer distributions of the proteinogenic AAs are considered to be the best estimate of intracellular flux distributions. Estimation results are represented only for net flux values. Flux values are normalized by the measured glucose uptake rate, which has been set equal to 100%. One-fourth of the 95% confidential interval value is used to characterized the precision of the calculated flux parameters [22]. Confidential intervals have been determined by the "discarding" strategy of a Monte-Carlo-based approach [21]. In the case of the pyruvate dehydrogenase reaction, only the upper and lower boundaries of the possible flux values could be calculated (displayed values represent the 95% confidence interval) because of the presence of two possible, but not distinguished, pathways of acetate synthesis: Pta-Ack and Pox.

The first example of the primary use of the ED pathway in glucose catabolism in *Xanthomonas campestris* pv. campestris was shown by ^{13}C-MFA. This bacterium produces polysaccharide xanthan gum, which used in the food industry as a stiffener and in cosmetology as a skin moistener. In this study, flux analysis was based on a simplified metabolic model that possessed all three glycolytic pathways (EMP, PP and ED) that were proposed in a genome-scale metabolic reconstruction of this bacterium [31].

^{13}C-MFA is useful for verifying the presence of metabolic pathways that are hypothesized to exist in poorly studied organisms. Two studies that were performed in the group of Prof. Antoniewicz are interesting. The first study is the metabolic characterization of the extremely thermophilic bacterium *Thermus thermophilus* strain HB8, which is one of the most well-characterized bacterium from a protein structure point of view [32]. The simplified metabolic model was proposed based on a list of all potential biochemical reactions; this list had been derived from a metabolic genome-scale reconstruction that contained additional typical glycolytic pathways (the ED pathway and the oxidative branch of the PP pathway) that have no protein homologs in the genome of this bacterium. ^{13}C-MFA revealed that there was no carbon flux in these hypothesized pathways, confirming the absence of the corresponding enzymes. Biomass precursors

