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RESEARCH PAPER

Improved 1,3-propanediol Production by *Escherichia coli* from Glycerol Due to Co-expression of Glycerol Dehydratase Reactivation Factors and Succinate Addition

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Abstract *Escherichia coli* was engineered to produce 1,3-propanediol (1,3-PDO) from glycerol, an inexpensive carbon source. This was done by introducing a synthetic pathway consisting of glycerol dehydratase, glycerol dehydratase reactivation factor, and 1,3-propanediol oxidoreductase isoenzyme. The JM-30BY15AB harboring pQE30/*dha*B123, *yqh*D and pQE15A/*gdr*A, *gdr*B produced 1,3-PDO (7.2 g/L) from glycerol, at a level higher than that produced by JM-30BY harboring pQE30/*dha*B123, *yqh*D (4.1 g/L). When 10 mM succinate was added to the medium, the titer of 1,3-PDO and the glycerol consumption increased to 9.9 and 23.84 g/L, respectively. In addition, the ratio of NADH to NAD^+ increased by 43%. The titer of 1,3-PDO and glycerol consumption were 145.6 and 86.6% higher, respectively, than those from the control which harbors one vector system without *gdr*AB and did not include succinate addition. Under fed-batch fermentation conditions, the titer of 1,3-PDO and its conversion yield from glycerol were 13.11 g/L and 0.49 g/g, respectively. This dual-vector system may be a useful approach for the co-expression of recombinant proteins. Further, succinate addition is a promising route for the biotechnological production of NADH-dependent microbial metabolites.

Keywords: dual-vector system, engineered *E. coli*, glycerol, *Klebsiella pneumoniae* AJ4, 1,3-propanediol, succinate

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1. Introduction

In recent years, global markets for the biodiesel, oleochemical, bioethanol, and soap industries have expanded. The production of glycerol is increasing as a major by-product of these industries, and this trend has generated a huge surplus of glycerol, which is considered an inexpensive and abundant feedstock for bioprocess industries. Glycerol is a highly reduced carbon source; hence, as a feedstock, it can be converted to various high-value reduced chemicals such as 1,3-propanediol (1,3-PDO) and generate higher product yields than those obtained using glucose [1,2].

1,3-PDO is a chemical with two hydroxyl groups that has a variety of applications in both medicine and industry, especially in the production of biodegradable and biocompatible synthetic polymers such as polytrimethylene terephthalate (PTT) [3,4]. Monomer generation is currently based on chemical production, but much attention has been paid to biological methods that can use renewable feedstock and are friendlier to the environment [5-7]. Many microorganisms such as *Klebsiella*, *Clostridium*, and *Citrobacter* have been studied for their ability to convert glycerol into 1,3-PDO [2,8-12]. However, industrial applications of these microorganisms are restricted because they are either opportunistic pathogens or strict anaerobes.

E. coli is generally regarded as a safe, well-studied microorganism for heterologous expression and is easy to culture. However, the bacteria cannot naturally produce 1,3-PDO by glycerol consumption. We constructed recombinant *E. coli* by introducing a synthetic pathway consisting of glycerol dehydratase complex (*dha*B123) and glycerol dehydratase reactivation factors (*gdr*AB) from *Klebsiella pneumoniae* AJ4 [9] and 1,3-propanediol oxidoreductase isoenzyme (*yqh*D) from *E. coli*. The production of 1,3-PDO by

