Reversible Hydrogenase Activity Confers Flexibility to Balance Intracellular Redox in *Moorella thermoacetica*

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Hydrogen (H2) converted to reducing equivalents is used by acetogens to fix and metabolize carbon dioxide (CO2) to acetate. The utilization of H2 enables not only autotrophic growth, but also mixotrophic metabolism in acetogens, enhancing carbon utilization. This feature seems useful, especially when the carbon utilization efficiency of organic carbon sources is lowered by metabolic engineering to produce reduced chemicals, such as ethanol. The potential advantage was tested using engineered strains of *Moorella thermoacetica* that produce ethanol. By adding H2 to the fructose-supplied culture, the engineered strains produced increased levels of acetate, and a slight increase in ethanol was observed. The utilization of a knockout strain of the major acetate production pathway, aimed at increasing the carbon flux to ethanol, was unexpectedly hindered by H2-mediated growth inhibition in a dose-dependent manner. Metabolomic analysis showed a significant increase in intracellular NADH levels due to H2 in the ethanol-producing strain. Higher NADH level was shown to be the cause of growth inhibition because the decrease in NADH level by dimethyl sulfoxide (DMSO) reduction recovered the growth. When H2 was not supplemented, the intracellular NADH level was balanced by the reversible electron transfer from NADH oxidation to H2 production in the ethanol-producing strain. Therefore, reversible hydrogenase activity confers the ability and flexibility to balance the intracellular redox state of *M. thermoacetica*. Tuning of the redox balance is required in order to benefit from H2-supplemented mixotrophy, which was confirmed by engineering to produce acetone.

**Keywords:** acetogen, metabolic engineering, ethanol production, hydrogen inhibition, hydrogen production, redox balance, mixotrophy

**INTRODUCTION**

There is a growing interest in chemical production derived from sources other than fossil fuels. Due to increasing levels of carbon dioxide (CO2) in the atmosphere, low-carbon emissions are required to eliminate environmental threats, such as global warming. Technology to capture and utilize CO2 as a resource is in progress worldwide, and bioprocessing of renewable feedstocks...
is one promising candidate. However, economic cost is a bottleneck in bioprocessing applications of bulk chemicals. A means to reduce the cost is to maximize carbon conversion of feedstock to the product.

Acetogens are a group of microorganisms capable of autotrophic growth on CO₂ and hydrogen (H₂) and are thus promising chassis for utilizing CO₂ by bioprocesses (Ljungdhal, 1986; Wood, 1991; Drake, 1994; Drake et al., 2008). The main product is acetate, but some acetogens produce other valuable chemicals, such as ethanol. These by-products can be utilized for industrial production from waste materials, such as off-gas from steel mills. This process, called gas fermentation, has attracted worldwide attention (Bengelsdorf et al., 2016; Liew et al., 2016; Bengelsdorf and Dürre, 2017; Teixeira et al., 2018; Omar et al., 2019; Jin et al., 2020; Kopke and Simpson, 2020; Bourgade et al., 2021; Fackler et al., 2021). On the other hand, acetogens are also capable of heterotrophic growth on various carbohydrate substrates and are good candidates for bioconversion of biomass to useful chemicals. Utilization of acetogens is especially effective for carbon utilization because processing by acetogens emits much less CO₂ due to the nature of their CO₂ fixation pathway. When acetogens metabolize hexose to acetate, two molecules of CO₂ are produced, then reassimilated into the CO₂ fixation pathway by utilizing reducing equivalents from glycolysis. Therefore, acetogens can theoretically convert one hexose molecule to three acetate molecules (Fontaine et al., 1942; Schuchmann and Müller, 2014, 2016).