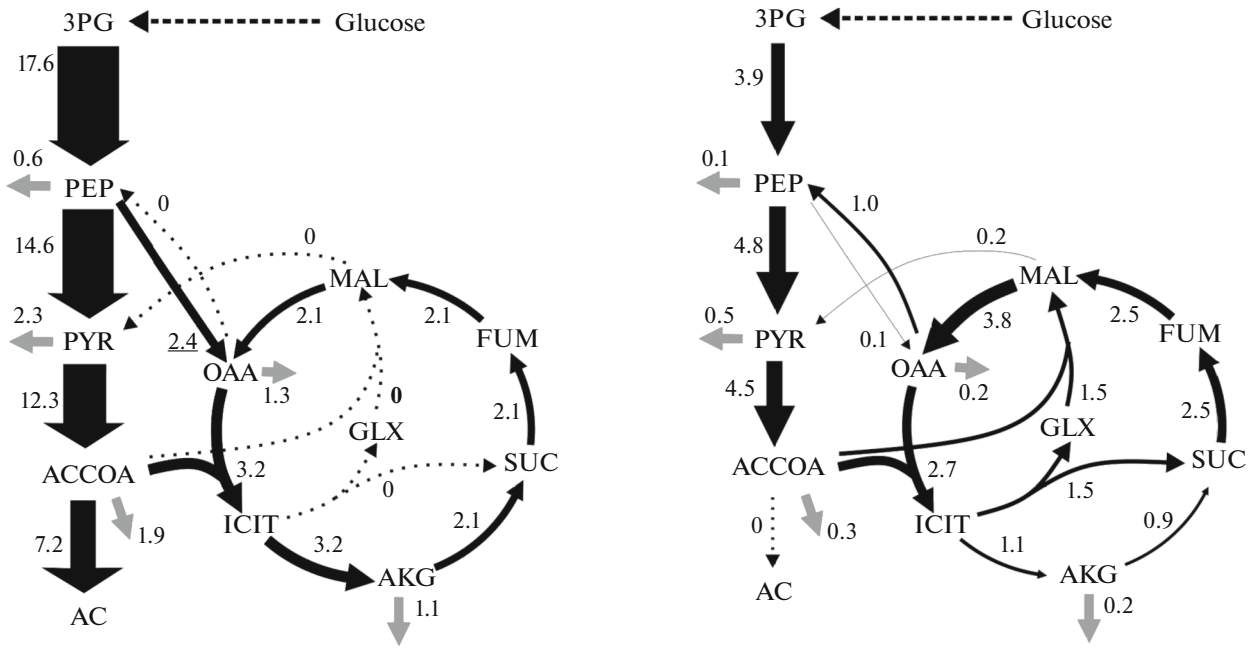


Fig. 3. Results of a ^{13}C-MFA of a carbon flux distribution in *E. coli* strain MG1655 that was grown on minimal media with glucose in glucose-available batch (left) and glucose-limited chemostat (right) cultivation conditions [24]. Part of the central metabolism including TCA, glyoxylate shunt, PEP-carboxylase, PEP-carboxykinase and malic enzyme reactions is represented. Specific glucose uptake rates were 11.3 ± 0.2 and 2.3 ± 0.1 mmol g^{-1} h^{-1}, respectively. Calculated fluxes are displayed as their absolute values (mmol g^{-1} h^{-1}).

(E4P, P5P) are synthesized by the non-oxidative PP pathway in this bacterium. Meanwhile, carbon flux through the glyoxylate shunt and MAL → PYR and OAA → PEP reactions, which could be catalyzed by enzyme homologs that have been identified in the *T. thermophilus* genome, was almost zero (within confidence intervals). Thus, it could be concluded that corresponding pathways are inactive under the tested conditions.

The other example of using ^{13}C-MFA to verify a metabolic model is related to the metabolism of *Clostridium acetobutylicum* cells [33]. Here, Parallel Labeling Experiments (PLEs) were used for model verification. In a PLE approach, cells are cultivated under the same conditions except for the use of different tracers in different samples. Then, all experimental data are used in flux calculations via a unique metabolic model. The verification of a metabolic model was accomplished by the step-by-step modification of the model (addition of biochemical reactions) to minimize weighted residuals between the measured experimental data and the data that has been simulated using the model (see [2]). After the optimal model has been identified, the model is minimized by eliminating reactions that exhibit "zero" flux.

Flux analyses that were based on minimized model provided interesting insights into *C. acetobutylicum* metabolism. Despite the fact that TCA cycles that operate in the "oxidative direction" have been reported, no carbon flux was observed between alpha-ketoglutaric acid (AKG) and succinyl-CoA (SUCCOA), succinate (SUC) and fumarate (FUM). The conversion of SUCCOA to SUC is also catalyzed outside the TCA cycle [33], and no MAL to OAA conversion occurred. Instead, PYR-FUM interconversion was detected to occur via aspartate. As a result, a gene that codes for citramalate synthase has been identified; this enzyme probably catalyzed the first stage of isoleucine synthesis in *C. acetobutylicum*. This study illustrates the great potential of PLE-based ^{13}C-MFA not only to confirm the adequacy of proposed metabolic models but also to allow model modification to occur independently from biochemical studies.

Two types of cultivation conditions are routinely used for ^{13}C-MFA: continuous cultivation under glucose-limited conditions and batch cultivation in cultures with glucose. Cell responses to different perturbations strongly depend on cultivation conditions. For example, 24 *E. coli* single knock-out mutants possessed rather stable flux distribution profiles (i.e., minor differences in flux parameter values) under chemostat cultivation conditions regardless of which gene had been knocked out [14]. Greater differences were detected in cultures of the same mutant under batch cultivation conditions [34]. The dependence of fluxes on cultivation conditions complicates the analysis of effects of genetic or environmental perturbations on carbon flux distribution [18]. However, ^{13}C-MFA of genetically modified strains is the only method to in vivo characterize their metabolic state. It is necessary to note that a continuous increase in ^{13}C-MFA precision [22] has provided the opportunity to perform label experiments on a miniaturized scale with high reproducibility [35, 36].

