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Elevated production of 3-hydroxypropionic acid by metabolic engineering of the glycerol metabolism in *Escherichia coli*

Won Seok Jung, Jin Ho Kang, Hun Su Chu, In Suk Choi, Kwang Myung Cho*

Biomaterials Lab, SAIT, SEC, 560 Maetandong, Youngtonggu, Suwonsi, Gyunnggido 443-370, South Korea

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ABSTRACT

3-Hydroxypropionic acid (3-HP) is a renewable-based platform chemical which may be used to produce a wide range of chemicals including acrylic acid, 1,3-propanediol, and acrylamide. Commercialization of microbial 3-HP production from glycerol, which is produced inexpensively as a by-product of biodiesel production, could be expedited when global biodiesel production increases significantly. For enhancing 3-HP production, this study aimed to investigate metabolic engineering strategies towards eliminating by-products of 3-HP as well as optimizing the glycerol metabolism. The removal of genes involved in the generation of major by-products of 3-HP including acetate and 1,3-propanediol increased both 3-HP production level (28.1 g/L) and its average yield (0.217 g/g). Optimization of L-arabinose inducible expression of glycerol kinase GlpK, which catalyzes the conversion of glycerol to glycerol-3-phosphate, was also made to increase the metabolic flow from glycerol to 3-HP. To activate the whole glycerol metabolism towards 3-HP, the regulatory factor repressing the utilization of glycerol in *Escherichia coli*, encoded by *glpR* was eliminated by knocking-out in its chromosomal DNA. The resulting strain showed a significant improvement in the glycerol utilization rate as well as 3-HP titer (40.5 g/L). The transcriptional analysis of *glpR* deletion mutant revealed the poor expression of glycerol facilitator GlpF, which is involved in glycerol transport in the cell. Additional expression of *glpF* in the *glpR* deletion mutant successfully led to an increase in 3-HP production (42.1 g/L) and an average yield (0.268 g/g).

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1. Introduction

Environment and sustainability issues are main driving forces behind the development of fuels and chemicals from biomass that is renewable, abundant and reliable. Through biotechnology including recombinant DNA technology, we can use microorganisms in new ways to manufacture polymers, commodity chemicals, or transportation fuel. Biodiesel, one of the most attractive biofuels, can be produced from vegetable or algal oils by a simple transesterification reaction (Ana and Enoch, 2003). The rapid growth of biodiesel industries have resulted in the large production of crude glycerol as its by-product (Clomburg and Gonzalez, 2013). As a consequence, there has been a gradual decrease in glycerol price, creating new application of waste stream of biodiesel production (Yazdani and Gonzalez, 2007).

By using glycerol as carbon source, microorganisms can produce multiple fermentation products. Among them, one of the promising bio-based chemicals is 3-hydroxypropionic acid (3-HP). 3-HP has gained considerable attention owing to its applications in deriving a variety of commercially valuable chemicals, such as acrylic acid, 1,3-propanediol (1,3-PDO), methyl acrylate, propiolactone, malonic acid, acrylamide, and hydroxyamides (Kumar et al., 2013). One of the main target product of 3-HP is acrylic acid, which is easily converted from 3-HP via dehydration process and has a 4.5 million tons per year ($11 billion) worldwide market at the end of 2011 based on ICIS pricing (http://www.icispricing.com). The production of bio-acrylic acid through 3-HP will be cost-competitive as the conversion technologies are developed (Paster et al., 2003).

Theoretically biological process using glycerol via 3-HP was reported to produce bio-acrylic acid at 50% lower costs than petroleum-based acrylic acid and with a 75% reduction in greenhouse gas emissions (OPXBIO, 2011). On the basis of the theoretical yield (0.97 g/g) of 3-HP synthesized from glycerol, yield for dehydration of 3-HP (0.8 g/g), and $0.35/kg cost of crude glycerol (Quispe et al., 2013), the minimum cost of manufacturing bio-acrylic acid from glycerol can be estimated to be $0.98. Considering the current purchase price of petroleum-based acrylic acid ($2.0) at the end of 2012 (ICIS pricing), production of bio-acrylic acid via 3-HP seems cost-competitive and future-oriented technology. Through this bio-process, an aim of solving both the environmental and pricing issues could be pursed simultaneously.

