Challenges in the production of itaconic acid by metabolically engineered *Escherichia coli*

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Metabolic engineering allows the production of a variety of high-value chemicals in heterologous hosts. For example, itaconic acid (IA) has been produced in several microorganisms, such as *Escherichia coli*, *Aspergillus niger*, and *Synechocystis* sp. through the expression of cis-aconitate decarboxylase gene (*cad*) from *Aspergillus terreus*. Recently, we showed that inactivation of the isocitrate dehydrogenase gene and overexpression of the aconitase gene dramatically enhanced the production levels of IA in *E. coli* expressing *cad*. Furthermore, we demonstrated that it is possible to produce IA directly from starch by engineered *E. coli* that additionally expresses the α-amylase gene from *Streptococcus bovis*. In this study, we sum up our findings regarding the challenges of IA production in *E. coli*.

Metabolic engineering has opened a novel avenue for producing chemicals of interest, including biofuels and high-value commodities in heterologous hosts. In particular, itaconic acid (IA), a promising vinyl monomer that is industrially produced by *Aspergillus terreus*, has recently been produced in *Escherichia coli*.

**Keywords:** α-amylase, *Escherichia coli*, itaconic acid, metabolic engineering, starch

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**ADDENDUM**

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ADDENDUM
culture. HPLC analysis revealed that cells grown for 18 h at 20°C produced 0.03 g/L of IA, suggesting that cad expression in a heterologous host promote production of IA. However, cells grown at 37°C did not produce IA. To further investigate this result, we performed SDS-PAGE. While the Cad protein (54.0 kDa) was present in the soluble fraction of cells grown at 20°C, it was mainly found in the insoluble fraction when expression was performed at 37°C (Fig. 1). Soluble Cad purified from recombinant E. coli shows activity at 37°C, but it is rather unstable. It is likely that the protein has low stability in vivo and that it forms insoluble aggregates during expression at 37°C, thus, losing its activity. The result shown in Figure 2 suggests that expression of Cad at a moderate temperature, such as 20°C to 30°C, might be suitable for IA production in E. coli. To facilitate protein folding at 37°C, we co-expressed Cad with the molecular chaperons DnaK, GrpE, and GroESL, following a published protocol; however, this attempt was unsuccessful.

In our study, we also observed an increase of the concentration of acetic acid in the cell culture upon acn overexpression (9.04 g/L after 105 h of growth). Overexpression of acn likely suppresses the activity of the glyoxylate shunt, resulting in an increase of acetic acid concentration derived from acetyl-CoA. To further optimize the production of IA, we inactivated both the acetate kinase gene (ack) and the pyruvate oxidase gene (pox), which are involved in acetic acid production, in E. coli expressing cad. In this study, we introduced pAK12-cad into the engineered E. coli to express cad. Cells bearing these modifications grew slower than the control cells (Fig. 3A), but we observed similar Cad activity in E. coli BW25113 (pAK12-cad) (9.9 U/g-protein) and in BW25113 (Δack, Δpox, pJAK12-cad) (10.3 U/g-protein). As expected, inactivation of ack and pox resulted in nearly 1.5-times higher levels of IA compared to the control (Fig. 3B). These findings indicate that ack and pox inactivation enhances IA production in cad-expressing E. coli.

If IA could be produced by E. coli using starch as a substrate instead of glucose, production costs would be considerably lower. To reach this goal, Okamoto et al. expressed the α-amylase gene (amy) from Streptococcus bovis in E. coli expressing cad and inactivated idc. In this report, we constructed a new expression vector, pGV3, which is suitable for cell surface display of heterologous proteins in E. coli. Cell surface display represents a unique expression system to decorate protein on the cell surface. pGV3 consists of a fragment of the pVUB3 expression vector, which contains lacI, PTrc, opri, and rnrBT1T2, and a fragment of the pGBM1 cloning vector, which contains the Spc marker and the pSC101 origin. The amy gene was cloned into pGV3, resulting in pGV3-SBA.