^{13}C-MFA CONTRIBUTIONS TO APPLIED STUDIES

The usefulness of quantitating carbon fluxes in the design of producer strains was clear at the first stage of ^{13}C-MFA methodology development. However, the number of studies in which ^{13}C-MFA plays a crucial role in producer strain improvement is limited. The reason for this limit has been suggested [37]: "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."

However, the use of ^{13}C-MFA in applied research is becoming more frequent. Flux analysis helps to reveal "bottlenecks," co-factor disbalances, energy deficiencies and stress responses [28]. This information, in turn, supports the development of strategies to overcome these challenges and to improve strain performance (see for example [38, 39]).

In the 1990s, most of the high-volume microbe industry was based on *Bacillus* and *Corynebacterium* producer strains. It is not surprising that these bacteria became a platform for ^{13}C-MFA method development. It is not clear why no attention was paid to actinomycetes, which produce one of the main products of the microbiological industry: antibiotics. At the beginning, some studies in Great Britain were focused on the fluxome of actinomycetes [40]. At the same time, strong teams that had wide contacts among pharmaceutical companies investigated antibiotic producers. Nevertheless, studies from Germany and Switzerland contributed greatly to ^{13}C-MFA methodology development by partly funding from biotechnology companies [41–43]. Later, teams from USA [44–46], Japan [47, 48] and other developed countries initiated ^{13}C-MFA research.

However, it should be noted that American scientists began fluxome research and contribute greatly to the development of the mathematical approaches that are used in ^{13}C-MFA [51–54]. According to published literature, the flux analysis of actinomycetes was initiated later, after method verification had been performed on other organisms [55, 56].

An interesting fact is that the first investigations of flux distributions in *Corynebacterium* amino acid (AA) producers [41, 57, 58] appeared before the *Corynebacterium glutamicum* genome sequence [59] and genome-scale metabolism reconstruction [60] were

published. At this time, ^{13}C-MFA has been successfully used to analyze flux distributions in microorganisms that have been metabolically engineered for the production of AAs (lysine (Lys) [35, 39, 41, 61, 62]; glutamate [63, 64]; phenylalanine [65], methionine [66], valine [67]), 1,3-propadiol [68], ethanol [69, 70], ethyl acetate [71], glycerol [72], fatty [38] and organic acids (succinate) [73], vitamins (riboflavin [74]; carotenoids [75]), antibiotics (penicillin-G [76, 77]; nystatin [78]; tobramycin [79]; actinorhodin [55, 80]; undecylprodigiosin [80]), and recombinant proteins [81, 82].

In this review, we will highlight studies in which ^{13}C-MFA played a crucial role in 1) characterization of metabolic states to identify targets that can improve strain performance; 2) identification of by-product fluxes that reduce target product yields; and 3) bottleneck identification.

^{13}C-MFA OF AMINO ACID PRODUCERS

It was mentioned previously that *Carynobacterium* strains—the traditional platform for AA production [83], which they continuously do [39, 84]—and *E. coli* strains [85, 86] were common objects of ^{13}C-MFA. These investigations resulted in new insight into improving producer strains. Thus, application of the method to newly generated producer strains seems very promising [88, 89].

NADPH balancing that was based on a ^{13}C-MFA of a Val-producing strain resulted in a proposal to introduce the H^+-dependent transhydrogenase PntAB into a *C. glutamicum* strain that was deficient in pyruvate dehydrogenase (PDH^-). As a result, the NADPH deficiency that was present in the PDH^- strain was alleviated by NADPH synthesis performed by the heterologous transdehydrogenase, all without increased flux through the PP pathway that was previously observed. This genetic modification led to a drastic increase in Val yield by saving CO_2 that was lost in the oxidative branch of the PP pathway in the PDH^- strain [67].

