Construction of an aerobic isobutanol-producing strain of *Escherichia coli* based on enzymatic and metabolic engineering

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Research objective

Microbial production of isobutanol (IB), which is a raw material for polyethylene terephthalate, has attracted attention in response to the recent trend toward bio-based chemicals. Among them, almost all studies that have used *Escherichia coli* as a host have employed anaerobic conditions during the stationary phase as an evaluation target to ensure reduction power and for avoiding competition during growth. Under these conditions, while the IB production yield can be increased, both the low amount of biomass due to the anaerobic conditions and the low substrate uptake rate due to the stationary phase can substantially limit rate of IB production. Alternatively, although a substantial amount of biomass and a high substrate uptake rate can be guaranteed under aerobic conditions during the log phase, the IB production yield will decline because of the increase in CO_2 generated by complete substrate oxidation (**Fig. 1**). In the present study, I focused on pyruvate, the branching point of carbon flow, and aimed to improve the flux to the IB side by limiting the flux to the growth side. Specifically, I adopted a strategy in which the distribution efficiency of pyruvate was changed by weakening AceE and strengthening AlsS using the catalytic efficiency (k_{cat}/K_m) value as an index.

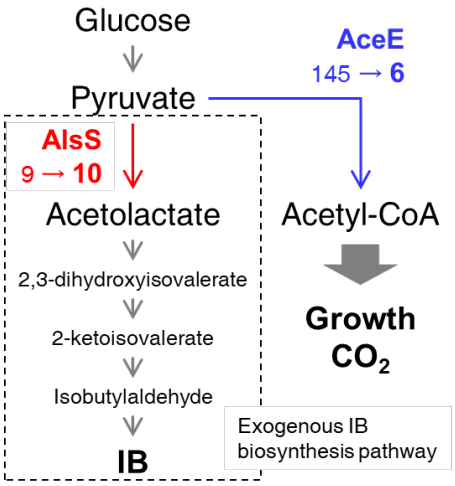

Fig. 1. A strategy focusing on the catalytic efficiency (k_{cat}/K_m) of enzymes involved in pyruvate distribution. The numbers in dicate k_{cat}/K_m values.

Methods

Escherichia coli MG1655 was transduced with T7 RNA polymerase gene using a λDE3 Lysogenization kit. A site-specific mutagenesis by pORTMAGE[1] was employed to construct the *aceE* mutant[2]. The plasmid for IB production (pIB) was constructed by linking five genes of the IB biosynthetic pathway[3] which were codon-optimized for *E. coli* into pIB under a T7 promoter. Site-specific mutagenesis by PCR was used to construct the *alsS* mutant[4]. *E. coli* harboring pIB was cultured aerobically in a modified M9 medium using

glucose as the sole carbon source. The supernatant of the culture was analyzed by HPLC to quantify the concentrations of glucose, IB, and organic acids. Intracellular pyruvate levels were measured using an EnzyChrom Pyruvate Assay kit.

Results

1) IB production using the *aceE* mutant strain

In a previous study, the k_{cat}/K_m value of the AceE variant ($AceE^{K403A}$) was approxymately 4% of AceE[2]. It is considered that the decrease in AceE activity leads to a reduced amount of flux to the TCA cycle, which is the main energy production pathway, thereby resulting in a lower growth rate. As expected, the specific growth rate of the $aceE^{K403A}$ strain decreased to approximately 17% of that of the wild-type strain. This result indicates that the weakening of AceE suppressed pyruvate distribution to the growth side. However, the growth rate as well as the IB production and glucose consumption decreased in the $aceE^{K403A}$ strain harboring pIB (**Fig. 2**). The reason for this is currently unknown; however, it is considered that certain enzymes supplemented the flux from pyruvate to acetyl-CoA instead of AceE because the levels of biomass and acetate production were increased relative to glucose consumption. Therefore, to improve the distribution efficiency of pyruvate to the IB side, further identification and suppression of the outflow pathway to acetate will be necessary to prevent pyruvate distribution to the growth side.

