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ORIGINAL RESEARCH PAPER

Engineering of itaconic acid pathway via co-localization of CadA and AcnA in recombinant *Escherichia coli*

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Abstract Itaconic acid is an excellent polymeric precursor with a wide range of industrial applications. The efficient production of itaconate from various renewable substrates was demonstrated by engineered *Escherichia coli*. However, limitation in the itaconic acid precursor supply was revealed by finding out the key intermediate of the tricarboxylic acid in the itaconic acid pathway. Efforts of enhancing the cis-aconitate flux and preserving the isocitrate pool to increase itaconic acid productivity are required. In this study, we introduce a synthetic protein scaffold system between CadA and AcnA to physically combine the two enzymes. Through the introduction of a synthetic protein scaffold, 2.1 g L^{-1} of itaconic acid was produced at pH 7 and 37 °C. By fermentation, 20.1 g L^{-1} for 48 h of itaconic acid was produced with a yield of 0.34 g g^{-1} glycerol. These results suggest that carbon flux was successfully increased itaconic acid productivity.

Keywords Metabolic engineering · *E. coli* · Itaconic acid · Scaffold protein · Fermentation

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Introduction

Itaconic acid is a white crystalline unsaturated C5 dicarbonic acid, which is widely used in the industrial synthesis of resins like polyesters and plastics, and the total market size of itaconic acid is about 30,000 ton per year (Steiger et al. 2013). In the itaconic acid production, due to high cost and relatively low yields, chemical synthesis of itaconic acid has been less competitive compared to the microbial fermentation processes. Among various itaconic acid producing strains, *Aspergillus terreus* is one of the dominant itaconic acid production hosts considering the high itaconic acid concentration (129 g L^{-1}) (Kuenz et al. 2012; Okabe et al. 2009). However, some characteristics of *A. terreus* such as the filamentous phenotype, slow growth rate, spore-forming life cycle and difficulty in genetic modification hinder the further engineering of *A. terreus* strain and process development.

The itaconic acid biosynthesis mechanism consists of the enzymatic decarboxylation of cis-aconitate to itaconic acid (Fig. 1). *cis*-Aconitate is an intermediate of the tricarboxylic acid cycle, and the itaconic acid synthesis is catalyzed by *cis*-aconitic acid decarboxylase (CadA). Since 2008, the sequence of the CadA of *A. terreus* was characterized, and the enhanced itaconic acid production studies were reported by overexpression of the heterologous CadA. For example, the overexpression CadA in *Saccharomyces cerevisiae* lead 0.17 g L^{-1} itaconic acid production from glucose (Blazeck et al. 2014). CadA has been

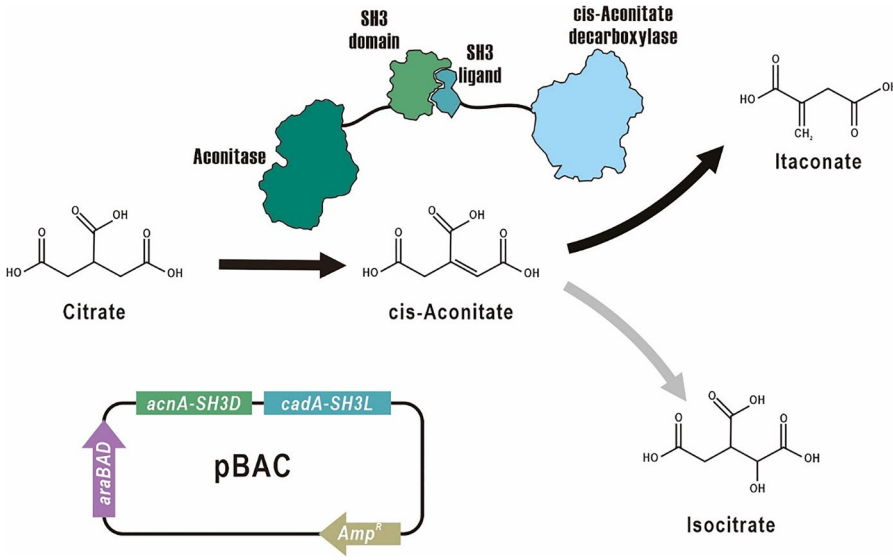

Fig. 1 Recombinant *E. coli* production of itaconic acid using a synthetic scaffold. Co-expression of AcnA-SH3D and CadA-SH3L to produce itaconic acid from glucose