* Corresponding author: Fax: +82 31 8061 1315.
E-mail address: km3.cho@samsung.com (K.M. Cho).

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Biologically, 3-HP can be produced from glycerol via two step pathway involving vitamin B_{12} dependent glycerol dehydratase (DhaB), which converts glycerol into 3-hydroxypropionaldehyde (3-HPA) as well as aldehyde dehydrogenase, which catalyzes the conversion of 3-HPA to 3-HP. Recently, a variety of research has been focused on the titer and yield enhancement of 3-HP from glycerol through balanced expression of two synthetic enzymes, DhaB and AldH (Rathnasingh et al., 2009; Raj et al., 2008), acid tolerance towards 3-HP (Warnecke et al., 2011), or reducing the usage of relatively expensive ingredient vitamin B_{12} (Ashok et al., 2013). The resulting strain showed 38.7 g/L production with an average yield of 0.35 g/g, which would be a commercially meaningful level (Rathnasingh et al., 2009). Although the main carbon source, glycerol, needs to get more attention for 3-HP-producing strain improvement due to low efficient glycerol utilization of *Escherichia coli*, there has been no remarkable report on engineering glycerol metabolism for enhanced 3-HP production.

In this study, we focused on the further developing and improving the 3-HP production capability via manipulation of structural genes that govern the glycerol metabolic pathway including glycerol kinase GlpK and glycerol facilitator GlpF. Especially, glycerol pathway repressor GlpR was revealed to be a major limiting factor in converting glycerol to 3-HP in *E. coli*. The 3-HP titer achieved by engineering glycerol pathway in this paper is 42.1 g/L from glycerol with a yield of 0.268 g/g.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and genetic manipulation

The genomic DNA isolation kit and pGEM-T vector were procured from Promega (Madison, WI). The high fidelity DNA polymerase and pETDuet-1 and pRSFDuet-1 vectors were acquired from Novagen (San Diego, CA). Miniprep and DNA gel purification kits were purchased from Qiagen (Mannheim, Germany). *E. coli* W3110 were obtained from American Type Culture Collection (ATCC 27325). W3110 (DE3) was used as host strains for 3-HP production, where W3110(DE3) was constructed using a λDE3 lysogenization kit (Novagen, San Diego, CA) for site-specific integration of λDE3 prophage into *E. coli* W3110. *E. coli* Top10 (Invitrogen) was used for general cloning and maintenance of plasmids. Recombinant *E. coli* cells were grown at 37 °C and 250 rpm in 14 mL test tubes containing 3 mL of LB medium supplemented with 100 μg/mL ampicillin (Sigma-Aldrich). 3-HP was purchased from Tokyo Kasei Kogyo Co. Ltd. Japan (TCI America). Genetic manipulations were carried out according to standard methods (Sambrook et al., 2001). The bacterial strains and plasmids used in this study are presented in Table 1. The primers used for gene cloning and knockout are listed in Table 2.