Two well-known anaplerotic reactions exist in *C. glutamicum*: PEP + CO_2 → OAA, which is catalyzed by PEP-carboxylase, and PYR + CO_2 → OAA, which is catalyzed by PYR-carboxylase. The enzymes are under metabolic regulation. ^{13}C-MFA revealed that Glu synthesis, which is promoted by biotin depletion or by detergent/antibiotic addition, is accompanied by an increase in the PYR-carboxylase flux. On the other hand, carbon flux through the PEP-carboxylase remains constitutive during both growth and Glu production stages [64]. This result clearly showed what reaction should be strongly controlled during Glu production and defined future targets for genetic modification of the producer strain.

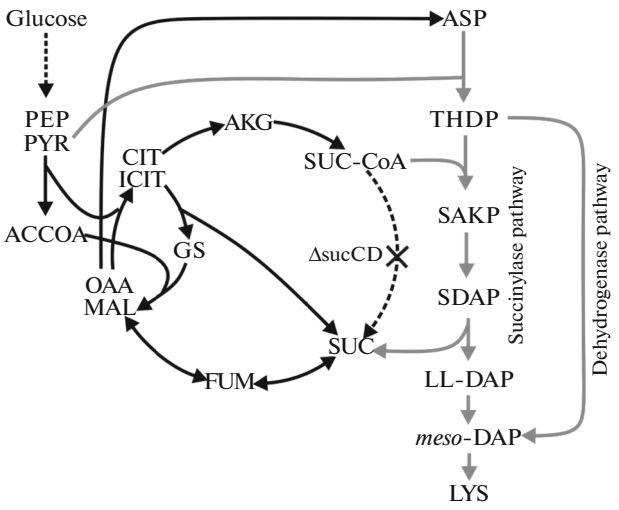


Fig. 4. New metabolic engineering strategy to increase Lys production by *C. glutamicum* cells [93]: coupling the TCA cycle with the succinylase pathway of Lys synthesis. Abbreviations: THDP—L-Δ^1-tetrahydrodipicolinate; SAKP—L-*N*-succinyl-2-amino-6-ketopimelate; SDAP—*N*-succinyl-LL-2,6-diaminopimelate; LL-DAP—L,L-diaminopimelate; *meso*-DAP—*meso*-diaminopimelate.

A set of very interesting studies that are related to *C. glutamicum* Lys producers have been published. For example, ^{13}C-MFA revealed a futile ATP-consuming PEP → PYR → OAA → PEP cycle during the growth of a Lys-producer on glucose. Eliminating the cycle by inactivating PEP-carboxylase led to a significant increase in Lys yield [91].

^{13}C-MFA confirmed that a minor increase in *fbp* expression in *C. glutamicum* increased the carbon flux through the oxidative branch of PP pathway. Increasing NADPH synthesis improved Lys production since NADPH is a co-factor in Lys biosynthesis.

A good example of using ^{13}C-MFA and then rationally designing a Lys producer has been described [39]. ^{13}C-MFA was then applied to the newly constructed strain. The use of flux analysis and genetic modification resulted in a flux redistribution that was very close to that predicted to be "ideal" for Lys production by flux balance analysis (FBA[2]). The most impressive result is that only six steps of targeted modifications were required to result in a strain whose performance is close to that of a strain that was generated for 50 years by traditional methods, including the use of selection and genetic engineering modifications.

A drastic increase in Lys production occurred when fluxes in the Lys biosynthetic pathway and a TCA cycle, that was broken by SUCCOA-synthase inactiva-

[2] FBA is a theoretical analysis of parameters of intracellular metabolic fluxes included in the stoichiometric model of the studied organism based on the solution of the linear programming problem in search for the extremum of a predetermined target function linear with respect to fluxes.

tion (Δ*suc*SD), were coupled [93]. In this case, a succinylase branch of Lys biosynthesis converted SUCCOA to SUC during aerobic growth (Fig. 4). Indeed, the Δ*suc*SD mutant of an advanced Lys-producer increased its Lys yield by 60%. ^{13}C-MFA confirmed the coupling of Lys biosynthesis and the TCA cycle.

