Metabolic engineering for commodity chemical production from lignin and CO_2 utilizing H_2 as an energy source

Junya Kato

National Institute of Advanced Industrial Science and Technology (AIST)

Research objective

Lignin derived from woody biomass is the second most abundant renewable carbon resource on Earth. However, its effective utilization remains a subject of extensive discussion, and various technologies for its application are currently under development. Lignin consists of methoxy-substituted aromatic structures. In this study, we aimed to develop microorganisms capable of low-CO_2-emission fermentation-based production by focusing on the abundant methoxy groups in lignin. I conducted fundamental research to obtain a synthetic metabolism that minimizes CO_2 emissions by utilizing hydrogen (H_2), which can be supplied as a renewable energy source. Regarding the host organism, I focused on the anaerobic microorganism *Neomoorella thermoacetica*[1], which can assimilate both methoxy aromatic compounds and H_2. This bacterium synthesizes acetate from methoxy compounds using acetyl-CoA. Acetyl-CoA is a highly versatile intermediate that can be further converted into various valuable compounds (such as ethanol, acetone, and isopropanol[2]) through metabolic engineering. In this metabolic pathway, methoxy groups are directly incorporated as the methyl group of acetyl-CoA, whereas other methoxy groups are oxidized to generate reducing power, which is then used to synthesize the carbonyl group of acetyl-CoA. However, oxidation of methoxy groups generates CO_2; therefore, I aimed to replace this reducing power supply with H_2 metabolism through the construction of synthetic pathways (Fig. 1).

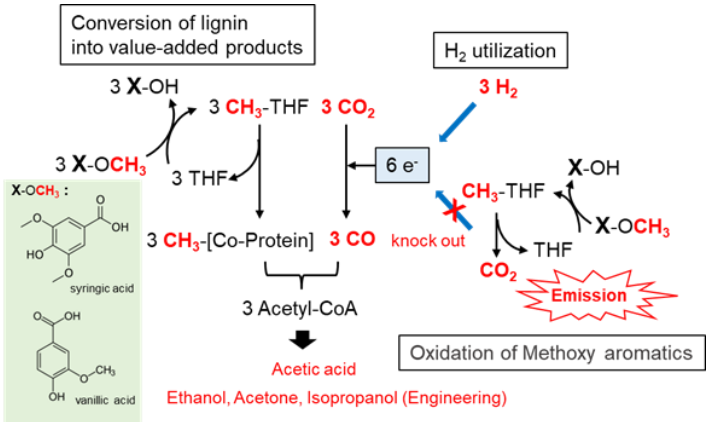

Fig. 1. Overview of the intended synthetic metabolic pathway.

Methods

1. Assimilation tests for methoxylated compounds and H_2

I conducted assimilation tests using small-scale anaerobic cultures with three representative methoxy aromatic compounds (syringic acid, vanillic acid, and *p*-hydroxybenzoic acid) and methanol as substrates. The effects of H_2 supplementation were evaluated under the same conditions.

2. Metabolic design and construction of gene knockout strains

To develop a microorganism capable of replacing the methoxy group oxidation pathway with H_2 metabolism, I designed a synthetic metabolic framework and attempted to construct recombinant strains. A metabolic design was devised that considered redox balance in the absence of the methoxy group oxidation pathway, and transformation experiments were performed to introduce the corresponding genetic constructs.

3. Transcriptomic analysis

To obtain fundamental insights for rational metabolic design, transcriptomic analysis was performed to examine gene expression under conditions where methoxylated compounds, H_2, or sugars served as primary substrates. Methanol was used as the substrate to ensure sufficient biomass for experiments involving methoxy metabolism. For H_2 metabolism, CO_2 was supplied as the sole carbon source, whereas fructose was used for sugar metabolism. RNA was extracted from cells harvested during the logarithmic growth phase under each condition, and transcriptomic analysis was performed to compare and evaluate changes in gene expression profiles.

Results

Cultivation tests using various methoxy aromatic compounds as substrates revealed that *N. thermoacetica* assimilated syringic acid, vanillic acid, and *p*-hydroxybenzoic acid, exhibiting a particularly high syringic acid assimilation capacity (Fig. 2A). Because syringic acid contains two methoxy groups per molecule, it is presumed that equimolar amounts of this substrate resulted in higher acetate production and enhanced growth. Furthermore, when methanol, which has high solubility, was supplied at elevated concentrations, both acetate production and growth increased, indicating a correlation with the number of methoxy groups. Additionally, co-feeding H_2 and CO_2 along with syringic acid suggested that simultaneous metabolism of methoxy groups and H_2 did not exhibit mutual inhibition (Fig. 2B). Inhibition occurs when pathways generating H_2 owing to redox imbalance are involved[3)]. Based on these findings, I proceeded with the construction of strains designed to disrupt the methoxy group oxidation pathway while maintaining redox balance, aiming to establish a synthetic pathway in which methoxy group oxidation is replaced by H_2 metabolism, as intended in this study.