Autotrophic and heterotrophic metabolism can be combined for mixotrophic growth, which enables the enhancement of carbon utilization and conversion of extra CO₂ using H₂ as the source of reducing power (Fast et al., 2015; Maru et al., 2018). Mixotrophy is a general trait of acetogens and is effective in fermentation, especially for products that are more reduced than acetate. A previous report succeeded in increasing overall metabolite yields by supplying H₂ to sugar-based cultures of Clostridium ljungdahlii (Jones et al., 2016). In this case, a shift in the metabolite profile was observed by providing H₂ with ethanol as the primary metabolite, over less-reduced products. H₂ supply for the industrial applications of mixotrophic fermentation would be supported by the development of technology to provide CO₂-free H₂ using renewable energy-based approaches, such as water splitting, biomass gasification, and ammonia reforming (Hosseini and Wahid, 2016; Aryal et al., 2018). Thus, together with this technology development to provide H₂, mixotrophic fermentation would contribute to the low-carbon emitting and economically feasible bioprocesses.

In addition to natural by-products, acetogens can also be engineered to produce chemicals other than acetate. Genetic engineering of acetogens is challenging because of their genetic barrier, such as restriction-modification systems and physical barriers by gram-positive cell walls; however, development of engineering tools has substantially improved the efficiency of engineering acetogens (Minton et al., 2016; Jin et al., 2020; Bourgade et al., 2021). It is also possible to apply metabolic engineering for pathway optimization to enhance the production of target metabolites. Metabolic engineering has begun to highlight the potential of acetogens for chemical production from CO₂.

Moorella thermoacetica is a thermophilic acetogen (Drake and Daniel, 2004; Pierce et al., 2008). Due to its thermophilic nature, M. thermoacetica can be used to establish an advantageous bioprocess for the recovery of products, especially volatile chemicals (Taylor et al., 2009; Abdel-Banat et al., 2010; Basen and Müller, 2017; Redl et al., 2017). However, Moorella thermoacetica is categorized as a homoacetogen that produces acetate exclusively. Therefore, the metabolic pathway must be modified to produce other chemicals for industrial applications (Iwasaki et al., 2013, 2017; Kita et al., 2013). We previously succeeded in engineering M. thermoacetica to produce ethanol and acetone from sugars and syngas, as well as to enhance yields, by adjusting the carbon flux (Rahayu et al., 2017; Kato et al., 2021; Takemura et al., 2021a). Disruption of the major acetate production pathway enables near-exclusive ethanol production from sugars.

In this study, we attempted to apply H₂-supplemented mixotrophy to enhance ethanol yield. Unexpectedly, we found that H₂ supplementation inhibited the growth of a high-ethanol-producing strain. Metabolomic analysis revealed that the engineered strain balanced the intracellular redox status by producing H₂ to oxidize NADH during heterotrophic growth. Reversible hydrogenase activity, which oxidizes H₂ in the wild-type strain under standard conditions, plays a vital role in the redox maintenance of metabolically engineered strains. It is necessary to avoid this reverse reaction to fulfill H₂-supplemented-mixotrophic bioproduction.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

*Moorella thermoacetica* ATCC 39073 and its derivatives were used in this study (Table 1). Modified ATCC1754 PETC medium comprising 1.0 g of NH₄Cl, 0.1 g of KCl, 0.2 g of MgSO₄·7H₂O, 0.8 g of NaCl, 0.1 g of KH₂PO₄, 0.02 g of CaCl₂·2H₂O, 2.0 g of NaHCO₃, 10 ml of trace elements, 10 ml of Wolfe’s vitamin solution (Tanner, 1989), and 1.0 mg of resazurin/L of deionized water was used as the basal medium (Tanner et al., 1993). The pH of the solution was adjusted to 6.9. The medium was prepared anaerobically by boiling and cooling under an N₂–CO₂ (80:20) mixed-gas atmosphere. After cooling, the medium was dispensed into 125 ml glass culture vials (serum bottles) under an N₂–CO₂ mixed-gas atmosphere. The vials were crimp-sealed and autoclaved.

Before starting the culture, fructose, yeast extract, and L-cysteine-HCl·H₂O were added to reach final concentrations of 2.0, 1.0, and 1.2 g/l, respectively. The final volume was adjusted to 50 ml with water. To provide H₂, the headspace pressure in the vials was adjusted to 0.12 MPa by using N₂–CO₂.