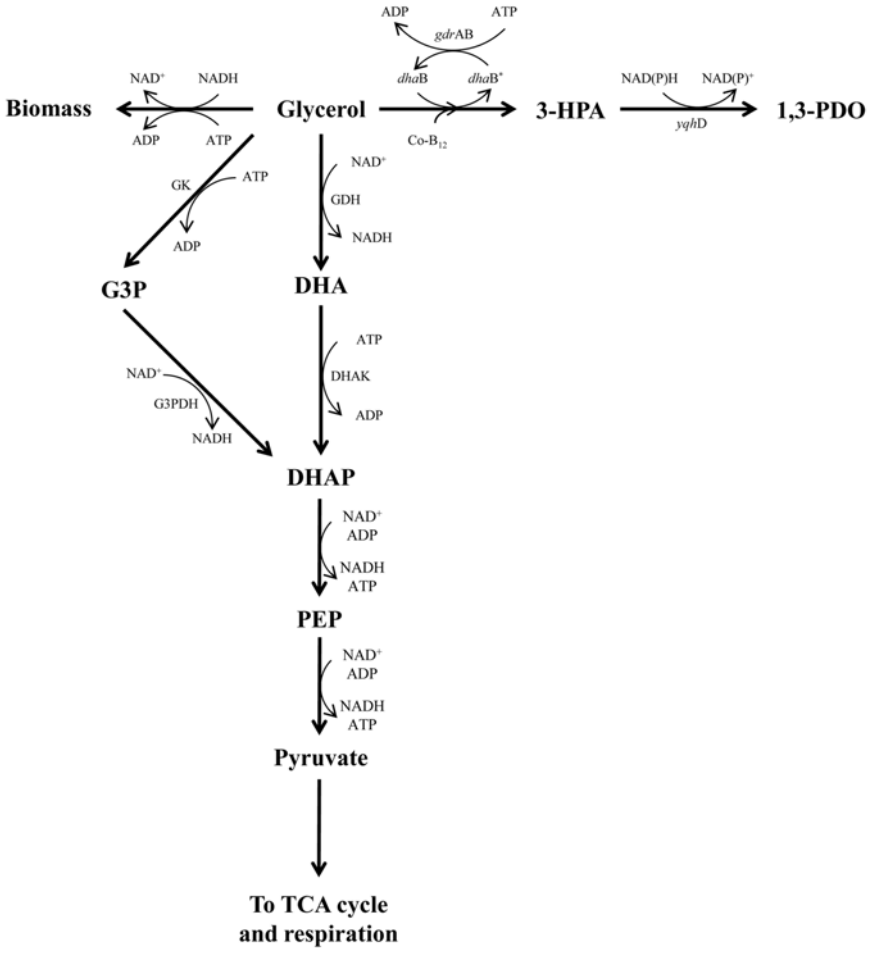

Fig. 1. 1,3-Propanediol production pathway from glycerol in recombinant *E. coli* over-expressing glycerol dehydratase (*dha*B123), glycerol dehydratase reactivation factors (*gdr*AB), and 1,3-propanediol oxidoreductase (*yqh*D). *dha*B* represents the inactivated glycerol dehydratase, which can be reactivated by *gdr*AB in the presence of ATP, coenzyme B_{12}, and Mg^{2+}. GK, glycerol kinase; G3PDH, glycerol-3-phosphate dehydrogenase; GDH, glycerol dehydrogenase; DHAK, dihydroxyacetone kinase; G3P, glycerol-3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetonephosphate.

recombinant *E. coli* under aerobic conditions eliminates the large volume of nitrogen gas consumption. This reduces the costs of general 1,3-PDO production. In 1,3-PDO production, *dha*B123 converts glycerol to 3-hydroxypropionaldehyde (3-HPA) in the presence of coenzyme B_{12}, and *yqh*D catalyzes the conversion of 3-HPA to 1,3-PDO (Fig. 1) [13-16].

Glycerol dehydratase undergoes mechanism-based inactivation by its physiological substrate, glycerol [17,18]. Glycerol dehydratase can be reactivated by a glycerol dehydratase reactivation factors in the presence of ATP, coenzyme B_{12}, and Mg^{2+} [18,19]. To investigate the effect of *gdr*AB during 1,3-PDO fermentation, we tested three commonly used laboratory *E. coli* strains for their ability to convert glycerol into 1,3-PDO when transformed with either one plasmid (pQE30/*dha*B123, *yqh*D) or two plasmids (pQE30/*dha*B123, *yqh*D and pQE15A/*gdr*A, *gdr*B). Then we identified a suitable strain as a host for the production of 1,3-PDO from glycerol. Finally, the flask-culture experiments of the selected *E. coli* strain JM109 were performed to study its efficiency when tricarboxylic acid (TCA) cycle intermediates were added. Bioreactor fed-batch experiments were carried out to evaluate JM109's potential for increased production of 1,3-PDO from glycerol.