Replacing native *C. glutamicum* GAP-dehydrogenase, which is NAD^+-specific, by a mutated GAP-dehydrogenase (GAPDH), which has double (NAD^+/$NADP^+$) specificity, could generate NADPH through an additional pathway [94].

Integrating the mutated GAPDH gene into the genome of a Lys-producing strain increased yields of the target AA. Despite increasing the flux toward Lys synthesis, no flux redistribution was detected by ^{13}C-MFA at the branch points of the EMP/PP pathways. Consequently, the oxidative branch of the PP pathway could not be the source of increased NADPH synthesis. The TCA cycle flux concurrently decreased by 10%, as carbon was redirected to anaplerotic pathways and Lys biosynthesis. The carbon flux through the C_4-decarboxylating reaction also decreased. Thus, neither NADP-dependent isocitrate dehydrogenase nor malic enzymes could provide additional NADPH for increased Lys production. As other pathways for NADPH synthesis have not been described for *C. glutamicum*, ^{13}C-MFA strictly confirmed that the only source of increased NADPH synthesis that was necessary to improve Lys production was the mutated GAPDH that has double co-factor specificity.

At the end of the twentieth century, *E. coli* became the platform organism for AA production because of its features: fast growth; the existence of effective genetic tools to precisely modify chromosome; and well-characterized genetic and metabolic regulation.

A new ^{13}C-MFA approach that is based on LC-MS/MS analysis of free intracellular AAs labeling has been applied for *E. coli* Lys production by Dr. Iwatani S. under the supervision of Prof. Shimizu K. [95]. For intracellular metabolite pools, isotopic steady-state conditions are reached rather quickly, making it possible to analyze steady-state isotopomer distributions of free AAs several hours after a tracer impulse (e.g., it is possible to characterize different stages of fed-batch fermentation). Following Prof. Wittmann's group, who applied GC-MS-based analysis of free AA labeling to produce Lys in *Corynebacterium*, Iwatani and co-authors used impulse labeling to perform steady-state ^{13}C-MFA of the exponential and stationary phases of an *E. coli* fed-batch culture. Flux redistribution between two cultivation phases has been confirmed. The shift to the stationary phase is characterized by a drastic increase of PEP-glyoxylate cycle activity that is accompanied decreased anaplerotic flux (PEP/PYR) + CO_2 + (–/ATP) → OAA + (–/ADP). Additionally, increased activity of NADPH-generated pntAB was recommended to further increase Lys production.

Wahl and co-authors [65] performed a series of ^{13}C-based experiments on an *E. coli* Phe producer in 300-liter fermenters that mimicked real industrial fermentations. It was suggested that strong flux from PEP to PYR could restrict yields of Phe by limiting the availability of the precursor, PEP. Considering this data and the known kinetic properties of enzymes that participate in PEP metabolism, the authors determined that PEP-synthase, because it catalyzes the PYR to PEP conversion, is the most promising target for improving the strain performance [97].

^{13}C-MFA OF PRODUCERS OF VITAMINS, ANTIBIOTICS AND OTHER BIOLOGICALLY ACTIVE COMPOUNDS

^{13}C-MFA has also been used to analyze the metabolisms of producers of vitamins and antibiotics. In many cases, one of the first steps that is performed to increase target compound synthesis is to reduce the formation of by-products. Well-known pathways such as ethanol synthesis in yeast, acetate synthesis in *E. coli*, and lactic acid synthesis in mammalian cells can limit target production. By-product elimination is sometimes a very complicated task that is solved by metabolically engineering a strain and/or optimizing a fermentation process [37]. ^{13}C-MFA of *E. coli* cells revealed a drastic flux redistribution during octanoic acid (C_8) production [98]. The fluxes trough the TCA cycle and pyruvate dehydrogenase reaction (PDH) decreased together with decrease of CO_2 production. Additionally, oxidation of pyruvate to acetate increased. It was suggested that the observed flux distribution resulted from C_8-induced stress. Octanoic acid synthesis can alter membrane structures, reduce electron-transport chain efficiency, and lead to activation of the PDH transcriptional regulator: PdhR. Based on this hypothesis, a strategy for the genetic modification of a strain and the optimization of cultivation conditions was implemented and resulted in increased octanoic acid production [98].