**Table 1** | Strains used in this study.

| Strain name | Relevant characteristics | References |
|-------------|--------------------------|------------|
| Wild type   | ATCC 39073               | ATCC       |
| M₄-aldh     | pyF::aldh                | Rahayu et al., 2017 |
| M₄-ΔpduL2::aldh | Δaldh | Rahayu et al., 2017 |
| M₄-ΔpduL1ΔpduL2::aldh | Δaldh | Rahayu et al., 2017 |
| M₄-ΔpduL2::acetone | Δaldh | Kato et al., 2021 |

**REFERENCES**

- Drake, 1994
- Pensabene et al., 2018
- Fackler et al., 2021
- Moorella thermoacetica ATCC 39073 and its derivatives were used in this study (Table 1). Modified ATCC1754 PETC medium comprising 1.0 g of NH₄Cl, 0.1 g of KCl, 0.2 g of MgSO₄·7H₂O, 0.8 g of NaCl, 0.1 g of KH₂PO₄, 0.02 g of CaCl₂·2H₂O, 2.0 g of NaHCO₃, 10 ml of trace elements, 10 ml of Wolfe’s vitamin solution (Tanner, 1989), and 1.0 mg of resazurin/L of deionized water was used as the basal medium (Tanner et al., 1993). The pH of the solution was adjusted to 6.9. The medium was prepared anaerobically by boiling and cooling under an N₂–CO₂ (80:20) mixed-gas atmosphere. After cooling, the medium was dispensed into 125 ml glass culture vials (serum bottles) under an N₂–CO₂ mixed-gas atmosphere. The vials were crimp-sealed and autoclaved.

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(80:20) mixed-gas. H₂ gas was then injected at the desired pressure. For example, when 0.01 MPa of H₂ was tested, the total pressure was adjusted to 0.13 MPa by the H₂ gas injection. Cells were grown at 55°C with shaking at 180 rpm.

**Analytical Methods**

We sampled and analyzed 1 ml of the culture medium at each time point and calculated the dry cell weight using the optical density (OD) at 500 nm [OD₅₀₀; g (dry cell weight)/L = 0.383 OD₅₀₀; Iwasaki et al., 2017]. The culture supernatant was analyzed for the amount of fructose, formate, acetate, ethanol, and acetone using high-performance liquid chromatography (HPLC; LC-2000 Plus HPLC; Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031 Plus; Jasco), Shodex RSpak KC-811 column (Showa Denko, Kanagawa, Japan), and Shodex RSpak KC-G guard column (Showa Denko) at 60°C. Ultrapure water containing 0.1% (v/v) phosphoric acid was used as the mobile phase at a flow rate of 0.7 ml/min, and crotonate was used as the internal standard (Miura et al., 2014). The gas composition in the headspace of the culture vials was analyzed using GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a stainless steel column packed with activated carbon at 70°C. Argon was used as the carrier gas (Miura et al., 2014). The total gas pressure in the headspace was measured using a differential pressure gauge (DMC-104N11; Okano Works, Tokyo, Japan).

**Metabolome Analysis**

Strains were grown to reach the exponential phase between 0.5 and 0.7 OD₅₀₀. The culture was immediately filtered to collect cells equivalent to a total count of 20 OD₅₀₀ (volume [mL] × OD₅₀₀ ≈ 20). Filtration was performed using hydrophilic PTFE, 1 μm pore size, and a 90-mm-diameter filter disk (Omnipore; Merck KGaA, Darmstadt, Germany). Harvested cells were immediately immersed in pre-chilled methanol containing 100 μM ribitol and 100 μM (+)-10-camphorsulfonate to quench the metabolic activity. This procedure was quickly performed, within 45 s after opening culture vials, to avoid metabolites from artifacts, such as those caused by oxygenation, degradation, and other modifications. Subsequently, intracellular metabolites were extracted using the chloroform–water–methanol method (Bolten et al., 2007). The supernatant was then concentrated using a centrifugal concentrator (CC-105; Tomy, Tokyo, Japan). According to a previous study, pre-treatment and analysis of the dried samples were performed (Wada et al., 2022).