2. Materials and Methods

2.1. Bacterial strains and plasmid constructs

All bacterial strains, including engineered strains and plasmids, and primers used in this study are listed in Table 1. *E. coli* XL1-blue, *E. coli* JM109, and *E. coli* W3110 were used as host strains. The plasmids pQE30 and pQE15A were used as expression plasmids. The plasmid pQE15A was constructed by overlap-extension PCR using primers P30 F-P30 R and P15A F-P15A R. A procedure used to replace the ampicillin-resistance and ColE1-origin genes of

Table 1. Bacterial strains, plasmids, and primers used in this study

Strains	Genotype	Source
E.coli XL-blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F' [::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Stratagene
E.coli JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) e14-[F' traD36 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	NEB
E.coli W3110	F$^-$ λ$^-$ rph-1 INV(rrnD, rrnE)	ATCC27325
Klebsiella pnumoniae AJ4	Wild type, isolation from soil	Laboratory collection
XL-30BY	Recombinant *E. coli* XL-blue harboring pQE30/*dha*B123, *yqh*D	This study
XL-30BY15AB	Recombinant *E. coli* XL-blue harboring pQE30/*dha*B123, *yqh*D and pQE15A/*gdr*A, *gdr*B	This study
JM-30BY	Recombinant *E. coli* JM109 harboring pQE30/*dha*B123, *yqh*D	This study
JM-30BY15AB	Recombinant *E. coli* JM109 harboring pQE30/*dha*B123, *yqh*D and pQE15A/*gdr*A, *gdr*B	This study
W-30BY	Recombinant *E. coli* W3110 harboring pQE30/*dha*B123, *yqh*D	This study
W-30BY15AB	Recombinant *E. coli* W3110 harboring pQE30/*dha*B123, *yqh*D and pQE15A/*gdr*A, *gdr*B	This study
Plasmids	**Description**	**Source**
pQE30	ColE1*ori*, P_{T5}, Amp^R	Qiagen
pACYCDuet-1	P15A *ori*, P_{T7}, Cm^R	Novagen
pQE15A	pQE30 derivative replaced Amp^R and ColE1*ori* genes with Cm^R and P15A *ori* genes of pACYCDuet-1	This study
p30-BY	pQE30 derivative containing *dha*B123 and *yqh*D genes	This study
p15-AB	pQE15A derivative containing *gdr*A, *gdr*B genes	This study
Primers	**Sequence (5' to 3')**	**Source**
P30 F	CCTATAAAAATAGGCGTATCACGAGGC	This study
P30 R	ATTGACTACCGGATAGCTTGGATTCTCACCAATAAAAAACGCC	This study
P15A F	GAGAATCCAAGCTATCCGGTAGTCAATAAACCGGTAAACCA	This study
P15A R	GGGCATGACTAACATGAGAATTACAACTTATAT	This study
DB S1 F	CGAGCTCATGAAAAGATCAAAACGATTTGCAGTAC	This study
DB K1 R	GGGGTACCTTAGCTTCCTTTACGCAGCTTATG	This study
YD K1 F	GGGGTACCATGAACAACTTTAATCTGCACACCC	This study
YD H3 R	CCCAAGCTTTTAGCGGGCGGCTTCGTATATA	This study
GA S1 F	CGAGCTCATGCCGTTAATAGCCGGGATTGAT	This study
GA X1 R	TCCCCCGGGTTAATTCGCCTGACCGGCCAG	This study
GB X1 F	TCCCCCGGGATGTCGCTTTCACCGCCAGG	This study
GB H3 R	CCCAAGCTTTCAGTTTCTCTCACTTAACGGCAG	This study

pQE30 with chloramphenicol-resistance and p15A-origin genes of pACYCDuet-1. The *dha*B123 complex gene from *K. pneumoniae* AJ4 and the *yqh*D gene from *E. coli* were amplified by PCR using primer the sets DB S1 F-DB K1 R and YD K1 F-YD H3 R, respectively. The amplified *dha*B123 fragment was cloned into the *Sac*I and *Kpn*I sites of pQE30 to generate pQE30/*dha*B123. Then, the amplified *yqh*D fragment was introduced into the *Kpn*I and *Hind*III sites of pQE30/*dha*B123 to construct pQE30/*dha*B123, *yqh*D (p30-BY) (Supplementary Fig. 1A). To construct pQE15A/*gdr*AB (p15-AB) (Supplementary Fig. 1B), the *gdr*A and *gdr*B genes from *K. pneumoniae* AJ4 were amplified using the following two pairs of primers, respectively: GA S1 F, GA X1 R; GB X1 F, GB H3 R. The *gdr*A PCR fragment was inserted into the *Sac*I and *Xma*I sites of pQE15A to generate pQE15A/*gdr*A. Then, the *gdr*B PCR fragment was cloned into the *Xma*I and *Hind*III sites of pQE15A/*gdr*A to construct pQE15A/*gdr*AB. The constructed plasmids were transformed into *E. coli* strains to generate engineered strains.