introduced into various strains and the constructed recombinant strains were cultured with various carbon substrates for the itaconic acid production. Recombinant *Yarrowia lipolytica* produced 4.6 g L^{-1} itaconic acid from glucose (Blazeck et al. 2015), recombinant *Candida lignohabitans* produced 4 g L^{-1} from glucose (Bellasio et al. 2015), recombinant *Escherichia coli* produced 7 g L^{-1} from glycerol (Jeon et al. 2016), recombinant *Corynebacterium glutamicum* produced 7.8 g L^{-1} from glucose (Otten et al. 2015) and recombinant *Synechocystis* sp. PCC6803 produced 0.015 g L^{-1} from CO_2 (Chin et al. 2015). In spite of the intensive studies, the overall itaconic acid titer obtained from the recombinant hosts still remain low level compare with *A. terreus*. Therefore, development of the novel itaconic acid production strategy was required for further improvement of itaconic acid production. *E. coli* have many advantages as a production host, including well-established molecular tools for genetic modification and relatively easy operating conditions for fermentation. Recently *E. coli* is considered as promising non-natural hosts that produces itaconic acid (Becker and Wittmann 2016; Harder et al. 2016).

Enzyme co-localization is a novel strategy used to engineer the intracellular metabolism via protein–protein interaction between the domains and ligands. The co-localizing metabolic pathway enzymes can lead the decrease of the intermediates transit time, the prevent of intermediates loss to diffusion or competing pathways and the prevention of unstable intermediates (Miles et al. 1999). Therefore, the cellular metabolic flux can be redirected toward desired metabolic networks and metabolites. The enzyme co-localization strategy can improve metabolite production with low expression levels of pathway enzymes, and it has been used for the development of various metabolite production systems (Kuenz et al. 2012). For example, the concentration of glucaric acid in the recombinant *E. coli* was increased by 5 folds via employing enzyme co-localization strategy (Moon et al. 2010).

In this work, we employed the enzyme co-localization strategy to for the enhanced itaconic acid production from various carbon source in recombinant *E. coli*. CadA and aconitase (AcnA) were co-localized, and the constructed recombinant srtain was cultured for itaconic acid production. Itaconic acid production conditions were optimized, and the significant increase of itaconic acid concentration was archived in fed-batch fermentation study.

Materials and methods

Strains and culture conditions

The bacterial strains used in this study are listed in Table 1. The single colonies were picked from Luria–Bertani (LB) plates and inoculated overnight in 10 mL LB medium (10 g L^{-1} bacto-tryptone, 5 g

Table 1 *E. coli* strains and the plasmids used in this study

Strain or plasmid	Description	References
Strains		
XL1_Blue	F'(proAB^{+}lacI^{q} lacZΔM15 Tn10 (Tet^{r})	Laboratory stock
Plasmids		
pBAD30	pBAD30, araC promoter, Amp^{r}	NEB[a]
pBAC	pBAD30, (*acnA-SH3D*, *cadA-SH3L*)	This work
pBNC	pBAD30, (*acnA*, *cadA-SH3L*)	This work

L^{-1} bacto-yeast extract and 5 g L^{-1} NaCl) at 37 °C in a rotary shaker (250 rpm), then inoculated (1%, v v^{-1}) into 100 mL LB supplemented with antibiotics (100 g mL^{-1} ampicillin) until the optical density of the suspension reaches 0.6 at 600 nm wavelength (OD600). For expressing genes, 0.5% arabinose was added to the culture medium. pH, temperature and, glucose (10 g L^{-1}) was tested to optimize the culture conditions.

