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Metabolomics-based development of bioproduction processes toward industrial-scale production☆

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Microbial biomanufacturing offers a promising, environment-friendly platform for next-generation chemical production. However, its limited industrial implementation is attributed to the slow production rates of target compounds and the time-intensive engineering of high-yield strains. This review highlights how metabolomics expedites bioproduction development, as demonstrated through case studies of its integration into microbial strain engineering, culture optimization, and model construction. The Design–Build–Test–Learn (DBTL) cycle serves as a standard workflow for strain engineering. Process development, including the optimization of culture conditions and scale-up, is crucial for industrial production. *In silico* models facilitate the development of strains and processes. Metabolomics is a powerful driver of the DBTL framework, process development, and model construction.

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Introduction

Microbial biosynthesis is promising for sustainably producing various chemicals, including fuels [1], natural plant products [2], and plastics [3,4]. However, to establish cost-effective industrial-scale production, engineering microbial strains and cultivation methods is essential. Advances in genetic engineering and screening techniques have facilitated the construction of highly engineered strains. The Design–Build–Test–Learn (DBTL) cycle is an effective workflow for creating high-producing microbial strains for target compounds [5–7]. The DBTL workflow begins with strain design, in which an appropriate host for target compound production is selected based on metabolic maps and past knowledge (Figure 1a). Potential modifications of genes and proteins that contribute to improved productivity are then proposed. Next, the designed strains are constructed to produce the target compound. Subsequently, constructed strains are evaluated by analysis methods, such as proteomic and metabolomic analyses, during the test phase. By studying the analysis data, metabolic rules are extracted, providing valuable feedback for the next design iteration.

Metabolomics is a powerful driver of the DBTL cycle [7,8]. Metabolomics has been utilized to identify bottleneck reactions in metabolic pathways from starting substrates to target compounds in various microorganisms, from heterotrophs to autotrophs [9,10]. This information enables the overexpression of bottleneck enzymes or enhancement of their activities through enzyme engineering, improving the production yield and productivity of target compounds. Additionally, metabolome data provide rational interpretations of the productivity changes of target compounds.

High-producing strains can be obtained through the metabolomics-guided DBTL cycle. However, the current lack of metabolic information frequently hinders expected productivity improvements. An additional issue is the lack of understanding of the functions and metabolic mechanisms of the unidentified proteins and genes necessary for linking the metabolome to the genome.

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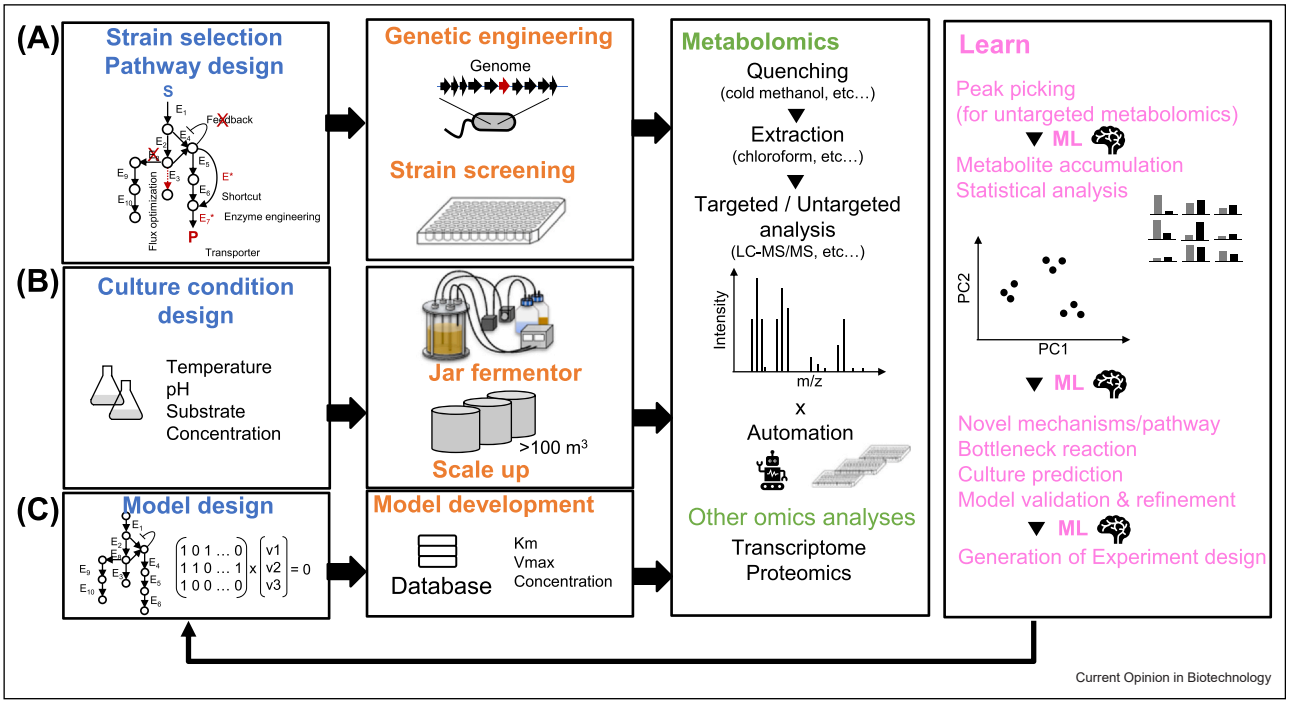
Figure 1