2.2. Flask cultures and fed-batch fermentation

The 1,3-PDO-producing *E. coli* strains were inoculated into 125-mL baffled Erlenmeyer flasks containing 20 mL Luria-Bertani (LB) broth at 37°C and 180 rpm. The cultures were supplemented with 100 mg/L ampicillin and 25 mg/L chloramphenicol, per requirement. When the cell density at OD_{600} reached 1.0, 30 g/L glycerol, 0.2 mM

isopropyl β-D-1-thiogalactopyranoside (IPTG), and 10 μM coenzyme B_{12} were added, and the culture was further incubated at 30°C for 40 h. All strains were analyzed for 1,3-PDO production. To screen the bacterial 1,3-PDO production efficiency with the addition of TCA cycle intermediates, such as malate, succinate, and fumarate; a 5, 10 or 20 mM intermediate was added to the medium at IPTG induction. All flask-culture experiments were performed in triplicate.

Fed-batch fermentation experiments were carried out at 37°C, 400 rpm, and at pH 7.0 ± 0.1 in a 1.5 L bioreactor (BioTron, Seoul, Korea), with an initial working volume of 1 L. The same LB medium as that in the flask culture experiments was used. The same concentrations of IPTG and coenzyme B_{12} were added when the cell OD_{600} reached at 1.0 ± 0.1. One exception was that the glycerol concentration was decreased to 5 g/L and the culture was further incubated at 30°C. The initial glycerol concentration was 5 g/L and feeding solution was added during 5 ~ 60 h to maintain glycerol concentration within 4 ~ 6 g/L with different feeding rates. The succinate concentration was initially 5 mM and when it decreased to 1 ± 0.5 mM, succinate was fed to increase the concentration to 5 ± 1 mM.

2.3. Analytical methods

The culture samples were centrifuged at 13,000 × *g* for 5 min and filtered through a 0.2-μm membrane filter. Organic compounds and carbon sources; including glycerol, 1,3-PDO, acetate, lactate, malate, succinate, and fumarate were measured using a Waters high-performance liquid chromatography system equipped with a refractive index detector, and an organic acid analysis column was used for separation (Aminex HPX-87H, 300 mm × 7.8 mm, 9 μm; Bio-Rad Co., Richmond, CA, USA). The column was eluted with sulfuric acid (4 mM) at a flow rate of 0.4 mL/min at 30°C [20,21]. Cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer. OD_{600} measurements were converted to the dry weight using a calibration curve to relate OD_{600} to dry cell weight (DCW). The correlation factor was DCW (g/L) = 0.45 × OD_{600}. The intracellular redox balance (represented by the ratio of NADH to NAD^+) was determined by NAD/NADH-Glo™ assay kit (Promega), according to the manufacturer's instructions.

3. Results and Discussion

3.1. 1,3-PDO production from glycerol varied with and without p15-AB or strain specificity

The glycerol dehydratase reactivation factors were assumed to serve as a reactivating factor for inactivated glycerol dehydratase [22]. To investigate the effects of *gdr*AB on 1,3-PDO fermentation, the expression plasmids p30-BY and p15-AB were used to construct several engineered *E. coli* strains (Table 1). The glycerol consumption of engineered *E. coli* strains harboring p30-BY alone was lower than *E. coli* strains harboring both p30-BY and p15-AB (Table 2). Due to inactivation of glycerol dehydratase in the fermentation process, it is necessary to co-express the *gdr*AB genes encoding glycerol dehydratase reactivating factor with *dha*B123 and *yqh*D in the engineered *E. coli* to produce 1,3-PDO efficiently. Thus, we constructed the pQE15A plasmid and a dual-vector system in *E. coli*.