For itaconic acid production, M9 medium (12.8 g L^{-1} $Na_2HPO_4{\cdot}7H_2O$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl, 1 mmol $MgSO_4$, 1 mg L^{-1} vitamin B1 and 0.1 mmol $CaCl_2$) was also used with various carbon sources (10 g L^{-1} of xylose or 10 g L^{-1} of glycerol).

Fed-batch fermentation was performed in the 5 L stirred-tank bioreactor. Initially, 2.5 L of the modified M9 medium was used with the addition of 10 g L^{-1} of glycerol, 5 g L^{-1} of yeast extract. The fresh medium was inoculated with 100 mL of the overnight pre-culture grown in M9, 0.5% arabinose was added at the time of inoculation. Dissolved oxygen (DO) was maintained at 10% with respect to air saturation by raising the stirrer speed (from 300 to 800 rpm). The aeration was set to 1 vvm (volume of gas per volume of liquid per minute). The pH was maintained at 7 by the automatic addition of 1 M NaOH. At the 12 h, 24 h, 36 h 48 h, and 60 h, 15.6 mL of glycerol feed with 625 g L^{-1} were fed to the bioreactor. All chemicals and reagents were purchased from Sigma–Aldrich (South Korea) unless otherwise specified.

Plasmid construction

The oligonucleotides used in this study were listed in Table 2. The codon-optimized *cadA* gene (Gene bank ID: KM464677) (Vuoristo et al. 2015) was chemical synthesized and *acnA* from *E. coli* genome were amplified using the Expand high-fidelity polymerase chain reaction (PCR) system (Roche Molecular Biochemicals, Mannheim, Germany) from XB chromosomal DNA. *SH3* domain and *SH3* ligand genes were PCR amplified from the laboratory stock plasmid. Then, the *SH3* domain gene was fused to the C-terminus of *acnA* by overlap PCR to make *acnA-SH3D*, The *SH3* ligand was attached to the C-terminus gene to make *cadA-SH3L*. The *acnA-SH3D* genes were cloned into pBAD30C using SacI and XmaI restriction sites. The *cadA-SH3L* gene was cloned

Table 2 Primer used in this study

Primer	Sequence
acnA-F	GC**CCCGGG**AGGAGGATGCCTAACA TTACCTGGTG
acnA-SH3D-R	AGGGCCCGCACATACTCTGCGCTGCCGCCGCTGCCGCTGCCGCTGCCCTTCTGATTCAGGCTGGC
acnA-SH3D-F	GCCAGCCTGAATCAGAAGGGCAGCGGCAGCGGCAGCGGCGGCAGCGCAGAGTATGTGCGGGCCCT
SH3D-R	GC**GGTACC**TTATTAATACTTCTCCACGTAAGGGA
acnA-R	GC**GGTACC**TTATTACTTCTGATTCAGGCTG
cadA-SH3L-F	CC**GTGAAA**TCGCCACTGGTAGGCGCGCCGGGCTCTGGCGTGAAAGAATCTCTGGTGTAATAATCTAGACC
cadA-SH3L-R	GG**GCATGC**TTATTACACCAGAGATTCTTTCACGCCAGAGCCCGGCGCGCCACTTCCCACCTTTACCG

Restriction enzymes sites are shown in bold

downstream of *acnA-SH3D* genes using XmaI and XbaI restriction sites to construct the pBAC expression plasmids. Restriction enzymes, T4 DNA polymerase were obtained from New England Biolabs (Ipswich, MA). Oligonucleotides were purchased from Genotech (Daejeon, South Korea).