**RESULTS**

**H₂ Supplementation Increases Carbon Utilization in Mixotrophic Growth by Producing Acetate, Not Ethanol, in Engineered Strains**

*Moorella thermoacetica* can convert one hexose molecule to three acetate molecules in theory (Figure 1A; Fontaine et al., 1942; Schuchmann and Müller, 2014, 2016). The engineered strains were designed to produce ethanol from acetyl-CoA in two steps (reducing reactions; Rahayu et al., 2017; Table 1). The reducing equivalents provided by glycolysis were assumed to be properly consumed (Figures 1A,B). In a model, the Ech complex, HydABC complex, and NfnAB complex would convert the reducing equivalents to NADH and NADPH. These NADH and NADPH would be consumed by the Wood–Ljungdahl pathway to convert CO₂ to acetate in the wild-type strain, whereas reduction of acetyl-CoA would consume the NADH and NADPH in the ethanol-producing strains. Therefore, the redox conditions in the engineered strains producing ethanol should be balanced. In fact, we previously observed that all engineered strains (Table 1) grew and produced ethanol on hexose sugars. However, one molecule of CO₂ is released to produce one molecule of ethanol because of the requirement for extra reducing equivalents (approximately 33% of the carbon is released from hexose sugars in theory). Therefore we supplied H₂ to increase carbon utilization by mixotrophy.

We used a culture containing fructose as the carbohydrate substrate. H₂ was added to the headspace of the culture vial at a partial pressure of 0.08 MPa (equivalent to 40% of the gas phase). The gas phase also contained CO₂ and therefore extra CO₂ could be incorporated in addition to the released CO₂. Of the injected gas, CO₂ was 12% of the total, and the medium contained NaHCO₃ to supply CO₂. First, we tested the effect of H₂ on the wild-type strain. Acetate was produced as the end product and the carbon molar yield improved from 0.74 to 0.82, as expected (Figures 2A,B). The optical density increased similarly while fructose was consumed, and decreased after the complete consumption of fructose in both conditions, indicating no significant effect on the growth (Figure 2C). We then tested an ethanol-producing strain, Mt-*aldh*, in which the * aldh gene encoding aldehyde dehydrogenase was expressed by a constitutive promoter. The main product was acetate, accompanied by a small amount of ethanol. This trend was similar in the H₂-supplied culture, and yield improvement was only observed for acetate production from 0.78 to 0.88 (Figure 2A). The change in ethanol production was not significant (0.02 and 0.03; Figure 2B). No significant effect on the growth was observed (Figure 2D). Although we expected to enhance the yield of reduced products, we reasoned that the abundant activity of the acetate production pathway in the Mt-*aldh* strain decreased the effect of H₂. We then tested another ethanol-producing strain, Mt-*ΔpduL2:aldh*, which showed less acetate production due to deletion of one of the two genes (* pduL2*) encoding phosphoacetyl transferase in the acetate production pathway. Despite the dominant production of ethanol over acetate, product yield enhancement was observed with only acetate from 0.19 to 0.25 (Figures 2A,B). The carbon molar yield for ethanol did not change (0.37). The rest was released as CO₂. The strain grew similarly in both conditions (Figure 2E). The effect of H₂ was in contrast to the results of a previous study, in which the supplementation of reducing power with H₂ was reflected in the production of more-reduced chemicals (Jones et al., 2016).
H₂ Supplementation Causes Growth Inhibition of the Engineered Strain, Which Exclusively Produces Ethanol

We tested the Mt-ΔpduL1ΔpduL2::aldh strain, which produces almost exclusively ethanol, because of the complete knockout of both two genes (pduL1 and pduL2) encoding phosphoacetyl transferase. In this case, we observed an enhancement in the ethanol yield. The carbon molar yield for ethanol was increased from 0.47 to 0.54 (Figure 2B). Approximately 50% of the carbon from fructose was released as CO₂ in the absence of H₂, and H₂ supplementation supported capture and conversion of CO₂. However, the supplemented fructose was not completely consumed in the H₂-supplied condition after 60 h of cultivation, whereas the same strain in the H₂-unsupplied condition, or the other strains in both conditions, consumed fructose completely (Figures 2C–F). Only 40.2% of the supplemented fructose was consumed in the H₂-supplied condition, and the growth was significantly reduced in a correlated manner (Figure 2F). The volumetric amount of ethanol was also significantly less in the H₂-supplied condition, showing only 45.5% against the no-H₂ condition (17.6 mM in the absence of H₂ and 8.0 mM in the presence of H₂), reflecting the amount of consumed fructose. Therefore, although the Mt-ΔpduL1ΔpduL2::aldh strain showed increased carbon molar