Table 2 also shows the appropriate strains used as a host for the production of 1,3-PDO. Under the dual- vector system, we tested three commonly used laboratory *E. coli* strains, *E. coli* XL1-blue, *E. coli* JM109, and *E. coli* W3110, for their ability to convert glycerol into 1,3-PDO. *E. coli* JM109 was able to produce the maximum amount of 1,3-PDO (7.2 g/L) compared to that production by *E. coli* XL1-blue (4.0 g/L) and *E. coli* W3110 (0.7 g/L) (Table 2). The expression levels and plasmid stability of three laboratory *E. coli* strains were similar (data not shown). However, the rates of substrate uptake per cell g (dry cell weight) were different. The maximum substrate uptake rate (8.5 g-glycerol/g-DCW) by *E. coli* JM109 was obtained compared to that rate by *E. coli* XL1-blue (6.1 g-glycerol/g-DCW) and *E. coli* W3110 (5.0 g-glycerol/g-DCW). Therefore, *E. coli* JM109 was selected for further studies. The results show that glycerol permeability in different *E. coli* stains may vary significantly and therefore affect production of 1,3-PDO. This research can be applied to other studies where *E. coli* are used to convert glycerol to alcohols and bio-chemicals.

3.2. The addition of TCA cycle intermediates improved 1,3-PDO production from glycerol

The conversion of glycerol to 1,3-PDO is coupled with the

Table 2. 1,3-PDO production in *E. coli* strains harboring pQE30/*dha*B123, *yqh*D (p30-BY) with and without pQE15A/*gdr*A, *gdr*B (p15-AB)

Mother strain	Plasmid(s)	Glycerol consumption (g/L)	1,3-Propanediol titer (g/L)	Dry cell weight (g/L)
E. coli XL1-blue	30BY	11.12 ± 0.53	3.10 ± 0.17	1.73 ± 0.04
	30BY15AB	11.19 ± 1.19	4.04 ± 0.04	1.84 ± 0.01
E. coli JM109	30BY	11.49 ± 0.32	4.06 ± 0.21	1.93 ± 0.01
	30BY15AB	17.21 ± 2.23	7.80 ± 0.37	2.03 ± 0.08
E. coli W3110	30BY	6.20 ± 0.42	0.38 ± 0.01	1.13 ± 0.03
	30BY15AB	6.71 ± 0.63	0.73 ± 0.05	1.33 ± 0.02

conversion of NADH to NAD^+, requiring the regeneration of NADH for continuous 1,3-PDO production. NADH regeneration requires extra glycerol consumption, which could reduce the conversion of glycerol into 1,3-PDO. In order to overcome these problems, TCA cycle intermediates (malate, succinate, and fumarate) were added to manipulate the intracellular redox balance. By manipulating the ratio of NADH to NAD^+, production of 1,3-PDO can be improved [23,24].

TCA cycle intermediates were added over a range of 0 ~ 20 mM, and the shake-flask fermentation experiments were carried out for 40 h with engineered *E. coli* JM109 (JM-30BY15AB). As shown in Table 3, when TCA intermediates were added to the medium, the glycerol consumption and 1,3-PDO production increased compared to those observed from control (without the addition of TCA cycle intermediates). Addition of 10 mM succinate caused increased titer of 1,3-PDO (9.9 g/L) compared to that obtained with malate (8.2 g/L) and fumarate (9.4 g/L). Therefore, succinate was selected for manipulating the intracellular redox balance. The optimal succinate concentration was 10 mM.

The time profile of glycerol consumption, 1,3-PDO production, and intracellular redox balance by the engineered *E. coli* JM109 (JM-30BY15AB) was monitored with or without the addition of 10 mM succinate. After 40 h, the

Table 3. Glycerol consumption, 1,3-PDO titer, and dry cell weight of engineered *E. coli* JM109 (JM-30BY15AB) under different TCA cycle intermediate concentrations

	Concentration (mM)	Glycerol consumption (g/L)	1,3-propanediol titer (g/L)	Dry cell weight (g/L)
Control	Non-feeding	18.11 ± 1.23	7.74 ± 0.17	2.88 ± 0.03
Malate	5	18.98 ± 1.11	7.88 ± 0.42	2.86 ± 0.04
	10	20.95 ± 0.15	7.96 ± 0.16	2.99 ± 0.07
	20	21.20 ± 0.83	8.17 ± 0.52	2.93 ± 0.01
Succinate	5	20.14 ± 0.67	8.24 ± 0.49	2.94 ± 0.02
	10	23.84 ± 0.55	9.91 ± 0.32	2.97 ± 0.01
	20	24.69 ± 1.05	9.81 ± 0.46	2.97 ± 0.03
Fumarate	5	21.14 ± 1.65	8.62 ± 0.71	2.84 ± 0.12
	10	24.72 ± 0.47	9.33 ± 0.29	3.02 ± 0.03
	20	25.02 ± 0.77	9.46 ± 0.18	3.01 ± 0.01