Analytical method

The concentrations of substrates and organic acids were determined by HPLC using the Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad). Samples were centrifuged at 12,000 rpm for 5 min. Then, the 1 mL supernatant was filtered through a 0.2 µm millipore filter and analyzed on HPLC system using RID detector. 0.08 N H_2SO_4 was used as a mobile phase. The temperature of the column was set at 35 °C and the flow rate of the mobile phase was 0.6 mL min^{-1}. The standard curves were determined using the same procedure for seven standard solutions: 0.1, 0.5, 1, 2, 3, 5, and 10 g L^{-1} of glucose, xylose, glycerol and organic acids (Sigma, Missouri, USA).

Result and discussion

Construction of the itaconic acid production strain

To overcome the low productivity of itaconic acid and achieve the economic feasibility of itaconic acid production process, the novel strategy allowing the redirection of pathway intermediate to itaconic acid network should be developed (Chang et al. 2017). In the TCA cycle, citrate is converted to isocitrate via *cis*-aconitate through two sequential reactions which are catalyzed by a single enzyme, AcnA. For the efficient itaconic acid production, the intermediate cis-aconitate need to redirected to itaconic acid by CadA (Fig. 1). If CadA was co-localized with AcnA by ligand-domain interaction, *cis*-Aconitrate produced by AcnA will have more chance to contact with CadA and can be converted to itaconic acid with higher chance.

To realize this idea, the codon-optimized *cadA* gene with SH3 ligand and *acnA* with SH3 domain were cloned to construct the plasmid pBAC, then introduced into *E. coli*.

By the overexpression of scaffold consist with AcnA-SH3D and CadA-SH3L under the control of *araC* promoter, the recombinant *E. coli* strain produced about 2.1 g L^{-1} of itaconic acid in the culture in 48 h (Fig. 2). The control strain without scaffold produced 0.19 g L^{-1} of itaconic acid. These data showed that itaconic acid production was increased by 11 folds by introduction of protein scaffold between AcnA and CadA, and TCA cycle carbon flux was redirected to itaconic acid pathway.

Optimizing condition of itaconic acid production

In order to optimize the conditions for itaconic acid production, the various pH (5, 6, 7, 8 and 9)

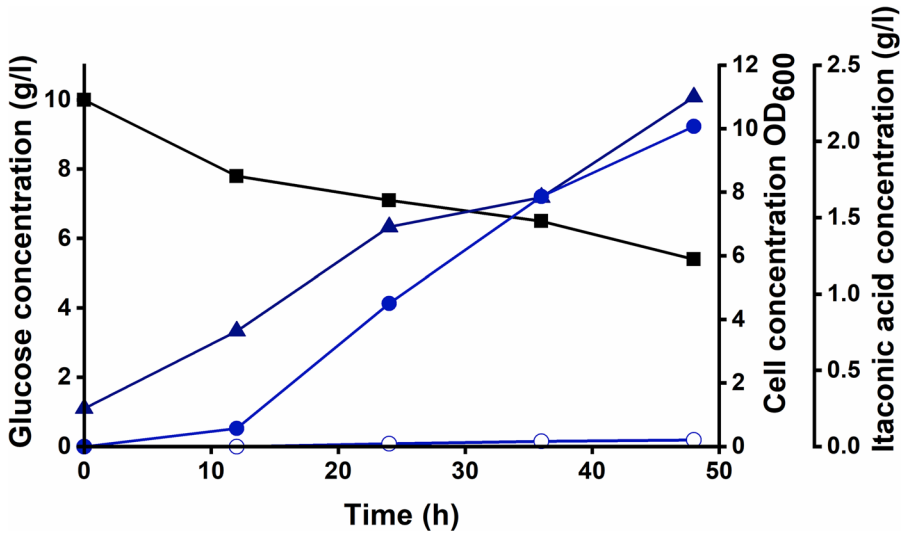

Fig. 2 Itaconic acid production by the strains with and without the AcnA and CadA scaffold. Itaconic acid concentration obtained with control strain *E. coli* (pBNC) (unfilled circle). Concentrations of itaconic acid (filled circle), glucose (filled square), and cell (filled triangle) obtained with *E. coli* (pBAC)