Figure 1. SDS-PAGE analysis of the Cad protein in E. coli. E. coli BW25113 (DE3) pLysS harboring pETHis (control) or pETHis-cad (cad) were grown at 20°C or 37°C in 2 mL of LB medium after 0.1 mM IPTG induction. After 18 h of incubation, cells were harvested, disrupted, and separated into soluble (S) and insoluble (IS) fractions. SDS-PAGE analysis was performed on a 12.5% polyacrylamide gel. 10 μg of sample from the soluble and from the insoluble fraction (one-fourth of precipitants) were loaded. The corresponding molecular masses (in kDa) of protein markers (M) are indicated on the left. The expressed Cad protein (54.0 kDa) is indicated by the arrow.

Figure 2. Plot of growth temperature (°C) versus itaconic acid (IA) production (g/L). The overnight cultures of recombinant E. coli (open squares, BW25113 (DE3) (pLysS, pETHis-cad); closed squares, BW25113 (DE3) (Δcad, pLysS, pETHis-cad)) cultivated in LB medium at 37°C were inoculated in fresh 2 mL of LB medium to an OD600 of 0.1. The cultures were subsequently incubated at 10, 20, 25, 28, 30, 33, 35, or 37°C for 18 h. When the cultures reached an OD600 of 0.4–0.6, IPTG was added at a final concentration of 0.1 mM to induce cad expression. The IA production in the resulting strains was analyzed after growth with IPTG induction.

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OprI'-based fusion proteins were anchored into the outer membrane of *E. coli*. pGV3 is compatible with plasmids containing the origins for ColE1 and p15A. Similar to pGV3, we constructed pAV3 from a fragment of pVUB3 and from a fragment of the pACYC184 cloning vector. The pAV3 plasmid contained the Cm' marker and the p15A origin. The *amy* gene was cloned into pAV3, resulting in pAV3-SBA. When *E. coli* cells containing pAV3-SBA were induced, they expressed the OprI'-Amy fusion protein, as confirmed by SDS-PAGE. Amylase activity was also confirmed in the *E. coli* cells expressing *amy*. These results suggest that pAV3 is suitable for cell surface display of heterologous proteins in *E. coli*, and that it is compatible with plasmids containing ColE1 and pSC101. The technique constructed herein for direct production of value-added chemicals from biomass by *E. coli* expressing biomass-degrading enzymes may reduce total incurred costs, including the production costs due to carbon source.

In our study, we also showed that *E. coli* cells inactivating *icd*, which are auxotrophic for glutamate, showed no growth in a minimal medium lacking glutamate, but that they recovered upon addition of 0.5% glutamate. Table 1 shows cell growth and production levels of IA in *E. coli* BW25113 (DE3) (*Δicd*, pLYsS, pETHis-cad, pGV3-SBA) grown for 78 h in 2 mL of M9 minimal medium with and without 0.5% glucose, or 0.5% starch. Despite the fact that the engineered *E. coli* showed hardly any growth without glutamate, the levels of IA increased, indicating that *cad*-expressing *E. coli* showed non-growth associated production of IA. This suggests that carbon yield during IA production in *E. coli* can be improved by using resting *E. coli* cells.

The engineered *E. coli* produces IA less efficiently than *A. terreus*. However, itaconic acid production in *E. coli* can be further enhanced by metabolic approach, such as inactivation of undesired metabolic pathways involved in ethanol and lactate production. Further research and a deeper understanding of the metabolic properties of *E. coli* might indicate a novel approach to improve IA production in this organism.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

### Table 1. Numbers of cells yield (X), itaconic acid (IA) production (P), and specific product yield (YP/X) from engineered *E. coli* grown for 78 h in different culture media

| Media | Glutamate | Glucose | Starch | X (g-cell/L) | P (g-IA/L) | YP/X (g-IA/g-cell) |
|-------|-----------|---------|---------|-------------|------------|-------------------|
| M9 (Gluc−) | + | − | − | 0.10 | 0.00 | 0.00 |
| | + | + | − | 0.13 | 0.00 | 0.00 |
| | + | + | + | 0.14 | 0.12 | 0.86 |
| | + | + | − | 0.34 | 0.08 | 0.24 |
| | + | − | + | 0.15 | 0.09 | 0.60 |
| | + | − | + | 0.57 | 0.02 | 0.04 |

*a*Engineered *E. coli* cells were washed twice with M9 (Gluc−) after IPTG induction and subsequently inoculated in 2 mL of fresh media until they reached an OD600 of 0.1.

*b*M9 minimal medium without glucose.
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