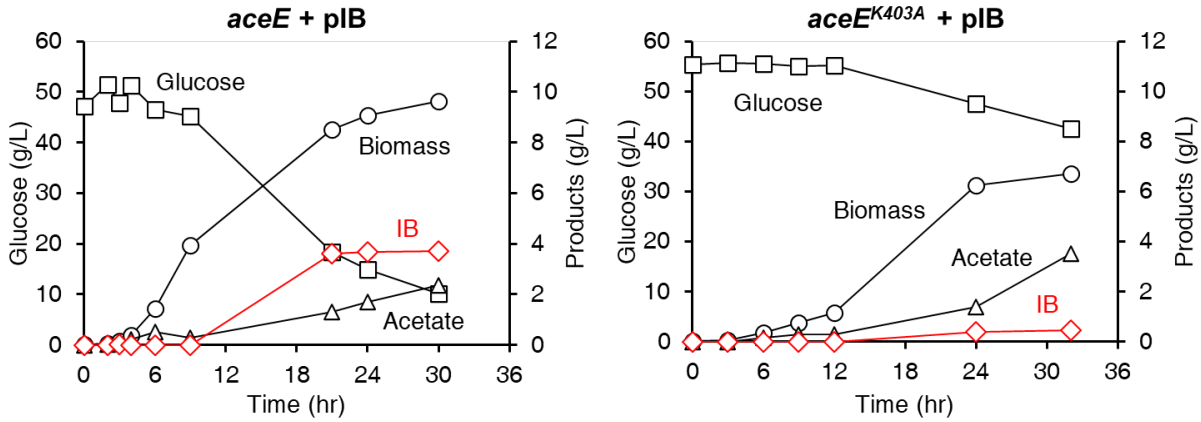

Fig. 2. Effects of *aceE* mutation on IB productivity.

2) IB production using the *alsS* mutant strain

AlsS, which catalyzes the initial reaction to the IB production pathway, uses pyruvate as a substrate in a similar manner to AceE catalyzing the initial reaction of the TCA cycle. The intracellular pyruvate concentrations of the *alsS* strain constructed in the present study under aerobic and anaerobic conditions were 1.2 mM and 3.1 mM, respectively. In contrast, it was reported that the K_m and k_{cat} values of AlsS were 13.6 mM and 121 s^{-1}, while those of the AlsS variant ($AlsS^{Q487S}$) were 1.1 mM and 10 s^{-1}, respectively[4]. From these results, it is hypothesized that the high k_{cat}/K_m value of $AlsS^{Q487S}$ can be utilized under

aerobic conditions because the intracellular pyruvate concentration was close to the K_m value of $AlsS^{Q487S}$ (1.1 mM) and was considerably lower than the of AlsS. In fact, the *alsS*^{Q487S} strain showed higher IB productivity than the *alsS* strain despite the low biomass (**Table 1**; biomass: 0.9-fold, IB production: 1.2-fold, specific IB production rate: 1.5-fold). This result indicates that the increase in k_{cat}/K_m contributes to the enhancement of the pyruvate distribution to the IB side. However, the amount of IB produced in the log phase was approximately 13% of that produced in the entire culture phase. This result is consistent with the fact that the k_{cat}/K_m values of AlsS and $AlsS^{Q487S}$ are extremely low compared to those of AceE, implying that IB production under aerobic conditions during the log phase must be integrated with AceE weakening.

Strain	Biomass (g/L)	IB production (g/L)	Specific IB production rate (mg/gDCW/h)
alsS	8.07 ± 0.07	4.69 ± 0.46	43 ± 4
alsS^{Q487S}	6.97 ± 0.08	5.49 ± 0.46	63 ± 6

Table 1. Effects of *alsS* mutation on IB productivity.

Conclusion

As expected, the growth rate of the *aceE*^{K403A} strain decreased to 17%. The specific IB production rate of the *alsS*^{Q487S} strain was 1.5-fold higher than that of the *alsS* strain, and 13% of the final titer of IB was obtained during the log phase. Further investigation is warranted to understand the mechanisms regulating the decrease in glucose consumption and IB production in the *aceE*^{K403A} strain harboring pIB. Additionally, it is also necessary to evaluate the IB productivity of strains harboring the *aceE*^{K403A} and *alsS*^{Q487S} mutation.

References

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