and temperature (25, 30 and 37 °C) were tested. As shown in Fig. 3a, the strain cultured in 30 °C generally provided higher itaconic acid concentration. The strain cultured in pH 5 and 25 °C did not showed the increase of itaconic acid concentration (0.1 g L^{-1}) compare with wild type strain. When the *E. coli* (pBAC) was cultured in pH 7.0 at 30 °C, the highest itaconic acid concentration of 2.1 g L^{-1} was achieved in 48 h with 10 g L^{-1} glucose as a carbon source (Fig. 3a).

To evaluate the effect of culture media, *E. coli* (pBAC) strain was cultured in M9 minimal media supplemented with glucose (10 g L^{-1}). During culture, the itaconic acid concentration gradually increased and reached 3.6 g L^{-1} at 48 h (Fig. 3b). This result supports that M9 minimal media is more suitable for itaconic acid than LB media.

To improve itaconic acid production, various carbon sources were tested since different carbon source can affect intracellular metabolism by producing different amounts of metabolites including NADH, ATP, etc. (Fig. 4). Glycerol is converted to glyceraldehyde-3-P (G3P) via glycerol-3-P, and then joins glycolysis pathway and further converted to itaconic acid. Xylose is converted to fructose-6-P and G3P via pentose phosphate pathway, and enters glycolysis pathway. When the strain was cultured in M9 medium supplemented with 10 g L^{-1} of glycerol, 4.8 g L^{-1} of final itaconic acid concentration was achieved. Xylose and glucose supplemented medium provided 3.4 and 3.5 g L^{-1} of itaconic acid concentration, respectively (Fig. 4). Time profiles of cell densities were also compared. When glycerol was used as substrate, cell density (OD_{600}) was increased to 12.4 at the end of culture while 15.9 of OD_{600} was achieved with glucose. In combination with itaconic acid concentration data, it can be deduced that intracellular carbon flux was directed toward itaconic acid pathway more efficiently when glycerol was used carbon substrate. Therefore, it was suggested that glycerol is good substrate candidate for itaconic acid biosynthesis in *E. coli*.

The effect of glycerol in organic acid production has been investigated in various studies for long time (Gao et al. 2016; Le Meur et al. 2014; Zambanini et al. 2016). It has been reported that glycerol can be considered as suitable substrate for the production of certain product considering its highly reduced state (Dharmadi et al. 2006). Glycerol is also an inexpensive and abundant carbon source, which makes glycerol more attractive carbon source.

Fed-batch fermentation for itaconic acid production with glycerol

To achieve higher itaconic acid concentration, fed-batch fermentation study was carried out in 5 L reactor using glycerol as carbon source (Fig. 5). Since it has been reported that controlling pH is important for itaconic acid production, fed-batch fermentation was carried out at pH 7. During 60 h of fermentation, itaconic acid concentration continuously increased as well as cell density. The highest itaconic acid concentration of 20.1 g L^{-1} was obtained at 48 h, and it decreased after that point. Through fed-batch fermentation study, 5 times higher cell concentration was obtained compare with flask culture and 4 times higher itaconic acid concentration was obtained (Fig. 5). These results suggest that itaconic acid concentration can be elevated by feeding of carbon substrate such as glycerol.