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**FIGURE 1** | Redox-balanced pathways for acetogenesis (A), ethanol production (B), and acetone production (C) from hexose in wild-type and engineered strains for ethanol production. NADH (shown in blue) and the reduced forms of ferredoxin are produced during glycolysis and the conversion of pyruvate to acetyl-CoA. The reduced ferredoxin is converted to NADPH (shown in green) via hydrogenases and electron-bifurcating enzymes, that is, the Ech complex, HydABC, and NfnAB complexes (shown in orange).
yield for ethanol under \( \text{H}_2 \)-supplemented mixotrophic conditions, growth inhibition emerged as an unexpected bottleneck.

We also tested the addition of different amounts of \( \text{H}_2 \) to the \( \text{Mt-}\Delta\text{pduL1}\Delta\text{pduL2}::\text{aldh} \) strain. In addition to the condition of partial pressure 0.08 MPa, we tested 0.04, 0.02, and 0.01 MPa, because the pressure of \( \text{H}_2 \) is correlated with dissolved \( \text{H}_2 \) in the culture medium. All cases with \( \text{H}_2 \) at any concentration showed growth inhibition effects. Interestingly, the effect of growth inhibition was dose-dependent, showing more potent inhibition by a higher concentration of \( \text{H}_2 \) in the culture medium, rather than by a certain threshold.

![FIGURE 2](image-url)
This tendency was clear when the growth rate was plotted against the \(H_2\) pressure, showing a linear correlation (Figure 2H).

**Metabolome Analysis Identifies Specific and Significant Enhancement of Intracellular NADH Level by \(H_2\) Supplementation in the Ethanol-Producing Strain**

To investigate the \(H_2\)-dependent growth inhibition mechanism of the ethanol-producing strain, we assessed intracellular metabolism by metabolome analysis. We used \(H_2\) at 0.02 MPa, because a high dose of \(H_2\) (such as 0.08 MPa) inhibited the growth almost completely, and hence might have significant effects on multiple metabolic pathways. We sampled the cells from the exponential phase and analyzed intracellular metabolites using GC–MS and LC–MS following the sample preparation method we developed. We succeeded in quantifying 19 intracellular metabolites, including seven cofactors (Figures 3A,B).

We then compared the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain and the wild-type strain, with or without \(H_2\) supplementation. Among all analyzed metabolites, NADH levels were strikingly lower in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain under \(H_2\)-supplied conditions. The NADH level increased by approximately four times compared to that in the no \(H_2\) condition, whereas \(H_2\) supplementation did not affect the NADH level in the wild-type strain. There was no such significant difference specific to \(H_2\) supplementation in the other metabolites in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain or metabolites in the wild-type strain (Figure 3B).

When the overall metabolite profiles were compared in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh and wild-type strains in the no \(H_2\) condition, the levels of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, 3-phosphoglyceric acid, glutamate, and ATP were lower, and the levels of acetyl-CoA, AMP, and NADPH were higher in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain. The ATP level was lowered in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh due to the knockdown of acetate production coupled with substrate-level phosphorylation, but the ATP level was enough to maintain the growth (Figure 2F). In contrast, the AMP level was higher in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh, and this may be related with the change of ATP level. The higher level of acetyl-CoA probably reflects a difference of conversion rate of acetyl-CoA to ethanol and acetate. The higher level of NADPH in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain suggests that the redox balance in this strain was altered by metabolic engineering. Metabolome analysis indicated that the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain suffered redox imbalances due to both metabolic engineering and \(H_2\) supplementation.