Metabolomics streamlines the DBTL workflow. Metabolomic data provide information on pathway bottlenecks, culture profile, and metabolic mechanisms. Systems biology model development can be accelerated by integrating automated high-throughput metabolomics and ML.

Therefore, in the current DBTL approach, acquiring high-producing strains may require multiple rotations of the DBTL cycle, which can be time-consuming and present a challenge. Metabolomics can also help resolve these issues. For example, metabolite profiling from genetic mutants or recombinants can be applied to assign novel enzyme candidates [11]. Metabolomic footprints and phenotype help discover causative genes or metabolic pathways in plants, microbiome, and human [12–15]. In addition, metabolomics plays a vital role in unraveling metabolic mechanisms [16,17].

Toward industrial-scale production, optimizing culture conditions and scale-up is also necessary. Using metabolomics, including other omics approaches, can characterize these effects [18] (Figure 1b). Mathematical models based on omics data, including metabolomics, are used to perform rational strain design and understand the effects of large-scale bioreactors [19]. Recent advances in machine learning (ML) have expanded the use of metabolome data to improve mathematical models [20–22] (Figure 1c). Metabolomes represent phenotypes. Using genomic information and environmental conditions, accurately predicting metabolome changes has become possible, thereby maximizing the productivity of target compounds [23]. This review discusses the roles of metabolomics in the development of microbial chemical production.

Metabolomic analysis process

Metabolomics is a comprehensive analytical method that enables the simultaneous identification and quantification of multiple metabolites within a sample. Targeted metabolomics aims to quantify essential intermediates of interest and is a key component of metabolic engineering workflows. The metabolomic analysis outcome depends heavily on sample preparation. To depict the intracellular metabolic state, cells are rapidly quenched to arrest metabolism, followed by the extraction of intracellular metabolites. Commonly employed methods include cold methanol quenching and methanol/chloroform extraction [24]. Acidic acetonitrile has been shown to be effective in the accurate quantification of redox cofactors [25]. Recently, phenol-based extraction has shown promise for precise cyanobacterial metabolomics [26]. The selection of suitable extraction methods may be species-dependent, necessitating tailored sample preprocessing strategies.

Samples are separated by liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis and subjected to comprehensive mass spectrometry (MS) analysis. Nuclear magnetic resonance-based metabolomics is also employed, with a comparative overview of the analytical techniques available in the literature [10,27]. Before conducting metabolomic tests, high-throughput analyses, such as supercritical fluid

Table 1

Application of metabolomics to strain engineering.