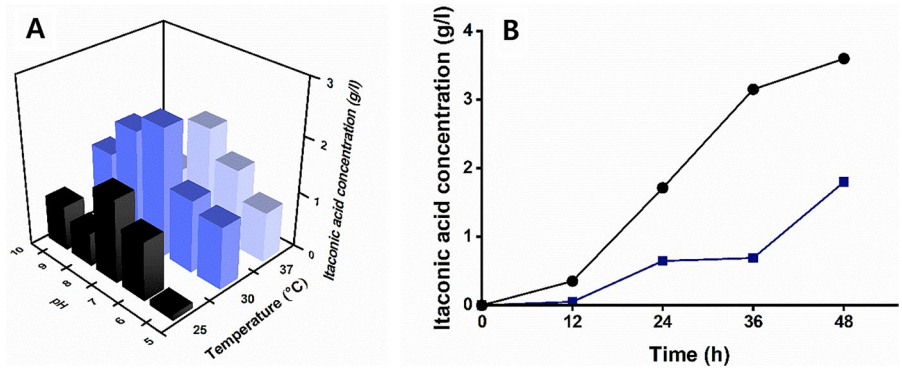

Fig. 3 **A** Itaconic acid concentration obtained at various temperatures and pH conditions. The strain was expressed at various temperatures (25 ºC, 30 ºC, and 37 ºC) and pH (5.5, 6.5, 7.5, 8.5, and 9.5). **B** Itaconic acid concentration produced in LB (filled square) and M9 (filled circle) media