**Hydrogen Production by the Ethanol-Producing Strain**

Metabolic analysis suggested a strong relationship between growth inhibition by \(H_2\) and increased levels of intracellular NADH. In *M. thermoacetica*, NAD⁺ is reduced to NADH by the electron-bifurcating hydrogenase HydABC complex (Wang et al., 2013). The HydABC complex reduces NAD⁺ and ferredoxin using electrons from \(H_2\). This reaction is reversible and produces \(H_2\) from NADH and reduced ferredoxin in *vitro*. Therefore, we measured the amount of \(H_2\) in the headspace of culture vials (Figure 4). The amount of \(H_2\) in the headspace was traced in conditions with or without \(H_2\) supplementation, and the wild-type strain and the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain were compared. \(H_2\) was supplied at 0.02 MPa of a partial pressure, in addition to fructose as a carbohydrate substrate, which was the same condition for our metabolome analysis. There was almost no \(H_2\) production by the wild-type strain, and the supplied \(H_2\) was consumed over time (Figure 4A). In contrast, the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain apparently did not consume \(H_2\) under the same conditions (Figure 4B). Moreover, when \(H_2\) was not supplied, the \(H_2\) level increased in the culture vial of the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain, in contrast to that in the wild-type strain. \(H_2\) production is usually attributed to the disposal of excess electrons from metabolism. In this case, this was most likely due to the increased level of NADH. However, \(H_2\) formation would require a sufficiently low level of \(H_2\) as the product. Therefore, the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain would have produced \(H_2\) for the clearance of the excess electrons from catabolizing fructose, and \(H_2\) supplementation would inhibit the \(H_2\) production, causing the growth inhibition due to the redox imbalance.

**NADH Consumption by DMSO Reduction Prevents the Growth Inhibition by \(H_2\)**

The results of the \(H_2\) measurement strongly indicated that the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain produced \(H_2\) using excess reducing equivalents and balanced the intracellular redox. Our metabolome analysis showed imbalanced redox in the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain, manifested as an increased level of intracellular NADH. If a high level of intracellular NADH is the direct cause of growth inhibition, the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain should recover its growth in the presence of \(H_2\) by lowering the intracellular NADH level. *M. thermoacetica* uses dimethyl sulfoxide (DMSO) as an electron acceptor, and the reported cases of bacterial DMSO reduction are NADH-dependent reactions (Zinder and Brock, 1978; De Bont et al., 1981; Drake and Daniel, 2004; Takemura et al., 2021b; Rosenbaum et al., 2022). We attempted to oxidize intracellular NADH via DMSO reduction by supplementing the culture medium with DMSO. We set up a culture of the Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain with \(H_2\) supplied at 0.08 MPa of partial pressure in addition to fructose, which showed strong growth inhibition (Figures 2F–H). At 24 h, the culture was supplied with 10 mM DMSO. The Mt-\(\Delta\)pduL1\(\Delta\)pduL2::aldh strain showed very slow growth with \(H_2\) supplementation, but the growth rate dramatically increased upon DMSO supplementation (Figure 5A). The fructose supplied was completely consumed after 55 h (Figure 5B). The supplied DMSO was readily consumed within 20 h after the DMSO addition, consistent with the recovery of growth and fructose consumption.
We analyzed the effect of DMSO on the intracellular metabolome in the presence of H₂ (Figure 3B). We used the same culture conditions as in the metabolome analysis (H₂

(Figure 5C). The addition of DMSO also improved total ethanol production, whereas acetate production remained minor (Figures 5D,E).
partial pressure = 0.02 MPa), except for DMSO supplementation, which was provided when the cells entered the exponential phase. As expected, the intracellular level of NADH was lower than that in the H\(_2\) condition. Therefore, growth recovery correlated with intracellular NADH levels.