Host organisms	Targets	Role of metabolomics	Product's titer	References
Escherichia coli	*O*-succinyl-L-homoserine	Elucidating the metabolic mechanism of the *O*-succinyl-L-homoserine high-producing strain	24.1 g/L	[4]
Escherichia coli	Succinic acid	Comparing the metabolomes of metabolically engineered strains	15 mM	[39]
Escherichia coli	1-Propanol	Elucidating accumulation of upstream by-products	5.1 g/L	[43]
Corynebacterium glutamicum	L-pipecolic acid	Comparing the metabolomes of engineered strains	93 g/L	[33]
Corynebacterium glutamicum	3-amino-4-hydroxybenzoic acid	Elucidating the effects of different culture conditions (different dissolved oxygen levels) on metabolism	5.6 g/L	[38]
Streptomyces nodosus	AmB	Elucidating the metabolic mechanism of high amphotericin-B production in mutant strains	5.03 g/L	[41]
Synechococcus elongatus	3-HP	Exploring the metabolic bottleneck of 3-HP production in an intermediate strain	91.3 mg/L	[40]
Synechococcus elongatus	Glucose	Elucidating the metabolic mechanism of a high-glucose-producing strain	5.0 g/L	[31]
Synechocystis sp. PCC 6803	Malic acid	Investigating the effects of genetic engineering on metabolism	3.2 g/L	[30]
Saccharomyces cerevisiae	Isoprenol	Exploring the metabolic bottleneck of isoprenol production in an intermediate strain	383.1 mg/L	[37]
Saccharomyces cerevisiae	SAM	Comparing the metabolomes of metabolically engineered strains	40.5 mg/L	[35]
Saccharomyces cerevisiae	Squalene	Elucidating the effects of media components on squalene production	408.9 mg/L	[42]
Pichia pastoris	resveratrol	Elucidating the metabolic mechanism of a high-resveratrol-producing strain	1825 mg/L	[34]
Pichia stipitis	Resveratrol	Elucidating the effects of different carbon sources on the metabolism of a resveratrol-producing strain	668.6 mg/L	[57]
Rhodosporidium toruloides	3-HP	Investigating the effects of media differences on the metabolism of 3-HP-producing strains	45.4 g/L	[36]

extraction–supercritical fluid chromatography or imaging MS, can streamline target strain selection [28,29]. These techniques offer valuable insights into selecting candidates before conducting metabolomic investigations.

Metabolomics for characterization of synthetic strains

In conventional metabolic engineering, efforts are directed toward enhancing productivity through genetic manipulation (Table 1). For example, the impact of the overexpression of bottleneck reaction enzymes has been elucidated through the metabolomic analysis of malic acid production using *Synechocystis* sp. PCC 6803 [30]. Glucokinase deficiency in *Synechococcus elongatus* PCC 7942 promotes glucose excretion, prompting a metabolomic investigation of its effect on carbon metabolism [31]. In an engineered *Escherichia coli* strain producing *O*-succinyl-L-homoserine via *sucD* knockout and *sucA* and *metA* overexpression, significant alterations in the tricarboxylic acid cycle and amino acid accumulation were observed [32]. In *Corynebacterium glutamicum*, the L-pipecolic acid biosynthetic pathway was introduced to probe the intracellular concentration of lysine along with the NADH/NAD+ ratio, a substrate and cofactor for L-lysine 6-dehydrogenase, and validate its *in vivo* enzymatic activity. These results indicated inadequate enzyme activity, leading to pathway replacement and higher production [33].

Metabolomic strain characterization is also useful for the strain engineering of eukaryotic microbes, such as yeast. Metabolomics revealed the characteristic accumulation of shikimate pathway intermediates in engineered *Pichia pastoris* for aromatic secondary metabolite production [34]. To explore the mechanisms of enhanced productivity in *S*-adenosyl-L-methionine (SAM)-producing yeast, metabolomics suggested a relationship between ATP generation and SAM synthesis pathways [35]. Upon expression of the 3-hydroxypropionic acid (3-HP) synthesis pathway in *Rhodosporidium toruloides*, intracellular accumulation of 3-HP was revealed, with production potential further anticipated through transporter expression [36].

Metabolomics for pathway bottleneck identification and pathway optimization

Bioproduction involves multistep reactions involving numerous metabolic pathways to produce the target compound. The accumulation of intermediates suggests that the conversion reaction is a bottleneck. In several cases, increasing enzyme expression or replacing enzymes with higher activity for the conversion reaction resolves this bottleneck, improving the production of the target compound (Table 1). For example, metabolic analysis of the isopentenyl diphosphate-bypass pathway revealed accumulation of isopentenyl phosphate, indicating that the final step of hydrolysis to isoprenol was a bottleneck.