2.2. Construction of 3-HP producing strain

In order to express *dhaB* (*dhaB1*, *dhaB2*, *dhaB3*) and *gdrAB* genes (*gdrA*, *gdrB*), pETDuet-1 vector with the T7 promoter was used. To clone the *dhaB* and *gdrAB* genes, the genomic DNA of *Klebsiella pneumonia* DSM2026 was isolated, and the coding regions of *dhaB123* and *gdrA* were amplified by PCR (Bio-Red, Hercules, CA) using the primer set dhaBAF-dhaBAR. The PCR fragment was cut by BspHI/EcoRI and ligated with NcoI/EcoRI digested pETDuet-1 vector (Novagen). A EcoRI-SalI fragment containing *gdrB* gene was amplified using the primer set gdrBF-gdrBR. The PCR fragment was digested by EcoRI/SalI and ligated with the same enzyme sites of the pETDuet-1 vector containing *dhaB123-gdrA* genes, generating pET-BAB. The *aldH* gene (Genbank: AE000228) was amplified from *E. coli* K-12 using the primer set aldHF-aldHR. The resulting NdeI-BglII fragment was cloned into the same enzyme sites of pET-BAB, generating pET-BAB-H. The *E. coli* W3110(DE3) was transformed with pET-BAB-H to construct SPC001.

Table 1
Bacterial strains and plasmids used in this study.

Strains and plasmids	Description	Source
Plasmids		
pETDuet-1	Expression vector, ampR	Novagen
pACYCDuet-1	Expression vector, CmR	Novagen
pET-BAB-H	*dhaB123*, *gdrAB*, *aldH* in pETDuet-1 vector	This study
pACara	L-Arabinose inducible promoter AraBAD in pACYCDuet-1	This study
pACara-*glpK*	*glpK* gene in pACara vector	This study
pACara-*glpK*/*glpF*	*glpK* and *glpF* genes in pACara vector	This study
pACara-*glpF*	*glpF* gene in pACara vector	This study
Strains		
W3110	Expression host	ATCC
SPC001	Recombinant host W3110 harboring pET-BAB-H	This study
SPC002	Δ*ackA-pta*, Δ*yqhD* of SPC001	This study
SPC003	Δ*ackA-pta*, Δ*yqhD*, Δ*glpK* of SPC001	This study
SPC003-1	Δ*ackA-pta*, Δ*yqhD*, Δ*glpK* of SPC001transformed with pACara-*glpK*	This study
SPC004	Δ*ackA-pta*, Δ*yqhD*, Δ*glpK* of SPC001 transformed with pACara-*glpK*/*glpF*	This study
SPC005	Δ*ackA-pta*, Δ*yqhD*, Δ*glpR* of SPC001	This study
SPC006	Δ*ackA-pta*, Δ*yqhD*, Δ*glpR* of SPC001 transformed with pACara-*glpF*	This study

2.3. By-product pathway knockout mutants

Mutants lacking *ackA-pta* or *yqhD* were developed according to the Red recombinase method described by Datsenko and Wanner (2000). The PCR used for *ackA-pta* knockout construction was carried out with oligonucleotides primer set ackAKF-ackAKR. Plasmid pKD4 (Datsenko and Wanner, 2000) was used as a template that contains an FRT-flanked kanamycin resistance element. Each PCR product carrying a 45-bp homologous region at each end was recombined into the chromosome of *E. coli* W3110 (DE3) by using the Red plasmid pKD46 and selecting km^R transformants as described elsewhere (Datsenko and Wanner, 2000). The plasmid pCP20 containing the thermally induced Flp recombinase gene (Cherepanov and Wackernagel, 1995) was transformed into *ackA-pta*::km strain to eliminate the km^R gene. The double crossover mutant Δ*ackA-pta* was identified on the basis of its genotype by PCR. The *yqhD* mutation was made by amplifying a PCR product on pKD4 (Datsenko and Wanner, 2000) as template with the following primer set yqhDKF-yqhDKR. The resulting PCR fragment was transformed into *E. coli* Δ*ackA-pta* by electroporation and select double crossover mutants to yield Δ*ackA-pta* Δ*yqhD*. Transformation of Δ*ackA-pta* Δ*yqhD* strain with pET-BABH led to generation of SPC002.

2.4. Glycerol pathway knockout mutants

The PCR used for *glpK* knockout construction was carried out with oligonucleotides primer set glpKKF-glpKKR. Plasmid pKD3 (Datsenko and Wanner, 2000) was used as a template and each PCR product carrying a 45-bp homologous region at each end was

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