**H\(_2\) Enhances Target Metabolite Production by a Metabolically Engineered Strain With Balanced Redox**

We found that the ethanol production pathway designed to balance the redox reaction requires tuning the imbalanced...
redox by producing $\text{H}_2$. This means that our ethanol-producing strains need to be re-engineered to balance the redox reaction to benefit from $\text{H}_2$-supplemented mixotrophy. However, it is possible that artificial modifications in the genome can affect metabolic activities in an unpredictable manner. We previously succeeded in engineering $\text{M. thermoacetica}$ to produce acetone (Kato et al., 2021; Table 1). The acetone synthesis pathway does not require any oxidoreductases to convert acetyl-CoA to acetone, which has the same redox balance as that of the native acetate pathway (Figure 1C). Therefore, acetone production has completely the same redox balance as acetate production, and the redox balance should not be affected. On the other hand, the pduL2::acetone strain has the same elements of genetic modification as an ethanol-producing strain, Mt-$\Delta$pduL2::aldh, using a pyrF marker for selection and a constitutive G3PD (glyceraldehyde 3-phosphate dehydrogenase) promoter to express $\text{aldh}$, disrupting the acetate pathway. Therefore, we examined whether the introduction of an oxidoreductase affected the redox balance by testing the $\text{H}_2$-supplemented mixotrophic acetone production of the pduL2::acetone strain.

We set up cultures of the pduL2::acetone strain with fructose as the carbohydrate substrate and tested the effect of $\text{H}_2$ supplementation, as was performed for ethanol-producing strains. $\text{H}_2$ was supplied at 0.08 MPa of partial pressure, the highest dose used for the ethanol-producing strains. The pduL2::acetone strain grew in the presence of $\text{H}_2$ in the same manner as in its absence (Figure 6A). The optical density increased while fructose was consumed, and decreased after the complete consumption of fructose in both cases. Furthermore, the produced acetone and acetate increased with $\text{H}_2$ supplementation (Figure 6B), in contrast to ethanol production. Acetone production was enhanced by 13%, and the total carbon molar yield (the sum of acetate and acetone) was greater than one, indicating that extra CO$_2$ was converted to these metabolites. CO$_2$ was externally provided in the headspace gas and from NaHCO$_3$ in the medium, in addition to CO$_2$ released from metabolism. Engineering to introduce a non-reductive pathway did not erase the effect of mixotrophy. Therefore, we concluded that introducing oxidoreductase reactions to convert acetyl-CoA to ethanol caused $\text{H}_2$ production by extra electrons and $\text{H}_2$ inhibition.

**DISCUSSION**

Metabolic engineering to divert acetate to more reduced chemicals lowers carbon utilization and releases more CO$_2$ in acetogens due to the loss of the reducing power to fix and convert CO$_2$ for oxidoreductase reactions. One strategy for overcoming this issue is $\text{H}_2$-supplemented mixotrophy. However, our engineered strains, Mt-$\text{aldh}$ and Mt-$\Delta$pduL2::aldh, produced an increased amount of acetate instead of ethanol. Growth inhibition was observed in the Mt-$\Delta$pduL1$\Delta$pduL2::aldh strain. Therefore, the strategy for enhancing carbon utilization by $\text{H}_2$ is not effective for metabolically engineered strains in terms of ethanol production.

Growth inhibition due to $\text{H}_2$ supplementation has been reported in several studies. For example, one classical case involves Clostridium cellubiovarum, an $\text{H}_2$ producer. When $\text{H}_2$ was added to the culture, C. cellubiovarum growth was inhibited in an $\text{H}_2$ dose-dependent manner (Chung, 1976). This trend was similar to that of our ethanol-producing strain, Mt-$\Delta$pduL1$\Delta$pduL2::aldh. The study also reported that the removal of $\text{H}_2$ recovered the growth using a catalyst, gassing out, or co-culture with methanogenic microorganisms.
C. cellubiovarum is a resident of the bovine rumen, living together with methanogens; therefore, the H₂ level remains low and does not affect their growth in situ (Hungate, 1967; Hungate et al., 1970). Another example was observed in the case of a thermophilic microorganism for H₂ bioproduction, *Caldicellulosiruptor saccharolyticus* (Willquist et al., 2011). A high level of H₂ produced by *C. saccharolyticus* inhibits its own growth, demanding continuous stripping of the produced H₂ from fermentation. Interestingly, the desired by-product for high H₂ yields is acetate, because more-reduced products, such as ethanol, drain electrons from H₂ production. When H₂ levels increase, *C. saccharolyticus* produces lactate and ethanol instead of H₂ to oxidize NADH and maintain the NADH/NAD ratio. In contrast, our metabolically engineered ethanol-producing strain of *M. thermoacetica* produced H₂ instead of ethanol to oxidize NADH. *M. thermoacetica* has been reported to evolve H₂ under certain conditions, such as in a CO-supplemented culture with glucose (Martin et al., 1983). The intracellular activity level of hydrogenases is significantly enhanced by CO, but not by other gas phases, including H₂ (Kellum and Drake, 1984). *M. thermoacetica* does not evolve H₂ in a standard culture under heterotrophic conditions, as seen in our experiment.