Screening for promiscuous phosphatases significantly improved isoprenol titers [37]. Furthermore, rewiring metabolic pathways from accumulated metabolites to target compounds enhances productivity. For example, in *C. glutamicum*, the accumulation of pyruvate led to by-product formation; however, *ldh* knockout resulted in increased 3-amino-4-hydroxybenzoic acid production [38]. An increase in relative mannitol concentration was observed in a succinic acid-producing *E. coli* strain. To redirect carbon flux from mannitol to malic acid production, the *mtlD*-encoding mannitol dehydrogenase was deleted. The M4-ΔgndΔmtlD mutant showed reduced mannitol synthesis and a 20% increase in malic acid production compared to that of M4-Δgnd [39]. Metabolomic analysis revealed the accumulation of intermediates, such as those in the oxidative pentose phosphate pathway, when xylose was utilized in *Synechococcus elongatus* UTEX 2973. By rewiring the native glycolytic pathway via heterologous phosphoketolase gene expression combined with phosphofructokinase gene knockout and fructose-1,6-bisphosphatase gene overexpression, greater carbon flux from xylose to acetyl-CoA was achieved [40]. Moreover, comparative metabolomics enables the estimation of crucial metabolic pathways that influence target compound production, providing guidelines for metabolic engineering. In the production of amphotericin B (AmB), a comparison of the metabolomes between the producing strain and the wild type revealed the association of AmB production with one carbon pool by folate. Overexpression of the methionine synthesis enzyme metH, which is involved in the formation of one carbon pool by folate, increased AmB production [41]. Correlation analysis revealed the impact of specific metabolic pathways on squalene production and was utilized in metabolic engineering design [42]. In case of 1-propanol production in *E. coli*, rational metabolic engineering based on combined metabolomics by GC/MS and ion-pair LC–MS/MS improved the titer and yield [43].

In addition to intracellular metabolomics, metabolomic analyses for excreted metabolites in culture medium are also helpful for metabolic engineering. Extensive exometabolomic analyses showed a strong correlation of the extracellular metabolite concentration and the cellular metabolic state among four biotechnologically relevant model microorganisms [44]. As hundreds of metabolites are secreted into culture medium during growth, exometabolomic measurement will provide valuable insight for rational metabolic engineering strategy [45].

Elucidating metabolic mechanisms using metabolomics

A thorough understanding of metabolic mechanisms is crucial to precisely control metabolic flux and increase the productivity of target compounds through metabolic engineering. *S. cerevisiae* has been studied as a model eukaryotic organism. However, even in central metabolic pathways, several unknown metabolic control mechanisms remain. In a recent study, multi-omics data, including the metabolome, collected under nine different chemostat conditions, were integrated and analyzed using a combination of hierarchical analysis and mathematical modeling [46]. This study revealed that glycolytic flux increased with specific growth rates: this increase was explained by the allosteric effect and phosphorylation level of glycolytic enzymes. Scott et al. [47] used random flux sampling and statistical methods, as well as the experimental flux data of extracellular metabolites, to characterize predicted differences in intracellular metabolic states among strains. The results revealed that metabolic differences were most pronounced in fluxes associated with transaminase and hexokinase reactions in the two strains with different volatile component productivities, suggesting that *S. cerevisiae* has different metabolic control mechanisms depending on the strain. *S. cerevisiae* dynamically switches its metabolism from glucose fermentation to ethanol production for respiratory mitochondrial metabolism, using ethanol when grown on glucose in an aerated culture. Brunnsåker et al. [48] used high-throughput metabolomics to gain insights into the metabolic changes that occur during the transition, called the diauxic shift, and how they are regulated by gene expression. They also investigated the consequences of gene deletions on the metabolic network and identified key regulatory nodes.

Aside from yeast, Wang et al. [49] revealed through metabolome and proteome analysis that induction of the glycerol synthesis pathway in *E. coli* reduces fructose-1,6-bisphosphate (FBP) levels and activates transcription factor Cra, leading to growth inhibition. This was confirmed by constructing a kinetic model. Rados et al. [50] cultured multiple strains of *E. coli* under various conditions and measured the concentrations of 101 metabolites under each condition. Numerous amino acids and nucleic acids showed minimal concentration changes. This study revealed that several feedback mechanisms in biosynthetic pathways contribute to end-product homeostasis.