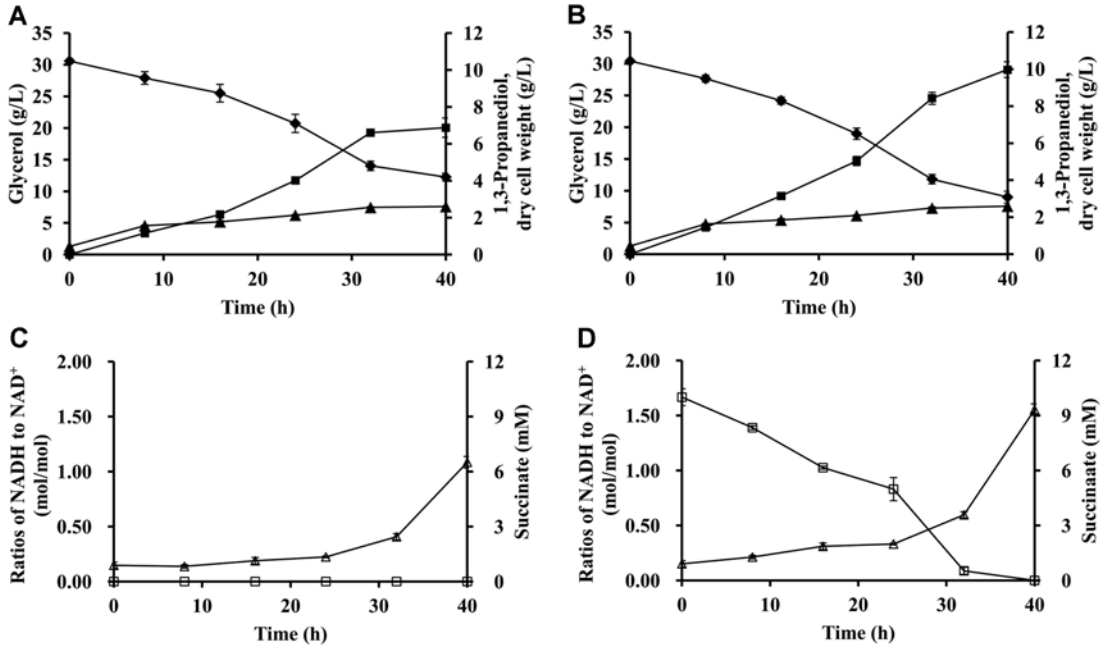

Fig. 2. Time profile of glycerol consumption, 1,3-PDO titer, dry cell weight, ratios of NADH to NAD^+, and succinate consumption in flask fermentation supplemented with 30 g glycerol/L at 30°C for 40 h. (A and C) 0 mM succinate, (B and D) 10 mM succinate. Error bars indicate one standard deviation, as determined from triplicate experiments. *Closed diamonds*, residual glycerol level; *closed squares*, titer of 1,3-PDO; *closed triangles*, dry cell weight; *open squares*, residual succinate level; *open triangles*, ratio of NADH to NAD^+.

ratio of NADH to NAD^+ increased by 43% with succinate was addition (Fig. 2). There was an increase of glycerol consumption and 1,3-PDO production by 17 and 38%, respectively, at the time of succinate addition compared to control. Conversion yields of 1,3-PDO from glycerol were similar at 41% regardless of succinate addition. These results show that succinate can be used to increase the ratio of NADH to NAD^+ and to speed up the metabolic flux of 1,3-PDO. Therefore, succinate is a key factor that influences 1,3-PDO production.