Metabolic analysis revealed that the Mt-ΔpduL1ΔpduL2::aldh strain could maintain NADH levels in the absence of H₂. This was due to H₂ production for NADH oxidation, and might also be due to NADPH production using the NfnAB complex. The NfnAB complex transfers electrons from reduced ferredoxin and NADH to NADP⁺ (Huang et al., 2012). However, because the Mt-ΔpduL1ΔpduL2::aldh strain possesses a high NADPH level, the conversion of reduced ferredoxin and NADH to NADPH would be inhibited or difficult. It is unclear why the basal level of NADPH in the Mt-ΔpduL1ΔpduL2::aldh strain was higher than that in the wild-type strain. One possibility is the slow conversion of acetyl-CoA to ethanol, because metabolome analysis showed that the intracellular level of acetyl-CoA was higher in the Mt-ΔpduL1ΔpduL2::aldh strain (Figure 3B). The reaction speed may have limited the consumption of cofactors NADH and NADPH, and the cofactors of the reduced forms accumulated. Although NADPH production can be balanced by H₂ formation, NADPH production cannot be balanced. The Mt-ΔpduL1ΔpduL2::aldh strain could grow and produce ethanol in non-H₂-supplemented conditions, but NADH could not be balanced upon H₂ supplementation due to the blockage of H₂ production. Another possibility is unregulated NADPH production by the pentose phosphate pathway caused by unknown mechanisms due to metabolic engineering. Although a high level of NADPH remained, even in the presence of DMSO to consume NADH under H₂-supplemented conditions (Figure 3B), the NADPH level did not inhibit growth. Growth inhibition was caused by high NADH levels. We assume that the target reaction influenced by NADH level is glyceraldehyde 3-phosphate dehydrogenase, because this is NADH-dependent (Thauer, 1972; Huang et al., 2012). In studies on ethanol tolerance, glyceraldehyde 3-phosphate dehydrogenase was found to be inhibited by high levels of NADH in *Clostridium thermocellum* (Tian et al., 2017).

Finally, we confirmed that the increased acetate formation, but not ethanol formation, was due to the redox balance itself in the Mt-ΔpduL2::aldh strain. An engineered strain for acetone production produced higher levels of acetate and acetone with H₂ supplementation. Therefore, our strategy for gene manipulation itself did not affect redox balance, and the usefulness of H₂-supplemented mixotrophy was confirmed. The introduction of oxidoreductases affected the redox balance in ethanol-producing strains; hence, H₂ supplementation only enhanced acetate or inhibited growth. Although the metabolic pathway was designed to be redox-balanced by choosing oxidoreductases with appropriate cofactors (Figure 1), fine-tuning and a different design for properly balanced redox is required to derive benefits from H₂-supplemented mixotrophy for ethanol production. In contrast, if H₂ production is the aim of engineering, a metabolic design to increase the intracellular NADH level is one strategy to exploit the hydrogenase reaction.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

JK and YN conceived and designed the experiments. ShK, JK, and KW performed the experiments. ShK, JK, KW, KT, SeK, TF, YI, YA, TM, AM, and KM analyzed the data. ShK, JK, and KW visualized the data. JK prepared the manuscript. YN supervised the project. All authors have contributed to the manuscript and approved the submitted version.

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