Application of untargeted metabolomics for a more comprehensive test cycle

Untargeted metabolomic analysis enables the comprehensive exploration of the metabolic profile of organisms instead of focusing on specific metabolites. This approach provides novel biological insights into the effects of cultivation conditions and genetic modifications on metabolism [51,52]. During data processing, ML assists with peak selection and normalization [53,54]. In untargeted metabolomics, challenges arise from the difficulty of metabolite identification and the complexity of data interpretation owing to the generation of extensive data. Various ML-based algorithms are being developed

to annotate metabolites in MS data. Recently, a metabolite annotation tool for LC–ion mobility (IM)–MS data processing was developed [55].

Untargeted metabolomics offers the possibility to identify key metabolites for strain and bioprocess improvement, which are often missed by targeted methods based on prior biological knowledge. In an *E. coli* succinate production bioprocess, the application of metabolic pathway enrichment analysis using time course of targeted and untargeted metabolomics data revealed three significantly modulated pathways [56]. Among them, ascorbate and aldarate metabolism is a newly identified target for improving succinate production. Data processing and analyzing tool is necessary to deal with untargeted metabolomics data set and extract useful information.

Metabolomics for characterization of culture conditions

For industrialization, cultivating optimized strains under ideal conditions is crucial. Metabolomics aids the assessment of different culture conditions for their optimization. For example, distinct metabolic changes were noted in resveratrol-producing yeasts subjected to diverse culture conditions [57]. As secondary metabolite production depends significantly on culture conditions, extensive exploration of such conditions and metabolic states is crucial. Microscale metabolomics platforms streamline and expedite the study of secondary metabolism, enabling a broader analysis of microenvironmental cues and offering key insights into their features [58]. Nonetheless, even if laboratory-scale culture conditions are established, large-scale fermenters may suffer from imperfect mixing, leading to fluctuations in pH, temperature, and substrate gradients that can unexpectedly impair strain performance. Two avenues to enhance cell performance in large-scale bioprocesses include (1) optimizing bioreactor design and operational conditions to minimize gradients and (2) developing more robust strains capable of withstanding these conditions. Notably, the latter requires a mechanistic understanding of the (metabolic) responses to dynamic environments. Vasilakou et al. [59] characterized the dynamic metabolic responses of *E. coli* under a repetitive feast–famine regime. Czajka et al. [60] employed stable isotope tracing analysis during the high-cell-density fermentation of a terpenoid-producing *Yarrowia lipolytica* strain in benchtop bioreactors, suggesting limitations in energy supply and strain performance due to electron transport and oxidative phosphorylation. Accordingly, metabolomics provides guidance for robust strains and culture conditions, even in large-scale cultures (Figure 1b).

Conclusions

Metabolomics is an effective tool for constructing microbial strains for bio-based chemical production. Profiling the entire metabolic landscape helps identify bottleneck pathways, leading to increased production of target compounds. Analytical techniques within metabolomics are advancing, for example, LC–IM–MS enables the analysis of compounds that are traditionally challenging to separate, such as isomers.

Employing metabolomic data for modeling and ML can reveal hidden data characteristics, leading to the discovery of novel mechanisms [61]. A closed-loop workflow integrating an ML-guided experimental design with automated systems improved the diauxic shift model [62]. High-throughput untargeted metabolomics analysis of mutants lacking functionally unknown genes related to the diauxic shift identified by the developed model suggested that metabolomics could effectively design and validate the model [48] (Figure 1c). Kinetic models can be used to identify key gene targets for metabolic engineering in *Pseudomonas putida* [63]. The integration of high-throughput strain engineering workflows, omics analysis, and kinetic modeling led to the successful fermentation scale-up of the carbon-negative production of acetone and isopropanol [64]. Thus, the synergy between ML-based model construction and automation in high-throughput metabolomic analysis holds promise for the streamlined development of strain and industrial-scale processes (Figure 1).

CRediT authorship contribution statement

Kenya Tanaka: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Takahiro Bamba:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Tomohisa Hasunuma:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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