While investigating the metabolic state of riboflavin-producing *B. subtilis* strains in continuous growth cultures as they shifted from high to low production, Zamboni and co-authors [74] revealed that under conditions of high riboflavin production, these strains synthesized riboflavin by a non-oxidative PP pathway. This result would be difficult to predict because three molecules of pentose are necessary to synthesize one molecule of riboflavin. Nevertheless, increased flux through the oxidative PP pathway branch drastically decreased riboflavin production. From an analysis of ATP and NADPH requirements for riboflavin synthesis, it was suggested that the excessive amount of NADPH that was synthesized by the activated oxidative PP pathway branch was toxic. Co-factor require-

ments were also fully satisfied under the conditions of riboflavin synthesis via the non-oxidative branch. Thus, it was recommended to decrease the flux through the oxidative branch of the PP pathway in riboflavin-producing strains to levels below that of the wild-type strain.

The opposite case was found in a ^{13}C-MFA of an astaxanthin-producing strain of *Phaffia rhodozyma* that was grown on glucose [75]. Glucose catabolism in this strain is accomplished via an oxidative PP pathway branch, which mainly reflects the increased requirement of NADPH as a co-factor in astaxanthin synthesis.

In the 2000s, the number of studies that involved a ^{13}C-MFA of *Streptomyces* strains that produce antibiotics increased. The main aim of the investigation was to characterize the metabolic states of the strains and propose new methods to improve the producer strains. Antibiotics are secondary metabolites that are synthesized by specific metabolic pathways from precursors and co-factors that are generated in central metabolism. Thus, for effective antibiotic production, a comprehensive understanding and the rearranging of primary metabolism are important.

To generate a model of *Streptomyces* metabolism, researchers used all available information. For example, for the traditional species *Streptomyces coelicolor* – a producer of actinorhodin [55] and undecylprodigiosin [80] – the metabolic model constructing was based on genome reconstruction [99], transcriptome [100] and proteome [101] data. The mutant strain of *S. coelicolor* that was unable to produce actinorhodin grew faster and exhibited greater flux through the PP and anaplerosis (PEP + CO_2 → OAA) pathways. Strong flux through the PP pathway provided increased NADPH generation, which was utilized for biomass synthesis and other processes. The glucose consumption rate and TCA cycle flux also decreased in the mutant strain relative to that of the wild type (which produced actinorhodin). The amount of NADPH that was produced exceeded the cellular requirements, which indicated a curtailed role of nicotinamide nucleotide transhydrogenase in redox homeostasis in *Streptomyces*. Maintaining a high level of energy could be required based on the redundantly high amounts of ATP that were generated in both strains. Also competition between actinorhodin and triacylglycerol biosynthesis for a common precursor, acetyl-CoA (ACCOA), was proposed.

A good example of how modifying central metabolism may influence antibiotic production has been described [80]. Inactivation of *pfk*A2 (one of three homologs of *pfk*A) decreased total phosphofructokinase activity and increased actinorhodin and undecylprodigiosin production. ^{13}C-MFA that was based on a proposed metabolic model revealed that this genetic modification increased PP pathway flux and increased flux toward antibiotic synthesis from pyruvate via ACCOA.

Other specific metabolic changes have been detected by using ^{13}C-MFA in *Streptomyces* during antibiotic production fluctuations and shifting physiological states. During increased nystatin production in *S. noursei*, increased PP pathway flux accompanied decreased TCA flux and biomass yield [78]. A shift from growth to tobramycin production in *S. tenebrarius* is characterized by increases in EMP- and PP-pathway fluxes and a decrease in ED pathway flux [79].