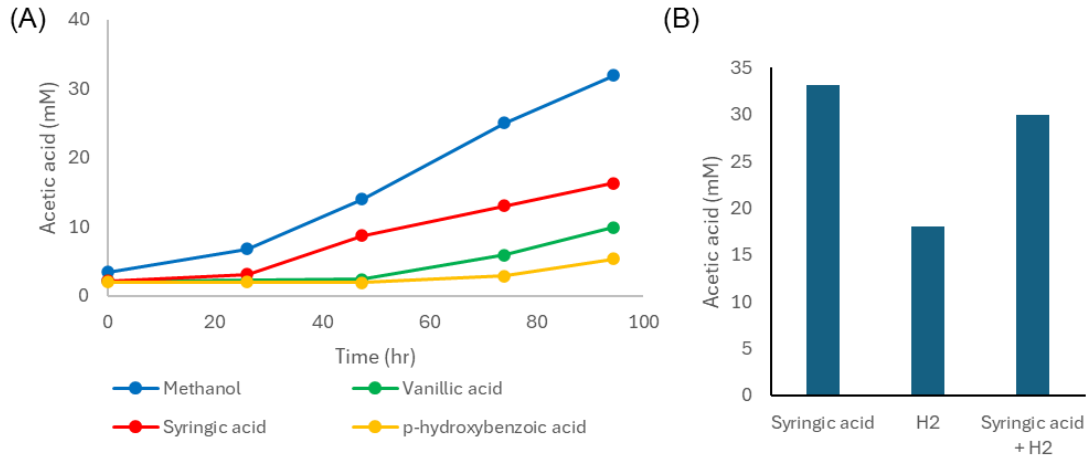

Fig. 2. Assimilation tests of various methoxy compounds and evaluation of the effects of H_2 supplementation.

To eliminate the methoxy oxidation pathway, I designed gene disruption targeting the key enzyme, methylenetetrahydrofolate reductase. Stoichiometric analysis under sugar-based growth conditions predicted the generation of excess NADH in the absence of this pathway. To address this, I introduced a synthetic pathway for NADH consumption along with gene disruption. Although the DNA constructs were successfully assembled and transformed, all recovered clones were false positives. Additional strategies, including supplementation with alternative electron donors and acceptors to mitigate unexpected redox imbalances, failed to produce the desired recombinant strains.

Transcriptomic analysis under different substrate conditions revealed that the enzyme targeted for gene disruption showed lower expression during sugar metabolism than under methoxylated compound or H_2 conditions, with no clear link to knockout difficulty. In contrast, genes encoding methyltransferases and cobalamin-related proteins were strongly upregulated during methoxy compound metabolism. Hydrogenase gene expression remained relatively constant across all conditions.

Conclusion

N. thermoacetica, selected as a host for low-CO_2-emission bioproduction through co-assimilation of lignin-derived methoxylated aromatics and H_2, efficiently utilized representative methoxy compounds without metabolic interference from H_2 supplementation, confirming its suitability as a platform organism. Attempts to construct a synthetic pathway that replaces methoxy oxidation with H_2-derived reducing power for low-CO_2-emission metabolism proved challenging, indicating the need for revised design strategies. Transcriptomic analysis further suggested that the regulation of methyl group utilization

pathways is critical for improving the efficiency of synthetic metabolism. Overall, this study provides a foundation for developing metabolic systems that enable the efficient utilization of renewable resources.

References

1) Drake, H.L. and Daniel, S.L. (2004) Physiology of the thermophilic acetogen *Moorella thermoacetica*. *Res. Microbiol.* **155**:869-883.

2) Kato, J.*, Matsuo, T.* *et al*. (2024) Isopropanol production via the thermophilic bioconversion of sugars and syngas using metabolically engineered *Moorella thermoacetica*. *Biotechnol. Biofuels. Bioprod.* **17**:13.

3) Kobayashi, S.*, Kato, J.* *et al*. (2022) Reversible hydrogenase activity confers flexibility to balance intracellular redox in *Moorella thermoacetica*. *Front. Microbiol.* **13**:897066.