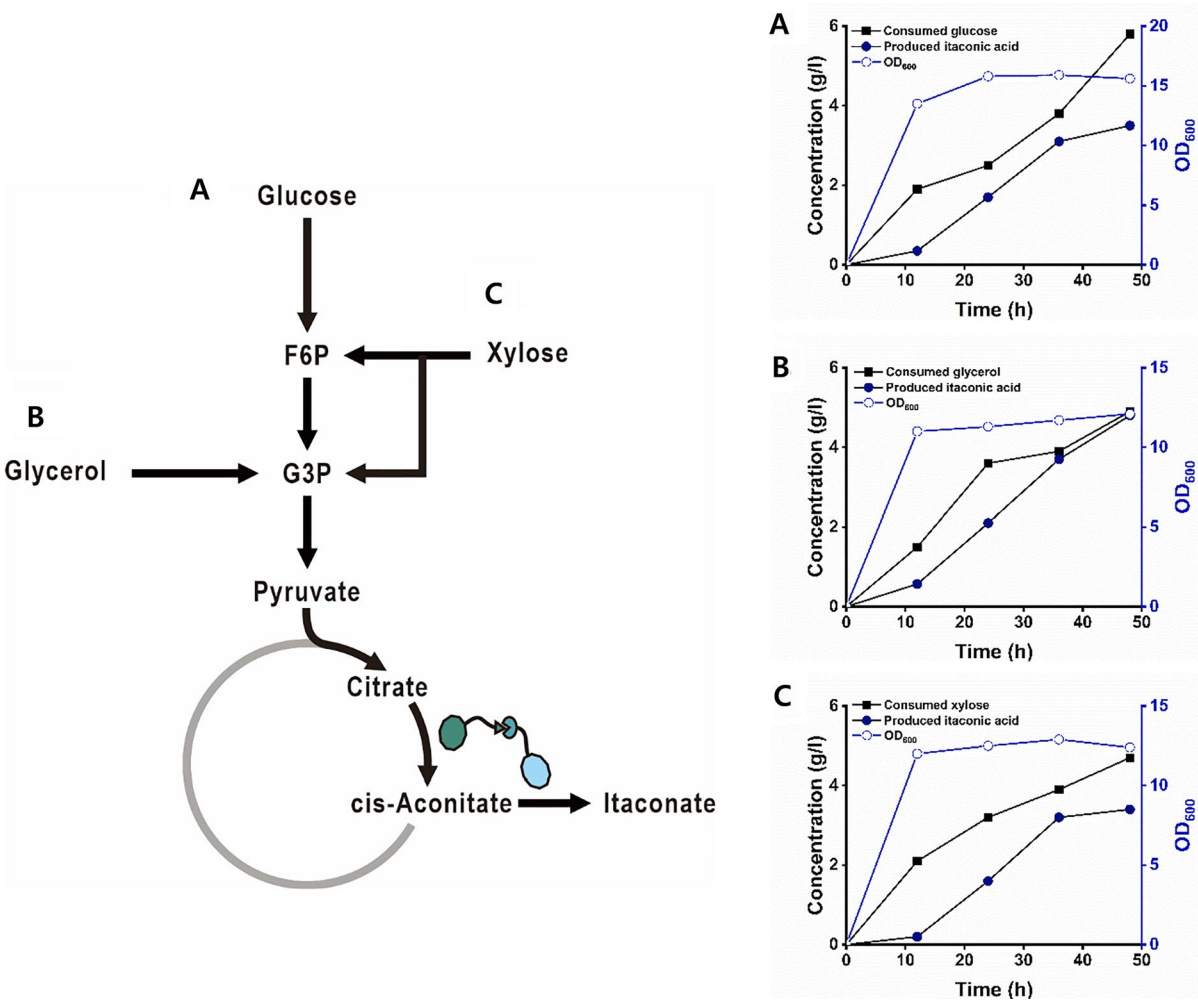

Fig. 4 Time profiles of itaconic acid concentration (filled circle), consumed substrates (filled square) and cell densicy (unfilled circle) when **A** glucose, **B** glycerol, and **C** xylose were used as substrate

Conclusion

In this study, the synthetic scaffold was employed as a novel strategy to construct more efficient itaconic acid producing recombinant strain. Through the synthetic protein scaffold strategy, a stable complex of several enzymes can be formed and co-localized in specific space. That leads to decreasing the diffusion of the product and increase metabolite production rate and yield. By scaffold, CadA is physically connected to AcnA. Therefore, *cis*-aconitate produced by AcnA can have more chance to react with CadA than other competing enzymes and can be converted to itaconic acid more efficiently. By the introduction of synthetic protein scaffold, itaconic acid concentration was increased 90.9% in the *E. coli* XB (pBAC) strain compared with that obtained with *E. coli* XB (pBNC), which does not have synthetic protein scaffold. By fed-batch fermentation, itaconic acid concentration was further increased to 20.1 g L^{-1}. The strategy developed in this study can be applied for the bio-refinery host improvements, and might enable the generation of an *E. coli* strain as an industrial itaconic acid producer.

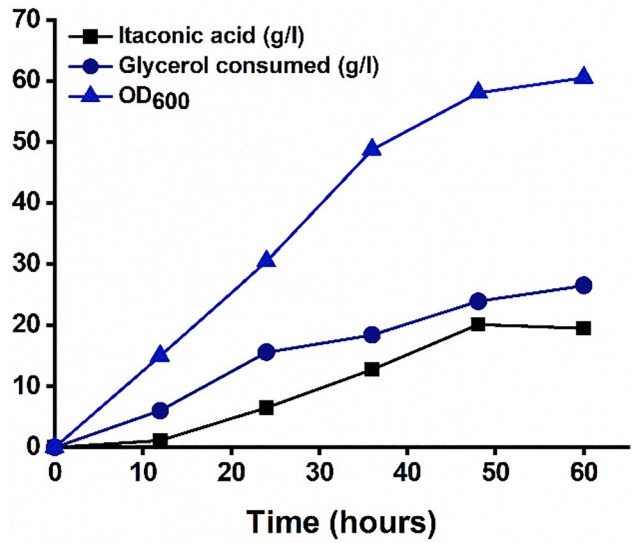

Fig. 5 Itaconic acid concentration (filled square), consumed glycerol concentration (filled circle) and cell density (filled triangle) obtained from fed-batch fermentation

Authors contributions Kim-Ngan T. Tran and Hong Soon Ho designed the study. Kim-Ngan T. Tran and Jaehoon Jeong performed the study. Kim-Ngan T. Tran, Jaehoon Jeong and Hong Soon Ho performed the study commented on draft versions of the article. All authors have approved the final article.

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Availability of data and materials The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to publish This article does not contain any studies with human participants or animals performed by any of the authors.

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