Thus, the characterization of cellular metabolic states by ^{13}C-MFA can provide the basis for a strategy to use either traditional or newly selected microorganisms for biotechnology applications.

^{13}C-MFA FUTURE PERSPECTIVES

At present, most results are obtained by using steady-state ^{13}C-MFA that is based on proteinogenic AA labeling data. This approach can only be applied to cells grown under continuous cultivation or grown to the exponential phase when batch cultivated (i.e., under conditions of active protein synthesis). However, an analysis of flux kinetics while the behavior of strains and/or environmental conditions are changing is more interesting from a practical point of view. It has been shown that steady-state ^{13}C-MFA can successfully characterize changes in flux. In this case, a tracer impulse is introduced at different time points during strain cultivation. To shorten the time that is necessary to achieve steady-state conditions, isotopomer distributions of free AAs (with corrections for AAs that result from protein degradation [95]), intracellular metabolites [102] and their fragments are measured by LC-MS/MS [103] and used as the sources of labeling data for ^{13}C-MFA. In contrast to proteinogenic AA labeling analysis, which is performed using GC-MS [42, 104–108], measuring intracellular metabolite isotopomer distributions requires more costly and sensitive equipment such as CE-TOFS [102], GC-MS/MS [109–111] and LC-MS/MS [95, 103, 112, 113].

One of the goals of the "analytical chemistry" part of ^{13}C-MFA is to develop methods that allow more labeling information (up to the complete isotopomer distribution, in the ideal case) to be extracted. Characterization of systems under non-steady-state isotopic conditions at the ultrashort time periods is very complicated and an interesting mathematical task that is currently being solved [68, 87, 114–116]. Kinetic analysis of label incorporation into the metabolite pool during non-steady-state ^{13}C-MFA can increase the precision of flux calculations, decrease the cost of ^{13}C-experiments and provide opportunities to investigate transient cell states [87]. On the other hand, using the steady-state ^{13}C-MFA approach, which is based on free metabolite pools, as described above, is quite

appropriate for evaluating flux kinetics to achieve the practical aims of metabolic engineering.

Certainly, more detailed metabolic models should be developed in ^{13}C-MFA to account for the compartmentalization of eukaryotic systems [5, 117–119]; the presence of metabolons in bacterial cells [120, 121]; the creation of multienzyme complexes, i.e., scaffolds, which enable substrate tunneling [122–124]; and the targeted modification of compartments where the desired product is synthesized [125]. At least several pioneering investigations of substrate tunneling have been reported, and they demonstrate gratifying results. The evaluation of such new metabolic states requires the use of comprehensive methods such as ^{13}C-MFA.

Until recently, the modification of cellular metabolism to the increase production of a target compound has been accomplished by each team intuitively, based on personal experience. However, several special software packages have been developed to predict all possible genetic modifications that are necessary to change carbon flux distributions in the characterized metabolic model that should result in high-performance production of target compounds [126, 127]. For example, one such software package, OptForce, helped generate a non-trivial strategy to modify the metabolism of an *E. coli* fatty acid-producing strain [128]. As a result, the yield of the target $C_{14\text{-}16}$ fatty acids increased from a native level of 11% of theoretical yield (in the wt strain) to 39% in the modified strain.

Finally, the increase in the precision of flux calculations by using genome-scale metabolic models of traditional and poorly studied organisms is one of the benefits of ^{13}C-MFA. The other important area of ^{13}C-MFA application is the analysis of flux kinetics during producer-strain cultivation using PLEs with different tracers and then measuring the proteinogenic AA and/or intracellular metabolite labeling by GC(LC)-MS/MS. These directions, together with the broad implementation of different algorithms to achieve statistically significant ^{13}C-MFA results and to be able to target flux manipulation via genetic engineering during the producer strain development process, are the main future roles of ^{13}C-MFA in fundamental and applied studies.

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