3.3. 1,3-Propanediol production by the engineered *E. coli* in a bioreactor

Based on the above results, strain JM-30BY15AB exhibited the highest 1,3-PDO titer at 9.9 g/L among the six engineered *E. coli*. For further optimization of fermentation conditions, a fed-batch experiment was performed in a 1.5 L bioreactor. During 1,3-PDO fermentation, feeding solution was continuously added with different feeding rates to maintain low glycerol concentrations (5 ± 1 g/L). Cell growth and 1,3-PDO production were inhibited when the concentration of glycerol was high [25], so fed-batch experiment with low substrate concentrations were studied. As shown in Fig. 3, the titer of 1,3-PDO and its conversion yield from glycerol were 13.11 g/L and 0.49 g/g, respectively. Under fed-batch fermentation conditions, the titer of 1,3-PDO and its conversion yield were enhanced to 32 and 20%, respectively, compared to those observed under flask culture conditions. This result suggests that fed-batch fermentation is a more efficient way to produce of 1,3-PDO.

Several papers describing construction of *E. coli* capable of producing 1,3-PDO have been published. Skraly *et al.* [14] engineered a recombinant *E. coli* strain that harbored genes from the *dha* operon from *K. pneumoniae*, and produced 6.3 g/L of 1,3-PDO. Rujananon *et al* constructed a recombinant *E. coli* strain by assembling the genes *dha*B1 and *dha*B2 from *Clostridium butyricum* and *yqh*D from *E. coli* [26]. The current study establishes the optimum conditions for 1,3-PDO production using the response surface methodology, and has produced the maximum values of 1.65 g/L 1,3-PDO production reported in the literature to date. Przystalowska *et al.* [27] constructed an *E. coli* strain harbored six heterologous glycerol catabolism pathway genes from *C. freundii* and *K. pneumoniae*, which used 32.6 g/L of glycerol to produce 10.6 g/L. The engineered *E. coli* JM-30BY15AB did not give satisfying results due to low cell density (2.67 g/L) because the optimization of medium constituents or culture conditions was not performed. If medium constituents and culture conditions were optimized, the cell concentration may increase which could result in a higher titer of 1,3-PDO production.

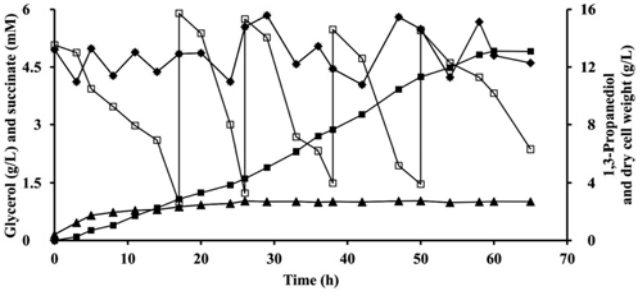

Fig. 3. Time profile of 1,3-propanediol fed-batch fermentation by recombinant *E.coli* JM109 (JM-30BY15AB) at 30°C for 65 h. *Closed diamonds*, residual glycerol level; *closed squares*, titer of 1,3-PDO; *closed triangles*, dry cell weight; *open squares*, residual succinate level.

4. Conclusion

In the metabolic pathway of glycerol conversion to 1,3-PDO, the enzyme *dha*B123 dehydrates glycerol to 3-HPA and then the enzyme *yqh*D reduces 3-HPA to 1,3-PDO. In this study, we achieved efficient production of 1,3-PDO from glycerol by engineered *E. coli* JM109 *via* the co-expression of *gdr*AB and addition of succinate. Construction of a dual-vector system and succinate addition during flask culture enhanced the glycerol consumption and titer of 1,3-PDO. The glycerol consumption and titer of 1,3-PDO were 86.6 and 145.6% higher, respectively, than those from the control (without co-expression of *gdr*AB and no addition of succinate), and the engineered *E. coli* JM-30BY15AB exhibited the highest 1,3-PDO titer at 13.11 g/L under fed-batch fermentation conditions. There are two possible reasons for these results. First, co-expression of *gdr*AB using a dual-vector system could effectively activate the inactivated glycerol dehydratase. This means that a high level of activated glycerol dehydratase could be achieved during 1,3-PDO fermentation. Second, the ratio of NADH to NAD^+ could be increased by addition of succinate, such that more reducing power is used for converting glycerol to 1,3-PDO. The dual-vector system constructed in this work suggests a useful approach for co-expressing heterologous proteins in *E. coli*, and succinate addition to the medium is a promising route for the production of NADH-dependent bio-chemicals.

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